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Cell cycle stage-specific accumulation of mRNAs encoding tubulin and other polypeptides in *Chlamydomonas*

(mitosis/cDNA cloning/blot hybridization technique/regulation of mRNA accumulation/synchronous cells)

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ABSTRACT **The accumulation pattern of a number of mRNAs during the cell cycle of *Chlamydomonas* was examined by two-dimensional gel analysis of *in vitro* translation products and by RNA blot hybridization analysis. Two-dimensional gel analysis revealed that 10–15% of the 300 most abundant translation products are differentially synthesized from RNA obtained at various cell cycle stages. RNAs that direct the synthesis of α- and β-tubulins and that hybridize to cloned α- and β-tubulin probes accumulate coordinately during the predivision period of the cell cycle, reaching peak levels before or during division. Other RNAs represented by selected cloned cDNA probes show a number of different cell cycle patterns of accumulation. The accumulation patterns of these RNAs are not directly influenced by ongoing illumination conditions, even though alternating light–dark illumination cycles are used to synchronize *Chlamydomonas* cells. The results suggest that there may be a complex program of gene expression correlated with cell cycle progression in *Chlamydomonas*.**

Considerable controversy surrounds the question of whether differential gene expression plays a significant role in progression of cells through the cell cycle. Studies of cell division cycle mutants in yeast and other organisms (for review see ref. 1) have demonstrated that certain gene products are required to act at particular cell cycle stages. However, when the synthesis of specific polypeptides has been examined by two-dimensional gel electrophoresis, as in yeast (2) and HeLa cells (3), it has been found that the rate of synthesis of most polypeptides does not vary or varies no more than a few fold during the cell cycle.

Other studies, which have focused on rates of synthesis of single gene products, have produced more convincing evidence for gene regulation during the cell cycle. A common target for such studies has been the histones. Hereford *et al.* (4) have shown that histone mRNA is accumulated only during the S phase of the yeast cell cycle and that inhibition of DNA synthesis abolishes this accumulation. Similar studies of histone synthesis during the mammalian cell cycle are not as conclusive. Groppi and Coffino (5) reported that mouse lymphoma S49 cells and Chinese hamster ovary cells synthesize histones at equivalent rates in G_1 and S, whereas others showed that histone synthesis is tightly coupled to DNA synthesis in HeLa cells (6–8) and hamster fibroblasts (9) and that histone mRNA accumulates only during S phase in HeLa cells (10).

The synthesis of microtubule proteins also seems to be regulated during the cell cycle. In *Physarum*, polypeptides identified as tubulins increase in relative rate of synthesis about 30-fold just prior to mitosis (11). Tubulins are among the few polypeptides shown to vary a few fold in relative rate of synthesis during the HeLa cell cycle (3). During the *Chlamydomonas* cell cycle, a number of polypeptides are synthesized in stage-specific fashion (12), including α- and β-tubulin (13), which are preferentially synthesized before and during mitosis. In this study we describe changes in functional level of a number of mRNAs, including those for α- and β-tubulin, during the cell cycle. In addition, using cloned probes, we have measured changes in physical level of a number of specific poly(A)$^+$ RNAs, including tubulin mRNAs, during the cell cycle. The results suggest that there may be a program of differential gene expression correlated with progression of cells through the cell cycle.

MATERIALS AND METHODS

Cell Culture. *Chlamydomonas reinhardi* 137c *mt*$^+$ *cw*15$^-$ was grown in HS medium (14) bubbled with 3% CO_2 and illuminated with fluorescent light (≈4,000 lux) at 21°C. Cultures were synchronized by three cycles of 12 hr of light and 12 hr of dark (15). Cells were then released into continuous light; during this time they completed a synchronous cell division cycle similar to that observed during the light–dark cycle (see Fig. 5). We have chosen to study the cell cycle in continuous light in order to avoid gene regulation events associated with the ongoing illumination conditions.

Protein Labeling. ^{35}S-Labeled flagellar proteins used to identify tubulin subunits on two-dimensional gels were isolated by first deflagellating cells (16), incubating deflagellated cells in label as described (17) for the period of reflagellation, and subsequently purifying newly synthesized flagella (16).

RNA Purification. RNA was isolated as follows: Approximately 10^9 cells were suspended in 10 ml of ice-cold 100 mM Tris·HCl, pH 8.5/400 mM LiCl/10 mM EGTA/5 mM EDTA/2% $NaDodSO_4$/proteinase K at 40 μg/ml, and the mixture was shaken at 4°C for 10 min. The mixture was extracted three times with phenol/$CHCl_3$, 1:1 (vol/vol), and the aqueous phase was mixed with ethanol to precipitate RNA. Ethanol precipitates were washed twice with 10 ml of 2 M LiCl to remove polysaccharides, dissolved in H_2O, and precipitated with ethanol after addition of NaOAc (pH 5.0) to 0.1 M. RNA was further purified by centrifugation through a CsCl cushion according to Glisin *et al.* (18) with modification (C. Silflow, personal communication) to account for the high G+C content of *Chlamydomonas* nucleic acids. The CsCl purification step appears to be important if the RNA is to be used in certain hybridization reactions such as hybrid-release translation. Poly(A)$^+$ RNA was isolated by two cycles of oligo(dT)-cellulose chromatography as described by Weeks and Collis (19). *Chlamydomonas* mRNA complementary to chicken α- and β-tubulin clones was isolated by hybrid selection as described by Silflow and Rosenbaum (20).

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Abbreviation: kb, kilobase(s).

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***In Vitro* Translation and Two-Dimensional Gel Electrophoresis.** *In vitro* translation was performed as described by Altenbach and Howell (21). Translation mix was prepared for isoelectric focusing as described by Lefebvre *et al.* (22) and gels were run essentially as described by O'Farrell (23). We have learned that different preparations of $NaDodSO_4$ affect the relative migration of *Chlamydomonas* α- and β-tubulin on $NaDodSO_4$ gels. Preparations from Baker were used because they appear to give the best separation of tubulin subunits. Gels were fixed and treated for fluorography (24), dried, and exposed to Kodak XAR-5 film. Rough quantitation of the relative amount of individual translation products was achieved by tracing the center of the autoradiographic spots through the $NaDodSO_4$ dimension with a Joyce–Loebl densitometer.

Preparation of Clones Homologous to Cell Cycle Regulated mRNAs. Embryonic chicken α- and β-tubulin clones, pT1 and pT2, respectively (25), were kindly provided by Don Cleveland and co-workers. Double-stranded cDNAs representing *Chlamydomonas* mRNA sequences were synthesized from 14-hr poly(A)$^+$ RNA by using the methods described by Buell *et al.* (26) and Wickens *et al.* (27). After treatment with S1 nuclease and size selection of fragments larger than 400 base pairs on neutral sucrose gradients, a homopolymer tract of ≈50 residues of dA was added to the cDNAs (28). The tailed cDNAs were annealed with (dT)-tailed, *Bam*HI-digested plasmid pBR322 and used to transform *Escherichia coli* C600 SF8, and ampicillin-resistant tetracycline-sensitive colonies were picked for further study. Clones were screened by colony-filter hybridization (29), using ^{32}P-labeled cDNA (specific activity ≈3.8 × 10^7 dpm/μg) synthesized (26) from cell cycle stage specific poly(A)$^+$ RNA, using $(dT)_{10}$ as a primer. Clones showing differential hybridization to one or another of the probes were picked for further study. Plasmid DNA was isolated and purified according to Kahn *et al.* (30). DNA from interesting clones was used to transform *E. coli* HB101 for further work, because we had noticed instability of some of our plasmids in SF8.

Blot Hybridization. Analysis of *Chlamydomonas* restriction fragments complementary to chicken α- and β-tubulin sequences was performed according to Southern (31), using the dextran sulfate-accelerated hybridization procedure of Wahl *et al.* (32). Cell cycle RNA was size fractionated on CH_3HgOH gels (33), transferred to nitrocellulose (Schleicher & Schuell, BA83) (34), and hybridized with nick-translated plasmid DNA (specific activity 0.2–1 × 10^8 dpm/μg) (35) according to Wahl *et al.* (32), except that *E. coli* or *Micrococcus lysodeikticus* DNA replaced salmon sperm DNA. Blots were washed (30 min, 50°C, 15 mM NaCl/1.5 mM trisodium citrate/0.1% $NaDodSO_4$), wrapped wet, and exposed to Kodak XAR-5 film at −70°C with an intensifier screen. Autoradiograms exposed in the linear response range of the film were traced with a Joyce–Loebl densitometer for quantitation of the autoradiographic signal. Probe was eluted after autoradiography by boiling 1 min in 15 mM NaCl/1.5 mM trisodium citrate/0.1% $NaDodSO_4$ in a microwave oven. Blots from which probe had been eluted were rinsed in 3 M NaCl/0.3 M trisodium citrate, baked 2 hr at 80°C under reduced pressure, and reused.

RESULTS

To survey changes in functional level of many mRNAs during the cell cycle, poly(A)$^+$ RNAs from different cell cycle stages were translated in the rabbit reticulocyte lysate system, and the translation products were displayed on two-dimensional gels (Fig. 1). Of the approximately 300 translation products detected by this analysis, the synthesis of only about 10–15% varied significantly during the cell cycle. Although most of the polypeptides observed on these gels are unidentified, one family, the tubulins, α1, α3, and β, can be recognized by comparison to the migration of α- and β-tubulins from purified flagella. We have observed the same slight shift in migration of the α1-tubulin translation product compared to mature flagellar α-tubulin as that reported by Lefebvre *et al.* (22). The appearance of functional tubulin mRNA is stage specific. Very little tubulin is synthesized from RNA obtained from cells early in the cell cycle (L2); however, levels of functional tubulin mRNA rise and peak before or during division (L14). We estimate from densitometry analysis of all tubulin spots that relative levels of translatable tubulin mRNAs vary 10- to 20-fold during the cell cycle.

In addition to the tubulins, the synthesis of a number of other major translation products is stage specific. The fold change in mRNA activity coding for these products (including tubulins) was measured relative to mRNA activity coding for polypeptide R, which appeared not to change during the cell cycle. By this criterion, the change in mRNA activity represented by spot 2 was 20-fold; spot 4, 24-fold; spot 6, 27-fold; spot 7, 6-fold; spots 9, 10, and 12, 40-fold; and spot 13, 12-fold. What is striking are the differences in pattern for each mRNA activity, both in terms of time of peak appearance and fold variation. Some spots, like the tubulins, represent the products of mRNAs that are coordinately accumulated during the cell cycle. An example are spots 9, 10, and 12, which appear together in early stages of the cell cycle.

Hybridization Probes for Cell Cycle-Regulated mRNAs. To quantitate physical levels of mRNAs coding for cell cycle-regulated proteins, and to begin studying the structure and function of cell cycle-regulated genes, we obtained hybridization probes for cell cycle-regulated mRNAs. Clones corresponding to chicken α- and β-tubulin mRNAs were provided by Don Cleveland and co-workers (25). These clones hybridize *only* to *Chlamydomonas* DNA restriction fragments of the same size as those reported by Silflow and Rosenbaum (20) for homologous *Chlamydomonas* tubulin clones (unpublished data). In addition, the chicken α- and β-tubulin clones select functional mRNAs coding for *Chlamydomonas* α- and β-tubulin, respectively, in hybrid release-translation experiments (unpublished data). To obtain hybridization probes for other mRNAs regulated during the cell cycle, a library of cDNA clones representing poly(A)$^+$ mRNAs present during the 14th hr of the cell cycle (predivision) was prepared. Clones were identified as "cell cycle-regulated" by differential colony-filter hybridization (29) of ^{32}P-labeled cDNAs synthesized from RNA present in cells during the 6th, 14th, and 22nd hr of the cell cycle. We chose for further study those clones that showed the most dramatic differential response at the time of division (L14), either "off" or "on." About 10% of the analyzed clones showed a significant differential cell cycle response.

Changes in Physical Level of Poly(A)$^+$ RNAs During the Cell Cycle. To measure changes in physical level of cell cycle-regulated mRNAs, poly(A)$^+$ RNA was isolated from cells at different times during the cell cycle, size fractionated on denaturing gels, and transferred to nitrocellulose filters. Radiolabeled plasmid DNA from each of the clones was hybridized to the blots, and specifically hybridized probe was detected by autoradiography. Fig. 2 shows the pattern of change in level of RNAs hybridizing to tubulin probes during the cell cycle. The α-tubulin plasmid, pT1, hybridizes to two RNAs, about 2.05 and 2.20 kb in length, of equivalent abundance. These two RNAs are coordinately accumulated during the cell cycle. Quantitation of the autoradiographic signal by densitometry indicates that the α-tubulin mRNAs increase ≈8-fold in relative abundance, peaking during or just before mitosis. The β-tu-

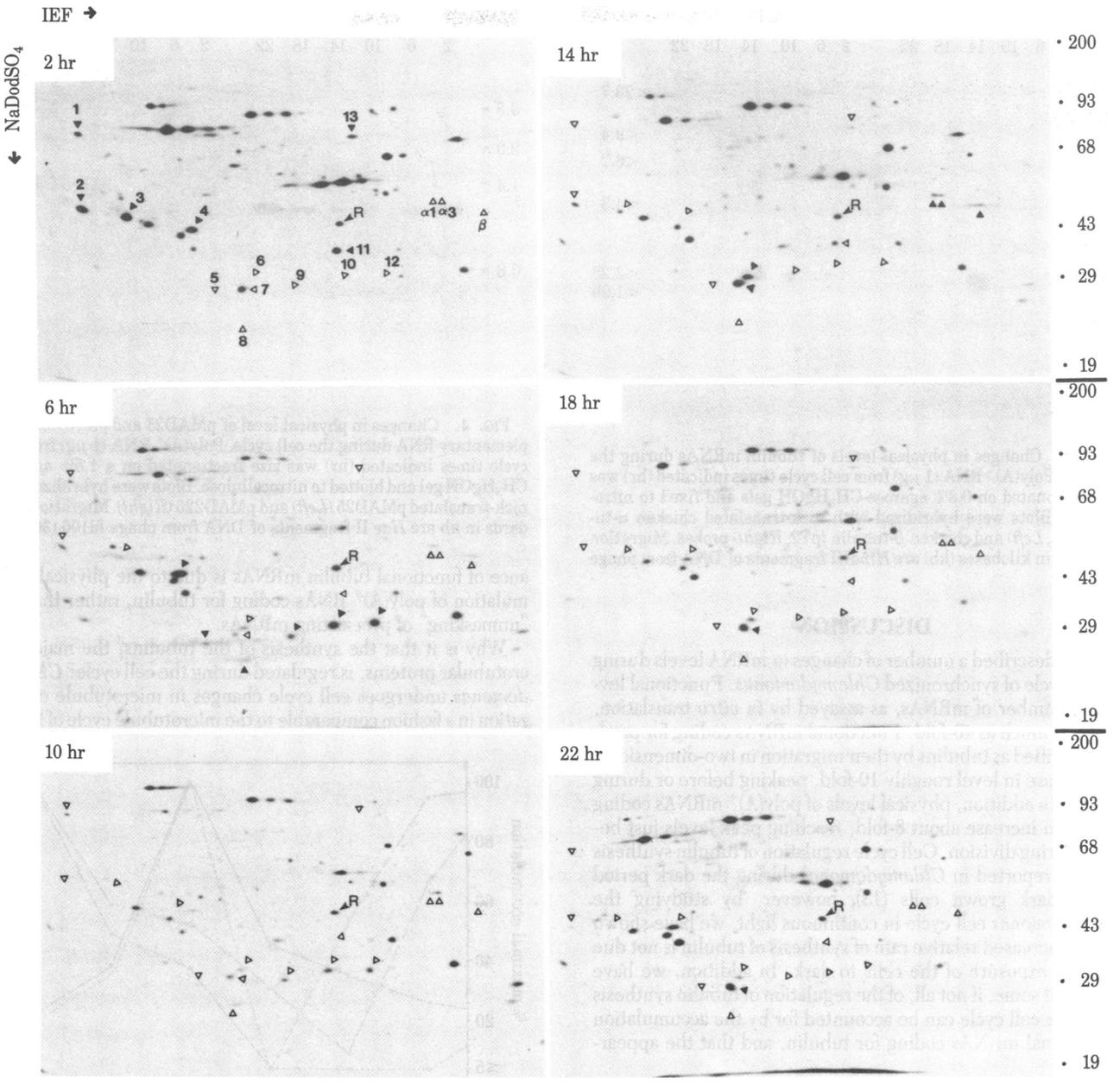

FIG. 1. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled translation products directed by cell cycle RNAs. Poly(A)$^+$ RNA (1 μg) from each of the times indicated was translated, and equal fractions of the translation mix were subjected to electrophoresis. The basic end of the isoelectric focusing (IEF) gel is at top left. Molecular weight markers (second dimension, indicated on the right $\times 10^{-3}$) were myosin heavy chain (200,000), phosphorylase *b* (93,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (19,000). Arrows indicate the position of migration of examples of polypeptides for which *in vitro* mRNA activity changes during the cell cycle. Filled arrows indicate peak activity. Product R represents a reference mRNA activity that appears not to vary during the cell cycle. R has been used as a standard to quantitate changes in mRNA activity during the cell cycle.

bulin clone, pT2, also hybridized to two mRNAs of equivalent abundance (poorly resolved in this figure) about 2.3 and 2.35 kb in length, which are also coordinately accumulated during the cell cycle and increase ≈8-fold in relative abundance. Therefore, all four tubulin mRNAs appear to be coordinately accumulated and increase ≈8-fold in relative abundance during mitosis.

The cell cycle pattern of accumulation of a poly(A)$^+$ RNA complementary to a *Chlamydomonas* cDNA probe, pMAD27, is shown in Fig. 3. This 2.5-kb RNA is not detectable early in the cell cycle; it accumulates at least 20-fold just before division. Two other cell cycle patterns of RNA accumulation are represented in Fig. 4. A 1.2-kb RNA complementary to pMAD25 is present early in the cell cycle, disappears by the 10th hr, and begins to reaccumulate in cells before division. This RNA changes in relative abundance by at least a factor of 20 during the cell cycle. A 1.3-kb RNA complementary to pMAD226 appears early, is lost by the 6th hr, and reaccumulates to its previous level by the 14th hr of the cell cycle. The variation in relative abundance of this RNA is ≈10-fold. In addition, a much lower abundance 1.65-kb mRNA recognized by pMAD244 is detectable in cells only during the 10th–14th hr of the cell cycle (data not shown). A summary of these data is presented in Fig. 5.

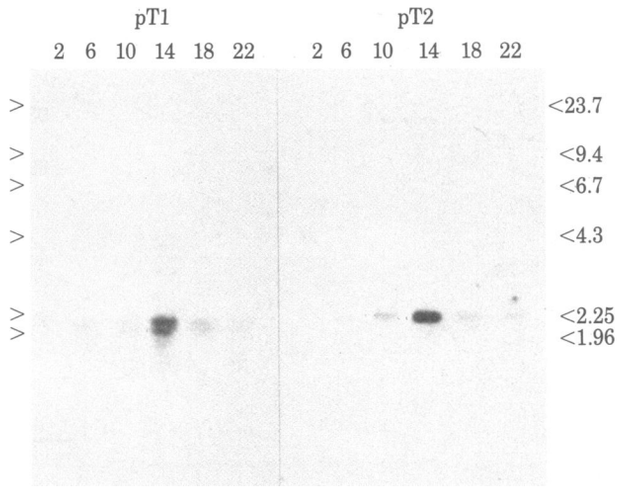

FIG. 2. Changes in physical levels of tubulin mRNAs during the cell cycle. Poly(A)$^+$ RNA (1 μg) from cell cycle times indicated (hr) was size fractionated on 0.8% agarose-CH_3HgOH gels and fixed to nitrocellulose. Blots were hybridized with nick-translated chicken α-tubulin (pT1, *Left*) and chicken β-tubulin (pT2, *Right*) probes. Migration standards in kilobases (kb) are *Hin*dIII fragments of DNA from phage λc*I*857.

DISCUSSION

We have described a number of changes in mRNA levels during the cell cycle of synchronized *Chlamydomonas*. Functional levels of a number of mRNAs, as assayed by *in vitro* translation, change as much as 40-fold. Functional mRNAs coding for products identified as tubulins by their migration in two-dimensional gels increase in level roughly 10-fold, peaking before or during division. In addition, physical levels of poly(A)$^+$ mRNAs coding for tubulin increase about 8-fold, reaching peak levels just before or during division. Cell cycle regulation of tubulin synthesis has been reported in *Chlamydomonas* during the dark period of light–dark grown cells (13); however, by studying the *Chlamydomonas* cell cycle in continuous light, we have shown that the increased relative rate of synthesis of tubulin is not due simply to exposure of the cells to dark. In addition, we have shown that some, if not all, of the regulation of tubulin synthesis during the cell cycle can be accounted for by the accumulation of functional mRNAs coding for tubulin, and that the appearance of functional tubulin mRNAs is due to the physical accumulation of poly(A)$^+$ RNAs coding for tubulin, rather than the "unmasking" of preexisting mRNAs.

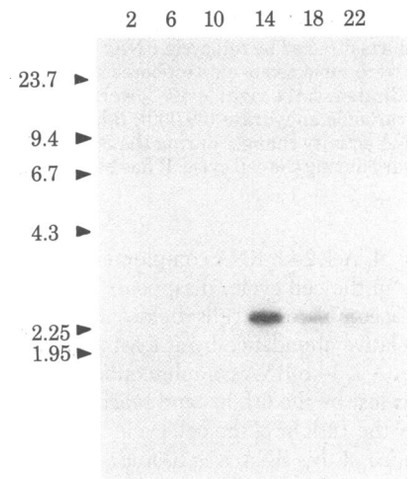

FIG. 3. Changes in physical level of pMAD27 complementary RNA during the cell cycle. Blot was same as in Fig. 2 except that nick-translated pMAD27 was used as probe.

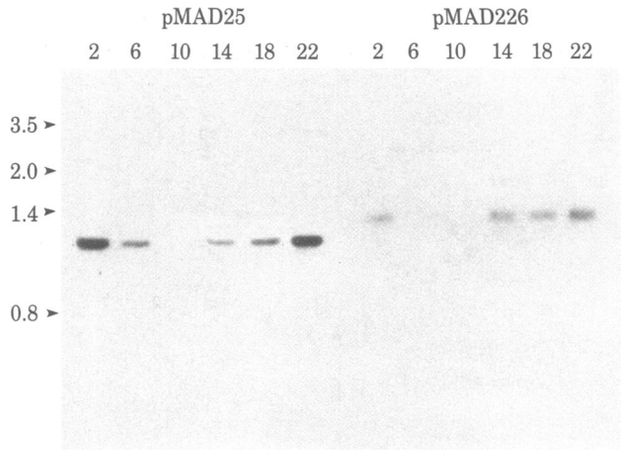

FIG. 4. Changes in physical level of pMAD25 and pMAD226 complementary RNA during the cell cycle. Poly(A)$^+$ RNA (1 μg) from cell cycle times indicated (hr) was size fractionated on a 1.5% agarose-CH_3HgOH gel and blotted to nitrocellulose. Blots were hybridized with nick-translated pMAD25 (*Left*) and pMAD226 (*Right*). Migration standards in kb are *Hae* II fragments of DNA from phage fd106 (36).

Why is it that the synthesis of the tubulins, the major microtubular proteins, is regulated during the cell cycle? *Chlamydomonas* undergoes cell cycle changes in microtubule organization in a fashion comparable to the microtubule cycle of higher

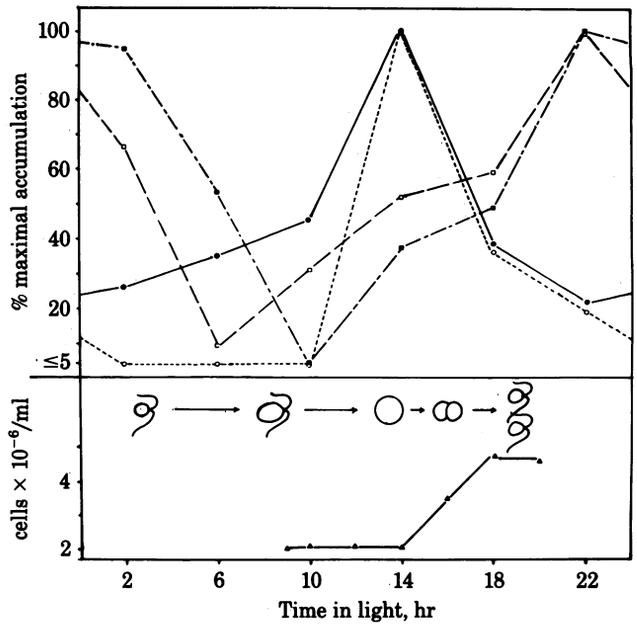

FIG. 5. Timing and extent of accumulation of a number of cell cycle-regulated RNAs relative to cell division. Cell number (▲——▲), tubulin mRNAs (●——●), pMAD25 RNA (■-·-■), pMAD27 RNA (○– – –○), and pMAD226 RNA (□——□) are shown. Autoradiographic signals from the RNA blots shown in Fig. 2, 3, and 4 were measured with a densitometer. Where a signal could not be detected (e.g., pMAD25 at 10 hr), the area of a peak with the same half-width as that derived from the maximal signal, and with a height representing twice the average noise of the trace, was taken as an estimate of the largest possible undetectable signal, and was never more than 5% of the maximal signal. Cell number was determined with a hemocytometer. Stages of the cell cycle are shown above the growth curve.

cells in that flagella are withdrawn (rounding up), the mitotic apparatus is formed, and after division flagella are regenerated (37). Work by a number of groups has shown that, during the period of flagellar regrowth after experimentally induced deflagellation of either asynchronous vegetative cells (38) or gametes (19) of *Chlamydomonas*, tubulin protein synthesis (17, 39) and tubulin mRNA accumulation (19, 20, 22, 40) are dramatically stimulated. It is possible that the cell cycle induction of tubulin synthesis simply results from the activities that occur during the normally scheduled flagellar regeneration period. Because the events of mitosis occur over a shorter period of time in individual cells than in the culture as a whole [i.e., mitosis takes ≈45 min (37), whereas the culture divides over about a 4-hr period (see Fig. 5)], we cannot unambiguously correlate the period of tubulin synthesis with either mitotic apparatus construction or flagellar regeneration. However, similar cell cycle changes in tubulin synthesis in *Physarum* syncitia (11) and HeLa cells (3), two cell types that do not have flagella, argue that there may be other requirements for tubulin synthesis during the cell cycle, perhaps in construction of mitotic apparatus.

Besides tubulin mRNA, the levels of a number of other messages vary during the cell cycle (Figs. 1 and 5). Though we have not identified functions encoded by other cell cycle-regulated mRNAs, their existence demonstrates a variety of patterns, with respect to both timing and extent of induction, of mRNA accumulation during the cell cycle. We conclude that there is a complex pattern of mRNA accumulation correlated with progression of cells through the cell cycle.

Why is it that specific RNAs appear only during particular times in the cell cycle? It may be that *Chlamydomonas* simply has found it advantageous or necessary to relegate certain functions to particular periods during the cell cycle, and that the timing or stage-specific nature of the event is unimportant to cell cycle progression. On the other hand, the stage-specific accumulation of certain mRNAs may be important in governing the orderly progression of cells through the cell cycle.

The cloned probes for cell cycle-regulated mRNAs provide an additional handle for studying the cell cycle of *Chlamydomonas*. A problem in studying the cell cycle has been an inability to identify biosynthetic events or "landmarks." The mRNA accumulation events we have described provide landmarks, other than mitosis and DNA synthesis, by which cell cycle progression can be measured. The cloned cDNAs complementary to cell cycle-regulated mRNAs will also be useful for determining how genes might be regulated during the cell cycle and at what levels cell cycle control of gene expression might take place. In addition, the probes will simplify the isolation and analysis of cell cycle-regulated genes and may provide insight into how cells might control their own division.

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