CONTROL OF HISTONE GENE EXPRESSION IN PHYSARUM POLYCEPHALUM

I. PROTEIN SYNTHESIS DURING THE CELL CYCLE

P. N. SCHOFIELD AND I. O. WALKER

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

SUMMARY

Synchronous cultures of Physarum polycephalum were pulsed with [3H]lysine hydrochloride in S and G2 phases of the cell cycle. Plasmodial extracts were separated into nuclear, ribosomal and acid-soluble post-ribosomal cytoplasmic fractions. Core histones could be detected by staining in the nuclear fractions of both S and G2 phases, but were not detected by staining in the cytoplasmic fractions. Newly synthesized histone was present in S-phase nuclei but not in S-phase cytoplasm. The specific activity of newly synthesized histone in G2-phase nuclei decreased by at least 95% compared to S phase and no newly synthesized histone was observed in G2-phase cytoplasmic fractions. Thus histone synthesis is restricted to S phase. There are no free pools of histone in the cytoplasm of Physarum in either S or G2 phases of the cell cycle.

INTRODUCTION

Despite the manifest changes in morphology and metabolic patterns observed in eukaryotic cells as they progress through the cell cycle, the number of specific proteins whose pattern of synthesis changes is relatively small for both higher eukaryotes such as mammalian cells (Milcarek & Zahn, 1978), and lower eukaryotes such as yeast cells (Elliot & McLaughlin, 1978) and the slime mould, Physarum polycephalum (Sauer, 1978). By contrast, the histones are thought to be one of the few classes of proteins whose synthesis is apparently regulated during the cell cycle. The experiments of Robbins & Borun (1967) showed that in synchronized HeLa cells synthesis and deposition of histones onto DNA in the nucleus was restricted to S phase and did not occur in G2. This also appears to be the case in Physarum (Jockusch, Brown & Rusch, 1970). More recently, Groppi & Coffino (1980) have examined the distribution of histones in the nucleus and cytoplasm of Chinese hamster ovary cells and mouse S49 cells and concluded that histones are synthesized in G2 and S phases, but that the association of newly synthesized histones with DNA takes place only in S phase.

Most of the observations on the synthesis of histone protein and associated experiments on the transcription and translation of histone messenger RNA (Melli, Spinelli & Arnold, 1977; Stein et al. 1977) have been restricted to G2 and S phases of the cell cycle, apparently because synchrony in mammalian cell cultures is lost soon after the end of S phase (Stein et al. 1977).

The slime mould, P. polycephalum, can be grown under conditions in which it is
naturally synchronous in its nuclear divisions. Furthermore, this synchrony is
maintained over several cell cycles. In contrast to the mammalian cell lines described
above, the cell cycle in \textit{Physarum} is simpler in that there is no \textit{G}_0 or \textit{G}_1 phase, the
nuclei proceeding into \textit{S} phase immediately after mitosis (Mohberg & Rusch, 1971).
\textit{Physarum} therefore provides an opportunity to study the synthesis of proteins during
and after DNA replication. In the experiments described below we examine the
synthesis of histones during the cell cycle in \textit{Physarum}.

\textbf{MATERIALS AND METHODS}

\textbf{Growth labelling of the mould}

\textit{P. polycephalum}, strain M\textsubscript{1}CVIII, was routinely grown in liquid culture at 26 °C as described
previously (Daniel & Baldwin 1964). Synchronous surface plasmodia were grown on filter
papers in Petri dishes using the method described by Guttes & Guttes (1964). An inoculum
(0.4 ml) of microplasmodia harvested from liquid culture was layered onto the filter paper;
this was starved in the dark for 1.5 h and then fed with culture medium and grown at 26 °C.
The growth of the mould was followed by examining smears using phase-contrast microscopy
as described by Guttes & Guttes (1969). The cell cycle measured between mitosis I and
mitosis II varied between 10 and 11 h.

In each experiment involving the labelling of \textit{S} or \textit{G}_1-phase cultures, three identical cultures
were inoculated. Each culture reached mitosis I within a period of about 20 min. One culture
was then labelled about 15-20 min after it had passed through mitosis, with \textit{H}\textit{[lysine hydro-
chloride (0.5 ml; 0.5 mCi, Radiochemical Centre, Amersham, England) by adding the solution
directly to the surface of the plasmodium. It was grown for a further 4 h and then harvested.
This was the \textit{S}-phase-labelled culture. The second culture was then labelled with \textit{H}[lysine
hydrochloride (0.5 ml; 0.5 mCi) as before but 6 h into interphase after
mitosis I. It was harvested 3 h later. This was the \textit{G}_1-phase-labelled culture. The growth of
the third culture (as well as the labelled \textit{S} and \textit{G}_1 cultures) was observed at regular intervals
in the microscope. It reached \textit{M}_II about 10.5 h after \textit{M}_I.

\textbf{Isolation of plasmodial fractions}

Plasmodia were harvested by scraping the culture off the filter paper into ice-cold 0.25
M-sucrose, 10 mM-EDTA (pH 7.0); it was then washed once in this solution, then in ice-cold
distilled water, resuspended in 0.25 M-sucrose, 10 mM-Tris (pH 8.0), 10 mM-MgCl\textsubscript{2}, 0.1 %
Triton X-100 (20 ml), 0.5 mM-phenyl methyl sulphonyl fluoride and then lysed with 20 strokes
in a glass/Teflon homogenizer. This procedure disrupts the plasma membrane but the nuclear
membrane remains intact (Mohberg & Rusch, 1971).

Nuclei were sedimented by centrifugation at 2000 \textit{g} for 20 min through a 2 ml underlay of
1 M-sucrose, 5 mM-MgCl\textsubscript{2}, 10 mM-Tris (pH 8.0), 0.1 % Triton X-100. The nuclear pellet was
then resuspended in an equal volume of sample buffer (2 × conc.) containing 0.2 % sodium
dodecyl sulphate (SDS), 8 M-urea, 0.02 M-glycine, 0.1 % 2-mercaptoethanol (pH 10.0).

The cytoplasmic supernatant, which was free of nuclei as judged in the microscope, was
made 100 mM in MgCl\textsubscript{2}, 100 mM in KCl by adding solid salt and was then centrifuged at
160,000 \textit{g} for 120 min in a Beckmann 50 rotor. This procedure sedimented the ribosomes and
polysaccharide (slime) as a two-layered pellet. The lower ribosomal layer was resuspended
directly in an equal volume of sample buffer (2 × conc.).

The post-ribosomal supernatant was made 66 % in acetic acid and left at 2 °C for 0.5 h. It
was then centrifuged at 2000 \textit{g} for 20 min to remove insoluble material. The clear supernatant
is referred to as the acid-soluble, post-ribosomal supernatant fraction.
Histone synthesis in P. polycephalum

Gel electrophoresis

Polyacrylamide (18%)/SDS slab gels were used to resolve proteins in one dimension (Thomas & Kornberg, 1978). The procedure for two-dimensional gels was as follows. The first dimension was a tube gel in acid/urea (pH 2-7, 2.5 M-urea) prepared according to Panyim & Chalkley (1969). After running the proteins into this gel, the gel was cut in half longitudinally and equilibrated for 20 min in glycerol (10%, w/v), 0.1% β-mercaptoethanol, 0.02 M-glycine, 8 M-urea, 0.2% SDS, pH 10.0 at room temperature and then frozen at −20 °C overnight. One half of this gel was then placed in a slot cut in a polyacrylamide (18%)/SDS slab gel, and stuck using 1% agarose in 4% SDS, 0.05 M-Tris (pH 6.8), 0.005% bromophenol blue. The proteins were then electrophoresed into the polyacrylamide/SDS gel. The optical density of stained protein bands was measured on a Joyce–Loebl Mark III double-beam densitometer.

Fluorography

Fluorography of gels dried down onto filter paper was carried out as described previously (Bonner & Laskey, 1974). One-dimensional gels were exposed for 8 days and two-dimensional gels for 9 weeks. The density of film blackening was measured on a Joyce–Loebl Mark III double-beam densitometer.

RESULTS

Preliminary experiments showed that calf thymus histones (core histones plus H1) when added to the plasmodium before homogenization, were extracted in the acid-soluble post-ribosomal supernatant fraction, as expected. There was no evidence of degradation of the exogenous histones.

Analysis of S-phase plasmodia

A solution of [3H]lysine (0.5 ml; 0.5 mCi) was added directly to the surface of a synchronous plasmodium 0.5 h after mitosis I. The plasmodium was allowed to grow for 4 h after which it was harvested and homogenized. The nuclear, ribosomal and acid-soluble post-ribosomal supernatant fractions were prepared as described above.

The nuclei were dissolved in an equal volume of sample buffer and electrophoresed on one-dimensional polyacrylamide/SDS gels. The gels, stained with Coomassie Blue, are shown in Fig. 1 A. The core histones migrate as three distinct bands since H2B and H3 co-migrate as one in this gel system. H1 is not visible in the S-phase nuclear gels. The four major bands migrating behind the core histones are proteins associated with nucleolar precursor ribosomal RNA (K. S. Davies & I. O. Walker, unpublished observations). These are shown as RNP proteins in Figs. 1 and 2. All the histones detectable by staining in the nucleus are combined with DNA in the chromatin complex, since no free histone can be detected in isolated nuclei at salt concentrations less than 0.25 M in ionic strength. Even then the only histone found not complexed to DNA up to ionic strengths of 0.7 is H1 (H. M. Cruickshank & I. O. Walker, 1981, and unpublished observations).

The fluorogram of this gel is shown in Fig. 1 B. The major radioactive bands correspond to the core histones and the proteins associated with nucleolar precursor rRNA. No bands corresponding to the histone H1 doublet could be seen by eye,
neither were there bands visible in the densitometer scan of the gel (Fig. 2). We conclude that core histones are synthesized during the 4 h period following mitosis I; that is, during S phase. *Physarum* histone H1 appears to be degraded very rapidly using the extraction conditions described here and consequently no information on its synthesis was obtained.

The specific activity of the core histones was estimated in arbitrary units by dividing the total sum of the areas in the densitometer scan of the radioactive gels by
Histone synthesis in P. polycephalum

Fig. 2. A. Densitometer scan of track (c) in Fig. 1B, S-phase. B. Densitometer scan of track (f) in Fig. 1B, G1-phase. The hatched areas in the G1 scan have been attributed to histones. RNP proteins are the proteins associated with the nucleolar ribonucleoprotein particles.

Fig. 3. The variation of the area depicted in the densitometer scans of Coomassie Blue-stained gels shown in Fig. 1A and the area in the densitometer scans of the fluorogram shown in Fig. 1B as a function of concentration for the three core histone bands: (▲) H2B/H3; (□) H2B/H3; (●) H4; (○) H4; (△) H2A.
the total area under the core histones in the stained gels. Precautions were taken to ensure that the amount of stain on the gel (and hence the area recorded on the densitometer) was directly proportional to the amount of protein, and that on the fluorograph, the film blackening caused by the radioactive decay was, again, proportional to the amount of protein. That this is so is shown by the graphs in Fig. 3 where...
Histone synthesis in P. polycephalum

stain density and film blackening (both measured in terms of optical density by the densitometer and converted to area) are plotted as a function of protein concentration. By this method the specific activity of the S-phase core histones averaged over the three histone bands is $2.2 \times 10^{-5}$ c.p.m./unit area.

One-dimensional polyacrylamide/SDS gels of the S-phase-labelled ribosomal pellet, solubilized in SDS, are shown in Fig. 4A, together with a total nuclear lysate and a control of the ribosomal pellet, plus nuclear lysate to identify the positions of the histones. A comparison of these three gels shows that there are no bands corresponding to histones H2A and H4 in the ribosomal pellet. There is a band that co-migrates with the histones H2B/H3, which run together in this system. Since the lysine-rich histone pair (H2A/H2B) and the arginine-rich pair (H3/H4) are strongly associated in vitro, and almost certainly in vivo, and since one of each of these pairs is absent from the ribosomal pellet gels, it is unlikely that the faint band co-migrating with H2B/H3 in the ribosomal pellet proteins represents these histones. We conclude that histones cannot be detected by stain in the ribosomal pellet fraction and therefore do not exist in the cytoplasm in the form of a complex with acidic protein, as found in nuclei of Xenopus oocytes (Earnshaw, Honda, Laskey & Thomas, 1980), since this would have pelletted under the experimental conditions used here.

The fluorogram of the ribosomal pellet proteins is shown in Fig. 4B, together with the densitometer scan of the gel in Fig. 5. The pattern of radioactive intensity closely matches the pattern of bands on the stained gel. There is no evidence for radioactively labelled histones in this fraction.

The two-dimensional gel of the acid-soluble post-ribosomal supernatant proteins from an asynchronous culture is shown in Fig. 6A. About 70 different proteins can be identified. Comparing this with the same gel to which calf thymus histones have been added (Fig. 6B) shows that endogenous histones cannot be detected by staining in
Fig. 6. A. Two-dimensional gel of acid-soluble post-ribosomal supernatant proteins isolated from an asynchronous culture. B. The same extract with added calf thymus histones (176 μg extract and 5 μg histones); both stained with Coomassie Blue.

C. Fluorogram of two-dimensional gel of acid-soluble post-ribosomal supernatant proteins labelled in S-phase. The area where core histones should appear is circled by a solid line.

D. Fluorogram of two-dimensional gel of acid-soluble post-ribosomal supernatant proteins labelled in G₄. The area where core histones should appear is circled by a solid line. Proteins u and v (together with r, s and t) have been used to locate the positions of the core histones in each gel.
Histone synthesis in P. polycephalum

The amount of histone that should be present, relative to total protein, if there were a free pool of histone in the cytoplasm may be calculated as follows. The DNA/protein ratio in the plasmodium is 1:40 (Mohberg & Rusch, 1969). As there is 1 pg of DNA per nucleus and the histone/DNA ratio is 1:1, (w/w) (Mohberg & Rusch, 1971), 2-5 % of cell protein is histone. Given that the quantity of histone must double during the cell cycle, this amount of histone must exist as a pool if synthesis is continuous through the cycle. This quantity of histone (i.e. 2-5 %) should have been detectable, since in the control gel exogenous histones have been added to 2-8 % and are clearly visible. We conclude that histones are not detectable by staining in interphase cytoplasm. Consistent with this, no histones could be detected by staining in the two-dimensional gel of a synchronous culture in S phase. In all respects the pattern of spots seen on the S-phase gel was similar to the asynchronous culture shown in Fig. 6A. The fluorogram of the S-phase gel is shown in Fig. 6C. The position expected to be occupied by radioactive histones is blank. We conclude that histones cannot be detected by staining or radioactive labelling in the cytoplasm of S-phase plasmodia.

Analysis of G₂ phase plasmodia

A solution of [³H]lysine (0.5 ml; 0.5 mCi) was added to the surface of a synchronous plasmodium 6 h after mitosis I. The plasmodium was allowed to grow for 3 h and was harvested about 1 h before mitosis II. The nuclear ribosomal and post-ribosomal supernatant fractions were prepared as described above.

The one-dimensional polyacrylamide/SDS gels of the nuclear fraction, stained with Coomassie Blue, are shown in Fig. 1. Similar amounts of protein were loaded into each gel slot as in the S-phase experiment. As expected, the six major polypeptides visualized on the gel are the four core histones and the three proteins associated with the nucleolar precursor rRNA. The stained patterns of S- and G₂-phase nuclear proteins are identical. As before, with the S-phase gel, H1 histone is not detectable.

The fluorogram of G₂-phase-labelled nuclear proteins (Fig. 1B) and the densitometer scans (Fig. 2) show that, whereas radioactive lysine is incorporated into the three proteins associated with the nucleolar precursor rRNA, the incorporation into the core histones is extremely low. The specific activity, measured in the same way and expressed in the same units as the S-phase-labelled histones, is 1.25 x 10⁻⁴ c.p.m./unit area. This value is maximal because of the difficulty in drawing the baseline for the areas under the very small peaks presumed to represent the histone bands (see Fig. 2). Thus, we conclude that there is at least an 18-fold decrease in newly synthesized histone protein appearing in the nucleus between S and G₂.

The one-dimensional polyacrylamide/SDS gels of the G₂-phase-labelled ribosomal pellet are shown in Fig. 4A. The stained gel pattern is almost identical to the gel pattern obtained in S phase. Qualitatively, the only difference appears to be a high molecular weight band (marked x in Fig. 4A, which is present in the G₂-phase gel but absent in S phase. The fluorogram of this gel (Fig. 4B) and the corresponding scans (Fig. 5) show that the newly synthesized protein patterns are again similar but
not identical. One high molecular weight protein appears to change its relative specific activity \((y)\) and a new protein appears in \(G_2\) (\(x)\). As in \(S\) phase, there are no bands that can be attributed unequivocally to core histones in either the stained gel or the radioactive gel.

The two-dimensional gel, of the acid-soluble post-ribosomal supernatant fraction stained with Coomassie Blue, was qualitatively similar to that obtained for the asynchronous interphase plasmodium (Fig. 6A). No histones could be seen on the gel. The fluorogram of the \(G_2\)-phase gel shows a pattern very similar to that obtained for the \(S\)-phase gel (Fig. 6B). The relative intensity of the spots \(r, s\) and \(t\) compared to that of spot \(u\) apparently decreases, though as all three are detectable in the \(G_2\)-phase pattern it suggests that incorporation of label, i.e., protein synthesis, is reduced but not switched off, on going from \(S\) phase to \(G_2\). Other small differences in the patterns can be seen, but because of fainter intensity these are not as unambiguous as the three major changes. No radioactive histones are visible on the fluorogram. We conclude, as with the \(S\)-phase experiment, that histones cannot be detected by staining or radioactive labelling in the cytoplasm of \(G_2\)-phase plasmodia.

**DISCUSSION**

*P. polycephalum* has several advantages over other experimental systems for studying events during the cell cycle. Firstly, it is naturally synchronous in its mitotic divisions and is therefore presumed to be synchronous in interphase. The synchrony is maintained over at least three cell cycles. It is easily grown, has a cell cycle of 9–10 h and rapidly takes up exogenous substrates. These properties have been exploited in the present study to investigate the synthesis of histones during the cell cycle. Replication of DNA (\(S\) phase) in *Physarum* commences immediately after mitosis (there is no \(G_0\) or \(G_1\) phase) and continues for 3–4 h of a 9–10 h cycle. This is followed by \(G_2\), which lasts to the next mitosis.

In the experiments described above we added a radioactive pulse of \([\text{H}]\)lysine for 4 h in \(S\) and for 3 h in \(G_2\) phase. The plasmodia were then separated into three fractions: nuclei, ribosomal pellet and acid-soluble post-ribosomal supernatant in order to simplify the protein gel patterns and also to gain information on the distribution of histone pools in the mould. An analysis of the fractions isolated after pulsing in \(S\) phase showed that: (1) cold histones could be detected in the nucleus only where they are bound to DNA in chromatin; (2) histones were newly synthesized in \(S\) phase; and (3) these newly synthesized histones could only be detected in the nucleus. These results show that there is no free pool of histones in the cytoplasm during \(S\) phase. They imply that newly synthesized histone is rapidly transported into the nucleus where it combines with replicating DNA to form chromatin. Our results do not rule out the possibility of a fraction of the newly synthesized histone existing as a free pool in the nucleus during part of the cell cycle. An analysis of the plasmodial fractions isolated after pulse-labelling in \(G_2\) showed that the amount of newly synthesized histone in the nucleus had decreased to less than about 5% of its value in \(S\) phase; no newly synthesized histone could be detected in the cytoplasmic fractions. As in
Histone synthesis in *P. polycephalum*

*S* phase, cold histones could not be detected in the cytoplasmic fractions but only in the nucleus, where they were combined with DNA in chromatin.

These observations, taken together, lead to the following conclusions. At all stages of the cell cycle, histones are found only in the nucleus of *Physarum*, where they are associated with DNA in the chromatin complex; there are no detectable amounts of histones in free pools in the cytoplasm. Over 95% of the newly synthesized histones are made in *S* phase and their synthesis would therefore appear to be tightly coupled to the replication of DNA. After synthesis in the cytoplasm these histones must move very rapidly into the nucleus. Furthermore, the histones are metabolically stable and there is no significant turnover during the cell cycle.

The small amount of new histone synthesis that can be detected in *G₂*, and that amounts to no more than 5% of the synthesis detected in *S* phase, has several possible explanations. It may represent the basal histone synthesis reported by Wu, West & Bonner, (1981) in cycling Chinese hamster ovary cells in tissue culture. On the other hand, it more likely represents, at least in part, histone synthesis associated with the replication of nucleolar ribosomal DNA, which occurs in *Physarum* independently of the rest of the DNA and takes place in both *S* and *G₄* phases (Newlon, Sonenshein & Holt, 1973).

The pattern of histone synthesis in *Physarum* thus appears to be similar to that first reported by Robbins & Borun (1967) for HeLa cells. It is also in agreement with the results of Jockusch *et al.* (1970), who looked at synthesis of protein in the nuclei of *Physarum*. On the other hand the absence of measurable pools of histone protein in the cytoplasm of *Physarum* contrasts with the situation found in S49 and CHO cells, where large cytoplasmic pools of histones occur throughout *G₁* and *S* phases of the cycle (Groppi & Coffino, 1980) and equivalent rates of synthesis take place during these phases. However, the behaviour of *Physarum* and the mammalian cell lines may not be strictly comparable. One of the difficulties in comparing these various systems is that observations on synchronized mammalian cells have usually been confined to *G₁* and *S*, apparently because the cells have begun to lose synchrony by the time they reach *G₄*. In *Physarum* there is no *G₁*, the system proceeding into *S* immediately after mitosis. It is clear that in the slime mould, net synthesis of histones only takes place during *S* phase and that these newly synthesized histones move rapidly into the nucleus. These observations suggest that the production of histone protein is strictly coupled to the synthesis of DNA. Just how this control is exerted, whether at the level of transcription or translation, is at present under study.

The authors wish to acknowledge the excellent technical assistance of Mrs A. Watson.

REFERENCES


(Received 16 February 1982)