Histone gene expression in *Physarum polycephalum* 2

Coupling of histone and DNA synthesis

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The addition of hydroxyurea to synchronously growing macroplasmodia of the slime mould, *Physarum polycephalum*, inhibited DNA synthesis by up to 90%. Histone protein synthesis was inhibited to the same extent. The synthesis of other nuclear proteins was not inhibited even though total RNA synthesis was inhibited. The results suggest that histone synthesis and DNA replication are tightly coupled.

Histone synthesis *DNA replication* Physarum polycephalum

1. INTRODUCTION

In many living systems the synthesis of histones appears to be tightly coupled to the synthesis of DNA [1–4]. The control of this coupling process is poorly understood but is thought to take place mainly at transcription, although there is some evidence to suggest that it is exerted at a post-transcriptional level in many somatic cells [5–8], possibly depending on the availability of cytoplasmic histone mRNA.

In a previous study we have shown that histone protein synthesis in *Physarum polycephalum* is temporally restricted to S phase of the cell cycle. Furthermore, no free pools of histone could be detected at any stage of the cell cycle [9]. Here we describe the results of an investigation to see how tightly coupled histone synthesis is to DNA replication in *Physarum*. We conclude that the two processes are closely linked together.

2. MATERIALS AND METHODS

Physarum polycephalum, strain M3C VIII, was routinely grown in liquid culture [10]. Synchronous cultures were prepared as in [11] and the cell cycle monitored by biopsy using phase contrast microscopy. The cell cycle time between mitosis II and mitosis III varied between 9 and 10 h.

Radiolabelling of DNA was carried out by direct additions of 1 mCi of [^{3}H]thymidine (20 Ci/mmol, Amersham International) onto the surface of the synchronously growing mould at mitosis II. Small samples of labelled culture were cut from the surface of the plasmodium using a circular cutter. These were lysed in 5% trichloroacetic acid and the solution centrifuged at $10000 \times g$ for 15 min. The precipitate was washed extensively with cold trichloroacetic acid, dried, redissolved in water (200 μl) and counted in 10 ml of Unisolve scintillant (Koch-Light). The results are expressed as counts incorporated per unit mass of mould.

The amount of mould is determined from the absorbance at 360 nm of the supernatant from the trichloroacetic acid precipitation. This is the maximum of the absorption curve of the yellow pigment which colours the mould [12]. It is assumed that the amount of pigment is directly proportioned to the mass of the mould.

Radiolabelling of RNA was carried out by adding 0.5 mCi of [^{3}H]uridine (45 Ci/mmol, Amersham) directly onto the surface of the mould at times described in section 3. After the labelling period, samples were frozen in liquid nitrogen,

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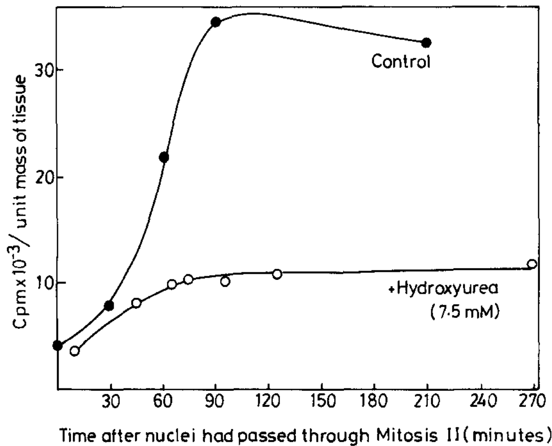

Fig.1. Incorporation of [^{3}H]thymidine into synchronous cultures. [^{3}H]Thymidine and hydroxyurea were added at mitosis II.

homogenised and digested for 2 h at 37°C in 1% SDS, 2.5% Sarkosyl (BDH), 2 mM EDTA, 0.1 M NaCl, 20 mM Tris, 1 mg/ml Protease K (Boehringer) pH 7.6, before ethanol precipitation at −20°C. The specific activity of the RNA was determined by measurement of the absorbance at 260 nm and scintillation counting.

Radiolabelling of histone was performed by adding 200 µCi of [^{3}H]lysine hydrochloride (92 Ci/mmol, Amersham) directly onto the surface of the mould. Nuclei were prepared as in [9] after a given period of labelling and the pellet was immediately resuspended in sample buffer for electrophoresis.

Electrophoresis on 18% polyacrylamide gels and quantitative analysis of the bands were as in [9]. Fluorography of gels dried onto Whatman no.3 paper was as in [14].

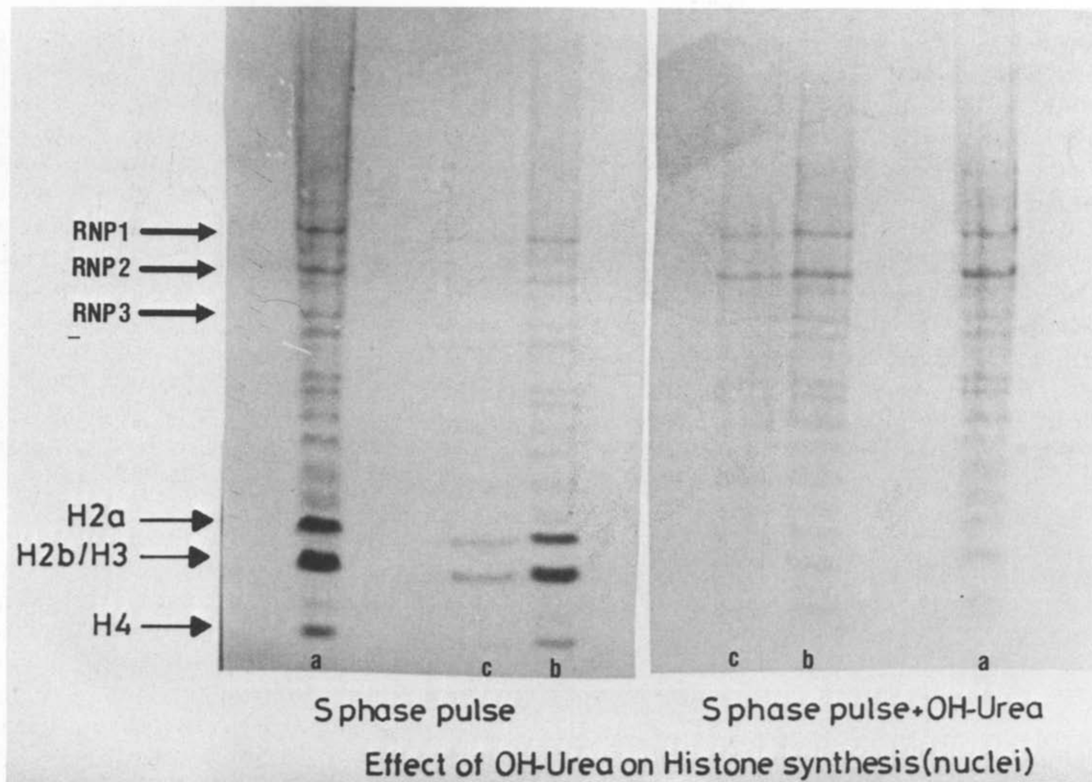

Fig.2. Fluorographs of whole nuclei displayed on 18% SDS gels. Three separate loadings are shown for the control and hydroxyurea (OH-urea)-treated cultures: (a) 100 units; (b) 50 units; (c) 25 units of protein concentration (in arbitrary units).

3. RESULTS AND DISCUSSION

The incorporation of [^{3}H]thymidine into DNA is shown in fig.1. The curve is sigmoidal and tends to a maximum between 2 and 4 h. The relatively low rate of incorporation at early times presumably reflects, at least in part, the rate of uptake of the label into the plasmodium. In all respects the curve is similar to previously reported data [15–17]. The effect of hydroxyurea, added at mitosis, is to reduce the incorporation of [^{3}H]thymidine into DNA by about 80% at 7.5 mM and 90% at 40 mM. Synthesis of DNA is completely inhibited after 75 min. These observations are in good agreement with previously reported data [17]. The lag between application of the drug and its effect on DNA replication even after extensive preincubation was also noted in [17].

The mechanism by which hydroxyurea affects DNA synthesis is generally accepted to be the inhibition of the enzyme ribonucleoside diphosphate reductase [17,18]. However, the low level of DNA synthesis observed during the 45-min period after adding hydroxyurea cannot be completely explained simply by the utilisation of the endogenous pool of deoxyribonucleotides, and it is possible that hydroxyurea has a more complex effect than simple inhibition of the enzyme. Authors in [24] suggested that hydroxyurea interferes with the processing of early replication products preventing the formation of longer intermediates. Whatever the mechanism, it is clear that hydroxyurea has a dramatic effect on DNA synthesis in *Physarum* which is quantitatively similar to that found in other organisms.

The incorporation of [^{3}H]lysine into core histones at 7.5 and 40 mM hydroxyurea is shown in fig.2,3 and table 1,2. The label was added 45 min after mitosis II and the nuclear proteins were isolated 3.25 h later. As shown previously the core histone bands in the control incorporate lysine during this period, confirming that histone synthesis takes place during S phase [9]. In the hydroxyurea-treated plasmodium, the incorporation of label into the histones is greatly reduced. For example, the specific activity of H2a is 24% of control values at 7.5 mM hydroxyurea, and 11% at 40 mM.

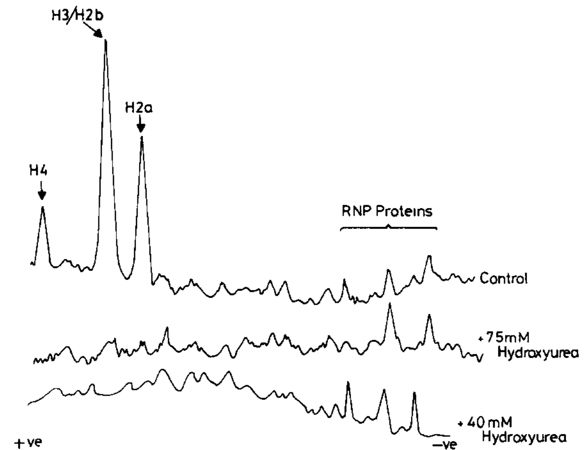

Fig.3. Densitometric traces of nuclear fluorographs shown in fig.2 (track (b) in each case) together with the experiment carried out with 40 mM hydroxyurea (at a protein concentration equivalent to (b) in fig.2).

The specific activity of the proteins associated with the nucleolar ribonucleoprotein particles (labelled as RNP proteins in fig.2) doubles at

Table 1

Relative activities of major proteins

Conditions	RNP_1	RNP_2	RNP_3	H2a	H2b/H3	H4
Control	0.59	0.60	0.85	4.3	3.6	2.6
7.5 mM Hydroxyurea	1.74	1.34	1.81	1.05	0.532	–
40 mM Hydroxyurea	0.72	0.97	1.008	0.47	0.27	–

Relative activities were obtained by integrating the peak areas on densitometric traces as in [9] and are in arbitrary units. The values for H4 in hydroxyurea were not determined. RNP_1, RNP_2 and RNP_3 are the 3 proteins associated with the nucleolar ribonucleoprotein particles [9]

Table 2

Residual synthesis of major proteins

Conditions	% Residual synthesis	
	H2a	H2b/H3
7.5 mM Hydroxyurea	24	15
40 mM Hydroxyurea	10.9	7.5

7.5 mM hydroxyurea and is the same as the control at 40 mM hydroxyurea.

Previous experiments [9] have shown that during a pulse of this time, the specific activity of the RNP proteins remains about the same in both S and G2. The perturbation observed here at low hydroxyurea concentrations could possibly reflect an accelerated transport of newly-made RNP proteins from the nucleus or a decreased rate of loss of labelled proteins from the nucleus, caused indirectly by the effect of the drug on RNA synthesis [17]. Another possibility is that it results from a difference in specific activity of precursor pools.

These results strongly suggest that hydroxyurea does not inhibit nuclear protein synthesis in general. This in turn would imply that RNA synthesis was unaffected by hydroxyurea. However, it has been found [17] that 40 mM hydroxyurea (the higher of the two concentrations used in this study) strongly inhibits RNA synthesis in early S but has a smaller effect in late S. In order to see whether a similar pattern of inhibition was present at the lower concentration of hydroxyurea here (7.5 mM), plasmodia were pulsed with 0.5 mCi of [^{3}H]uridine as described in the legend to table 3. There was a marked decrease in the incorporation of label into RNA in early S corresponding to 65% inhibition. In the late S phase, the inhibition had decreased to 30%. These results exactly parallel those found in [17] and show that the pattern of RNA inhibition at both low (7.5 mM) and high (40 mM) concentrations of hydroxyurea are the same. In our experiments we have not taken into account the small proportion of labelled uridine (15%) which is incorporated into DNA and which will co-precipitate with RNA in the trichloroacetic acid-insoluble fraction. Neither have we distinguished between label incorporated into mRNA and rRNA. However, given the close parallels between our results and those in [17] for both DNA and RNA synthesis, it is reasonable to conclude that there will be some inhibition of both mRNA and rRNA synthesis, at least in the early S phase. Nevertheless, this does not explain the exaggerated depression in histone protein synthesis as compared to other nuclear proteins. Given this, it appears that either there is a specific mechanism controlling translation or transcription of histone mRNA, or that histone mRNAs have an extremely short half life during S phase as compared to other nuclear proteins. We conclude that DNA and histone synthesis are closely coupled. Our findings are not consistent with those in [23] who showed that histone:DNA ratios increased as much as 3-fold after prolonged treatment with FUDR. Bearing in mind that there are no free pools of histone in the nucleus or cytoplasm [9] their observations suggest that histone and DNA synthesis are uncoupled in *Physarum*.

These results, taken together, suggest that there is a continual monitoring of the rate of DNA synthesis to allow the control of parallel histone synthesis. Thus an increased degree of inhibition of DNA synthesis is paralleled by an increased inhibition of histone synthesis. Furthermore, at both concentrations of hydroxyurea used, there exists a lag between administration of the drug and complete cessation of DNA synthesis. Whatever the cause of this lag, its existence enables us to differentiate between the effects of initiation of DNA replication and the effect of ongoing replication, on histone synthesis. In the presence of the drug,

Table 3

Inhibition of RNA synthesis by hydroxyurea

Labelling time	Specific activity of RNA (cpm/μg)	
	+7.5 mM OH-urea	Control
30 min	6035	17886
150 min	15869	22723

7.5 mM Hydroxyurea was added to one of two synchronous cultures at mitosis. 15 min later 0.5 mCi of [^{3}H]uridine was added to each; 30 min later half of each plasmodium was harvested and RNA prepared; 150 min after labelling, the remaining half plasmodia were harvested

even though initiation of apparently normal S phase events in terms of DNA replication has occurred, once DNA synthesis is inhibited, histone synthesis is also effectively inhibited.

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