Distinct Replication-Independent and -Dependent Phases of Histone Gene Expression during the *Physarum* Cell Cycle

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During the S phase of the cell cycle, histone gene expression and DNA replication are tightly coupled. In mitotically synchronous plasmodia of the myxomycete *Physarum polycephalum*, which has no G1 phase, histone mRNA synthesis begins in mid-G2 phase. Although histone gene transcription is activated in the absence of significant DNA synthesis, our data demonstrate that histone gene expression became tightly coupled to DNA replication once the S phase began. There was a transition from the replication-independent phase to the replication-dependent phase of histone gene expression. During the first phase, histone mRNA synthesis appears to be under direct cell cycle control; it was not coupled to DNA replication. This allowed a pool of histone mRNA to accumulate in late G2 phase, in anticipation of future demand. The second phase began at the end of mitosis, when the S phase began, and expression became homeostatically coupled to DNA replication. This homeostatic control required continuing protein synthesis, since cycloheximide uncoupled transcription from DNA synthesis. Nuclear run-on assays suggest that in *P. polycephalum* this coupling occurs at the level of transcription. While histone gene transcription appears to be directly switched on in mid-G2 phase and off at the end of the S phase by cell cycle regulators, only during the S phase was the level of transcription balanced with the rate of DNA synthesis.

The synthesis of core histone proteins is primarily limited to the S, or DNA synthetic phase, of the cell cycle. While the rates of synthesis of most proteins remain constant over the course of the cycle, rates of histone protein synthesis are 10-fold greater in the S phase than in the G2 phase (12). An accumulation of histone mRNAs during the S phase has been described in numerous systems, ranging from yeast cells to human cells (for reviews, see references 5 and 11). In HeLa cells, histone mRNA levels are 15-fold higher in the S phase than during the remainder of the cycle. Both the increased rates of histone gene transcription and the increased stability of histone mRNA during the S phase appear to drive this accumulation (3).

Histone gene expression is tightly coupled to ongoing DNA synthesis (for a review, see reference 14). Inhibition of DNA synthesis with drugs such as hydroxyurea or aphidicolin causes a rapid decrease in the synthesis of histone proteins. This decrease is mirrored by a decline in the levels of translatable histone mRNA. The depletion of histone mRNA is produced by alteration of both gene transcription and messenger turnover rates. In synchronized mouse myeloma cells, the S-phase rate of histone gene transcription is reduced by 70 to 80%, while messenger half-lives decrease approximately fivefold in response to drug treatment (15).

The coupling phenomenon depends on ongoing protein synthesis. Treatment of cells with cycloheximide prior to inhibition of DNA synthesis negates the coupling effect (1, 16). Several models of histone gene regulation have been put forward to explain these observations, including autoregulation of histone expression (1), synthesis of histone message-specific RNases (13), and the production of labile cell-cycle-regulated repressor proteins (17).

The tight coupling of histone gene expression and DNA replication in mammalian cells makes it difficult to determine

whether periodic histone gene expression is subject to direct cell cycle regulation or merely responds to increased DNA synthesis via this coupling mechanism. In yeast cells, histone expression may begin in very late G1 phase, just before the S phase begins (4). The case for direct cell cycle control is much stronger in the myxomycete *Physarum polycephalum*. *Physarum* plasmodia exhibit natural mitotic synchrony, thereby eliminating the need for artificial synchronization procedures. This provides an excellent system in which to study cell cycle periodicities. The *Physarum* cell cycle has been the subject of two recent reviews (8, 10).

We have recently described the pattern of α -tubulin and histone H4 mRNA accumulation and gene transcription during the *Physarum* cell cycle (2, 8). Transcription of both genes increases dramatically in mid-G2 phase in preparation for mitosis and entry into the S phase. Because *P. polycephalum* lacks a G1 phase, the late G2 accumulation of histone mRNA can be considered analogous to the late G1 expression of histone genes in yeast cells. Transcription of both the tubulin and histone genes is activated before there is any evident increase in demand for the proteins they encode. This suggests that they are under direct cell cycle control. Although synthesis of the histone proteins is strictly limited to the S phase, the histone mRNA that accumulates in the cytoplasm during late G2 phase can be translated in vitro (8a). Translation of mature, cytoplasmic mRNA appears to be prevented until S-phase DNA synthesis creates a demand for new histones.

Here we extend these studies to examine histone gene transcription during the S phase and its relationship to ongoing DNA synthesis. Using various inhibitors, we confirmed the coupling of histone gene expression with DNA synthesis in *P. polycephalum* at the nucleic acid level. The transition from replication-independent to -dependent expression mapped to the beginning of the S phase. In addition, our data suggest that coupling occurs mainly at the level of histone gene transcription.

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MATERIALS AND METHODS

Synchronous cultures. Cultures of strain M₃CV plasmodia were propagated as previously described (9). To obtain synchronous cultures, microplasmodia grown in shaken cultures were concentrated by centrifugation, suspended in water, plated on filter paper supported by a stainless steel grid over a modified soy medium, and incubated at 26°C. The third mitosis postplating occurred at approximately 20 h and was monitored by phase-contrast microscope observation of ethanol-fixed smears.

Inhibitor experiments. For inhibitor studies, a single growing plasmodium was divided, and segments were incubated for the appropriate times on either modified soy medium or this medium supplemented with various metabolic inhibitors. Concentrations of inhibitors were 25 mM and 10 µg/ml, respectively, for hydroxyurea and cycloheximide.

DNA labeling. Plasmodial disks (7 mm diameter) were excised at various times, overlaid with 20 µCi of [³H]thymidine, and incubated for 10 min at 26°C. Disks were harvested in 0.25 ml of 20 mM Tris hydrochloride (pH 8.0)–0.1 M EDTA–0.1 mg of proteinase K per ml–0.5% sodium dodecyl sulfate (SDS) and incubated for 15 min at 70°C. To assay radioactivity, 0.02 ml of homogenate was added to 0.5 ml of 0.5 M NaOH and hydrolyzed at 37°C for 4 h. Nucleic acids were precipitated by the addition of 0.5 ml of 20% trichloroacetic acid–0.5 N HCl and collected by filtration on glass fiber filters. Samples were counted in 5 ml of scintillation cocktail.

Northern blot analysis. Total cytoplasmic RNA was isolated at various times by using the same guanidine isothiocyanate-cesium chloride method used previously (2). Samples were denatured with formaldehyde, electrophoresed through 1% agarose in 10 mM sodium phosphate (pH 7), and blotted to nylon filters (Biodyne A; Pall Corp., Glen Cove, N.Y.) or nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.). Blots were prehybridized for 4 h at 42°C in a solution of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, and 250 µg of herring sperm DNA per ml. Hybridizations were done in an identical solution containing 4 × 10⁵ cpm of probe per ml for 16 to 20 h at 42°C. Blots were washed twice at room temperature in 2× SSC–0.1% SDS and once in 0.2× SSC–0.1% SDS at 50°C. Exposures were from 3 to 18 h at –80°C with intensifying screens. Probes were ³²P labeled by nick translation of plasmid DNA. Plasmid pCR1H2B-05, provided by M. Birnstiel, carries a sea urchin histone H2B gene (7). The histone H4 plasmid, provided by F.-X. Wilhelm (18), contains a 591-base-pair *Hind*III fragment carrying an H4 genomic sequence. The actin probe was derived from an *ardA* clone kindly provided by W. Nader (12).

Quantitation of mRNA levels. RNAs were denatured with formaldehyde and blotted to nylon membranes (Biodyne A; Pall Corp.) by using a dot blot manifold as previously described (2). Blots were hybridized as described above for 48 h at 42°C. Filters were washed twice in 2× SSC–0.1% SDS at room temperature and twice in 0.1× SSC–0.1% SDS at 60°C. To quantitate radioactivity, dots were excised from the filters and counted in 5 ml of scintillation cocktail.

Nuclear transcriptions. Isolation of nuclei, nuclear transcriptions, and plasmid dot blotting were carried out as described previously (2). To prepare ³²P-labeled RNA, transcription reactions were treated with 20 µg of RNase-free DNase per ml for 15 min at 26°C. After DNase treatment,

TABLE 1. Effect of inhibitors on DNA replication^a

Time (min) before or after mitosis	cpm (%) of labeled DNA after treatment		
	Control	Hydroxyurea	Cycloheximide
–120	480	NA	NA
+15	57,000	NA	NA
+30	51,000	8,300 (16)	1,100 (2.1)
+60	47,000	9,000 (19)	1,300 (2.8)

^a A synchronous plasmodium was divided into three segments following the second mitosis after plating. Individual segments were transferred at 15 min after mitosis to plates containing either fresh medium or medium supplemented with 10 µg of cycloheximide per ml or 25 mM hydroxyurea. The rate of DNA synthesis was determined by [³H]thymidine pulse-labeling of 7-mm disks cut from these plasmodia. NA, Not applicable.

proteinase K was added to 100 µg/ml, and the reactions were incubated at 42°C for 30 min. Preparations were then extracted twice with phenol-chloroform (50:50) and once with chloroform alone. Unincorporated nucleotides were removed by chromatography through Sephadex G-50 (Pharmacia, Inc., Piscataway, N.J.), and the labeled RNA was precipitated with ethanol. Hybridizations were carried out as described before (2) by using 2 × 10⁶ cpm of labeled transcripts. Blots were exposed for 4 days without intensifying screens. The hybridization signal was quantitated by scanning-laser densitometry.

RESULTS

Inhibition of DNA synthesis. The inhibitory effects of both hydroxyurea and cycloheximide on plasmodial DNA synthesis are shown in Table 1. Hydroxyurea (25 mM) reduced the incorporation of [³H]thymidine by over 80%, while cycloheximide (10 µg/ml) inhibited incorporation by virtually 100%. The inhibitory action of either drug is rapid in onset, reaching the maximum effect within 15 min after addition. In control plasmodia, the rate of [³H]thymidine incorporation was over 100-fold higher at the beginning of the S phase than it was in late G₂ phase. This differential illustrates the degree of inherent synchrony of *P. polycephalum*.

Histone mRNA levels. At various times following treatment with either hydroxyurea or cycloheximide, plasmodial RNA was isolated, and histone H4 levels were examined by Northern blot analysis. The effect of hydroxyurea on histone H4 mRNA accumulation is shown in Fig. 1. A rapid decrease in H4 mRNA levels was observed in treated plasmodia when compared with either untreated controls or actin mRNA (a nonperiodic control). By 45 min posttreatment, virtually no H4 mRNA could be detected on the Northern blots. The level of H4 mRNA decreased in the presence of hydroxyurea, with a half-life of approximately 15 min. By 45 min, a new steady state was established at 10% of the control level. The effect was not specific for the histone H4 mRNA (Fig. 2). Levels of histone H2B mRNA responded similarly to drug treatment, suggesting that the core histone genes were regulated coordinately.

The effect of hydroxyurea treatment on histone mRNA levels was limited to the S phase. Treatment of a plasmodium with hydroxyurea prior to mitosis had no effect on G₂-phase expression (Fig. 3). However, on completion of mitosis and entry into the S phase, histone H4 mRNA levels dropped and were undetectable by 40 min into the S phase. Levels of actin mRNA remained constant in both treated and control plasmodia. Since hydroxyurea had no effect in the G₂ phase, we can conclude that in the S phase its effect was mediated by inhibition of DNA synthesis rather than by

some other undocumented effect of this drug. There was little effect of hydroxyurea on the low level of G2-phase DNA synthesis (data not shown).

Cycloheximide had a radically different effect on histone mRNA levels. While cycloheximide treatment inhibited DNA synthesis by >90%, histone H4 mRNA levels were virtually unaffected by this drug (Fig. 4). Several studies suggest that cycloheximide treatment can produce a hyperaccumulation of histone mRNAs in other systems (16, 17). The hyperaccumulation is not due to simple polysome shielding of the message, since inhibitors of translation initiation are also able to negate coupling (16). Cycloheximide treatment does not appear to cause a short-term hyperaccumulation of histone mRNA in *P. polycephalum*. Levels of actin mRNA again remained constant.

FIG. 1. Sensitivity to hydroxyurea of S-phase levels of histone H4 mRNA. At 10 min after mitosis, synchronous plasmodia were transferred to medium supplemented with 25 mM hydroxyurea, and controls were transferred to fresh, unsupplemented medium. At various times thereafter, samples were taken, and RNA was prepared by the guanidinium isothiocyanate method. (A) Northern blot analysis. Samples (15 μ g) were fractionated on formaldehyde gels and blotted to nylon membranes, and the actin and histone sequences were detected by hybridization with homologous, nick-translated probes. Whereas the histone H4 transcripts disappeared after hydroxyurea was added, there was no effect on actin transcripts (a nonperiodic control). Times are in minutes after mitosis. C, Control; H, hydroxyurea. (B) Dot blot quantitation of messenger levels. Duplicate 2.5- μ g aliquots were denatured with formaldehyde and applied to a nylon filter using a dot blot manifold. Histone sequences were detected with a histone H4 probe, and the signal was quantified by liquid scintillation counting. Averages of the duplicate samples are plotted. $\circ$, Inhibited plasmodia; $\bullet$, control plasmodia.

FIG. 2. Parallel responses of histone H4 and histone H2b mRNA levels. H, Inhibited with 40 mM hydroxyurea; C, control. In lane 1, hydroxyurea was added at 120 min before the second mitosis after plating, and plasmodia were harvested at 60 min before the mitosis (G2 phase). In the other cases, the drug was added at 60 min before the mitosis. Other lanes (minutes after the second mitosis of plasmodium harvesting): 2, 15; 3, 30; 4, 45; 5, 60; 6, 75. Samples were prepared and analyzed as described in the legend to Fig. 1.

In accordance with previous observations (1, 16), inhibition of protein synthesis negated the hydroxyurea effect. The coupling of histone gene expression with DNA replication was dependent on ongoing protein synthesis (Fig. 5). A plasmodium was pretreated with cycloheximide for 5 min prior to incubation in medium containing both hydroxyurea and cycloheximide. This treatment protocol prevented the loss of histone H4 mRNA. Transcript levels remained elevated even to 45 min posttreatment, a time when none could be detected in plasmodia treated with hydroxyurea alone (Fig. 1A).

Transcription following drug treatment. A nuclear run-on assay was used to examine the effect of drug treatment on the rate of transcription of the histone H4 genes. Nuclei were isolated from S-phase plasmodia following treatment with either hydroxyurea or cycloheximide or both and utilized in the *in vitro* system. The effects of these drugs on histone gene transcription are shown in Fig. 6. After 10 min in the presence of hydroxyurea, histone H4 gene transcription was markedly reduced. Longer incubation times (30 min) produced a complete inhibition of histone gene transcription. The slight reduction in actin gene transcription in hydroxyurea-treated plasmodia was likely due to a nonspecific reduction of RNA polymerase II-dependent RNA synthesis

FIG. 3. Hydroxyurea had no effect until the S phase. Hydroxyurea (25 mM) was added at 75 min before the third mitosis (M). The third mitosis occurred at the same time in both inhibited and control cultures. Samples were prepared and analyzed as described in the legend to Fig. 1. Times are in minutes relative to mitosis. C, Control; H, hydroxyurea.

FIG. 4. Cycloheximide had no effect on histone H4 mRNA accumulation. Both panels parallel the experiment shown in Fig. 1, substituting 10 μ g of cycloheximide per ml for hydroxyurea. (A) Northern blot analysis. C, Control; Y, cycloheximide. (B) Dot blot quantitation.

in response to hydroxyurea. A similar reduction in total RNA synthesis has been previously noted (6).

Cycloheximide treatment appears to increase the rate of both histone and actin gene transcription. A similar nonspecific enhancement of gene transcription in the absence of protein synthesis has been previously described (16). The ratios of histone to actin gene transcription in controls and in cycloheximide-treated plasmodia showed little or no specific enhancement of histone expression in the absence of protein synthesis. Continuing protein synthesis was necessary for the coupling of histone mRNA synthesis to DNA replication. In agreement with the results shown in Fig. 5, prior treatment with cycloheximide blocked the effect of hydroxyurea on histone gene transcription. Since the levels of histone and actin transcription were comparable to those of the control,

FIG. 5. Inhibiting protein synthesis blocks the effect of hydroxyurea. Cycloheximide was added at 5 min after the third mitosis, and hydroxyurea was then added to the same culture at 10 min after the mitosis. Samples were taken and analyzed as described in the legend to in Fig. 1. Times are in minutes relative to the third mitosis. C, Control; HY, hydroxyurea plus cycloheximide.

the nonspecific effects of the two drugs appear to cancel each other out.

Taken together, these data suggest that the coupling of histone gene expression with DNA synthesis in *P. polycephalum* occurs primarily at the level of transcription and that this coupling requires continuing protein synthesis. Comparison of steady-state transcript levels before and after hydroxyurea treatment shows that modulation of transcription is sufficient to explain the reduction in messenger accumulation. However, our run-on data need to be confirmed by in vivo labeling experiments. Until then, the unlikely possibility that a histone-specific, pancreatic-RNase-inhibitor-insensitive RNase potentiates these homeostatic controls cannot be formally excluded.

DISCUSSION

Both direct cell cycle control and homeostatic coupling mechanisms control histone gene expression in *P. polycephalum*. From the beginning of histone gene transcription in mid-G2 phase to its end at the S-G2-phase transition, messenger synthesis was independent of continuing protein synthesis. This reflects a cycle-specific switching mechanism that is independent of histone protein utilization. However, the coupling of transcription to replication during the S phase reflects a homeostatic control mechanism that acts to balance supply with demand for the histone proteins. In the S phase, this mechanism may be superimposed on the first, when it is germane to balance supply and demand. Our data suggest that synthesis of the proteins that potentiate this coupling is limited to the S phase. Histones could serve this function themselves. A pool of free histones that increases when supply exceeds demand has the potential to act as a negative regulator of gene expression.

In most cell types examined, a combination of increased gene transcription and lowered messenger turnover drives histone mRNA accumulation. Although histone gene transcription in *P. polycephalum* increases dramatically in late G2 phase and remains consistently high throughout most of the S phase, transcript levels fall shortly after mitosis, declining gradually until the S phase is over (2). This suggests that histone messenger stability decreases shortly after entry into the S phase. The transcriptional periodicity

FIG. 6. Hydroxyurea inhibition affects the rate of histone mRNA synthesis. Nuclear run-on assays were performed as described in Materials and Methods. pBR322 was used as a control for nonspecific hybridization, and histone and actin transcripts were detected by homologous sequences cloned in pBR322. Hydroxyurea or cycloheximide or both were added to plasmodia at 15 min after the third mitosis, and samples were taken for nuclear isolation 10 and 30 min thereafter. The radioautographic signal was quantitated by scanning-laser densitometry. The histone-to-actin ratios are presented without normalization. H, Hydroxyurea; Y, cycloheximide; HY, both.

seems to be regulated in a simple on-off manner, while histone messenger levels may be additionally controlled by the modulation of turnover rates. This type of regulation would allow the plasmodial cell to fine tune levels of histone protein synthesis to changing rates of DNA synthesis during the S phase.

The data presented here demonstrate that coupling also occurred at the level of transcription. Histone H4 gene transcription was not detected by run-on assays after 30 min in the presence of hydroxyurea. The 15-min half-life of the H4 message observed in the presence of hydroxyurea is in good agreement with previous work (15). Presently, we are unable to address messenger turnover by in vivo experiments owing to an inability to equilibrate large nucleotide pools. While the effect on transcription alone is sufficient to account for the drop in mRNA level after replication was blocked by hydroxyurea, this may reflect an extreme situation. Messenger turnover rates may only be involved in the fine tuning of messenger levels, while transcriptional regulation provides a greater dynamic range when needed.

P. polycephalum differs from other cell systems in that hyperaccumulation of histone mRNA in the presence of cycloheximide was marginal and never exceeded a factor of two. The reason for this discrepancy is unclear. Perhaps the hyperaccumulation phenomenon in *P. polycephalum* is not as rapid in onset as it is in higher eucaryotic cells and therefore was undetectable in the time course experiments described here.

We have now documented three major transition points that define discontinuities in histone gene regulation. Replication-independent transcription is switched on in mid-G2 phase (2), the replication-dependent mechanism is switched on at the mitosis-S-phase transition, and transcription is switched off at the S-G2-phase transition. One possibility is that a second level of homeostatic control is superimposed on cell cycle control; another possibility is that a single set of controls is regulated in two ways. These transitions provide major foci for the future study of the developmental program that regulates periodic gene expression within the cell cycle.

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