Control of Ribonucleic Acid Synthesis in Eukaryotes. 3. The Effect of Cycloheximide and Edeine on RNA Synthesis in Yeast†

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ABSTRACT: The addition of cycloheximide to a thermosensitive conditional yeast mutant (t_s-187) before and after transfer to the nonpermissive temperature (36 °C) for initiation of protein synthesis produces the uncoupling of the RNA and protein synthetic machineries. Since the drug can produce this relaxation in the presence and absence of protein synthesis, it is concluded that the coupling of protein and RNA synthesis, which a temperature shift produces, is not exclusively related to the inhibition of protein synthesis. Support for this assumption has been obtained using the parental (A364A) strain. Transferring this strain to 36 °C produces inhibition of RNA synthesis in the presence of stimulation of protein synthesis. Furthermore, cycloheximide and edeine prevent this inhibition of RNA synthesis that temperature shift produces. It is, therefore, postulated that this inhibition of RNA synthesis results from the synthesis or activation of a factor(s) elicited by the increase in temperature whose function is to repress the transcriptional apparatus. Cycloheximide or edeine can prevent the function of this repressor-like factor by binding to the factor or by preventing its synthesis. The fact that inhibition of protein synthesis either by cycloheximide action or temperature shift in t_s-187 produces inhibition of RNA synthesis in isolated nuclei indicates that, in addition to the aforementioned repressor, other factor(s) having a promoter function may exist. Since a slight inhibition of protein synthesis produces nuclear template restrictions, it is postulated that the promoter-like factor(s) is a polypeptide different from the RNA polymerase and, at least in yeast, has a high turnover.

The results reported in the preceding communication, in conjunction with previous publications, strongly suggest that the RNA polymerases are not the rate limiting factors controlling RNA synthesis in eukaryotes (Gross and Pogo, 1974; Lampert and Feigelson, 1974; Benecke et al., 1973). Therefore, another element(s) functioning in cooperation with enzymes and templates must play the central role in controlling transcription. The findings which led us to postulate the existence of these elements were obtained with an experimental approach which used well-defined physiological conditions that alter RNA synthesis in isolated nuclei. Thus, starvation and inhibition of protein synthesis have proved to be powerful modifiers of the RNA synthetic machinery in nucleated cells. This approach permitted the discovery of stringency in ascites cells and stringency and relaxation in yeast (Franze-Fernández and Pogo, 1971; Franze-Fernández and Fontanive-Sengüesa, 1973; Gross and Pogo, 1974, 1976).

The detailed study of RNA metabolism in amino acid starved yeast indicated that the relaxation phenomenon can only be detected in biological systems which do not suppress the conversion of RNA precursors into ribonucleotide residues by feedback inhibition. This suggests that relaxation, like stringency, is a universal phenomenon but difficult to detect in some systems (Gross and Pogo, 1976). Moreover, two novel aspects of the stringent and relaxed phenomena have been observed using yeast. One is that they can be elicited by a temperature shift, lending support to the notion that in eukaryotes the function of the RC^{str} genes (Ryan and Borek, 1971) may be under the influence of other stimuli. Thus, hormones affecting RNA metabolism may exert such control by altering the activities of these genes (Tata, 1966). The other aspect is that in yeast and perhaps other eukaryotes there is a "nuclear" and a cellular relaxation. The former can only be observed in the presence of protein synthesis, but the latter can be obtained with or without protein synthesis (Gross and Pogo, 1976).

It became apparent that these two aspects of relaxation, as well as the stringent phenomenon, are ultimately related to protein synthesis or some elements of the protein synthetic machinery. We wish to present in this paper experiments which support the concept that the backbone of the stringent and relaxed phenomena lies in the action of at least two factors having antagonistic effects on RNA metabolism. One is a factor which switches off RNA synthesis and whose activity or synthesis is probably mediated by ribosomes but not by protein synthesis. The other is a factor which switches on the RNA synthetic machinery. Its functioning is dependent upon protein synthesis, and it is believed to be a polypeptide having the property of promoting the binding of the RNA polymerase to specific template initiation sites.

Materials and Methods

Yeast (Saccharomyces cerivisiae), Media, and Cell Growth. The haploid strains A364A and t_s-187 (*gal ade2 ura1 his7 lys2 tyr1*), kindly supplied by Dr. L. Hartwell (University of Washington, Seattle), were grown overnight to a final density of 2–3 × 10^7 cells/ml in complete medium as explained (Gross and Pogo, 1974, 1975). The cells were harvested by filtration onto Millipore filters, washed with distilled water, resuspended in fresh complete medium, and cultured for an additional hour before the start of the experiments.

Spheroplast Preparation. Metabolically active spheroplasts were prepared by digestion of the cell wall with a commercial snail gut enzyme (Glusulase; Endo Laboratories) as explained (Gross and Pogo, 1974, 1975).

Nuclear Isolation. Nuclei were isolated from A364A

† From the Laboratory of Cell Biology, The Kimball Research Institute of The New York Blood Center, New York, New York 10021. *Received October 17, 1975.* This work was supported by Grant HL-09011 from the National Heart and Lung Institute and Grant CA-17626 from the National Cancer Institute. This is the third paper in a series on control of RNA synthesis. The first was Gross and Pogo (1974).

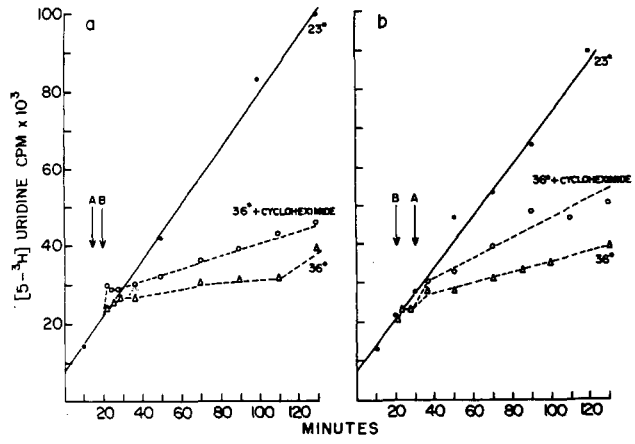


FIGURE 1: Incorporation of [5-^{3}H]uridine in the cycloheximide-treated t_s-187 cells during temperature shift from 23 to 36 °C. In (a) the cells were resuspended at a density of 2×10^7 cells/ml in 45 ml of complete medium and cultured at 23 °C for 50 min. Then 135 μCi of [5-^{3}H]uridine was added. Thirty minutes later 0.5-ml duplicate aliquots were withdrawn. Five minutes prior to transfer to 36 °C, cycloheximide was added to a 15-ml aliquot to make a final concentration of 100 μg/ml. Then 15-ml treated and nontreated aliquots were transferred to 36 °C and the remainder maintained at 23 °C. (b) is similar to (a) but cycloheximide was added to the 15-ml aliquots 10 min after the transfer to 36 °C. Duplicate aliquots (0.5 ml) were withdrawn at the indicated intervals and the radioactivities were determined in the cold 10% trichloroacetic acid precipitate as explained (Gross and Pogo, 1974, 1976). ●—●, culture maintained at 23 °C; Δ- - -Δ, culture transferred to 36 °C; O- - -O, culture treated with cycloheximide and transferred to 36 °C; A, the time of cycloheximide addition; B, the time of transfer.

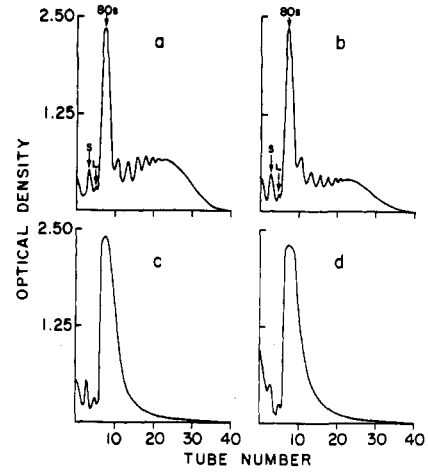


FIGURE 2: Polysomal distribution in t_s-187 spheroplasts cycloheximide-treated and transferred to 36 °C. Spheroplast culture (80 ml) was incubated at a density of 1×10^8 cells/ml in complete medium at 23 °C for 1 h after the recovery period. It was then divided into 20-ml aliquots. In (a) the aliquot was maintained at 23 °C for 25 min. In (b) 100 μg/ml of cycloheximide was added 5 min prior to transfer to 36 °C and then maintained for 20 min. In (c) the aliquot was transferred to 36 °C and maintained for 20 min. In (d) the aliquot was transferred to 36 °C and 10 min later 100 μg/ml of cycloheximide was added and maintained for 10 min. S, small ribosomal subunits; L, large ribosomal subunits.

spheroplasts according to published procedures (Gross and Pogo, 1974, 1975).

RNA and Protein Synthesis Measurements. [5-^{3}H]Uridine (29 Ci/mmol) and L-[^{3}H]lysine (7 Ci/mmol) (Schwarz/Mann) were added as explained in the corresponding figures and Table I. Incorporation of radioactivity was determined as explained (Gross and Pogo, 1974, 1975).

Spheroplast Fractionation and Sedimentation Velocity Gradients. Before harvesting, 100 μg/ml of cycloheximide was added for 1 min to prevent a runoff of ribosomes in the nontreated cultures (Figure 3), and the spheroplast suspension was quickly cooled by transferring it into a beaker maintained at −10 °C. Spheroplasts were collected by centrifugation at 3000*g* for 5 min and washed with cold 1 M D-sorbitol. The final pellet of spheroplasts was resuspended in 2 ml of a solution containing 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.6, 20 mM KCl, 3 mM $MgCl_2$, and 0.01% spermidine (lytic buffer). Triton X-100 was added to make a final concentration of 0.5% and the detergent-treated spheroplasts were homogenized with a Teflon homogenizer and centrifuged for 30 min at 10 000*g*. The supernatant which contained the majority of polysomes and ribosomes was fractionated in a linear sucrose gradient (10:45% w/v) containing the components of the lytic buffer without spermidine and centrifuged in a Spinco SW41 rotor at 40 000 rpm for 1 h. The gradient was fractionated in an ISCO density gradient fractionator with ultraviolet absorption recorder (254 mμ; 1-cm path length).

Results and Discussion

Effect of Cycloheximide on the Incorporation of Uridine in the Absence of Protein Synthesis. It has been demonstrated that nuclear RNA polymerase activities (EC 2.7.7.6) are inhibited by cycloheximide in yeast, rat liver, and lymphosarcoma (Muramatsu et al., 1970; Sajdel and Jacob, 1971; Lampert and Feigelson, 1974; Gross and Pogo, 1974, 1976). Yet the drug stimulates uridine incorporation when added to amino acid starved yeast cells (Gross and Pogo, 1974, 1976). A similar effect has been observed in temperature-shift experiments under conditions in which either protein synthesis is suppressed (t_s-187 transferred to 36 °C) or stimulated (A364A transferred to 36 °C) (Gross and Pogo, 1976). Therefore, inhibition of nuclear RNA polymerases and stimulation of RNA metabolism, two antagonistic and paradoxical effects, are not restricted to the addition of cycloheximide to amino acid starved cells.

Since cycloheximide is a powerful protein synthesis inhibitor it is important to determine the roles of protein synthesis and the drug in the relaxation phenomenon. To study this, cycloheximide was added before and after the t_s-187 was transferred to the nonpermissive temperature (Figure 1). It is clearly shown that cycloheximide can relax the system in the presence (Figure 1a) and absence (Figure 1b) of protein synthesis. These findings suggest that the drug may block the synthesis or the function of a repressor-like factor which is induced during temperature shift. The observation that in cycloheximide-treated cells (Figure 1a) the rate of uridine incorporation is less than that in untreated cells is consistent with the fact that the drug at the same time inhibits protein synthesis and produces template restriction (Gross and Pogo, 1974, 1976).

In vivo and in vitro studies support the notion that cycloheximide inhibits the translocation step of translation. This takes place by a direct effect on the ribosomes (Kaji, 1973). Therefore, it is important to know whether the drug produces relaxation only when the ribosomes are attached to the mRNA or whether it can produce relaxation when they are free. Addition of the drug before the transfer of the t_s-187 to 36 °C prevents a large loss of polysomes due to this temperature shift (Figure 2b). This is expected since in this condition a run-off of ribosomes is avoided as polypeptide elongation and termination are inhibited by cycloheximide. This is consistent with the assumption that the genetic defect of this mutant lies in one of the initiation factors for protein synthesis (Hartwell and McLaughlin, 1969). However, when the drug is added after the transfer to 36 °C very few polysomes are observed; in this

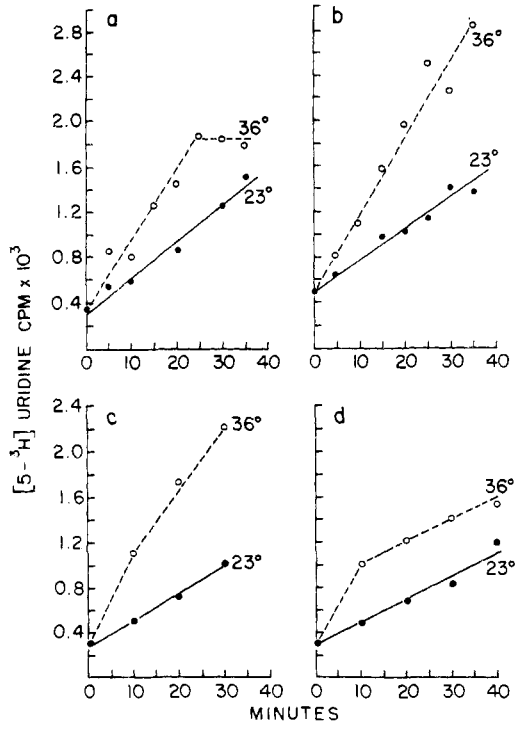


FIGURE 3: Effect of cycloheximide and edeine on uridine incorporation of A364A spheroplasts during transfer to 36 °C. A364A spheroplasts (230 ml) were cultured at a density of 5×10^7 cells/ml for 30 min. Then 770 μCi of [5-^{3}H]uridine was added, incubated for an additional 30 min, and divided into 40-ml aliquots. In (a) no drug was added, in (b) and (c) cycloheximide was added to a concentration of 1 $\mu g/10^7$ cells and 0.01 $\mu g/10^7$ cells, respectively, and in (d) edeine (Galen Laboratories, Houston, Texas) to a concentration of 200 $\mu g/10^7$ cells. The cycloheximide-treated cultures were further incubated at 23 °C for 5 min and the edeine-treated culture for 10 min. Then, the cultures were divided into 20-ml aliquots; one was maintained at 23 °C and the other transferred to a 36 °C prewarmed flask. Duplicate aliquots (1 ml) were withdrawn at the indicated intervals and radioactivities measured as explained in Materials and Methods.

condition the rate-limiting factor is some of the initiation factors for translation (Figure 2d). Nevertheless, relaxation has been obtained in both conditions, i.e., with or without polysomes (Figure 1). It is concluded then that active translocation is not a prerequisite for cycloheximide relaxation. A logical implication is that this relaxation is independent of the status of ribosomes and protein synthesis.

Effect of Cycloheximide and Edeine on the Uridine Incorporation During Temperature Shift in A364A. As has been shown previously, temperature shift of A364A produces a transitory inhibition of RNA synthesis (Gross and Pogo, 1976). This yeast strain reacts by stimulating instead of inhibiting protein synthesis at 36 °C. It is therefore important to know if cycloheximide can also prevent this inhibition of RNA synthesis. The experiment described in Figure 3 clearly indicates that the drug produces relaxation in conditions where protein synthesis is completely (b) or slightly (c) inhibited.[1] This is consistent with the results obtained in t_s-187 cultured at 36 °C and strongly supports the concept that cycloheximide relaxation is linked neither with the inhibition of protein synthesis nor with the synthesis of a polypeptide.

In the same figure, a similar but less efficient relaxation by edeine is shown (Figure 3d). Edeine is another protein synthesis inhibitor which affects yeast. The drug inhibits the aminoacyl-tRNA binding to ribosomes and the dissociation of ribosomes. It appears that the drug binds to the large and the small ribosomal subunits and has a specific action on the donor site of the ribosomes (Kurylo-Borowska and Hierowski, 1965). The fact that relaxation can be obtained with two distinct protein synthesis inhibitors which bind to different sites of ribosomes indicates that the relaxed phenomenon may depend upon a factor(s) associated with or synthesized by unaltered ribosomes. Similar results have been obtained in the relaxation of bacterial RNA synthesis (Kaplan et al., 1973). Nevertheless this is only a suggestion since other protein synthesis inhibitors, such as puromycin, which does not bind to ribosomes, cannot be used in these yeast strains (Gross and Pogo, unpublished results).

In our hands edeine proved to be a less efficient agent than cycloheximide. It is not known whether this is due to the drug per se or to a decreased permeability of spheroplasts to edeine. Since the drug is a powerful inhibitor of protein synthesis in cell-free systems its lower efficiency in intact spheroplasts may indicate a problem in permeability. The fact that edeine is a much weaker protein synthesis inhibitor than cycloheximide on a molar basis supports this assumption. Thus, in the experiments of Figure 3d it produces a 10–15% inhibition in lysine incorporation at a concentration 35 times higher than that of complete inhibition by cycloheximide (not shown).

Effect of Different Doses of Cycloheximide on the Nuclear RNA Polymerases of A364A Spheroplasts Maintained at 23 °C and Shifted to 36 °C. Table I depicts the effect of different amounts of cycloheximide on the α-amanitin-resistant and α-amanitin-sensitive activities of nuclei isolated from spheroplasts cultured at 23 °C and shifted to 36 °C.

As expected the drug does not prevent the inhibition in the nuclear enzymes, mainly α-amanitin-resistant, that a temperature shift produces. On the contrary, it enhances this inhibition. Furthermore, there is a correlation between the degree of inhibition of the α-amanitin-resistant activities and the inhibition of protein synthesis. Thus, 0.2 $\mu g/10^7$ cells which inhibits protein synthesis by about 60% in the 36 °C culture has a greater affect on the nuclear enzymes of these spheroplasts than 0.02 $\mu g/10^7$ cells which inhibits protein synthesis at 36 °C by about 15%. None of these concentrations affects protein synthesis at 23 °C. These observations are consistent with the assumption that cycloheximide produces a template restriction only when it produces inhibition of protein synthesis. The drug per se cannot restrict the RNA synthetic capacity of the nuclei.

Conclusion

The studies of control of transcription should focus on the identification of the elements which limit the rate of RNA synthesis. In general this rate is the product of two main processes: (a) the number of RNA chains under construction (chain initiation) and (b) the average rate at which ribonucleotide residues are added to each RNA chain (chain elongation). The former, which is essential for initiation of transcription, is directly correlated with the number of RNA polymerase molecules bound to the template. In eukaryotes RNA synthesis in isolated nuclei is a direct measurement of the number of RNA polymerase molecules bound to the template. Since yeast RNA polymerases are very stable molecules, it is evident that another component(s) of the transcriptional apparatus must be the rate limiting factor in the initiation of transcription. All the evidence indicates that either the existence or activity of this component is directly linked to

[1] The minimum amount of cycloheximide required to immediately suppress protein synthesis at 23 °C is 1–2 $\mu g/10^7$ cells (unpublished observation).

Table I: Effect of Cycloheximide on RNA Synthesis in Isolated A364A Nuclei from Spheroplasts Temperature-Shifted to 36 °C and Maintained at 23 °C.[a]

Expt	Incubation Time (min)	Cycloheximide[b] Per 10^7 Cells	pmol of UMP/10^8 Nuclei: α-Amanitin-Resistant 23 °C	α-Amanitin-Resistant 36 °C	α-Amanitin-Sensitive 23 °C	α-Amanitin-Sensitive 36 °C
1	15	—	61	46	50	53
		2.0 μg	21	29	34	55
2	15	—	75	51	74	41
		2.0 μg	42	36	58	51
3	20	—	79	60	69	70
		0.2 μg	75	36	62	48
4	20	—	75	36	37	41
		0.02 μg	68	30	39	28

[a] For the determination of RNA synthesis in isolated nuclei 1.4 l. of A364A spheroplasts at a density of 2×10^7 cells/ml was recovered at 23 °C for 60 min. Then, to 700-ml aliquots cycloheximide was added at one-half the amount per 10^7 cells as indicated and the cultures were maintained at 23 °C for 10 min. The treated and nontreated cultures were divided into two 350-ml aliquots and to each was stepwise added 350 ml of fresh medium prewarmed to 23 and 50 °C. Cycloheximide was then added to each of the treated cultures to have the indicated final concentration and the incubation was prolonged as indicated. For the determination of protein synthesis to a 60-ml A364A spheroplast culture at a density of 2×10^7 cells/ml 250 μCi of [^{3}H]lysine was added after the recovery period. Thirty minutes later the culture was divided into three aliquots of 20 ml each, cycloheximide was added to two of them at one-half the amount per 10^7 cells as indicated, and the cultures were maintained at 23 °C for 10 min. The treated and nontreated cultures were divided in half and to each an equal volume of fresh medium prewarmed to 23 and 50 °C was stepwise added. Additional cycloheximide was added to each of the treated cultures to have the indicated final concentration and the incubation was prolonged for 30 min more. Duplicate aliquots (2 ml) were taken at 10-min intervals. The degree of inhibition in [^{3}H]lysine incorporation between treated and nontreated cultures was maintained throughout the experiment. [b] At 2.0 μg/10^7 cells there is complete inhibition in the [^{3}H]lysine incorporation into spheroplasts cultured at 23 and 36 °C. At 0.2 μg/10^7 cells there is 55% inhibition in the [^{3}H]lysine incorporation into spheroplasts cultured at 36 °C and no inhibition in spheroplasts cultured at 23 °C. At 0.02 μg/10^7 cells there is 15% inhibition only in spheroplasts cultured at 36 °C.

protein synthesis, and therefore is believed to be a polypeptide. Moreover, the evidence strongly suggests that it operates as a positive control. A similar factor has been postulated for ribosomal RNA synthesis in HeLa cells (Chesterton et al., 1975). Since a slight inhibition of protein synthesis restricts the nuclear template (Table I), it is assumed that this component has, at least in yeast, a high turnover. It appears that there may be as many of these components as RNA polymerase species. Thus, the nuclear α-amanitin-resistant and α-amanitin-sensitive activities are asynchronically inhibited in starvation, inhibition of protein synthesis, or temperature shift (Gross and Pogo, 1974, 1976). The manner by which this component operates and its relationship to the protein factor which stimulates RNA polymerase II (or B) activity on native DNA remain to be discovered (Seifart et al., 1973; Lee and Dahmus, 1973; Sugden and Keller, 1973).

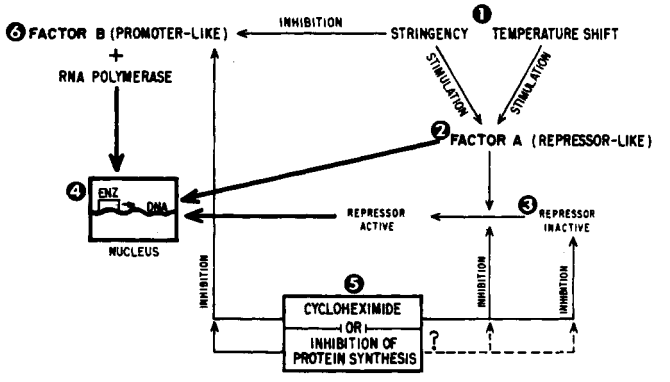
The fact that cycloheximide produces relaxation in the absence or presence of protein synthesis (Figure 1) strongly suggests that in addition to the postulated polypeptide, other component(s) must control the transcriptional apparatus. The experiments so far indicate that this second component also produces a restriction in the nuclear RNA synthesis, but by a different mechanism. The evidence suggests that it operates as a negative control, producing either early termination or prevention of the formation of the enzyme-template initiation complex. It appears that the synthesis or function of this component is elicited by amino acid starvation and temperature shift. Moreover, the evidence supports the concept that this component is associated with or synthesized by ribosomes and that drugs like cycloheximide inhibit its action. The drug could perhaps either bind to the factor preventing its functioning or inhibit an enzymatic reaction which synthesizes or activates the factor. An exhaustive study of yeast spheroplasts and/or cells failed to detect any accumulation of ppGpp and/or ppGppp as well as cAMP in stringent conditions (not shown). This is in agreement with observations in other nucleated cells (Buckel and Böck, 1973; Mamont et al., 1972; Alberghina et al., 1973). Whether this repressor factor is an unknown nucleotide or oligonucleotide remains to be seen. The possibility exists that this element does not directly operate as an inhibitor but activates an inactive form of an inhibitor. In the Appendix we present the proposed interrelationship of these two hypothetical factors and how they may function in the regulation of RNA synthesis.

Acknowledgments

We wish to thank Ms. Valerie Zbrzezna for her expert technical assistance.

Appendix: Schematic Representation of the Functions and Interactions of the Postulated Two Factors Modulating RNA Synthesis

The model that we wish to propose is based on antagonistic effects of at least two distinct factors on the activity of the transcriptional apparatus. One is a repressor-like component designated factor A(2). Its stimulation can be elicited either by stringency or temperature shift in the particular case of yeast (1). It has the property of restricting the activity of the transcriptional apparatus (4) by itself or through the activation of an inactive form of a repressor (3). In addition, a second factor designated factor B (6) has to be proposed. The function of this factor is to promote the formation of the enzyme-template complex. It seems to be a polypeptide. Therefore, inhibition of protein synthesis either by stringency (1), cycloheximide (5) or any procedure which inhibits the protein



synthetic machinery (5) has to inhibit the synthesis of this factor. Finally, a drug like cycloheximide (5) has the property of preventing the synthesis or the activation of factor A (2). In the presence of this drug factor A cannot operate and the transcriptional apparatus is released from repression. The possibility exists that inhibition of protein synthesis by means

other than the cycloheximide's effect might also prevent the function of factor A (5).

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Affinity Labeling of *Escherichia coli* DNA-Dependent RNA Polymerase with 5-Formyl-1-(α-D-ribofuranosyl)uracil 5′-Triphosphate†

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ABSTRACT: 5-Formyl-1-(α-D-ribofuranosyl)uracil 5′-triphosphate has been used to affinity label *E. coli* DNA-dependent RNA polymerase. It is a noncompetitive inhibitor of the enzyme with K_i = 0.54 mM. A short preincubation of the enzyme and α-fo^{5}UTP is required to achieve maximum inhibition, and the extent of the inhibition is dependent upon the α-fo^{5}UTP concentration. When a preincubation mixture of α-fo^{5}UTP/enzyme is diluted, the enzyme regains activity with time showing that the inhibition is reversible, presumably occurring by Schiff base formation between an amino group on the enzyme and the formyl group. Upon sodium borohydride reduction of an enzyme/α-fo^{5}UTP preincubation mixture the enzyme is irreversibly inhibited. α-fo^{5}UTP is more effective in inhibiting the enzyme than α-fo^{5}U, and the inhibition is decreased by the presence of ATP, UTP, or GTP in the preincubation mixture, suggesting that inhibition is occurring at a triphosphate binding site. The stoichiometry of binding of α-fo^{5}UTP to the enzyme was determined using the γ-^{32}P-labeled derivative. After a 20-s preincubation of enzyme/α-fo^{5}UTP followed by $NaBH_4$ reduction the stoichiometry of binding was 1.1:1 (α-fo^{5}UTP bound:inactivated enzyme), and this rose to 2.42:1 after a 10-min preincubation. After a 20-s preincubation the [γ-^{32}P]-α-fo^{5}UTP was shown to be located on the β subunit of RNA polymerase by cellulose acetate electrophoresis in 6 M urea.

Much interest has been focussed upon the role of the different subunits of DNA-dependent RNA polymerase in the transcription process. In order to determine which subunits contain the catalytic site of the enzyme the technique of affinity labeling has been employed. Thus the substrate analogue 4-thiouridine 5′-triphosphate (Frischauf and Scheit, 1973) was shown to be covalently attached to the β and β′ subunits after photooxidation. The periodate oxidation product of 6-methylthiopurine ribonucleoside proved to be a noncompetitive inhibitor of RNA polymerase (Nixon et al., 1972), and it was suggested that this was due to inhibition of the initiation mechanism. After sodium borohydride reduction it was found to be attached to the β subunit. Using a fluorescent derivative, AMPR-OP, of this compound in which the methyl group was replaced by *N*-(acetylaminoethyl)-1-naphthylamino-5-sul-

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