Transcription of α-Tubulin and Histone H4 Genes Begins at the Same Point in the *Physarum* Cell Cycle

John J. Carrino and Thomas G. Laffler

Department of Microbiology-Immunology, The Medical and Dental Schools, Northwestern University, Chicago, Illinois 60611

Abstract. In naturally synchronous plasmodia of *Physarum polycephalum*, both tubulin and histone gene transcription define periodic cell cycle–regulated events. Using a slot-blot hybridization assay and Northern blot analysis, we have demonstrated that a major peak of accumulation of both α-tubulin and histone H4 transcripts occurs in late G2 phase. Nuclear transcription assays indicate that both genes are transcriptionally activated at the same point in the cell cycle: mid G2 phase. While the rate of tubulin gene transcription drops sharply at the M/S-phase boundary, the rate of histone gene transcription remains high through most of S phase. We conclude that the cell cycle regulation of tubulin expression occurs primarily at the level of transcription, while histone regulation involves both transcriptional and posttranscriptional controls. It is possible that the periodic expression of both histone and tubulin genes is triggered by a common cell cycle regulatory mechanism.

In naturally synchronous plasmodia of the myxomycete, *Physarum polycephalum*, tubulin synthesis is limited to a discrete portion of the cell cycle. During the 2 h preceding mitosis the rate of tubulin biosynthesis increases by 40-fold and returns to basal levels within 1 h after mitosis (22). This applies to all four tubulin isotypes expressed in plasmodia: two α- and two β-tubulins (5). While the increase in the differential rate of tubulin protein synthesis is mirrored by an equivalent increase in the level of tubulin mRNA (32), messenger titration experiments cannot distinguish between transcriptional and posttranscriptional regulation.

In *Physarum* plasmodia, the sole function of microtubules may be as part of the mitotic spindle. Interphase microtubules have never been observed in plasmodia, and assembly of spindle microtubules begins ~30 min before metaphase (32). Since the increase in tubulin expression precedes spindle assembly, it is unlikely that expression is driven by microtubule polymerization. Rather, tubulin expression seems to be under the direct control of a "mitotic clock" mechanism that may not depend on microtubule assembly.

We have recently reported evidence that heat shocks can uncouple tubulin expression from the timing of mitosis (6). Since similar heat shock effects have been reported for thymidine kinase synthesis (41), it is possible that the same mechanism regulates genes involved in chromosome replication as well as those involved in mitosis. Since there is no G1 phase in the *Physarum* cell cycle, S phase immediately follows mitosis (35). Histone synthesis is essentially limited to S phase (33) and is coupled to DNA replication (21). Therefore, the coupling of tubulin and histone gene expression, while surprising, is not implausible.

In mammalian cells the accumulation of histone mRNA is subject to both transcriptional and posttranscriptional controls (1, 13–15, 30). Transcriptional regulation is most important in early S phase, while posttranscriptional regulation predominates later in S (30). These studies are limited by the synchrony of cell populations prepared by elutriation (1), mitotic detachment (30), or G1/S arrest-release methods (15, 30) because the high levels of histone mRNAs in S phase tend to obscure late G1 events. Pre–S phase events can be more clearly addressed in lower eukaryotes where better synchrony is possible. In yeast, it has been demonstrated that histone transcripts begin to accumulate late in G1 phase (16, 17), and in *Physarum* a preliminary report indicates that histone H4 transcripts accumulate in G2 phase (40). In both cases, the cells accumulate a dowry of histone transcripts in anticipation of chromosome replication. With the virtually perfect natural synchrony of mitosis in *Physarum* plasmodia, the timing of these events can be more carefully studied than in other systems.

This system is an excellent model for studying the regulation of gene action during the cell cycle. Two new findings will be presented in this report. First, the accumulation of histone transcripts begins at the same point in the cell cycle that tubulin transcripts begin to accumulate, and second, this is accompanied by a dramatic increase in the rate of transcription of both tubulin and histone genes. The significance of this temporal coupling of tubulin and histone transcription to the overall scheme of cell cycle regulation will be discussed.

Materials and Methods

Synchronous Cultures

Strain M_3CV plasmodia were cultured as previously described (22). To prepare synchronous cultures, microplasmodial shake cultures grown in simplified soy

medium (22) were harvested by centrifugation, the pellet resuspended in an equal volume of sterile water, 1 ml plated on filter paper supported by a stainless steel grid over simplified soy medium, and incubated at 26°C. The third mitosis after plating, determined by phase contrast observation of ethanol-fixed smears, usually occurred 20 h after plating.

RNA Titrations

At various times relative to the third mitosis after plating, approximately one-quarter of a plasmodium was harvested and RNA isolated by essentially the same guanidine isothiocyanate/CsCl method as described by Burland et al. (5). RNA was dissolved in RNAase free water and quantitated by absorbance at 260 nM. Aliquots were denatured with formaldehyde as described by White and Bancroft (38), adjusted to 15× standard saline-citrate buffer (SSC), and applied to a nylon filter (Biodyne A, Pall Corp., Glen Cove, NY) using a slot blot manifold (Schleicher & Shuell, Inc., Keene, NH). SSC (1×) is 0.15 M NaCl, 15 mM sodium citrate. Triplicate filters were prepared containing paired slots loaded with 2 and 0.5 μg RNA, respectively, for each timepoint. Filters were prehybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 250 μg/ml denatured salmon sperm DNA at 42°C for 2 h. Denhardt's solution is composed of 0.02% Ficoll (400,000 D), 0.02% polyvinylpyrrolidone (360,000 D), and 0.02% BSA. We did hybridizations in the same buffer with 2.5×10^6 cpm/ml probe for 2 d at 42°C. Under these conditions, the radioactive signal from each probe is directly proportional to the amount of RNA loaded. Filters were washed twice in 2× SSC, 0.1% SDS at room temperature for 30 min, and twice with 0.1× SSC, 0.1% SDS at 60°C for 30 min. One filter was hybridized to an α-tubulin probe, another to a histone H4 probe, and the third to a probe prepared from Ppc42 (32). Probes were ^{32}P-labeled by nick translation of plasmid DNA as described by Maniatis et al. (25). The α-tubulin plasmid NU62 carries a 1.1-kb Eco RI-Sac I fragment from Ppc-α125 cloned into pSP65. This fragment is internal to the α-tubulin coding region. The histone H4 plasmid NU81 carries a 591-bp HinIII fragment carrying an H4 genomic sequence (39). Plasmid Ppc42 is a cDNA clone of a presumptive "constituitive" transcript (32). However, our data show a doubling in transcript level before mitosis. Radioactivity was detected by radioautography and quantified by Cerenkov counting of excised slots. Plasmids Ppc42 and Ppc-α125 were kindly provided by T. Burland (University of Wisconsin) and F. X. Wilhelm (C.N.R.S., Strasbourg, FRG) provided the histone H4 segment.

Northern Blot Analysis

20-μg aliquots of total plasmodial RNA was denatured with glyoxal and dimethylsulfoxide (25), electrophoresed through 1% agarose in 12 mM Tris-HCl, pH 7.0, 6 mM sodium acetate, 0.3 mM EDTA, and blotted to Gene Screen Plus (New England Nuclear, Boston, MA) transfer membranes. Blots were prehybridized in 10 ml 50% formamide, 1 M NaCl, 1% SDS, and 10% dextran sulfate for 6 h with constant agitation. For hybridization, heat denatured salmon sperm DNA and nick-translated probe were added in 0.5 ml to a final concentration of 100 μg/ml and 5×10^5 cpm/ml, respectively. Hybridizations were performed overnight at 42°C with constant agitation. Blots were washed twice in 2× SSC for 5 min at room temperature, twice in 2× SSC, 1% SDS for 30 min at 60°C, and twice in 0.1× SSC for 30 min at room temperature. Exposures lasted 4 h at −80°C with intensifying screens. The actin probe was derived from an *ard*A clone (29) kindly provided by W. Nader (Max Planck Institute).

Nuclear Transcriptions

Nuclei were isolated by homogenization in 0.25 M sucrose, 10 mM $MgCl_2$, 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.2, essentially as described by Mohberg (28) and stored at −80°C in 40% glycerol, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ at a concentration of 2 mg DNA/ml. To prepare labeled transcripts, nuclear aliquots containing 100 μg DNA were incubated for 30 min at 26°C in the presence of 100 μCi α-^{32}P-guanosine 5′-triphosphate in a volume of 100 μl. The incubation buffer contained 60 mM $(NH_4)_2SO_4$, 35 mM KCl, 0.2 mM $MnCl_2$, 3 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.25 M sucrose, 20% glycerol, 1 mM ATP, 0.25 mM cytidine 5′-triphosphate and uridine 5′-triphosphate, 10 μM guanosine 5′-triphosphate, 75 mM Tris-HCl, pH 7.8. These conditions are similar to those reported elsewhere (11, 27), and we have confirmed the optima with our nuclei (data not shown). A probe was prepared as described by Groudine et al. (12). Linearized plasmids were denatured in 0.3 M NaOH at 60°C for 15 min, an equal volume of 2 M ammonium acetate was added, and 2 μg of each spotted in 5 μl onto nitrocellulose filters (BA85, Schleicher & Shuell, Inc.) presoaked in 1 M ammonium acetate. By baking 2 h at 80°C under vacuum, DNA was fixed to the filters. Each filter was hybridized to 10^6 cpm runoff transcript probe for 4 d (12), and washed as above except that the final washes were at 65°C. The hybridization solution was identical to that used in the slot-blot analysis except that SDS was omitted and yeast tRNA added at 125 μg/ml. Radioactivity was detected by radioautography. Under the conditions used, the signal is proportional to the amount of probe added (data not shown) and hybridization to pBR322 controls is minimal.

Results

Transcript Levels

Relative levels of tubulin transcripts are reported to increase by 40-fold late in G2 phase (32). These determinations were based upon dot hybridization assays using a heterologous β-tubulin probe and a homologous α-tubulin probe (cDNA clone Ppc-α125). We have confirmed these results using as a probe a subclone of Ppc-α125 containing a fragment internal to the α-tubulin coding region. In addition, we have followed histone H4 transcripts by hybridization with a homologous histone H4 genomic sequence (39). The sequence carried by Ppc42 was included as a non–cell cycle–regulated control. Fig. 1 shows the results of a representative slot hybridization experiment. α-Tubulin transcripts begin to accumulate ~2 h before mitosis and return to basal levels soon after mitosis. Histone H4 transcripts follow a more complicated pattern.

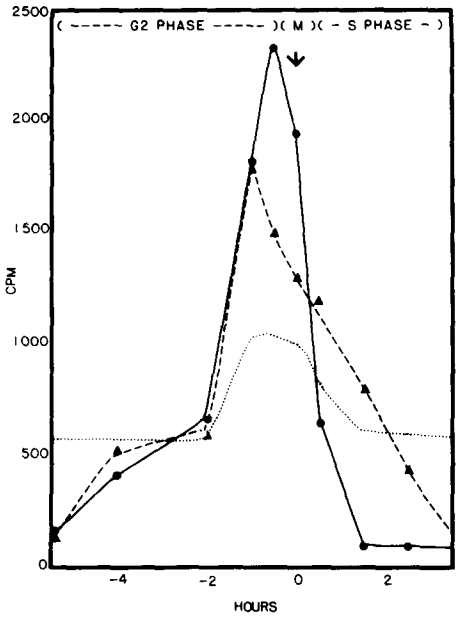

Figure 1. Analysis of α-tubulin, histone H4, and Ppc42 RNA levels over the cell cycle. Total plasmodial RNA was prepared from plasmodial samples taken at various cell cycle points relative to the third synchronous mitosis after plating. The arrow marks metaphase (t = 0). G2 phase runs from the end of the previous S phase (t = −6 h) to prophase (t = −0.5 h). S phase extends from telophase (t = 0.1 h) to the beginning of the next G2 phase (t = 3.5 h). RNA aliquots were denatured with formaldehyde and blotted to Biodyne A (Pall Corp.) paper using a slot-blot manifold. Blots were prehybridized as described and hybridizations carried out in identical buffer containing 2.5×10^6 cpm nick-translated probe (10^8 cpm/μg) for 48 h. Blots were exposed overnight without intensifying screens. After autoradiography, radioactive slots were excised and the cpm of probe hybridized determined by liquid scintillation counting. The data are presented as cpm hybridized to 2 μg total RNA vs. time relative to metaphase. Filled circles, hybridization with α-tubulin probe. Open circles, hybridization with Ppc42 probe. Triangles, hybridization with histone H4 probe.

While the major peak of accumulation parallels the tubulin peak, the basal level is only reached at the end of S phase. The transcript detected by Ppc42 describes a minor G2 peak. This same general result has occurred several times.

A Northern blot analysis was done with the same RNA preparations as were used in Fig. 1. The data shown in Fig. 2 indicate that, for actin, histone H4, and α-tubulin sequences, the signal represents a single size class of transcript. Our data fail to reproduce the difference in size between late G2- and S-phase histone H4 transcripts reported by Wilhelm et al. (40). This Northern analysis shows no systematic cell cycle variation in the level of actin mRNA. Since histone protein synthesis is limited to S phase (33), it is notable that the histone transcript level shown in Fig. 1 appears to peak before the beginning of S phase: the mRNA level is higher just before mitosis than it is at the beginning of S phase. It appears that the major accumulation of both tubulin and histone mRNAs begins at the same time in the cell cycle: mid G2 phase.

Transcriptional Activity

To distinguish transcriptional regulation from other levels of control, we followed messenger synthesis by nuclear run-on assays. Existing transcription protocols were modified to maximize the activity of isolated *Physarum* nuclei. The most significant modification was to include RNAsin (Promega Biotech, Madison, WI) to inhibit endogenous RNAase activity present in all *Physarum* nuclear preparations. As the data in Fig. 3 indicate, the period of α-tubulin gene transcription is limited to the 2 h preceding mitosis, while transcription of histone H4 genes begins at the same time in the cell cycle as that of the tubulin genes but continues through S phase.

Inhibitor studies were performed to test that these genes are being transcribed by RNA polymerase B. As the data in Fig. 4 indicate, incorporation can be inhibited by various concentrations of the transcription inhibitor α-amanitin. While about half of the incorporation is resistant to the inhibitor, 1 μg/ml α-amanitin completely inhibits histone, tubulin, and actin transcription, as expected for genes transcribed by RNA polymerase B.

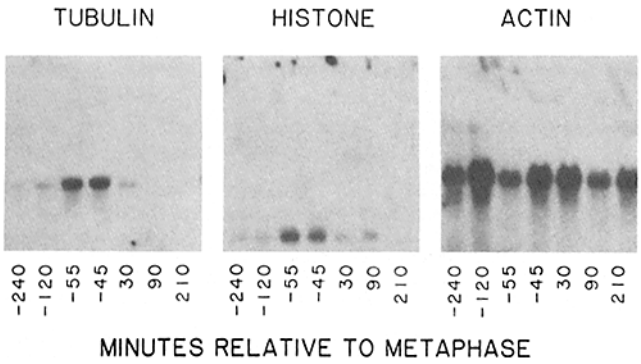

Figure 2. Northern blot analysis of alpha tubulin, histone H4, and actin RNAs. Total RNAs were isolated at timepoints relative to metaphase of the third synchronous mitosis after plating. Aliquots (20 μg) were glyoxalated, electrophoresed through 1% agarose gels, and blot-transferred to Gene Screen Plus (New England Nuclear) membranes. Hybridizations were carried out according to manufacturer's instructions in 10 ml of hybridization buffer containing 5 × 10^5 cpm/ml nick-translated probe. Blots were exposed at −80°C for 4 h with intensifying screens.

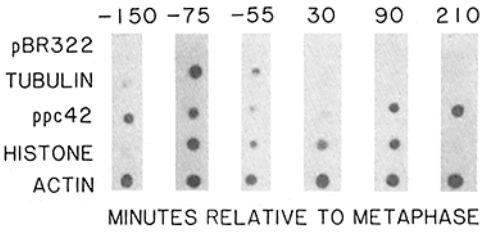

Figure 3. Transcription of α-tubulin, histone H4, Ppc42, and actin genes during the cell cycle. Nuclear transcription reactions were performed at 26°C for 30 min and labeled RNA isolated as described. The 2-μg aliquots of the indicated plasmid DNAs were denatured with NaOH and spotted on nitrocellulose filters. After overnight prehybridization, 10^6 cpm labeled transcript was added in four parts prehybridization solution/one part 50% dextran sulfate and hybridization carried out for 4 d at 42°C. Exposures were for 72 h at −80°C with intensifying screens.

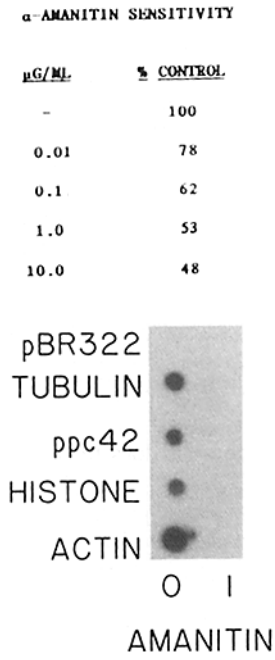

Figure 4. α-Amanitin sensitivity of transcription in vitro. The amount of labeled RNA synthesized in vitro in the presence of various concentrations of α-amanitin was determined by trichloroacetic acid precipitation. The values shown represent percent incorporation relative to untreated control reactions. Labeled transcripts from untreated reactions and reactions containing 1 μg/ml α-amanitin were used as a probe as described. After hybridization, blots were exposed at −80°C for 72 h with intensifying screens.

Our results show that the transcription of both tubulin and histone genes is activated at the same time in late G2 phase. However, tubulin transcription is turned off at mitosis while histone transcription continues until the end of S phase. By inspection of Fig. 3, modulation of transcription seems sufficient to explain the accumulation of tubulin mRNA, while posttranscriptional effects appear to play a significant role in controlling the level of histone mRNA. In particular, there are comparable levels of histone mRNA in early–mid-G2 phase (see Fig. 1, −2.5 h) and in mid–S phase (Fig. 1, +1.3 h), but the rates of transcription are radically different as shown by comparing the equivalent timepoints in Fig. 3. Rather, it appears that histone H4 transcription remains high from the time it is activated in mid-G2 phase until the end of S phase, when it stops. Hereford et al. (17) claim that in yeast, transcription of the histone genes stops early in S phase, and suggest that replication of the histone genes at that time might signal the end of their transcription. It has recently been reported that the *Physarum* histone H4 genes are replicated in early S phase (19). Our data indicate that in *Physarum* at

least histone H4 mRNA transcription continues unabated after the genes encoding those transcripts are replicated.

Discussion

Our data raise two questions: why is there cell cycle stage-specific transcription of the tubulin genes in *Physarum* but not in other cell types, and why is histone gene transcription activated in mid–G2 phase?

Tubulin Periodicities

Although no interphase or cytoskeletal microtubules are found in *Physarum* plasmodia, there are cytoskeletal microtubules in most other cells. It seems likely that in most organisms spindle microtubules could be assembled from subunits scavenged from the cytoskeleton (18). It makes good sense for tubulin mRNA synthesis to be periodic in cells that have a cyclic demand for microtubules but not in cells with constant demand. Thus, the lack of cell cycle periodicity in the synthesis of tubulin by HeLa cells (4) is not inconsistent with our findings.

The homeostatic regulation of microtubular protein synthesis first demonstrated by Ben-Ze'ev and Penman (3) couples the level of tubulin mRNAs to that of unpolymerized tubulin (8, 9). Since nuclear transcription studies failed to show any difference in the rate of tubulin gene transcription between colchicine-treated and untreated cells, it was suggested that tubulin mRNA levels may be regulated by a posttranscriptional mechanism (7). Since the premitotic burst of tubulin gene transcription anticipates spindle assembly in *Physarum*, control of transcription by the free tubulin pool seems unlikely. However, there are no data indicating the fine tuning of tubulin mRNA levels does not involve posttranscriptional controls in *Physarum* much as it does in mammalian cells.

Our data are the first clear example of cell cycle regulation of tubulin mRNA transcription. Earlier reports of periodicity in *Chlamydomonas* (2, 10, 36) appear to be synchronization artifacts, since the periodicity disappears when cultures are returned to normal growth conditions (31). There is precedent in *Chlamydomonas* for the direct induction of tubulin gene transcription during flagellar regeneration. After deflagellation, there is a burst of tubulin mRNA synthesis (20, 24, 26, 34) that is not directly dependent on flagellar regeneration (23, 37). Rather, some mechanism triggers gene activity in anticipation of flagellar assembly. In *Physarum*, synthesis of the tubulin mRNAs similarly is triggered in anticipation of spindle assembly.

We have recently presented data showing that the level of tubulin protein increases significantly before mitosis and drops soon after mitosis (6). Perhaps the periodicity of tubulin expression is part of a mechanism that keeps the intracellular tubulin concentration below a critical threshold during interphase, increasing the level when microtubule assembly is required.

Histone Periodicities

In *Physarum*, the accumulation of a dowry of histone mRNA during G2 phase permits the immediate synthesis of large amounts of histone at the beginning of S phase, at a time when the rate of DNA replication is maximal (21). Since histone protein synthesis appears to be limited to S phase, transcripts that accumulate in advance would be maintained in a translationally inactive state. As *Physarum* lacks a G1 phase, the G2 accumulation of histone transcripts we observe need not be viewed as substantially different from accumulation in the G1 phase of yeast (17). However, the phenomenon is far more pronounced in *Physarum*, where substantial amounts of histone mRNA are stored for hours, than it is in yeast, where transcription begins shortly before S phase (17). Our data indicate that the rate of histone mRNA turnover may actually increase at the beginning of S phase; this variance with other systems could stem from the exceptional stability of histone transcripts during late G2 phase.

The temporal coupling of tubulin and histone gene transcription is novel. The need for the periodicity of each is clearly distinct: tubulins are needed for mitosis and histones are needed for chromosome replication. Assuming that α-tubulin and histone H4 are representative of their respective families, both sets of genes may be activated by a common cell cycle trigger. The tubulin genes could be under simple transcriptional control, while the histone genes respond to multiple levels of control. Termination of expression is clearly different. Transcription of the tubulin genes is turned off at mitosis, while transcription of the histone genes is not turned off until the end of S phase. It is an intriguing possibility that transcription of both the tubulin and histone genes may be cell cycle–regulated in a simple on-off manner, while the levels of mRNA are coupled to demand for the proteins they encode by a homeostatic mechanism that acts at the level of messenger degradation.

This paper is dedicated to Harold P. Rusch on the occasion of his 77th birthday in recognition of his central contributions to the field of *Physarum* research and to cancer research in general.

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