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HISTONE H4 mRNA IS STORED AS A SMALL CYTOPLASMIC RNP DURING THE G_2 PHASE IN PHYSARUM POLYCEPHALUM

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In Physarum polycephalum the triggering of histone H4 gene transcription occurs in G_2 phase. The rate of synthesis of histone H4 mRNA was measured by in vivo pulse-labeling experiments. We show that it begins to increase in mid-G_2. During the second part of G_2 it increases approximately 20 fold over its minimum value and reaches a maximum at the end of G_2. After entry of the cells in S, histone H4 gene transcription rate begins to decrease and reaches a minimum value in early G_2. The histone H4 mRNA which accumulates in G_2 is not translated immediately into proteins but is stored in an inactive form until the beginning of the next S phase. Immediately after its transcription the H4 mRNA is transported to the cytoplasm where it is stored and stabilized as an inactive mRNP complex. This was shown by fractionation of cytoplasmic RNP in sucrose gradients and blot hybridization of subcellular fractions.

The synthesis of histone proteins and mRNAs occurs periodically during the cell cycle of eukaryotic cells (1-4). Translation of histone mRNA is essentially limited to the S phase and is tightly coupled to DNA replication. In mammalian cells the mRNAs for the replication-de-

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ABBREVIATIONS: DEPC - Diethyl pyrocarbonate, DTT - Dithiothreitol, VRC - Vanidyl ribonucleoside complex.

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pendent histone variants begin to accumulate at the end of G_1 or at the G_1-S border, reach maximum levels at mid-S phase and decline as DNA synthesis is completed.

It is now clearly established that in a certain number of cases histone gene transcription begins before entry of the cells in S phase (5-7). This was first shown in yeast (6), but has also been observed in mammalian cells (5,7). In naturally synchronous plasmodia of Physarum polycephalum this phenomenon is even more pronounced (8) since histone transcripts begin to accumulate during the second half of G_2 several hours before the mRNAs are actually used to make histone proteins. Histone messengers synthesized in G_2 constitute a pool of molecules in anticipation of the next S phase. A dowry of histone mRNA may be necessary at the beginning of S as Physarum lacks a G_1 phase. Thus large amounts of histone mRNAs are needed immediately after mitosis when DNA begins to be replicated at a high rate and histone proteins are rapidly required to package newly synthesized DNA into chromatin. The histone mRNAs which accumulate in G_2 are not translated immediately but are stored in an inactive form until the beginning of the next S phase. There must be a mechanism that prevents both translation and degradation of histone gene transcripts during the G_2 phase. In this report we show that the histone H4 mRNA is transported to the cytoplasm immediately after its transcription. It is stored as an inactive ribonucleoprotein complex and is subjected to a translational control that prevents it from being translated.

MATERIALS AND METHODS

Isolation of nuclear and cytoplasmic RNA fractions

RNAse-free glassware was used at all steps of the procedure. When possible the two RNase inhibitors VRC and RNasin were added to the buffers at a final concentration of 5 mM and 5 u/ml respectively. Nuclear and cytoplasmic RNA fractions were isolated as follows : macroplasmodia were harvested by scraping the culture off the filter paper and washed once in sterile water containing 0.2% DEPC. Each macroplasmodium was homogenized in 60 ml of buffer containing 0.25 M sucrose, 5 mM DTT, 5 mM $MgCl_2$, 10 mM Tris pH 8, 0.1% Triton X 100, VRC and RNasin. This procedure disrupts the plasma membrane but the nuclear membrane remains intact. The nuclei were sedimented over 10 ml of a sucrose cushion containing 1 M sucrose, 5 mM $MgCl_2$, 10 mM Tris, VRC and RNasin. The nuclear pellet was immediately resuspended in 10 ml of the lysis buffer used in the preparation of total RNA. All the following steps were as in the preparation of

total RNA. The cytoplasmic supernatant which was free of nuclei as judged by microscopic observation was made 5 M in guanidinium isothiocyanate by adding solid salt and all the following steps were as in the preparation of total RNA.

Cytoplasmic RNP fractionation

The cytoplasmic supernatant prepared as described above was applied to a linear 15% to 35% sucrose gradient in 10 mM Tris pH 8, 5 mM $MgCl_2$, 5 mM DTT, 5 mM VRC, 5 u/ml RNasin and centrifuged in a Beckman SW27 rotor for 17 h at 13000 rpm and 4°C. RNA was recovered from pooled gradient fractions by addition of sodium dodecyl sulfate to 0.5%, extraction with phenol, phenol-chloroform and precipitation with ethanol.

Measurement of the synthesis of H4 mRNA by in vivo 3H-uridine pulse labeling

To label the RNA, ^{3}H-uridine was added to the growth medium at a final concentration of 1 mCi/ml. Labeling was performed for 30 min and total RNA was immeditely extracted as indicated above. The specific activity of the RNA was approximately 2.10^4 cpm/µg.

The pulse-labeled RNA was hybridized in a 300 µl reaction volume to nitrocellulose filters to which pBR322 or pφH126 had been individually fixed. Plasmid pφH126 contains the pBR322 sequence and 600 bp of Physarum DNA containing the entire coding sequence of one of the H4 gene of Physarum and approximately 300 bp of non coding sequence. The hybridization conditions were identical to those used in the Northern blots. Each hybridization reaction contained approximately 10^6 cpm of RNA. After incubation for 40 h, the filters were washed, dried and counted in Beckman MP scintillation liquid. To evaluate the hybridization to the H4 gene the counts hybridized to pBR322 (between 20-40 cpm) were substracted from the counts hybridized to pφH126. The low number of counts bound to the filters to which pBR322 has been fixed indicates that in the hybridization conditions used very little non specific binding occurs. The rate of synthesis is expressed as the fraction of input cpm bound.

RESULTS

Transcription rates during the cell cycle

Transcriptional and post-transcriptional regulation of gene activity can be distinguished by comparing the accumulation of mRNA and its rate of synthesis. We have therefore analysed the rate of synthesis of

histone H4 message by in vivo pulse-labeling experiments. Physarum polycephalum macroplasmodia were pulse-labeled for 30 min with ^{3}H-uridine at time points falling in S phase, early G_2 or late G_2. After the pulse-labeling the radioactive RNA was extracted and hybridized to cloned DNA containing a histone H4 DNA sequence bound to nitrocellulose under conditions of DNA excess. The result of such an experiment is shown in figure 1. The rate of synthesis of H4 mRNA begins to increase in mid-G_2. During the second part of G_2 it increases approximately 20 fold over its minimum value in mid-G_2 and is maximum at the end of G_2. It decreases then immediately after entry of the cells into S. In figure 1 the level of histone H4 mRNA determined by Northern blot analysis is compared to the rate of

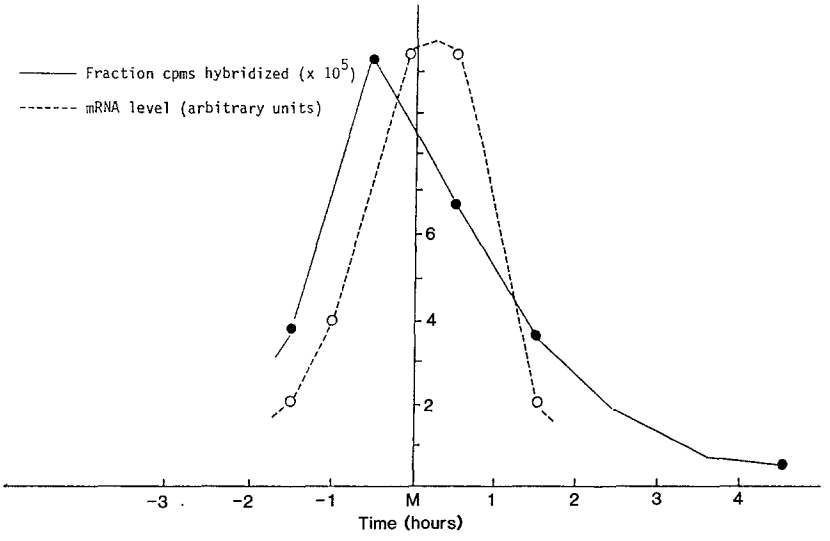

Figure 1

Periodic transcription and accumulation of histone H4 mRNA during the cell cycle.

The rate of synthesis was measured as described in materials and methods and is plotted against the mid-point of the 30 min pulse interval (———). The fraction cpm hybridized was measured as follows : the pulse-labeled RNA (imput cpm) was hybridized to nitocellulose filters to which pBR322 or pφH126 had been individually fixed (see materials and methods). After incubation the filters were washed, dried and counted. The counts hybridized to pBR322 (pBR cpm) were substracted from the counts hybridized from the counts hybridized to pφH126 (pφH cpm). The fraction cpm hybridized is the ratio (pφH cpm - pBR cpm)/input cpm. The data points shown are the average of duplicate points from two independent experiments The standard error varried between 10%-20%. The relative level of H4 mRNA was determined by densitometring the autoradiography of a Northern blot of aliquots of the RNA samples used to measure the rate of synthesis (----).

synthesis : the peak of accumulation occurs at the beginning of S, 30 min to 1 hour after the maximum rate of transcription. The H4 mRNA is still very stable in early S since it remains at its maximum level despite a decrease in the transcription rate. Only in mid-S phase is the histone mRNA destabilized. This result will be compared to previously published nuclear run-on experiments in the discussion section.

Storage of histone H4 mRNA

The histone H_4 mRNA which accumulates during late G_2 phase is not translated immediately. Therefore there must be a mechanism that allows the storage of the transcripts during the G_2 phase. We have tested several possibilities to find out how the histone messengers are stored and stabilized during the mitotic cycle of Physarum.

Polyadenylation

Total RNA prepared at three time points during the cell cycle (early S, mid-G_2 and late G_2) was fractionated into poly A^+ and poly A^- fraction by chromatography through a poly U-Sepharose column. Approximately 5% of the starting material was retained by the poly U-Sepharose and represents the poly A^+ fraction. The amount of H4 mRNA in poly A^+ and poly A^- RNA was determined by Northern blot analysis (figure 2A) : at the three time points more than 90% of the H4 mRNA was found in the poly A-fraction indicating that polyadenylation cannot explain the stability and inactivation of the histone mRNA during G_2.

Intracellular localization of histone H4 mRNA

The intracellular localization of histone H4 mRNA was determined by separating the cytoplasmic and nuclear RNA fraction as described in Material and Methods. The amount of H4 mRNA in each fraction was determined by Northern blot analysis. In figure 2B the result obtained with RNA isolated from macroplasmodia harvested in late G_2 (20 min before mitosis) and early S (20 min after mitosis) is shown. For both stages of the cell cycle the histone mRNA is mainly found in the cytoplasm. Thus histone mRNA is transported and stored in the cytoplasm immediately after its transcription. A nuclear localization of the message cannot explain the absence of translation during G_2.

Histone H4 cytoplasmic RNP

In (18), Laffler and Carrino have shown that the inactive histone transcripts stored in late G_2 phase are competent to be translated *in vitro* even though they are not translated *in vivo*. We have observed that in late G_2 the histone H4 mRNA is more resistant to the degradation by endogeneous nucleases. These observations suggest that in late G_2 the histone messenger RNA is protected from degradation by the inte-

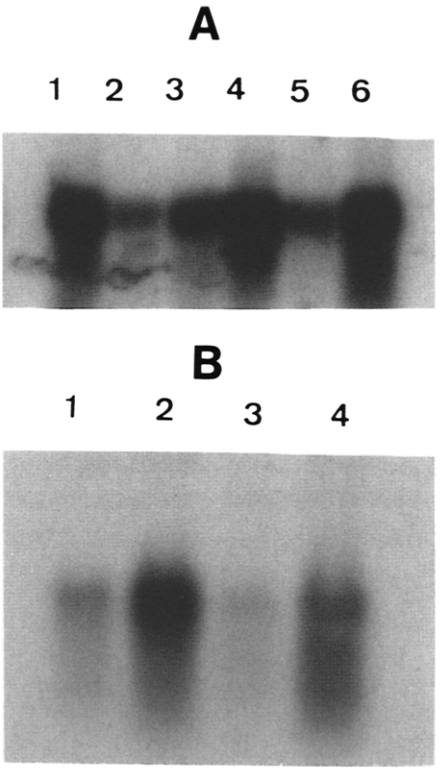

Figure 2

Northern blots of poly A^+ fractionated RNA and nuclear or cytoplasmic RNA fractions at different stages of the cell cycle.

A) Northen blot analysis of poly A^- and total cellular RNA at three stages of the cell cycle : late G_2 (mitosis - 20 min), mid G_2 (mitosis - 2 hours) and early S (mitosis + 30 min). Only the results obtained with poly A^- and total cellular RNA are shown since 5% or less H4 mRNA was found in the poly A^+ fraction.

Poly A^- RNA in late G_2 (1), mid G_2 (2) and early S (3) respectively. Total RNA in late G_2 (4), mid G_2 (5) and early S (6) respectively.

B) Intracellular localization of histone H4 mRNA in S and G_2. RNA was extracted from the nuclear or cytoplasmic fraction and analysed by Northern blot hybridization.

1 and 2 : G_2 phase nuclear and cytoplasmic RNA respectively.

3 and 4 : S phase nuclear and cytoplasmic RNA respectively.

raction with proteins and that the formation of a specific mRNA complex prevents the mRNA to be translated. To analyse the histone H4 ribonucleoproteins, the clear cytoplasmic supernatant obtained in G_2 or S was applied to a sucrose gradient as described in Materials and Methods. The gradient was collected and the RNA was prepared from pooled fractions along the sucrose gradient. The RNA was analysed by gel electrophoresis and blot hydridization with a histone H4 probe. In S phase the H4 mRNA is broadly distributed across the gradient (figure 3A). This

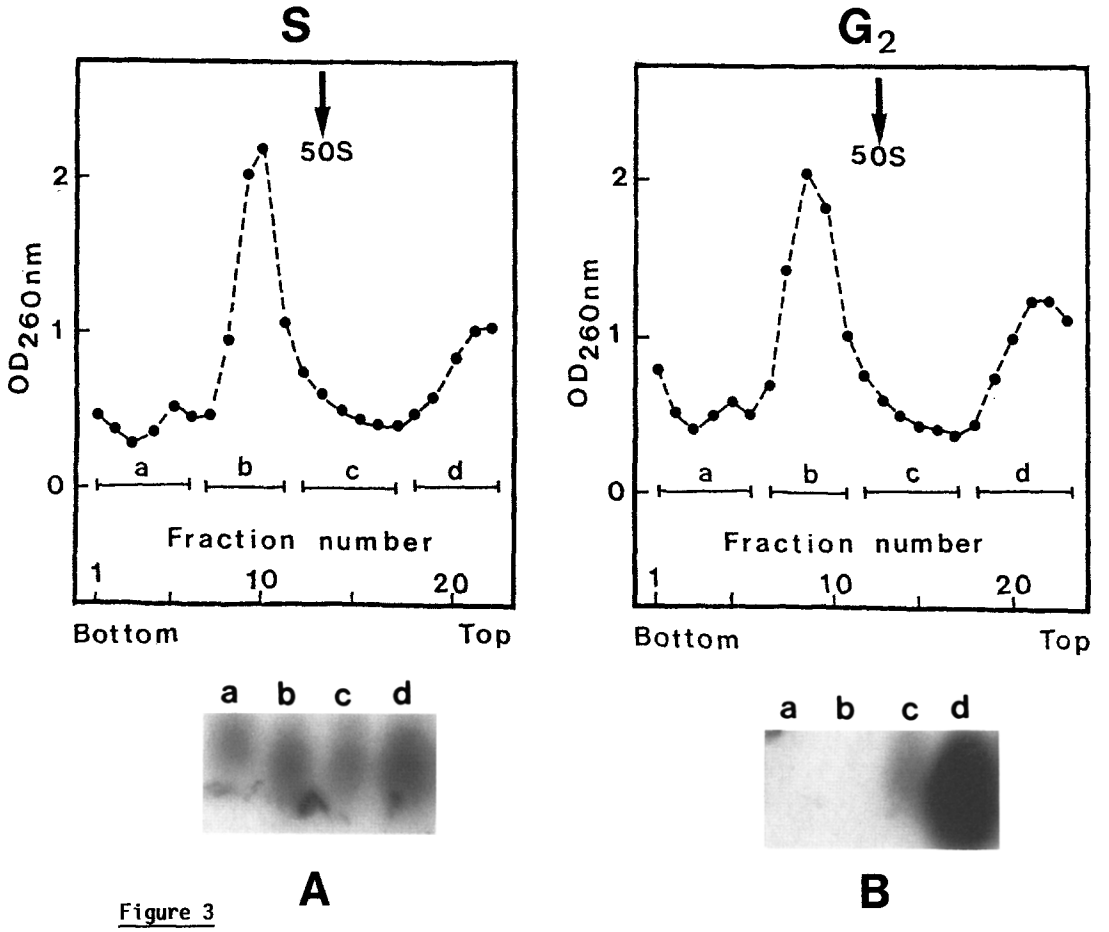

Figure 3

Sucrose gradient fractionation of S and G2 cytoplasmic fractions

S and G_2 phase cytoplasmic fractions were prepared and sedimented in a sucrose gradient as described in the text. The gradients were collected starting from the bottom. The optical density of each fraction was measured and the RNA of pooled fractions was analysed by gel electrophoresis and blot hybridization with a histone H4 probe. The peak of a 50 S marker of E. coli ribosomes is indicated by an arrow and was in fraction 13 of a parallel gradient.

A) S phase RNP. The Northern blot of pooled fractions 1-6 (a), 7-11 (b), 12-17 (c), 18-22 (d) is shown.

B) G_2 phase RNP. The Northern blot of pooled fractions 1-6 (a), 7-11 (b), 12-17 (c), 18-23 (d) is shown.

featureless distribution is expected if the histone mRNA is to be found in the polysomes. In contrast when late G_2 phase cytoplasmic supernatant is analysed in the same manner, the histone H4 mRNA sediments slower than 50 S (figure 3B). In view of the fact that this RNA is not translated and resistant to the degradation by endonucleases this result suggests that it is stored as a small ribonucleoprotein particle.

DISCUSSION

In Physarum polycephalum the triggering of histone H_4 gene transcription occurs during the G_2 phase of the mitotic cycle. This G_2 accumulation of histone transcripts is not substantially different from the observation that in other systems the transcription of histone mRNA increases at the end of G_1 (5-7). In yeast for example, activation of histone mRNA synthesis occurs in G_1 at a point that precedes or is concomitant with the cdc 7 function (6). In (5) it is shown that in a hamster fibroblast temperature sensitive cell cycle mutant (K1) the triggering of histone mRNA synthesis occurs late in G_1 and that it is synthesized at its maximum rate 3h to 5h before its peak of accumulation. Also lymphoma cells (S49) arrested in G_1 by cyclic AMP produce and contain significant levels of histone mRNA (7). Therefore the view (13) that events which normally occur in the G_1 period in most cell types are positionned in the latter part of the G_2 period in cell types which lack a G_1 phase, seems to be verified for histone gene expression in Physarum polycephalum.

We have measured the rate of transcription of the histone H_4 genes by in vivo pulse-labeling experiments and shown that it increases steadily during the second part of G_2. After entry of the cells in S, the histone gene transcription rate decreases and reaches a minimum value in early G_2. In a recent report Laffler and Carrino have measured histone H_4 messenger synthesis by an in vitro nuclear run-on assay and found that transcription continues until the end of S-phase. The variance between the in vivo and in vitro results may reflect an increase of histone mRNA turnover at the beginning of S which in turn explains the decrease of the level of histone mRNA after mid-S phase. This result, as well as others obtained in different systems (6-7, 9-12), indicates that transcriptional but also post-transcriptional mechanisms are used to control the histone mRNA level.

Taken together our results allow us to divide the cell cycle of Physarum polycephalum into three periods with respect to histone H_4 gene activity.

1) Histone gene transcription is initiated during the second half of G_2. During this first period the mRNA which accumulates is not translated into proteins and the amount of histone transcipts is mainly controled at the transcriptional level.

2) When the cells enter S-phase, histone proteins are synthesized. The level of histone transcripts is controled by post-transcriptional mechanisms. When newly synthesized histones are no more needed, the mRNA is destabilized and its level decreases to reach a basal level at the end of S.

3) Between the end of S-phase and the middle of G_2 (i.e. 5 hours after mitosis) the basal level of H_4 mRNA remains constant, the rate of H_4 mRNA synthesis is minimum and no newly synthesized histones can be detected.

Histone protein synthesis is limited to the S-phase. Thus transcripts that accumulate in late G_2 phase are stored in a translationally inactive state. A number of mechanisms are used in different species to store and stabilize histone messengers. In sea urchin the maternal mRNAs accumulate in the pronucleus of the haploid egg (14). It is stored as inactive nucleoproteins and released for translation during the early stages of embryogenesis (17). In amphibian oocytes the stored histone mRNAs exist in polyadenylated form. Polyadenylation may be partially responsible for the high stability of the histone mRNAs in the growing oocyte (15). Delayed accumulation of histone mRNA has also been reported during Drosophila oogenesis. In this latter case the stored mRNA appears to be associated with polysomes (16). We have shown that in Physarum the histone H_4 transcripts are transported to the cytoplasm immediately after their transcription and that they are stored as inactive small ribonucleoprotein particles. These specific mRNP complexes would prevent the histone mRNAs to be recruited in the polysomes and explain how histone gene expression is controled at the translational level in late G_2.

It would now be of great interest to identify the proteins which interact specifically with the histone mRNA and to understand how they contribute to the regulation of its translation.

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