CONTROL OF CHROMATIN TRANSCRIPTION BY A CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE-STIMULATED PHOSPHORYLATION IN *NEUROSPORA CRASSA*

Norberto D. JUDEWICZ and Héctor N. TORRES

Instituto de Investigaciones Bioquímicas 'Fundación Campomar' y Facultad de Ciencias Exactas y Naturales, Obligado 2490, 1428 Buenos Aires, Argentina

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1. Introduction

Evidence indicates that non-histone acidic proteins of chromatin play an important role in the control of gene expression in eucaryotic organisms [1,2]. The extent of phosphorylation of such proteins strongly influences transcriptional activity of reconstituted chromatin preparations [3–6].

On the other hand the extent of phosphorylation of such proteins has been correlated with cell maturation and responses to growth stimulants [7,8].

More recently, specific measurements of gene transcriptional activity indicated that phosphorylation of non-histone proteins regulates transcription of histone genes [9].

The morphological phenotype of the ascomycete fungus *Neurospora crassa* seems to be under the control of cyclic AMP [10]. The studies have been simplified by the discovery of mutant strains deficient in adenylate cyclase activity [11,12]. However the molecular basis of the cyclic AMP effect on *Neurospora* morphology remains unknown. An interesting possibility could be that the cyclic nucleotide might influence the extent of phosphorylation of chromatin proteins.

This paper reports studies indicating that cyclic AMP increases phosphorylation of non-histone proteins in *Neurospora* chromatin preparations. In turn, phosphorylation of such proteins stimulates endogenous transcriptional activity sensitive to α-amanitin.

2. Materials and methods

2.1. *Strains and culture media*

Slime strains of *Neurospora* having wild-type (FGSC 1118) or negligible levels (FGSC 326) of adenylate cyclase activity were used. These strains were grown in Vogel's medium [13] supplemented with 7.7 g per liter nutrient broth, 7.5 g/l yeast extract and 20 g/l sucrose, at 30°C with gentle shaking. After 48 h, cultures were collected by centrifugation.

2.2. *Preparation of nuclei*

The cell pellets were resuspended in 10 vol. 0.02 M sodium phosphate buffer (pH 6.5) containing 15% Ficoll (w/v) and homogenized using a glass Dounce-type homogenizer (3 strokes with the loose pestle and 3 strokes with the tight one). After centrifugation at 3000 × *g* for 15 min the supernatant containing the nuclei was purified by centrifugation on a Ficoll cushion as in [14].

2.3. *Phosphorylation of nuclear proteins*

The incubation mixture contained 50 mM Tris–HCl buffer (pH 6.8), 0.25 mM [γ-^{32}P]ATP (spec. act. 10–40 cpm/pmol), 8 mM $MgCl_2$, 10 mM NaF, 2.5 mM theophillyne, 0.05 mM cyclic AMP, 12.5% glycerol (v/v) and nuclei (~26 mg protein/ml incubation mixture, total vol. 0.1 or 1.0 ml). Incubations were performed at 30°C for the indicated periods and the reactions were stopped as described below.

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2.4. *Distribution of ^{32}P radioactivity in different nuclear protein fractions*

To measure total nuclear labeling, reactions (0.1 ml total vol.) were stopped by the addition of 2 ml of a cold 10% trichloroacetic acid solution. After centrifugation at 3000 × *g* for 10 min, the precipitated material was redissolved, reprecipitated and counted for radioactivity as in [15].

To study the distribution of ^{32}P in different protein fractions (1.0 ml reaction mixtures), incubations were stopped by cooling in an ice–water mixture and centrifugation on a 13 ml, 2 M sucrose cushion for 2 h at 24 000 rev./min (SW 40 Beckman rotor). The nuclear sediment was resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 25% glycerol (v/v), 5 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM dithiothreitol and centrifuged for 10 min at 7500 × *g*. The nuclear sediment thus obtained was homogenized in 10 vol. solution containing 50 mM Tris–HCl buffer (pH 6.8), 80 mM NaCl and 20 mM EDTA. 'Free', 'loosely bound' and 'tightly bound' nuclear protein fractions were obtained as in [16]. Histones were isolated by extraction with 0.2 M HCl for 30 min at 0°C.

For transcription experiments the material obtained after extraction of 'free' and 'loosely bound' nuclear proteins, was used as a source of enzyme and template activity.

Radioactivity in the different fractions was determined after precipitation with 10% trichloroacetic acid, filtering through Whatman GF/C filters and counting in a 0.4% Omnifluor (New England Nuclear) solution in toluene. Insoluble chromatin material was dissolved in 1 N NaOH and counted in Bray's solution.

2.4. *Measurement of RNA synthesis*

The incubation mixture contained: 50 mM Tris–HCl buffer (pH 8.0), 5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 5 mM dithiothreitol, 80 mM $(NH_4)SO_4$, 0.4 mM ATP, CTP (or UTP) and GTP, 0.004 mM [^{3}H]UTP (or CTP) (spec. act. 2 × 10^6 cpm/nmol), 12.5% glycerol (v/v) and chromatin (~1.0 mg protein). Incubations were carried out at 30°C for the indicated periods (total vol. 0.1 ml). Reactions were stopped by the addition of 2 ml 10% trichloroacetic acid. The insoluble material was collected on filters and counted for radioactivity as indicated above.

2.5. *RNA isolation and centrifugation on sucrose gradients*

Total cellular RNA was extracted by a modification of the procedure in [17]. Cells were homogenized in 10 vol. 0.01 M Tris–HCl buffer (pH 7.5) containing 1.0 mM EDTA. Immediately, 0.1 vol. 10% SDS plus 1 vol. 1:1 phenol–chloroform solution (v/v) were added and mixed. The mixture was extracted 3 times with a Tris–NaCl–SDS–EDTA solution as in [18]. The aqueous phase was then precipitated by the addition of 2 vol. cold ethanol after the addition of Na-acetate solution up to 0.2 M final conc. The mixture was left overnight at −15°C and thereafter centrifuged at 10 000 × *g* for 10 min. The pellets were resuspended in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl, 1 mM EDTA and 0.5% Sarkosyl (w/v) and sedimented through 10–30% sucrose gradients made in the same buffer solution. Centrifugations were performed at 100 000 × *g* for 210 min at 2°C in a SW 50.1 Beckman rotor.

For the analysis of radioactive RNA samples synthesized in vitro, the reaction mixtures containing EDTA and SDS were centrifuged at 3000 × *g* for 10 min to discard insoluble material and the supernatant fluid was mixed with an equal vol. 0.2 M sodium phosphate buffer (pH 7.0) containing 12% formaldehyde (v/v) and heated for 10 min at 65°C. The material was then centrifuged at 49 000 rev./min for 150 min in 10–30% sucrose gradients made in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, 3% formaldehyde (v/v), 0.1% SDS (w/v), and 1 mM EDTA, using a SW 50.1 Beckman rotor [19].

3. Results

3.1. *Phosphorylation of nuclei*

The effect of cyclic AMP on the extent and the rate of phosphorylation of isolated nuclei was studied. As shown in fig.1, total radioactive incorporation form [γ-^{32}P]ATP into trichloroacetic acid-insoluble nuclear material was proportional to the incubation time up to 15 min incubation. The reaction was slightly stimulated by the cyclic adenylate. Under these conditions 10^{-5} M cyclic AMP has been found to be the optimal concentration in bringing about the enhancement of this phosphorylation. No stimulation was obtained with cyclic GMP.

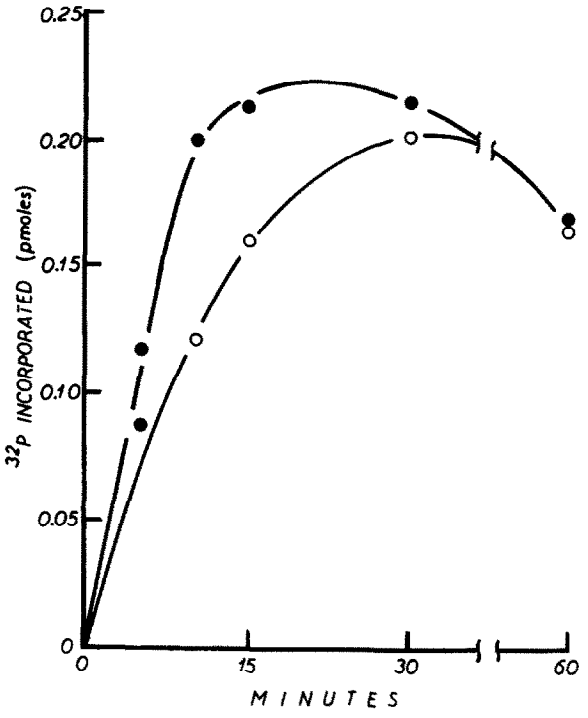

Fig.1. Time course of the phosphorylation reaction of *Neurospora* nuclei performed in the absence (○) or presence (●) of 10^{-5} M cyclic AMP. Conditions are in section 2.

3.2. *Distribution of radioactivity in phosphorylated nuclei*

Table 1 shows distribution of ^{32}P label in different protein fractions from a nuclear preparation phosphorylated in the absence or presence of cyclic AMP. Maximal radioactive incorporation was found in the non-histone protein fraction of chromatin. The cyclic nucleotide stimulated the incorporation into this fraction. This stimulation was more evident in the adenylate cyclase deficient mutant. The effect of cyclic AMP on the phosphorylation of other protein fractions was negligible.

3.3. *Effect of nuclear phosphorylation on transcriptional activity of chromatin*

Endogenous transcription by isolated chromatin has been rarely studied due to the scarce activity detectable in such type of preparation. Owing to this fact, several authors supplemented the assay systems with exogenous RNA polymerases [20]. However, in order to approach 'in vitro' conditions, it would be interesting to observe the influence of phosphorylation on the intrinsic transcription capacity of chromatin complexes.

Figure 2A shows the time course of the transcription reaction catalyzed by chromatin preparations isolated from non-phosphorylated and phosphorylated nuclei. It is evident that RNA synthesis is proportional to the incubation time up to 10 min. Thereafter the reaction reached a plateau. On the other hand, the level of total incorporation as well as the reaction rate, appeared to be higher in phosphorylated than in non-phosphorylated preparations.

It is well known that the fungal toxin α-amanitin is a rather selective inhibitor of nucleoplasmic RNA polymerase II, enzyme responsible for the transcrip-

Table 1
Distribution of ^{32}P radioactivity in different nuclear protein fractions after phosphorylation of *Neurospora* nuclei in the presence or absence of cyclic AMP

Protein fraction	^{32}P incorporation (pmol)			
	FGSC 1118		FGSC 326	
	Without cyclic AMP	10^{-5} M cyclic AMP	Without cyclic AMP	10^{-5} M cyclic AMP
Free	12.7	13.7	16.0	17.6
Loosely bound	17.8	16.0	15.0	14.8
Tightly bound:				
non-histone	49.0	58.0	51.0	67.0
Histone	19.0	20.0	22.5	25.0

Phosphorylations were performed for 30 min at 30°C. Other conditions are in section 2

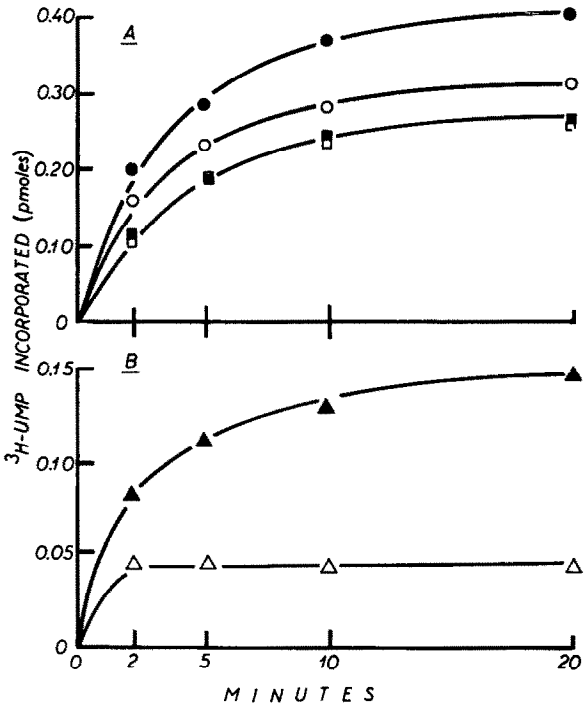

Fig.2. Effect of nuclear phosphorylation on the transcriptional activity of chromatins. (A) Transcriptional activity of chromatin preparations isolated from phosphorylated (closed symbols) or non-phosphorylated (open symbols) nuclei was measured in the absence (circles) or in the presence (squares) of α-amanitin (20 μg/ml). (B) Differences between assays not supplemented and supplemented with toxin. Closed and open triangles correspond to phosphorylated and non-phosphorylated preparations, respectively. Conditions are in section 2.

tion of mRNA sequences [21]. In the presence of such toxin (20 μg/ml), both the extent and the rate of chromatin transcription were depressed. Under such conditions, time courses corresponding to chromatin preparations from phosphorylated or non-phosphorylated nuclei were identical. Figure 2B shows the plot of differences between incubations in the presence or absence of the inhibitor. Such differences might be taken as an expression of the capacity of chromatin to transcribe mRNA sequences. It is evident that phosphorylation increased the capacity to transcribe such sequences.

3.4. *Characterization of in vitro transcriptional products*

[^{3}H]RNAs produced in transcriptional assays performed under conditions corresponding to those shown in fig.2, were characterized in sucrose gradients under denaturing conditions. The intention of such an experiment was to detect differences, if any, on size of RNA populations synthesized by chromatins from phosphorylated or non-phosphorylated nuclei. As shown in fig.3, sizes tended to increase with the reaction time. However no important differences were

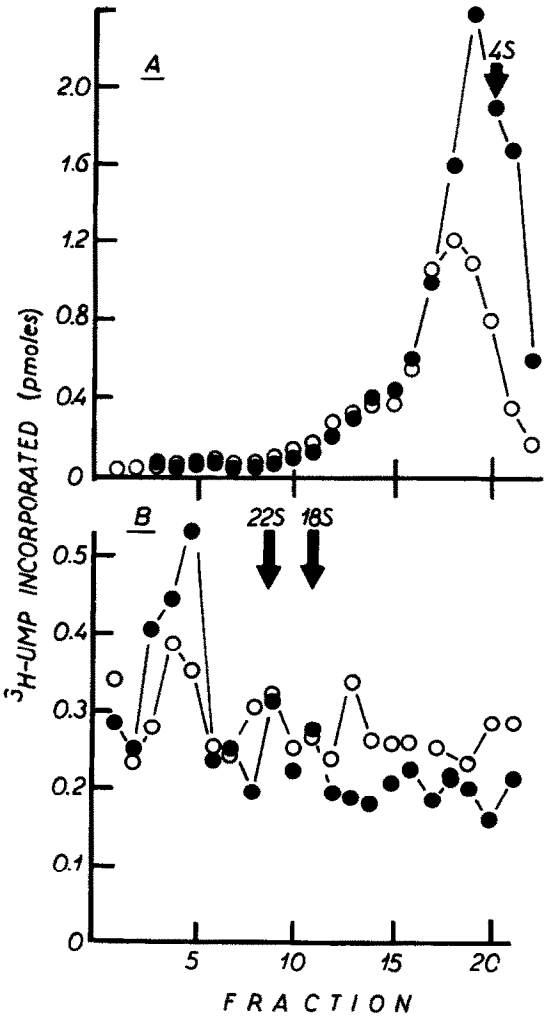

Fig.3. Characterization of in vitro chromatin transcripts by sucrose gradients centrifugation. [^{3}H]RNA coming from chromatin incubations with [^{3}H]UTP with the standard mixture for (A) 10 min (B) min corresponding to phosphorylated (•) and non-phosphorylated (○) nuclei were analysed as in section 2.

observed between products from both types of chromatin preparations.

4. Discussion

In *Neurospora crassa*, dependence of hyphae elongation on cyclic AMP has been demonstrated in cr-1 strains having a deficiency of adenylate cyclase activity. Such mutants have a strong impairment of the ability to form aerial hyphae, which is partially overcome by growing such strains in the presence of cyclic AMP or its dibutyryl derivative [10].

Among several possibilities, the existence of a putative regulatory step at the level of transcription of some genomic sequences could explain the dependence on cyclic AMP. Evidence reported in this article might indicate that cyclic adenylate influences the rate of phosphorylation of non-histone chromatin proteins. In turn, such phosphorylation rather specifically affects the transcriptional capacity mRNA sequences in chromatin preparations. On this basis it might be possible that fungal differentiation would be strongly dependent on the transcription of some genomic sequences.

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