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Accelerated poly(A) loss and mRNA stabilization are independent effects of protein synthesis inhibition on α-tubulin mRNA in *Chlamydomonas*

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ABSTRACT

In *Chlamydomonas*, the usual rapid degradation of tubulin mRNAs induced by flagellar amputation is prevented by inhibition of protein synthesis with cycloheximide. Evidence is presented that the ability of cycloheximide to stabilize α-tubulin mRNA depends on the time of addition. Addition of cycloheximide to cells before induction strongly stabilizes the induced mRNAs, while addition after their synthesis stabilizes them only transiently. Moreover, cycloheximide inhibition does not stabilize the same α-tubulin mRNA species in uninduced cells. These results suggest that cycloheximide is not acting to stabilize the induced α-tubulin mRNAs simply by preventing ribosome translocation. The stabilized state of tubulin mRNA was found to correlate with its occurrence on smaller polysomes but larger EDTA-released mRNP particles than the unstable state. A second effect of cycloheximide on the metabolism of induced tubulin mRNAs is to accelerate complete poly(A) removal. This effect of cycloheximide inhibition, unlike stabilization, occurs whenever cycloheximide is added to cells, and appears unrelated to stabilization. The effect is shown to be mRNA-specific; poly(A)-shortening on the rbcS2 mRNA is not altered in the presence of cycloheximide, nor do completely deadenylated molecules accumulate. Experiments in which cells were released from cycloheximide inhibition suggest that deadenylated α-tubulin mRNAs may be less stable than their polyadenylated counterparts during active translation.

INTRODUCTION

The observation that inhibition of protein synthesis can dramatically increase the stability of certain mRNAs is longstanding. There are now many examples of mRNA-specific stabilizations which reveal that a variety of mRNA degradation pathways are translation-dependent (recently reviewed in 1–3), although in no case is the actual mechanism of translation-linked degradation clearly defined. Proposed mechanisms by which protein synthesis inhibitors act can be placed into one of two, not mututally exclusive, categories: 1) that stabilization is due to inhibition of synthesis of a protein product required for degradation, or 2) that stabilization is due to interruption of a normal mRNA-ribosome interaction, i.e. inhibiting the act of translation itself. Indirect evidence for the first mechanism has been provided by studies which demonstrate that efficient translation of the substrate mRNA itself is not necessary for its degradation (4–6), as well as studies using nontranslating cell-free degradation systems (7, 8). On the other hand, degradation mechanisms requiring the translation of the substrate mRNA have been demonstrated for several mRNAs now, among them, mammalian cell-cycle regulated histone mRNA (9, 10), β-tubulin mRNA (11–13), and probably the c-*myc* mRNA (14) and the MATα1 mRNA in yeast (15). It is becoming clear that translation-dependent pathways for mRNA degradation may be diverse.

Tubulin and other flagellar protein mRNAs are induced to high levels in *Chlamydomonas* cells in response to flagellar amputation. The induced mRNAs normally persist for about an hour, after which rapid degradation ensues (16, 17). In the presence of cycloheximide (CX) the post-induction degradation does not occur and the mRNAs remain stable for hours (18). A second effect of inhibition of protein synthesis during the induction of tubulin mRNAs is to accelerate their rates of poly(A) loss (19). In the presence of CX, a large proportion of induced α-tubulin mRNAs accumulate as completely deadenylated molecules. Thus, active translation appears to normally protect these mRNAs from rapid deadenylation. A translationally-dependent rate of poly(A) removal is unusual. Many studies have found poly(A) shortening to occur independently of translation, and to be generally unaffected by inhibitors of protein synthesis (20–25). A translationally-dependent rate of deadenylation has also been demonstrated for the c-*myc* and c-*fos* mRNAs in mammalian cells (26, 27). Inhibition of protein synthesis, which also stabilizes both of these mRNAs, has been shown to *slow* their rates of poly(A) loss, the opposite effect of that observed for tubulin mRNA in *Chlamydomonas*. The parallel inhibition of both deadenylation and degradation is consistent with the hypothesis that poly(A) removal is an essential step in the

degradation pathways of the c-*myc* and c-*fos* mRNAs, and indeed, a variety of experiments support a tight linkage between these events (26–31).

However, the correlation between accelerated deadenylation and stabilization of the α-tubulin mRNAs in protein synthesis-inhibited *Chlamydomonas* cells is difficult to reconcile with a role for translationally-dependent rate of poly(A) removal in destabilizing mRNAs. In this report, the relationship between tubulin mRNA stabilization and altered poly(A) metabolism in CX is investigated. These studies show that extensive deadenylation is not invariably linked to the long term stabilization of these mRNAs, indicating that accelerated poly(A) loss and long term stabilization are independent effects of protein synthesis inhibition. Evidence is also presented that the deadenylated form of α-tubulin mRNA may be less stable than the polyadenylated form under conditions of active translation. It is proposed that tubulin mRNA in *Chlamydomonas* is degraded by two distinct pathways: one which may be sensitive to the polyadenylation status, and one which is not.

MATERIALS AND METHODS

Cell growth and sample preparation

Chlamydomonas reinhardtii, wild-type strains 125, MT^+ and 124, MT^- were grown as previously described (17). For experiments, cells were concentrated to 2×10^7 cells/ml in fresh medium and stirred under illumination. Cells were deflagellated by mechanical shear (32). Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was added to cells at a final concentration of 20 μg/ml, which inhibits cytoplasmic protein synthesis by greater than 95% (32). Anisomycin (Sigma Chemical Co.) was used at a final concentration of 0.4mM. Actinomycin D (Sigma Chemical Co.) was used at a concentration of 160 μg/ml. It is not known why this high concentration is needed to effectively inhibit RNA synthesis in *Chlamydomonas*, but the cells tolerate it well as evidenced by continued active swimming for the duration of experiments. *Protein synthesis rates*: Measurement of protein synthesis rates in the presence and absence of protein synthesis inhibitors was performed using 124 MT^- cells which had been grown in sulfur-free medium for 6–8 days. Incorporation of ^{35}S-methionine (710 Ci/mmol, New England Nuclear, Boston, MA) into total trichloroacetic acid-precipitable cpm over the course of a 2-hr labeling period was measured.

To wash cells out of cycloheximide-containing medium, cells were gently pelleted, washed once in fresh medium, and resuspended in fresh medium, the entire wash procedure taking 8–10 minutes to complete. Cells recover protein synthetic activity almost immediately after this wash procedure. Cells were examined by phase contrast microscopy after the wash to assure that the centrifugation did not result in significant amount of deflagellation. *Nucleic acid preparation*: For each sample preparation, cells were pelleted and lysed in RNA Extraction Buffer (0.3M NaCl-5 mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid-50 mM Tris hydrochloride (pH 8.0)-2% sodium dodecyl sulfate) at a concentration of $0.5-1\times10^8$ cells/ml. Lysates were quick-frozen in liquid nitrogen. Phenol/chloroform extractions of nucleic acid was performed as previously described (17), except the proteinase K treatment was omitted. Total nucleic acid was used for all northern blot and poly(A) analyses. This is possible because DNA constitutes a relatively small proportion of total nucleic acid in *Chlamydomonas* cells.

Extract preparation for polysome and mRNP analysis

Extracts were prepared from cell wall-less cells (strain CW15). One minute prior to beginning the preparation, cycloheximide (CX) and chloramphenicol (to maintain chloroplast polysome integrity) were added to cells to a final concentration of 100 μg/ml each. Thus, all extracts contain the same final concentration of CX. The cells were incubated for 1 minute, poured over 1 volume of crushed ice to rapidly chill, and pelleted. Cell pellets were resuspended in ice-cold lysis buffer at 2×10^8 cells/ml. Two different lysis buffers were used: lysis buffer A (150mM KCl, 25mM Tris, pH 7.8, 8 mM Mg-acetate, 240mM sucrose, 2.5mM dithiothreotol, 10mM vanadyl ribonucleoside complexes, 100μg/ml cycloheximide, 100 μg/ml chloramphenicol, 2% Triton X-100) and lysis buffer B (identical to lysis buffer A, except the 150mM KCl is replaced by 40 mM NH_4Cl). For some extract preparations, the suspension was drawn through a 22-gauge syringe needle several times over a 5-minute period to facilitate lysis, which was monitored by microscopy. Lysates were centrifuged at 15,000×g for 10 minutes at 3°C, quick frozen in 800 μl aliquots in liquid nitrogen, and stored at −80°C. *Sucrose gradients*. Extracts prepared in lysis buffer A (400 μl) were centrifuged in 15–50% sucrose gradients made in 200mM NaCl, 25mM Tris pH 7.8, 8mM MgOAc, 2.5mM dithiothreotol. Extracts prepared in Lysis buffer B were centrifuged in similar gradients in which the 200mM NaCl was replaced with 40mM NH_4Cl. For sucrose gradient analysis of EDTA-released mRNPs, Na_2EDTA was added to lysates to a final concentration of 50 mM, and to gradients, to 20 mM. Gradients were centrifuged for the speeds and times noted in the Figure 2 legend. The top 350 μl of each gradient was discarded and gradient fractions were collected by displacement with 60% sucrose, diluted in RNA extraction buffer, and phenol/chloroform extracted for northern blot analysis. Absorbance at 260 nm was monitored using a Gilford spectrophotometer equipped with a flow cell.

Poly(A) length analysis

Fragments of α-tubulin mRNAs were generated by RNase H digestion of oligo-mRNA hybrids as previously described (19). Briefly, an 18-nucleotide antisense DNA oligomer, complementary to a region near the 3′ end of the coding sequence of both α-tubulin mRNAs, was annealed, in a reaction with total *Chlamydomonas* nucleic acid, to α-tubulin mRNAs, and the mRNA-DNA hybrids digested with RNase H. Oligo dT was included in some digests to produce a deadenylated fragment. The digested samples were run on a 20 cm, 7M urea, 6% polyacrylamide gel made in TBE/SDS (90 mM-Tris base, pH 8.3, 90 mM-boric acid, 4 mM-Na_2EDTA, 0.1% sodium dodecyl sulfate), and the RNA electrophoretically transferred to nylon membranes (MagnaNT membrane, Micron Separations, Inc., Westboro, MA). Electroblots were hybridized to an RNA probe complementary to, and specific for, the 3′ untranslated region of α2-tubulin mRNA. The size of the expected 3′ fragment is 269 nucleotides + poly(A). A similar protocol was used for RNase H cleavage of the rbcS2 (ribulose bisphosphate carboxylase/oxygenase, small subunit 2) mRNA, except that the 18-nucleotide oligo and nucleic acid were heated together to 65°C and allowed to cool to room temperature before proceeding with the digestion protocol. The size of the expected rbcS2 3′ fragment is 217 nucleotides + poly(A). Autoradiograms were scanned and quantified using a GS300 densitometer and GS370 software (Hoefer Scientific Instruments, San Francisco, CA).

Standard Northern blots and hybridizations

Total nucleic acid samples were run on 1.4% formaldehyde-agarose gels, transferred to nylon membranes by vacuum-blotting (Hybaid, Vangard, International, Neptune, NJ) and hybridized with RNA probes at 60°C in 50% formamide hybridization buffer as previously described (19). In all cases, membranes were stained with methylene blue after blotting, and only blots which showed equal sample loading and transfer were used for quantitation. The RNA probe used to detect the rbcS2 mRNA was generated by SP6 transcription from plasmid pRubA, containing the complete cDNA sequence cloned into pGEM. The original cDNA source (pCS2.1) was a gift from M. Goldschmidt-Clermont (University of Geneva). The α2-tubulin-specific probes were described previously (19). Quantification of autoradiograms of northern blots was performed by densitometry as described above.

RESULTS

Extent of stabilization of induced tubulin mRNAs depends on time of cycloheximide addition

Tubulin mRNAs are synthesized in a transient transcriptional burst which immediately follows deflagellation (17). Tubulin mRNAs accumulate to peak levels—15–20-fold over steady state levels—within 60 minutes, and are then rapidly degraded with half-lives of about 15 minutes. This post-induction degradation is accelerated relative to the normal degradation of these mRNAs, the usual half-life of α-tubulin mRNA being 50–60 minutes (17). Degradation of induced tubulin mRNAs is prevented by inhibition of protein synthesis. When cells are deflagellated in the presence of cycloheximide (CX), the mRNAs accumulate normally, but then remain at high levels for at least several hours (e.g., Fig. 1A and 1B, CX−15′). This effect was shown to be due primarily to stabilization of the induced mRNA, not a prolongation of the transcriptional burst (18). In these studies, CX was added to cells shortly before deflagellation, and was present throughout the induction. It was of interest to determine whether the stabilization conferred by CX required inhibition during the induction period itself or whether translation-linked degradation is an intrinsic property of these mRNAs. Experiments were performed in which the effect of adding CX to cells before deflagellation was compared with effect of adding CX at 30 minutes after deflagellation. Because the tubulin mRNAs are synthesized in a transient burst of transcription, the bulk of synthesis is temporally separated from degradation. The 30 minute time point was chosen because, at 30 minutes after deflagellation, the transcriptional burst giving rise to these mRNAs is almost over, and at least 90% of the induced mRNA has already been synthesized (17–19); however, it is well before the onset of rapid degradation. Fig. 1A is a graph showing α-tubulin mRNA levels in cells during a normal induction (control, open circles), and in cells treated with CX before deflagellation (CX−15′, open squares), or at 30 minutes after deflagellation (CX+30′, closed circles). These data were generated by densitometric analysis of northern blots from four different experiments. In control cells, the onset of degradation of induced α-tubulin mRNA occurred between 40 and 60 minutes. When cells were deflagellated in the presence of CX (CX−15′), the α-tubulin mRNA remained at high levels for the duration of the experiments, three hours. When CX was added at 30 minutes after deflagellation (CX+30′), there was only a transient stabilizing effect. Within about 30 minutes, rapid degradation ensued. The rate of degradation of these presynthesized mRNAs (i.e., synthesized before CX addition) in the presence of CX corresponds to a half-life of about 20 minutes, only slightly longer than the rate of degradation following normal regeneration. In all four experiments, the transient maintanence of (or increase in) elevated mRNA levels following CX addition appears to be due to a short term stabilization of presynthesized transcripts, not to stimulation of transcription, since no mRNAs bearing the 90–120 residue poly(A) of newly synthesized mRNA appear during this period (see below, Fig. 3). These results demonstrated that simply slowing ribosome translocation was not sufficient for conferral of long term stabilization onto the tubulin mRNAs. It appears, then, that deflagellation-induced α-tubulin mRNAs synthesized in the presence of a protein synthesis inhibitor are substantially more stable than the same mRNAs subjected to translational inhibition after their synthesis. The drug need not be present before the deflagellation signal itself though, since addition at 5 minutes after deflagellation provides stabilization comparable to addition before deflagellation (not shown).

The dose of CX used in these experiments (20 μg/ml) inhibits cytoplasmic protein synthesis by greater than 95% in *Chlamydomonas* (32). Gay et al. (11) demonstrated, for the case of mouse β-tubulin mRNA, that opposite effects on stability may be produced by partial inhibition compared with near 100%

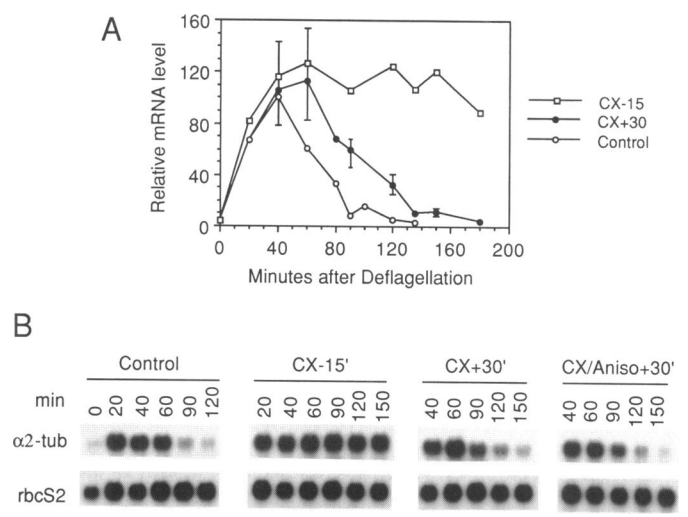

Figure 1. Effect of time of addition of cycloheximide (CX) on stability of induced α-tubulin mRNAs. Cells were deflagellated either in the absence (control) or presence of 20 μg/ml CX. CX was added either 15 minutes before (CX−15′) or 30 minutes after (CX+30′) deflagellation. Nucleic acid samples were isolated from time points throughout a 3 hour time course, and α-tubulin mRNA levels analyzed by northern blot. **A.** Quantification of northern blot data from 4 experiments. Autoradiograms from northern blots were quantified by densitometry, and values from the 4 experiments were normalized with respect to the 40 min time point in control cells (set equal to 100). For each time point, the average value is plotted. The error bars show the range of values obtained. To simplify the graph, error bars are shown only for the CX+30′ data points. Control and CX−15 curves have been previously reported and are highly reproducible. **B.** Autoradiogram of northern blot showing α-tubulin mRNA levels from one experiment in which the effect of addition of cycloheximide (CX+30′) is compared with the effect of addition of both 20 μg/ml cycloheximide and anisomycin (CX/Aniso+30′) at 30 minutes after deflagellation. The values above each lane correspond to time (in minutes) following deflagellation. 4 μg samples of total nucleic acid were run on a formaldehyde-agarose gel, and vacuum-blotted onto a nylon filter. The blot was hybridized simultaneously with ^{32}P-labeled RNA probes which recognize the α2-tubulin mRNA.and the rbcS2 (ribulose bisphosphate carboxylase small subunit) mRNA.

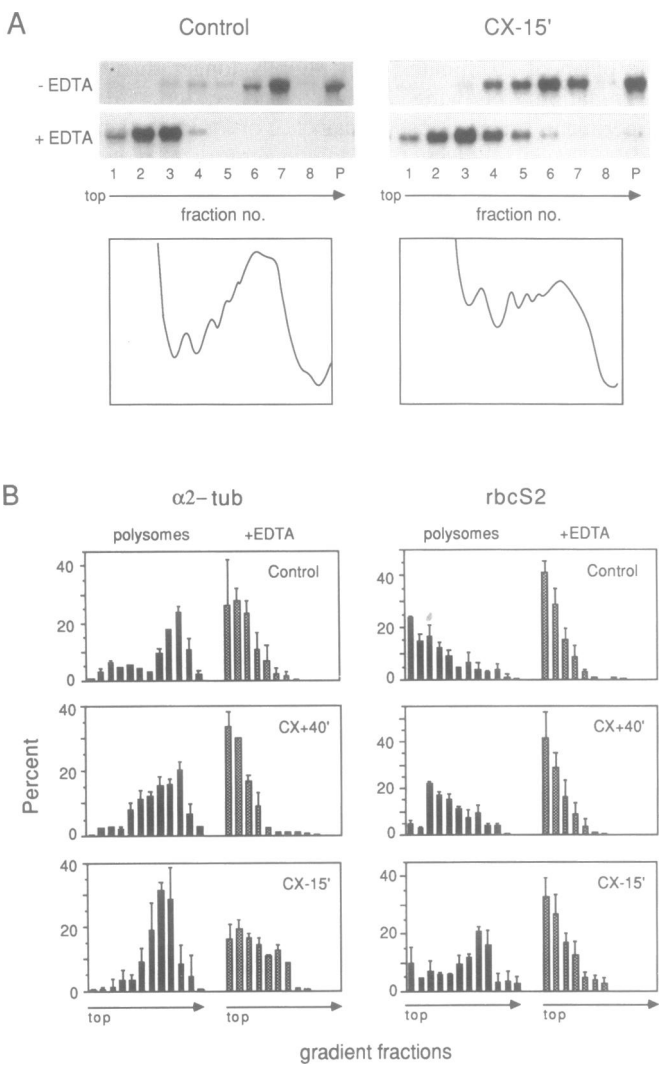

Figure 2. Sucrose gradient analysis of α2-tubulin mRNA and rbcS2 mRNA in polysomes and EDTA-released mRNPs. Extracts were prepared from deflagellated cells in the absence of CX (Control) or cells to which CX was added either before deflagellation (CX−15′) or 40 minutes after deflagellation (CX+40′). Extracts were centrifuged on 15−50% sucrose gradients in the absence or presence of EDTA to display polysomes or mRNP particles (see Methods). RNA extracted from gradient fractions was used for northern blot analysis. **A**—Northern blot of sucrose gradient fractions from one experiment, P = pellet. The extracts were prepared at 60 minutes after deflagellation in lysis buffer A (see Methods). Gradients were centrifuged for 2 hours at 45,000 rpm in an SW 50.1 rotor. Absorbance profiles show the polysome regions from gradients run in the absence of EDTA. **B**—Densitometric quantitation of northern blot data from sucrose gradient analysis of three extracts. Extracts were prepared at 70 minutes after deflagellation in lysis buffer B, layered on 15−50% sucrose gradients in the presence or absence of EDTA, and centrifuged at 40,000 rpm for 2 hours. The monosome peak falls in fractions 3−4 in these gradients. The total α2-tubulin mRNA or rbcS2 mRNA in each gradient was set equal to 100, and the percentage in each fraction graphed. Each bar in the graphs represents the averaged results from two separate determinations. The error bar indicates the upper range of the two values obtained. black bars, (−) EDTA; grey bars, (+) EDTA.

inhibition of translation elongation: 90−95% inhibition was found to enhance destabilization while 100% inhibition prevented it. Thus, it was possible that residual slow ribosome movement in 20 μg/ml CX was necessary and sufficient for rapid degradation of the presynthesized tubulin mRNAs (although apparently insufficient for mRNAs induced in the presence of CX). To examine this further, an experiment was performed in which a combination of protein synthesis inhibitors (which block elongation by interaction with different ribosomal components) was used to more closely approach 100% inhibition. A combination of CX and anisomycin (which together reduce cytoplasmic protein synthesis to undetectable levels) was added to cells at 30 minutes after deflagellation. Results are shown in Fig 1B. A constitutive cytoplasmic mRNA (rbcS2 mRNA, encoding the small subunit of ribulose bisphosphate carboxylase) serves as a control for mRNA extraction and gel loading. This experiment shows that the combination of CX and anisomycin added at 30 minutes after deflagellation (CX/Aniso+30′) does not stabilize the α-tubulin mRNA any more than does CX alone (CX+30′). Therefore, induced tubulin mRNAs can undergo rapid degradation under conditions in which ribosome translocation is severely impaired. In summary, the results shown in Figure 1 indicate that the long term stabilization of tubulin mRNAs induced in the presence of CX is not due simply to inhibition of ribosome movement along the substrate RNA, and further suggest that ribosome translocation may not be a requirement for the post-induction degradation process. However, it remains possible that the short term stabilization which occurs after CX addition at 30 minutes is due to inhibition of elongation itself.

Stabilized tubulin mRNAs are on smaller polysomes but in larger mRNP particles than unstable tubulin mRNAs

To begin to investigate the basis for the stabilized state of the tubulin mRNAs induced in the presence of CX, the sizes of tubulin mRNA-containing polysomes and EDTA-released mRNP particles were compared in control and CX−15′ cells. Extracts were prepared at 60 minutes following deflagellation, and centrifuged through 15−50% sucrose gradients. Results from one analysis are shown in Figure 2A. This analysis shows that induced tubulin mRNAs in control cells sediment with larger polysomes than the corresponding mRNAs in CX−15 cells. This is an expected outcome of the inhibition of elongation on newly synthesized transcripts; that is, initiation may be normal, but inefficient elongation would be expected to retard the acquisition of multiple ribosomes. The absorbance (260nm) profiles of the polysome region from these gradients indicate that there is a partial shift of material from larger to smaller polysomes in the CX−15′ cells, a highly reproducible observation. To what extent this shift is due to an apparent effect of CX on translation initiation (an effect reported previously; e.g., 11) versus an accumulation of newly synthesized−and thus relatively underloaded−transcripts isn't known. The size of EDTA-released mRNP particles was also observed to differ in control and CX−15 cells, but in the opposite manner to polysome size. The tubulin mRNPs from CX−15 cells show a larger average size and sediment over a broader size range than the corresponding particles from control cells.

It is possible that the difference in size of the tubulin mRNP particles is a factor in the observed difference in stability. If so, it might be predicted that the tubulin mRNP size in CX+30 cells would more closely resemble that of control cells than of CX−15 cells. Figure 2B shows this to be the case. In these experiments, CX was actually added at 40 minutes instead of 30 minutes, since the cell wall-less mutants used to prepare extracts show somewhat slower regeneration kinetics than wild-type cells. All three extracts shown in Fig. 2B were prepared at 70 minutes after deflagellation. This figure shows the percentage of either total α2-tubulin or total rbcS2 mRNA present in each gradient fraction

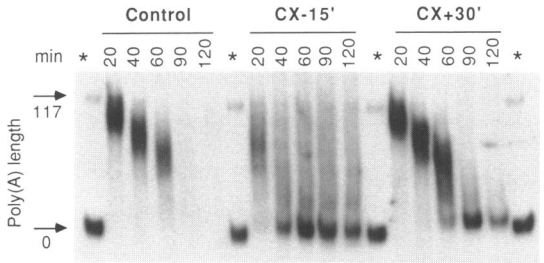

Figure 3. Northern blot analysis of poly(A) lengths on α2-tubulin mRNAs in control cells and cells to which cycloheximide was added at 15 minutes before deflagellation (CX−15′) or 30 minutes after deflagellation (CX+30′). α-Tubulin mRNAs were cleaved into 5′ and 3′ fragments by RNase H-oligonucleotide digestion as described in Methods. Digested RNA samples (15 μg total nucleic acid) were run on 7M urea-polyacrylamide gels and transferred electrophoretically to nylon membranes, which were hybridized with a ^{32}P-labeled RNA probe which recognizes α2-tubulin mRNA 3′ untranslated sequences. There is some cross-hybridization to the α1-tubulin mRNA 3′ fragment in this blot. The values above each lane correspond to time (in minutes) following deflagellation. The lanes marked with an asterisk (*) show the completely deadenylated α2-tubulin 3′ fragment (269 nucleotides) and the completely deadenylated α1-tubulin 3′ fragment (386 nucleotides), generated by including oligo dT in the RNase H digestions. These deadenylated fragments are used for size markers for poly(A) length: **0**, the deadenylated α2-tubulin 3′ fragment; **117**, the deadenylated α1-tubulin 3′ fragment.

(i.e., the sum of the values for the 12 gradient fractions is 100%). This study confirms the results shown in panel A that 1) the mean α-tubulin mRNA polysome size is smaller in CX−15 cells than in control cells, and 2) that α-tubulin mRNA-containing mRNP particles are larger in CX−15 cells. It also shows that the α2-tubulin mRNP particles from the CX+40′ cells are in the same size range as those from control cells, not those from CX−15′ cells, correlating with the relative stability of the tubulin mRNAs in these cells. The size distribution of α-tubulin mRNA polysomes in CX+40′ cells appears to be intermediate between that of control and CX−15′ cells.

RbcS2 mRNA in the same gradients (Fig. 2B) shows a different pattern. For this constitutive mRNA, the three extracts represent a time course in CX (i.e., control = 0, CX+40′ = 30′ and CX−15′ = 75′). In control extracts there is a substantial portion of nonpolysomal rbcS2 mRNA and most polysomes are small (an observation reproduced in a number of experiments). With increasing time in CX, there is gradual shift toward larger polysomes. The EDTA-released particles, however, show a similar size distribution in all extracts. There may be a slight shift toward larger RNP particles in CX−15′ extracts, which could indicate that the effect of CX on mRNP sedimentation may not be completely specific one. However, even considering the fact that the rbcS2 mRNA is only about one-half the length of tubulin mRNAs, the shift is clearly not of the same magnitude as that exhibited by the α-tubulin mRNA.

There are two arguments that the slower sedimentation of the mRNPs in the CX−15 cells is not due to a difference in the ability of 50 mM EDTA to strip control ribosomes versus CX-ribosomes from the mRNPs: 1) Analysis of both the absorbance (260nm) profiles in sucrose gradients and ribosomal RNA species in each gradient fraction (by methylene blue staining of blots) indicates a similar shift of the majority of ribosomal material to lighter fractions in control and CX-extracts treated with EDTA (not shown). 2) The extract from CX+40′ cells was prepared after the cells had been in CX for 30 minutes and certainly contained ribosomes which were as saturated with CX as ribosomes in the CX−15 extracts, but yielded smaller tubulin-mRNP particles. The possibility that there is a time-dependent modification of ribosomes in the presence of CX which alters the partitioning of ribosomal and mRNP proteins has not, however, been excluded.

Extensive deadenylation is not strictly correlated with long term tubulin mRNA stabilization

It has been previously shown that α-tubulin mRNAs induced in the presence of are subject to an accelerated rate of poly(A) loss (19). Under these conditions a large portion (about 50%) of α-tubulin mRNA becomes completely deadenylated, concommitant with its long term stabilization. Because the relationship, if any, between these events was unknown, it was of interest to determine whether inhibition of protein synthesis would result in extensive deadenylation of presynthesized mRNAs, which are only transiently stabilized by CX. Figure 3 shows an analysis of poly(A) tail lengths on α2-tubulin mRNA in control cells and in cells to which CX was added 15 minutes before or 30 minutes after deflagellation. This figure is an autoradiograph of a northern blot of α2-tubulin mRNA 3′ fragments generated by oligonucleotide-directed RNAse H cleavage. Newly synthesized poly(A) in *Chlamydomonas* is about 90−120 adenylates in length. In control cells, α2-tubulin mRNAs are subject to a gradual poly(A)-shortening process, in which poly(A) tails are shortened at a rate of about 40 adenylates per hour (19). In cells induced in CX, a substantial amount of completely deadenylated mRNA accumulates within 30 minutes of deflagellation, indicating a rate of poly(A) shortening exceeding 240 adenylates per hour (if exonucleolytic) for at least some of the mRNAs. This study shows that addition of CX to cells at 30 minutes after deflagellation also results in the rapid accumulation of completely deadenylated α2-tubulin mRNAs (CX+30′). In fact, under these conditions, nearly all of the α-tubulin mRNA undergoes complete poly(A) loss. Quantitation of the rate of poly(A) shortening from multiple similar experiments indicates that CX does cause an acceleration in the rate of shortening under these conditions (as opposed to simply transiently stabilizing mRNA deadenylated at the usual rate). For example, in this figure, if poly(A) shortening continued at its usual linear rate after CX addition, the modal poly(A) length at 90 minutes would be about 30−40 adenylates, not zero. Thus, the tubulin mRNAs need not be induced in the presence of CX in order to be subject to extensive deadenylation, although this is a requirement for long term stabilization. This finding suggests that the two phenomena are due to two different consequences of protein synthesis inhibition. It can also be concluded, not surprisingly, that it is not the deadenylated state *per se* which confers long term stability on mRNAs induced in CX.

The distribution of poly(A) lengths is quite different in cells treated with CX before versus after deflagellation. In cells treated with CX before deflagellation, a stable distribution of poly(A) lengths is attained within 60 minutes after deflagellation, in which about 40−50% of the α2-tubulin mRNA is completely deadenylated, while the remaining 50−60% carries poly(A) tails ranging from a few adenylate residues to full length. A ladder of discrete poly(A) lengths representing multiples of 25−30 residues is also apparent under these conditons (best illustrated in the autoradiogram shown in Fig. 6). However, in cells treated with CX at 30 minutes almost all of the mRNA is completely deadenylated within an hour after addition. This difference in the pattern of poly(A) loss is highly reproducible in different experiments, but its significance remains unknown.

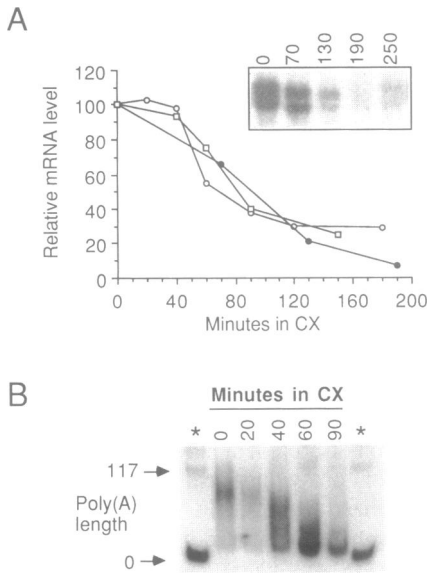

Figure 4. The effect of cycloheximide on steady state α-tubulin mRNA levels and poly(A) length in nondeflagellated cells. Cycloheximide (20 μg/ml) was added to nondeflagellated, vegetative cells and total nucleic acid samples isolated at times following addition. **A**—Graph showing α-tubulin mRNA levels as a function of time after cycloheximide addition. Values from three different experiments are plotted individually. Data were obtained by densitometric quantitation of northern blots, including the one shown in the inset. The northern blot shown in the inset (closed circles in graph) was probed with an RNA probe which recognizes both α1 and α2 tubulin mRNAs, while the other two blots were probed with an α2-tubulin-specific probe. **B**—Analysis of poly(A) tail length on α2-tubulin mRNA. α-Tubulin mRNA 3′ fragments were analyzed by northern blot as described in the legend to Fig. 3 (except that 30 μg of RNAse H-digested nucleic acid was loaded into each lane). The values above each lane represent time (in minutes) after addition of CX.

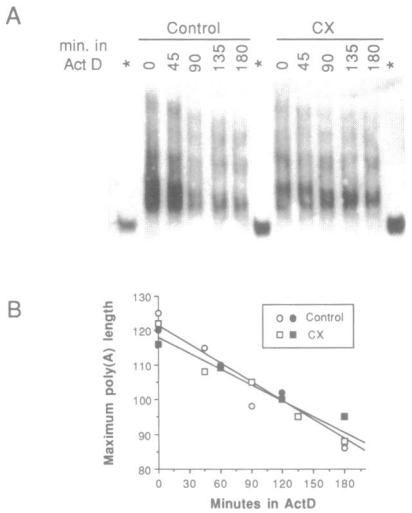

Figure 5. Northern blot analysis of poly(A) shortening on the **RbcS2 mRNA** in the absence (control) or presence of CX. Actinomycin D (160 μg/ml) was added alone to control cells, and in combination with cycloheximide (20 μg/ml) to CX cells at time 0. RNA samples from the time points indicated were subjected to oligonucleotide-directed RNAse H cleavage, gel electrophoresis, and transfer as described in Methods. The blot was hybridized to an RNA probe complementary to the 3′ UTR of the rbcS2 mRNA. **A.** Autoradiogram of the northern blot. The values above each lane correspond to time (in minutes) following actinomycin D addition. Lanes marked with an asterisk (*) show the completely deadenylated rbcS2 mRNA 3′ fragment (217 NTs). Polyadenylated fragment lengths were estimated based on SP6-transcript markers of 200, 245 and 339 NTs (not shown). **B.** Maximum poly(A) length on rbcS2 mRNA as a function of time after actinomycin D addition.

Cycloheximide does not stabilize constitutively expressed α-tubulin mRNAs, but does cause an accumulation of deadenylated molecules

In uninduced (nondeflagellated) cells, there is a constitutively-expressed population of tubulin mRNAs, which are products of the same genes as are the induced transcripts. The constitutive α-tubulin mRNAs in uninduced cells are more stable than the induced mRNAs, turning over with a 50–60 minute half-life, measured both by a continuous-labeling protocol (17) and by actinomycin D chase (unpublished data). (During an induction, these mRNAs represent only about 5% of the total α-tubulin mRNA. It is not known whether they maintain their higher stability during an induction since they are indistinguishable from the induced transcripts.) Here it is shown that the constitutive half-life is not lengthened by CX treatment, and may actually be shortened. Figure 4A is a graph which shows the effect of CX on the steady state population of α-tubulin mRNAs in three different experiments. The data were generated from densitometry of northern blot autoradiographs, including the one shown as an inset in this figure. If CX were stabilizing the mRNA (and transcription not being affected) the expected result would be a steady increase in tubulin mRNA levels with time. These experiments show that there is actually a decrease in the levels of the two α-tubulin mRNAs. Following a lag of about 40 minutes, the α-tubulin mRNAs disappear with a half-life of about 50 minutes. (Under these same conditions, the rbcS2 mRNA disappears slowly, with an apparent half-life of 3.5–4 hours, not shown.) One interpretation of the decrease in α-tubulin mRNA levels is that CX is destabilizing the mRNA. However, it is also possible that CX is simply failing to stabilize, and the decrease is due to the fact that CX treatment causes the cells to depress constitutive transcription, an effect for which evidence exists (36 and unpublished data). We tried to distinguish between these two interpretations by measuring the half-life of the α-tubulin mRNAs in the presence and absence of CX using an actinomycin D chase, but found that simultaneous addition of actinomycin D and CX invariably results in a transient 'superinduction' of these mRNAs (not shown), an mRNA-specific effect which is not observed in the presence of either drug alone. We have therefore avoided the use of actinomycin D in studies involving tubulin mRNA metabolism in these cells. Because the extent of transcriptional depression due to CX cannot be accurately quantified, neither can the extent of destabilization, if any. However, the limited conclusion can be drawn that CX is not significantly *stabilizing* the constitutive tubulin mRNAs. The 40-minute lag before the onset of disappearance of these mRNAs is consistent with the possibility that CX addition may cause a transient stabilization, as it does in CX+30′ cells.

Figure 4B shows an analysis of poly(A) lengths on constitutive α2-tubulin mRNAs in CX-treated cells. As in the case of deflagellation-induced α-tubulin mRNAs, deadenylated mRNA accumulates as a function of time in CX. There is normally very little deadenylated α-tubulin mRNA at steady state (0-time, lane 2). Therefore, although CX does not confer long term stabilization on the steady state tubulin mRNAs, it does stimulate deadenylation. It may be significant that the lag preceding the onset of disappearance of the mRNA corresponds to a time frame in which extensive deadenylation occurs.

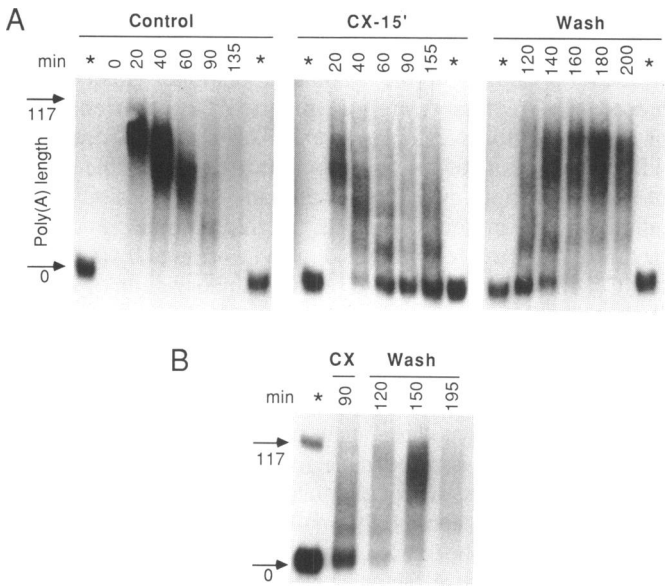

Figure 6. Effect of release from cycloheximide inhibition on α2-tubulin mRNA stability and poly(A) length distribution. Cells were deflagellated in the absence (Control) or presence of 20 μg/ml cycloheximide (CX−15'). A portion of the cycloheximide-treated cells were washed free of the drug at 100 minutes following deflagellation (Wash). Poly(A) length analysis was performed as described in the legend to Fig. 3. **A.** Results from an experiment performed at 20−21°C. Values above each lane represent minutes after deflagellation. **B.** Results from a similar experiments performed at 26−27°C.

Poly(A) shortening on the rbcS2 mRNA is unaffected by protein synthesis inhibition

Figure 5 shows an analysis of poly(A)-shortening on the rbcS2 mRNA in the presence and absence of CX. For this analysis, actinomycin D was used to halt transcription so that the kinetics of poly(A) shortening on this constitutively synthesized mRNA could be measured. Figure 5A shows a northern blot from one experiment. The 0-time points display the steady state distribution of poly(A) lengths, which includes tails ranging from about 12 to 120 adenylates. There is a similar pattern of poly(A) loss in both the presence and absence of CX: a gradual reduction in the maximum poly(A) length with time, but otherwise little alteration in the distribution of poly(A) lengths. (The particularly strong signals in the 0' and 45' control lanes are due to overloading, not to authentic quantitative differences, as evidenced by methylene blue staining patterns. The two 0-minute lanes show the same RNA sample.) Also apparent is a striking ladder of discrete poly(A) tail lengths, representing multiples of about 25 residues, and an accumulation of mRNAs bearing a minimum 12-adenylate tail. Figure 5B is a graph of data from two experiments showing the maximum poly(A) length on rbcS2 mRNA as a function of time following actinomycin D addition. There is no significant difference in the apparent rates of shortening in control and CX-treated cells; both are about 12 A/hr. The autoradiogram shown in 4A suggests that CX might slightly slow poly(A)-shortening, but this was not apparent in the other experiment. CX clearly does not accelerate the rate of poly(A) removal for this mRNA, and therefore, the accelerated shortening observed for the tubulin mRNAs in CX-treated cells is an mRNA-specific phenomenon. There are two other notable differences between the metabolism of poly(A) on the α-tubulin mRNAs and the rbcS2 mRNA: 1) The rate of poly(A) shortening on the rbcS2 mRNA, measured here and in other experiments (ca. 12 A/hr; Fig. 5B), is considerably slower than the rate measured for the α2-tubulin mRNA (ca. 40 A/hr; 19 and Figs. 1 and 3). It is possible that actinomycin D could slow the poly(A)-shortening process, although it does not have that effect on α-tubulin mRNA (not shown). 2) Little or no rbcS2 mRNA bearing poly(A) tails shorter than about 12 residues accumulates.

Preferential degradation of deadenylated α-tubulin mRNA after reactivation of protein synthesis

The stable accumulation of both polyadenylated and deadenylated mRNAs in cells treated with CX before deflagellation provided the opportunity to examine their relative stabilities once protein synthesis was allowed to resume. Cells were deflagellated in the absence or presence of CX, and a portion of the CX-treated cells was washed out of the drug at 100 minutes. Quantitative northern blots showed that α-tubulin mRNA levels remained elevated for about 90−100 minutes following reactivation of protein synthesis, after which rapid degradation occurred (not shown). Figure 6A shows the poly(A) length distribution of α2-tubulin mRNA in control cells, cells deflagellated in the presence of CX, and in cells released from CX inhibition (Wash). Most obvious in this autoradiogram is that release from inhibition results in 1) the immediate onset of disappearance of deadenylated mRNAs, and 2) an accumulation of mRNAs with long poly(A) tails (60−100 adenylate residues). The accumulation of long poly(A)-tailed mRNAs is due to a stimulation of new transcription, not readenylation of the deadenylated molecules (see below). Densitometry of the autoradiogram showed that the deadenylated mRNA disappeared with a half-life of 15−20 minutes in this experiment. On the other hand, mRNAs having short poly(A) tails (20−40 residues) disappeared with a half-life of about 75 minutes, 3.75 times more slowly than the deadenylated mRNAs. These data suggested that only the deadenylated form of the mRNA is subject to immediate rapid degradation following reactivation of protein synthesis. Because of the accumulation of the new, long poly(A)-tailed mRNAs, it is not possible to say with certainty from inspection of the Fig. 6A autoradiogram whether the deadenylated molecules are actually disappearing faster than all of the polyadenylated forms. For example, newly transcribed mRNAs could be contributing to the pool of mRNAs with short poly(A) tails via poly(A) shortening. Performing the experiment at higher temperature was found to temporally uncouple the loss of deadenylated mRNA from the accumulation of long poly(A)-tailed mRNA. In so doing, it provided a window of time in which it could be clearly seen that the deadenylated molecules disappear significantly more rapidly than the polyadenylated forms. Fig. 6B shows an analysis of α2-tubulin mRNA poly(A) lengths from one experiment performed at 26−27°C. (The experiment shown in Fig. 6A was performed at 20−21°C.) One time point from CX-treated cells is shown (CX−90 min), representing the poly(A) length distribution of mRNA before the wash. The first time point following the wash out of CX (Wash-120 min) shows a transition period in which most deadenylated mRNA had disappeared, but the new, long poly(A)-tailed material had not yet accumulated. This study provides evidence that the accumulation of long poly(A)-tailed mRNAs is due to new mRNA synthesis, since it demonstrates the lack of a precursor-product relationship between the disappearing deadenylated molecules and the accumulating polyadenylated molecules. It also suggests that the unadenylated form of α-tubulin mRNA is less stable than the polyadenylated

form during active protein synthesis. Repetitions of this experiment yielded similar results. In each case, the deadenylated mRNA disappeared about 3−4-fold faster than polyadenylated mRNA.

DISCUSSION

Effect of inhibition of protein synthesis on α-tubulin mRNA stability

Tubulin mRNAs induced by deflagellation are normally rapidly degraded, but the degradation is prevented when the tubulin mRNAs are induced in the presence of cycloheximide (CX). This is a property not only of the α and β-tubulin mRNAs, but also other induced mRNAs encoding flagellar structural proteins (unpublished data). Studies in this report demonstrated that the extent of stabilization depends on the timing of CX addition. Long term stabilization of α-tubulin mRNA occurs when CX is added before (or immediately after) deflagellation. When it is added at 30 minutes after deflagellation, i.e., to presynthesized tubulin mRNAs, only a transient stabilization results (Fig. 1). Slowing the rate of ribosome movement still further, by using a combination of elongation inhibitors, does not extend the period of transient stabilization nor slow the rapid rate of degradation of presynthesized tubulin mRNAs (Fig. 1B). CX inhibition also does not significantly stabilize the same α-tubulin mRNA species present at low steady state levels in nondeflagellated cells (Fig. 4). Therefore, the long term stabilizing effect of CX on tubulin mRNAs appears to be restricted either specifically to tubulin mRNAs *induced* in its presence, or possibly to any tubulin mRNA synthesized/transported in its presence (which would be only a fraction of constitutively synthesized tubulin mRNAs). These results also indicate that the stabilization of tubulin mRNAs induced in CX is very unlikely to be due (exclusively) to inhibition of ribosome movement, since induced mRNAs may be rapidly degraded in the continued presence of CX (in CX+30′ cells). The transient stabilization caused by addition of CX at 30 minutes is not necessarily due to an attenuated version of the same mechanism responsible for long term stabilization. Our data are consistent with the possibility that transient stabilization occurs whenever CX is added to cells, and could be due to inhibition of ribosome translocation itself. Two possible mechanisms by which inhibition of protein synthesis may preferentially stabilize tubulin mRNAs induced/synthesized in its presence are:

(1) mRNPs produced in CX-inhibited cells are structurally different from those produced under normal conditions. This difference need not be hypothesized to be specific for the induced tubulin mRNAs.

(2) The accumulation of a product of deflagellation-induced protein synthesis is required for the post-induction degradation of the tubulin mRNAs. Under conditions in which CX is added to presynthesized mRNAs, 30 minutes of induced protein synthesis has already occurred, which may be adequate to accumulate a protein product required for the destabilization of the coinduced tubulin mRNAs.

The finding that EDTA-released α-tubulin mRNPs from cells deflagellated in CX are larger than their counterparts from either control cells or cells to which CX is added after their synthesis is consistent with the possibility that this difference in RNA-binding protein composition underlies the difference in stability. If so, the question of whether this is an effect peculiar to the induced tubulin mRNPs or a reflection of a more general phenomenon remains to be answered. For example, any newly synthesized mRNA may require active protein synthesis for exchange of nuclear for cytoplasmic proteins. A change in mRNP structure in response to the action of an inhibitor is not without precedent. Dreyfuss et al. (42) reported the occurrence of a physical alteration in cytoplasmic mRNAs in the presence of two different inhibitors of transcription, due to the reversible binding of a pre-existing cytoplasmic protein. The possibility that inhibition of protein synthesis might promote a nuclear modification of newly synthesized mRNPs is raised by the finding that CX and anisomycin cause rapid phosphorylation of specific nuclear proteins in mammalian cells (43, 44). Interestingly, these modifications occur even at subinhibitory concentrations of the drug, indicating the effect may not be a consequence of protein synthesis inhibition *per se*. At this time, there is no direct evidence in favor of the hypothesis that inhibition of synthesis of a coinduced protein is responsible for long term stabilization of the tubulin mRNAs. Although inhibition of synthesis of a labile protein has been a commonly invoked explanation for specific mRNA stabilization, to date, no such cellular protein has been positively identified. A factor which binds to the AU-rich region of c-*myc* mRNA and promotes its degradation in an *in vitro* system (45) is a candidate for such a labile destabilizer, since extracts prepared from CX-treated cells apparently do not contain this activity (29). The two hypotheses offered above are not mutually exclusive, and are not necessarily even distinct. For example, a putative coinduced destabilizer may associate specifically with induced flagellar protein mRNPs. Bandyopadhyay et al. (46) have demonstrated the occurrence of nuclease activities tightly associated with both free and EDTA-released mRNPs in mammalian cells. We have found that tubulin mRNPs in extracts prepared from control and CX-treated cells maintain their differential stability *in vitro* (unpublished data), a feature which should allow a biochemical investigation of the significance mRNP size differences.

Poly(A) metabolism in control and CX-treated cells

Induction in the presence of CX results in tubulin mRNAs which are subject to an accelerated poly(A) removal process. It is not clear whether deadenylation occurs via the same pathway in control and CX-inhibited cells. In control cells, the pattern of poly(A) loss suggests an exonucleolytic shortening process to which the entire population of tubulin mRNAs is subject, i.e. there is a gradual length reduction and the distribution of lengths remains relatively narrow. In the presence of CX, the pattern of poly(A) loss suggests an endonucleolytic-like process, i.e., the length distribution rapidly becomes heterogeneous and includes poly(A) of all lengths. A ladder of poly(A) tails of discrete lengths, representing multiples of about 25 residues, is usually evident in CX-treated cells (Fig. 6), suggesting that phased poly(A)-binding proteins may confer some protection against this mode of degradation.

Addition of CX to cells at 30 minutes after deflagellation (Fig. 3) or to nondeflagellated cells (Fig. 4) also results in extensive deadenylation of the (presynthesized) tubulin mRNAs, demonstrating that the effects of protein synthesis inhibition on stability and poly(A) metabolism are not strictly correlated; i.e., CX can facilitate rapid poly(A) removal without necessarily conferring long term stabilization on the tubulin mRNAs. They also suggest that a translation-linked rate of poly(A) loss is an intrinsic property of the α-tubulin mRNAs. It has been observed in a number of experiments that there is a lag of at least 20 minutes following CX addition before the onset of rapid

deadenylation, although CX acts to inhibit protein synthesis much more rapidly than that. The lag is obvious in CX+30′ cells (Fig. 3) and nondeflagellated cells (Fig 4B). This observation is consistent with the possibility that rapid deadenylation may be stimulated by the degradation of a labile protein which normally protects against this activity. Rapid deadenylation of tubulin mRNA is also observed using other inhibitors of elongation (anisomycin and emetine, not shown), but it was not possible to determine whether inhibition of initiation stimulates rapid deadenylation, since tubulin mRNAs are very rapidly degraded under such conditions (unpublished data).

Poly(A) metabolism is clearly different for the rbcS2 mRNA. First, the rate of poly(A) removal from the rbcS2 mRNA is not accelerated in the presence CX (Fig. 5), indicating that translation-linked poly(A) removal is an mRNA-specific phenomenon. In addition, the usual rate of poly(A) loss is about 3−4× slower for the rbcS2 mRNA than for the α2-tubulin mRNA (ca. 12 A/hr vs. 40 A/hr). The observation that almost all rbcS2 mRNAs carry at least an oligo(A) tail suggests either that the poly(A) shortening process does not proceed to completion on this mRNA or that mRNAs which are completely deadenylated are destabilized. There is evidence supporting the latter explanation for a similar observation of a minimal oligo(A) status on the yeast MFA2 mRNA (49, 50). Recent analysis of the deadenylation process *in vitro*, using partially purified yeast components, indicates that poly(A) shortening to the oligo(A) state and terminal (complete) deadenylation are kinetically distinguishable processes which are independently affected by 3′ UTR sequences (50).

The significance of the translation-dependent rate (and/or mode) of poly(A) shortening on α-tubulin mRNA remains unknown. One model to explain its possible significance would be to propose that CX inhibition is mimicking a normal ribosome stalling event which occurs on the tubulin mRNAs. A model suggested by Cleveland and colleagues (11) to explain the destabilization of vertebrate β-tubulin mRNA proposed that binding of a protein to the amino terminus of a β-tubulin nascent chain causes a transient stalling of the ribosome, which leaves the mRNA in an RNase-susceptible conformation. Ribosome stalling at rare codons has been proposed to explain the destabilizing ability of a region of the yeast MATα1 mRNA which contains a high density of rare codons (15). It could be proposed that transient ribosome stalling on the *Chlamydomonas* tubulin mRNAs destabilizes them by facilitating poly(A) removal.

Role of poly(A) in tubulin mRNA stability

The results presented in this report may best be explained by the hypothesis that tubulin mRNAs in *Chlamydomonas* are degraded by two distinct pathways, a post-induction pathway and a constitutive pathway. There is no evidence to suggest that poly(A) removal is a necessary step in the post-induction degradation pathway for tubulin mRNAs. Perhaps the best evidence against such a requirement is the simple observation that, in control cells, the onset of rapid degradation occurs at a time when the induced population of tubulin mRNAs still have reasonably long poly(A) tails, 40 adenylates and greater (Figs. 3, 6). Normal translation seems not to be necessary for the specific degradation of these mRNAs via the postinduction pathway (i.e., they are degraded rapidly in CX+30′ cells). The final study presented in this report provided evidence that deadenylated α-tubulin mRNA (accumulated in the presence of CX) may be less stable than the polyadenylated form once protein synthesis is allowed to resume (Fig. 6). Resumption of normal translation was necessary (and sufficient) for triggering the rapid degradation of these deadenylated mRNAs.

A possible explanation of these observations is that relative instability of deadenylated α-tubulin mRNA during ongoing protein synthesis may be of significance in the constitutive pathway for tubulin mRNA turnover, but normally irrelevant to the post-induction pathway. The distribution of poly(A) tail lengths on steady state (constitutive) tubulin mRNAs (Fig. 4, 0-time) suggests that mRNAs completely lacking poly(A) do not accumulate. The rapid degradation of induced, deadenylated mRNA observed after release from CX inhibition could represent the activity of the constitutive degradation pathway on the induced mRNAs.

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