PHOSPHORYLATED GUANOSINE DERIVATIVES OF EUKARYOTES: REGULATION OF DNA-DEPENDENT RNA POLYMERASES I, II, AND III IN FUNGAL DEVELOPMENT[1].

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SUMMARY

Three phosphorylated guanosine derivatives designated HS-1, HS-2 and HS-3 synthesised during active protein synthesis in the water-mould, Achlya sp (1969) were shown to regulate the enzymatic activities of nucleoplasmic and nucleolar DNA-dependent RNA polymerases (RNAP-I, II and III) from both Achlya and another unrelated water-mould, Blastocladiella emersonii. These HS compounds were without effect on E. coli DNA-dependent RNA polymerase holoenzyme. The most potent of the three compounds was HS-3 which inhibited the activity of all enzymes completely at 100 µg/ml. HS-1, on the other hand, activated maximally at 1 to 10 µg/ml. HS-1 activation (3-fold) was restricted to enzyme III, and it had only partial inhibitory effects on enzymes I and II. The pattern of synthesis of HS-compounds throughout the 20-hour asexual growth cycle of the organism correlated with the detectable levels of the different RNA polymerases of Achlya.

INTRODUCTION

The problem of regulating transcriptional activities is a common feature of all living systems. The situation is even more complex in eukaryotic cells where at least three distinctly different DNA-dependent RNA polymerases (RNA-P) exist within the nucleus-nucleolar organelles. One currently accepted designation for these polymerases is RNAP-1 (nucleolar, for synthesising ribosomal RNA), RNAP-II (nucleoplasmic, for synthesising messenger RNA), and RNAP-III (nuclear-nucleoplasmic) whose role is unknown but considered as a good candidate for the synthesis of RNA primers for DNA replication or even tRNA and 5S RNA (1, 2). There are indications that subspecies of RNAP-I and RNAP-II exist (3, 4). In bacteria and bacteriophage transcribing systems, some of the complications observed in transcriptional regulation involve the elaboration of new enzymes,

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modifications of existing ones and incorporation of a modulating protein component such as sigma factor into the basic enzyme complex (5). It is also known that small molecules such as ppGpp and pppGpp may modulate the transcription of specific genes (6, 7, 8) in bacteria although some of the data (7, 8) are still in conflict.

Comparable regulatory studies of transcription in eukaryotes that are definitive do not yet exist in the literature. This communication provides evidence that transcriptional regulation (no evidence of gene specificity) in two primitive eukaryotes may be mediated by three phosphorylated guanosine derivatives that are synthesised during active protein synthesis. These compounds, dubbed HS-3, HS-2 and HS-1, inhibit and activate the various RNA polymerases of two fungi in a complex manner. The tentative structures of these compounds is the subject of a separate communication.

MATERIALS & METHODS

Organisms. The organisms used were Achlya sp (1969) and Blastocladiella emersonii. Conditions for growing and harvesting of cells have been described (9, 10).

Isolation of RNA Polymerases. The methods of Cain and Nester (11) and of Young and Whiteley (12) were followed. The former, to affirm the nuclear origin of the various RNA polymerases, and the latter to obtain high yields of the enzymes.

Enzyme Assays. The procedure of Cain and Nester (11) was used. In all cases, inhibition by actinomycin D and hydrolysis by 0.3 N KOH were parameters used to determine whether RNA was being synthesised. The following were used as templates, poly-d(A-T), undenatured and denatured calf thymus DNA, and undenatured DNA isolated from Achlya by a procedure communicated to us by Dr. P. A. Horgen, Erindale College, University of Toronto. The salient aspects are given in the footnote to table 2.

Enzyme Designation. Our designation of RNAP-I_A, I_B, II_A, II_B, II_C and III is based on the differential properties of the enzymes to the drugs cycloheximide, α-amanitin and rifampicin; order of elution from DEAE-cellulose and DEAE-Sephadex columns with increasing ionic strength of $(NH_4)_2SO_4$, template preference, and relative K_m values for $MnCl_2$ and $MgCl_2$ cofactors. Complete details will be presented for publication elsewhere.

Biochemicals. Fine biochemicals were obtained from Miles Laboratories, Indiana, Sigma Chemical, St. Louis, P-L Biochemicals, Milwaukee, and Calbiochem., California. Radioisotopes were from Amersham/Searle, Illinois and New England Nuclear, Mass.

DEAE-cellulose was purchased from Schleicher & Schuell, Keene, N. H., and DEAE-Sephadex from Pharmacia.

RESULTS

DNA-dependent RNA Polymerases from Achlya. RNA polymerases isolated from Achlya either by the Cain-Nester (11) method from nuclei or the Young-Whiteley (12) procedure from intact cells showed similar chromatographic properties on either DEAE-cellulose or DEAE-sephadex (A-25). Typical activity profiles are shown in Fig. 1A for enzymes extractable at 7.5 hr and 12 hr after germination of spores at 28°C. Twelve hours is about mid-log phase of growth. The relative

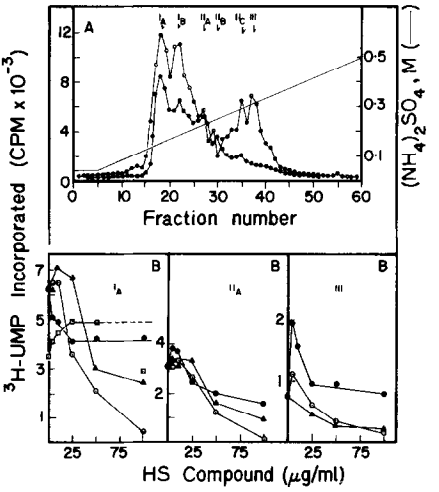

Fig. 1A. Activity profiles of DEAE-Sephadex A-25 chromatography of RNA polymerases from Achlya after 7.5 hr (⊙) and 12 hr (●) of growth from spores. The designations, I_A through III represent enzymes that are kinetically distinguishable from each other. Fig. 1B. Differential inhibition and activation of RNA polymerases I, II and III of Achlya by HS compounds. Left panel: inhibition of RNAP-I_A by HS-3 (⊙), HS-2 (△), HS-1 (●) and activation of RNAP-I_B by HS-1 (⊡). Centre panel: inhibition of RNAP-II_A by HS-3 (⊙), HS-2 (△) and HS-1 (●). Right panel: activation of RNAP-III by HS-1 (●) and inhibition by HS-3 (⊙) and HS-2 (△). Note: Large scale cultures in 15-litre medium composed of 5 g glucose and 0.5 g Difco yeast extract per litre distilled water were vigorously aerated and appropriate samples of cells in suspension withdrawn aseptically at the specified time. Medium was inoculated with 1 litre of spore suspension (10^5 cells/ml). Incubation temperature was 28°C. The cells were collected on Whatman #1 filter paper by suction filtration, washed with 1-litre of distilled water and used immediately for either nuclei isolation or total cell disruption. The procedures of Cain-Nester (11) and Young-Whiteley (12) were then used for RNA polymerase extraction.

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activities of the different enzymes shown in the figure are reproducible under the growth conditions outlined in the figure legend. The important feature is that in the early stages of growth, all five enzymes were present whereas RNAP-II_C and III were absent at 12 hr. Furthermore, the overall levels of RNAP-I_A and I_B were reduced at 12 hr. Available data (not shown) indicate that at various stages of the growth cycle, there were changes in the relative proportions of the five enzymes.

Concurrently, studies on the rates of RNA, protein and DNA syntheses, as well as the relative rates of labelling of the purine nucleotide pools of the cells during the growth cycle revealed (a) a reciprocal relationship between the rates of RNA synthesis and entry of ^{32}P-orthophosphate into certain forms of

unusual purine nucleotides; and (b) a direct correspondence between the rates of protein synthesis and ^{32}P labelling of the nucleotides. Full details will be given elsewhere. Because the levels of these unusual nucleotides fluctuated extensively during the course of growth in apposition to RNA synthesis, we set out to investigate what effects they might have on RNA synthesis *in vitro*.

Specificity of Unusual Nucleotide Effects. The unusual purine nucleotides purified (LéJohn, H. B., Cameron, L. E., McNaughton, D. R. and Klassen, G. R., accompanying manuscript) were tested for their effects on the activities of the different RNA polymerases of *Achlya* in an *in vitro* system. As shown in Fig. 1B, HS-3 strongly inhibited all three major enzyme types. HS-2 was moderately inhibitory whereas HS-1 was weakly inhibitory on RNAP-I and II, but activated RNAP-III about 3-fold at 10 μg/ml. One consistent feature was a small but persistent enhancement of the enzymatic activities of all the enzymes by the various HS compounds at the lower concentrations (10 μg/ml or less).

The complexity of the HS effects can be seen in HS-1 influence on the transcription system. This compound inhibited RNAP-I_A by 30% (optimal) and activated RNAP-I_B to the same extent (Fig. 1B, left panel). Also, whereas HS-3 completely inhibited all enzymes at a concentration of 100 μg/ml, HS-2 inhibited them by about 65% at the same concentration.

A comprehensive summary of the HS effects on RNA polymerases of *Achlya* and *Blastocladiella* is given in Table 1. A comparison with *E. coli* RNA polymerase holoenzyme showed that the *E. coli* enzyme was unaffected by HS-compounds within this concentration range (Table 1). Data not included here

Table 1. Effect of purified HS compounds on the activity of RNA polymerases from three organisms.

HS compound (1 to 100 μg/ml)	RNA polymerase							
	Achlya						*B. emersonii*	*E. coli*
	I_A	I_B	II_A	II_B	II_C	III	I_A & I_B	holoenzyme
HS-3	-*	-*	-*	nt	nt	-*	-*	0
HS-2	-*	-*	-*	nt	+	-*	-*	0
HS-1	-	+	-	nt	nt	+*	-	0

+, activation; -, inhibition; 0, no effect; nt, not tested.

*, pronounced effect observed.

are the observation that the 'magic spots' MSI and MSII of E. coli, (ppGpp and and pppGpp), their analogues ppppG and pppGp did not inhibit any of the fungal RNA polymerases when they were used in the same concentration range.

Template Preference. Although the majority of enzyme assays were done with calf thymus DNA as template, we have observed that the HS compounds inhibited the enzymes better when DNA from Achlya was used. Typical inhibition data for HS-3 on RNAP-I_A and I_B are shown in Table 2. In both cases, enzyme activity was inhibited 30% to 50% when HS-3 was supplied at 10 µg/ml. With calf thymus DNA as template, none of the enzyme activities were inhibited by 10 µg/ml HS-3 (see Fig. 1B). Poly-d(A-T) as template was as ineffective to HS-3 inhibition as calf thymus DNA within this concentration range. To what extent this template preference might be true for all of the enzymes is yet to be explored.

Table 2. Effect of purified HS-3 on RNAP-I_A and I_B of Achlya using Achlya DNA as template.

HS-3 (µg/ml)	RNA Polymerase activity (cpm) I_A	I_B
0	1273	1043
1	1081	916
2	987	847
5	977	772
10	875	580

Nuclei isolated as for enzyme purification was suspended in 8 M urea containing 1 M Na perchlorate and 0.12 M Na phosphate buffer, pH 7. Sodium dodecyl sulphate (1.25%) was added and sonicated for 2 min. An equal volume of a mixture of chlorform:isoamyl alcohol (9:1) was added to the suspension and vigorously mixed for 4 min. The mixture was centrifuged at 8,000 x g for 10 min and the aqueous layer drawn off and added to 2 g of hydroxylapatite (BioRad HTP) prewashed in the urea-phosphate buffer. The mixture was centrifuged for 10 min at 8,000 x g, the supernatant discarded and the pellet resuspended in the urea-phosphate buffer, then transferred to a 1 cm diameter glass column. The column was washed with the urea buffer until the effluent A_{260} reading was below 0.15. Thirty ml of 0.006M phosphate buffer, pH 7 was used to wash off urea from the column and then the DNA was eluted with 0.4M phosphate buffer. The DNA fractions detected by A_{260}/A_{280} readings were pooled then dialysed exhaustively at 4°C with an Amicon concentrator unit. The concentrated DNA (1 mg/ml or more) was stored with a drop of chloroform at 4°C until used. The preparative method yields DNA fragments of less than 10^6 mol. wt.

HS Pool Size and RNA Polymerases In Vivo. Using the 20-hr growth cycle condition for sampling and fractionating whole cells and nuclei to estimate the relative distribution of the various RNA polymerases and concurrent pool sizes of HS compounds at different times, the results obtained are summarized in Table 3. The important feature is that RNAP-I was predominant over all others during the first 12 hr of growth. RNAP-III was absent at 12 hr but re-appeared around 14 hr to 15 hr. RNAP-II_B, the predominant species of RNAP-II enzyme in eukaryotes (13) was present at all times while RNAP-II_A and RNAP-II_C fluctuated. During the 14 hr to 15 hr period when the HS pool size was maximal RNAP-I, believed to synthesise ribosomal RNA, was not detectable among the enzymes isolated from the nuclei. Whether this represents enzyme inactivation or absence remains to be determined.

DISCUSSION

The three HS compounds we have isolated from the two fungi Achlya and B. emersonii are unlike pppGpp and ppGpp of bacteria (14). But HS-3, HS-2 and HS-1 are nucleotide polyphosphates (accompanying manuscript) that may yet become another class of nucleotides with important regulatory functions in transcription.

Our determination of the pool size of the HS compounds (manuscript in preparation) has shown that the cellular concentration of HS-3 is between 0.25 g and 0.5 g/l of cell water while HS-2 and HS-1 are 0.2 g and 0.03 g respectively.

Table 3. Relative activities of Achlya RNA polymerases and pool sizes of HS compounds during the 20-hr growth cycle of the organism.

HS pool (total) % of maximum	RNA Polymerases						Growth (hr)
	I_A	I_B	II_A	II_B	II_C	III	
5 - 10	+	+	+	+	+	+	7.5
20 - 30	+	+	+	+	-	-	12.0
80 - 85	-	-	+	+	+	+	14.0
95 - 100	-	-	-	+	+	+	15.0
20 - 30	x	x	x	x	x	x	17.0 *
10 - 20	x	x	x	x	x	x	20.0**

+, enzyme activity detectable under optimum assay conditions.
-, enzyme activity not detectable under optimum assay conditions.
x, tests not yet made because,
*, hyphal tip differentiation into sporangia initiated at this time;
**, sporulation has occurred.

These figures are probably higher by 20% to 30% considering the solid volume of the cell. If they are localized in the nucleus, their effective concentration would be extremely high. Inhibition of the various RNA polymerases by HS-3 and HS-2, and activation of RNAP-III by HS-1 at concentrations less than 100 µg/ml therefore satisfy the requirements for the effects described here to have physiological meaning.

Another feature of physiological interest is the change in detectable activity of the various enzymes at different stages of the growth cycle. One correlation seen is that there is an alteration in the total pool sizes of the HS compounds (not a consequence of changes in the ability of the cell to take up ^{32}P-orthophosphate) and the predominant enzyme types (Table 3). When the HS pool was maximal at 15 hr, only RNAP-II_B, II_C and III could be isolated from cell nuclei. At 7.5 hr when HS levels were minimal, all enzymes were detected. The raison d'être surrounding this observation awaits the results of more refined experiments that are currently in progress. Whether or not the activating effect of HS-1 on RNAP-III is an indication that this particular enzyme may be responsible for synthesising short pieces of RNA 'primers' in DNA replication remains to be seen.

REFERENCES

1. Roeder, R. G. and Rutter, W. J. (1969). Nature 224, 234-237.
2. Goldberg, M., Perriard, J-C, Hager, G., Hallick, R. B. & Rutter, W. J. (1973). Control of Transcription (B. B. Biswas., R. K. Mandal., A. Stevens & W. E. Cohn., editors) Plenum Press, New York, pp. 241-256.
3. Chesterton, C. J. & Butterworth, P. H. W. (1971). FEBS Letters, 15, 181-185.
4. Kedinger, C., Nuret, P. & Chambon, P. (1971). FEBS Letters, 15, 169-174.
5. Cashel, M. (1970). Cold Spring Harbor Symposium Quant. Biol. 35, 407-413.
6. Aboud, M. & Pastan, I. (1975). J. Biol. Chem. 250, 2189-2195.
7. Travers, A., Kamen, R. & Cashel, M. (1970). Cold Spring Harbor Symposium Quant. Biol. 35, 415-418.
8. Haseltine, W. A. (1973). Nature, 235, 329-332.
9. LéJohn, H. B. & Jackson, S. (1968). J. Biol. Chem. 243, 3447-3457.
10. LéJohn, H. B. & Stevenson, R. M. (1970). J. Biol. Chem. 245, 3890-3900.
11. Cain, A. K., Nester, E. W. (1973). J. Bacteriol. 115, 769-776.
12. Young, H. A. & Whiteley, H. R. (1975). Exptl. Cell Res. 91, 216-222.
13. Biochem. Biophys. Res. Commun. 64, 625-632.
14. Cashel, M. & Kalbacher, B. (1970). J. Biol. Chem. 245, 2309-2318.