*PROTEIN SYNTHESIS AND THE TEMPORAL CONTROL OF GENETIC TRANSCRIPTION DURING SLIME MOLD DEVELOPMENT**

By M. Sussman

DEPARTMENT OF BIOLOGY, BRANDEIS UNIVERSITY, WALTHAM, MASSACHUSETTS

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In the cellular slime mold, *Dictyostelium discoideum*, the enzyme UDP-Galactose polysaccharide transferase, responsible for the incorporation of galactose into an acid mucopolysaccharide, is entrained by the over-all developmental program. This enzyme first appears at the beginning of the pseudoplasmodial stage, accumulates rapidly to a peak of specific activity near the end of fruit construction, is preferentially released by the cells, and then is quickly destroyed.[1, 2] Both the accumulation and subsequent disappearance are sensitive to the coincident inhibition of protein synthesis by cycloheximide and the prior inhibition of RNA synthesis by actinomycin.[3, 4] In strain FR-17, the period of actinomycin sensitivity begins 4.5 hr and ends 0.5 hr before the start of enzyme accumulation and, within it, a simple linear relationship exists between the enzyme activity which ultimately accumulates and the time at which actinomycin was added.

In the present communication, the consequences of interference with protein synthesis during the transcriptive process are described. The data indicate that: (*a*) any RNA that may be fabricated under these conditions is not subsequently translated into active UDP-Gal polysaccharide transferase when protein synthesis is allowed to resume while previously fabricated RNA is so translated; (*b*) the formation of transferase-specific RNA is not automatically and unconditionally limited to the prescribed period but may be extended considerably beyond it; (*c*) the time lag between the synthesis of transferase-specific RNA and the appearance of the enzyme, normally 4–5 hr, can be increased to at least 7–8 hr without detriment to the total activity which ultimately accumulates.

Methods.—Organism and cultivation: *D. discoideum*, mutant strain FR-17,[5] was grown with a bacterial associate, *Aerobacter aerogenes*, on SM agar plates.[6] For experiments involving assays of enzyme activity, the amoebae were harvested, washed, suspended at 2×10^8/ml, and dispensed in 0.5-ml aliquots on 2-inch black Millipores over pads saturated with streptomycin-salt solution. Under these conditions a very high degree of morphogenetic synchrony is achieved.[2] For experiments in which RNA synthesis was measured, the cells were harvested from growth plates and preincubated for 1 hr at 22°C in SM broth plus streptomycin (500 μg/ml) to sterilize the bacterial associate[7] before washing and incubating the amoebae on the Millipores.

Enzyme assays: The detailed assay procedure is given elsewhere.[1] Single Millipores were harvested with 3 ml of Tris-thioglycollate buffer and frozen. Immediately before assay, the suspension was thawed and treated in a Branson Sonifier (intensity = 1, time = 60 sec) to produce total cell breakage. The assay mixture contained a buffer-salt solution, a standard concentration of mucopolysaccharide acceptor, the enzyme extract, and UDP-Galactose C^{14} (918 cpm/mμmole; 30 mμmole/reaction mixture). The incorporation of C^{14}-galactose into the ethanol-insoluble fraction was measured after 1 hr incubation at 30°C. The specific enzyme activities are given as cpm of C^{14}-galactose incorporated/hr/mg protein, the latter estimated by the Folin procedure.[8]

Chemicals: Actinomycin D was graciously supplied by Merck, Sharp and Dohme, Inc. Cycloheximide (trade name: Actidione) was purchased from the Upjohn Co.

Sucrose gradient centrifugation: Single Millipores were harvested in cold water and the cells collected by centrifugation and frozen. The pellets were thawed rapidly in the presence of 0.1 ml 10% sodium dodecyl sulfate, diluted with 0.9 ml H_2O, and immediately layered over a 15–30% ucrose-SDS gradient. The tubes were spun in an SW-25 Spinco rotor 17 hr at 18°C, emptied

from below with a finger pump through the flow cell of a Gilford recording spectrophotometer, and collected in 1-ml fractions.[9] Each fraction was precipitated with TCA, deposited and washed with TCA on a Millipore filter, cemented to a planchet, and counted. RNase sensitivity controls have been run in previous experiments,[4] though not in those reported here, and have indicated that at least 95% of the counts were RNase-sensitive.

Results.—The actinomycin-sensitive period in FR-17: In a previous study[4] FR-17 amoebae were dispensed on Millipores, preincubated 16 hr at 15–16°C, and then shifted to 22°C to begin the morphogenetic sequence. (Preincubating for 16 hr at 15–16°C is equivalent to the first 2 hr at 22°C, i.e., developmental events occur 2 hr sooner in preincubated cells (including the rise and fall of transferase activity) and the terminal morphogenetic stage is reached in 12 hr versus 14 hr for cells not preincubated.) UDP-Gal polysaccharide transferase activity appeared at 6.5 hr, accumulated to a peak between 10 and 11 hr, and thereafter disappeared. Addition of actinomycin at 2 hr after the temperature shift prevented any enzyme accumulation; if added at 6 hr or later, slightly more (110%) than the normal peak specific activity was attained; if added between 2 and 6 hr, a linear relationship existed, between the peak enzyme activity and time of addition.

Effect of exposure to cycloheximide during the actinomycin-sensitive period: Figure 1*A* (*open circles*) shows the normal course of enzyme accumulation and disappearance in FR-17. When cycloheximide (250 μg/ml) was added to the Millipore support pads at 3 hr and removed at 5 hr (*triangles*), the enzyme activity did not appear at the usual time (6.5 hr) but only after 3 additional hr, i.e., 4.5 hr after removal of cycloheximide, and then accumulated to a level 85–90 per cent of that reached by the control cells. If, after cycloheximide was removed, actinomycin was added to prevent further RNA synthesis (*squares*), no enzyme accumulated at all. (In repetitions of this experiment, cells treated in this fashion did accumulate as much as 5–10% of the enzyme activity attained by the control cells.) In contrast, the addition of actinomycin at 5 hr to previously untreated cells permitted the normal course of enzyme accumulation to 75 per cent of the level reached by the control in agreement with previous results.[4]

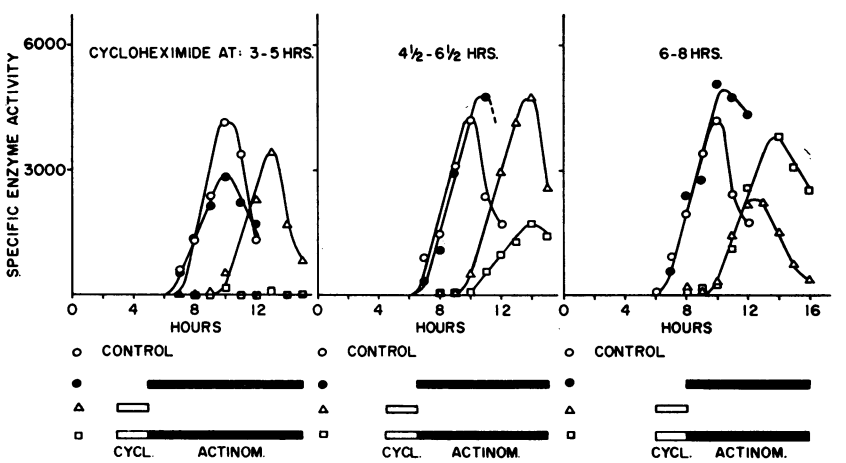

FIG. 1.—Effect of cycloheximide treatment during and after the period of transcription. *Ordinate:* Specific enzyme activity in cpm/hr/mg protein (918 cpm = 1 mμmole galactose incorporated). *Abscissa:* Time of incubation on Millipore filters after the temperature shift from 16 to 22°C.

Figure 1*B* illustrates the effect of adding cycloheximide at 4.5 hr and removing it at 6.5 hr. A lag of 3 hr occurred before appearance of the transferase, but the activity ultimately accumulated was approximately the same as that of the control cells. However, when actinomycin was added immediately after cycloheximide removal, the accumulated activity was only 40–50 per cent that of the controls, despite the fact that addition of actinomycin at 6.5 hr to previously untreated cells permitted them to accumulate the full complement of transferase activity.

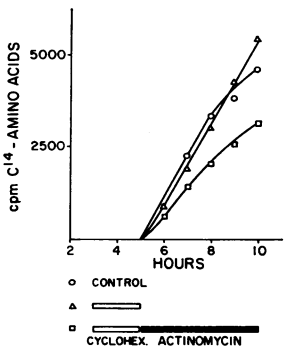

FIG. 2.—Amino acid incorporation after removal of cycloheximide. C^{14}-algal hydrolysate (1.4 mc/mg; 7.5 μc/ml saturating the Millipore support pads) was added at 5 hr. The cell samples were harvested from the Millipores in cold water and centrifuged. The pelleted cells were frozen, thawed, incubated with 10% TCA, and deposited on Millipores for counting.

Figure 1*C* shows that addition of cycloheximide between 6 and 8 hr, i.e., at the end of the normal period of transcription, had little or no effect on the amount of enzyme activity which subsequently accumulated. Thus, the peak activity attained by cells exposed to cycloheximide between 6 and 8 hr, and thereafter to actinomycin, was approximately the same as in the controls and in cells treated with actinomycin only. However, it should be noted that enzyme accumulation did not begin in the cycloheximide-treated cells until 1.5 hr after removal of the drug. This is in contrast with over-all protein synthesis as reflected by the rate of amino acid incorporation which recommences immediately.[3] The failure of cells treated with cycloheximide alone to accumulate the total complement of enzyme activity is due to the fact that transformation into spores and stalk cells was delayed only 1 hr by the late exposure to cycloheximide (unlike the previous experiments), and thus dormancy occurred prematurely with respect to the course of transferase accumulation.

Amino acid incorporation after cycloheximide removal: Figure 2 demonstrates the immediate resumption of amino acid incorporation after cycloheximide removal as previously shown.[3] It also indicates that cells exposed to cycloheximide for 2 hr and thereafter to actinomycin could incorporate amino acids at an appreciable rate without significant delay. Thus, the inhibition of transferase accumulation observed in Figure 1*A* and the lags in Figure 1*B* and *C* were preferential and not due to over-all interference with protein synthesis. Please note that the rate of incorporation, though appreciable, was still significantly lower than in the controls, suggesting that a considerable fraction of the mRNA contingent is subject to

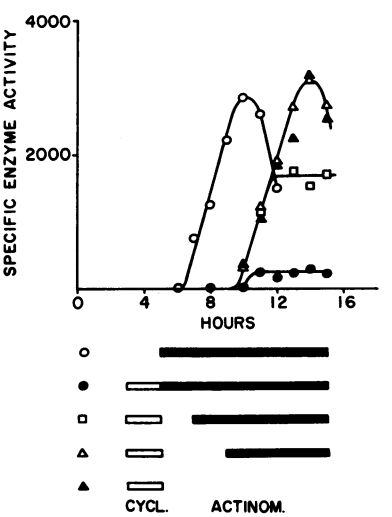

FIG. 3.—Dependence of enzyme accumulation on RNA synthesis after removal of cycloheximide. *Ordinate* and *abscissa* as in Fig. 1.

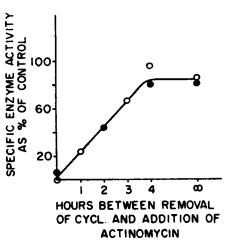

FIG. 4.—Relation between the amount of enzyme activity ultimately accumulated and the time between removal of cycloheximide addition of actinomycin.

the same fate in the presence of cycloheximide as that observed in the case of the transferase-specific RNA.

Further transcription after the removal of cycloheximide: Figure 3 shows the results of an experiment in which actinomycin was added at different times after the removal of cycloheximide. When actinomycin was added immediately, transferase activity appeared after a 4-hr lag, but accumulated to a level only 5 per cent that of the control. When actinomycin was added 2 hr after removal of cycloheximide, the enzyme accumulated to a level *ca.* 50 per cent of that reached in the control and, if added 4 hr afterward, 100 per cent.

Figure 4 shows the combined results of two experiments of this kind and demonstrates that, in the 4 hr subsequent to cycloheximide removal, a linear relationship existed between the time of actinomycin addition and the amount of enzyme activity ultimately elaborated. This relationship, set back by cycloheximide to a period between 5 and 9 hr, corresponds precisely to the relationship which was found to exist between 2 and 6 hr in normal, untreated cells,[4] i.e., presumably representing a second period of transcription of the transferase-specific RNA.

Please note also in Figure 3 that when actinomycin was added 2 hr after the removal of cycloheximide, the transferase activity which accumulated subsequently did not disappear. A similar temporal relationship between the actinomycin-sensitive periods for enzyme accumulation and disappearance was also observed previously in untreated cells.[4]

The effect of cycloheximide on the synthesis and/or stability of RNA: FR-17 amoebae on Millipore filters were incubated for 3 hr at 22°C and switched to new support pads containing 2 ml of salt solution plus carrier-free P^{32} orthophosphate (25 μc/ml) with and without cycloheximide (250 μg/ml). The cells were harvested after 2 hr incubation, treated as described in the *Methods* section, and the extracts spun through a 15–30 per cent sucrose gradient. Figure 5 shows the results of

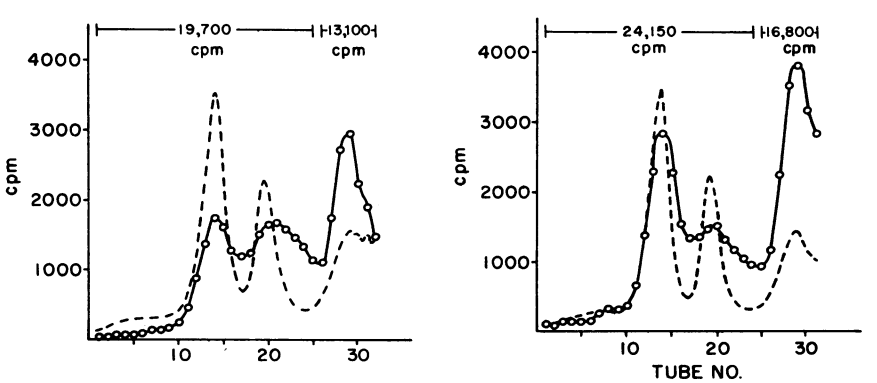

FIG. 5.—RNA synthesized in the presence of cycloheximide. See text for experimental details. *Left:* actinomycin-treated cells. *Right:* controls. *Dotted lines:* OD traces. *Solid lines:* radioactivity.

single runs, each representative of quadruplicate samples. (The profiles of the replicates were virtually identical, but a variation of ± 15% was encountered in the total amounts of P^{32} incorporated.) The poisoned cells incorporated almost as much label (*ca.* 85%) into high molecular weight (>4*S*) RNA as did the controls. This is consistent with the uridine incorporation data reported earlier.[3] However, considerable differences in molecular weight distribution were encountered. Thus, in the poisoned cells, there was a marked deficit of labeled 23*S* RNA after 2 hr, but a surplus of 4–16*S* RNA, so that the 16*S* peak was skewed toward lighter values and convex upwards on the light side. The data therefore show significant changes in the state of the RNA synthesized in the presence of cycloheximide very similar to effects observed previously in yeast by Fukuhara.[10] However, they do not permit a decision to be made between the possibility that cycloheximide (*a*) interfered differentially with the synthetic process, or (*b*) rendered the nascent RNA labile to nucleolytic attack.

Discussion.—The data represented in Figures 1, 3, and 4 are schematically summarized and interpreted in Figure 6. They indicate that, during the 2-hr exposure to cycloheximide, the RNA which is normally synthesized in this period *plus that fraction fabricated within the previous 30–60 min* did not subsequently give rise to active UDP-Gal transferase. Instead, a second round of RNA synthesis ensued, and this second round accounted for part or all of the transferase accumulation, depending on whether part or all of the first round had been rendered nontranslatable. Thus, the period of time during which this particular region of the genome is transcribed is not automatically restricted to the 2–6-hr period, but may be extended far beyond it.

Please note also that the amount of enzyme activity elaborated as a result of both synthetic rounds together approximated the total which accumulated in the undisturbed system. This may imply the operation of some sort of feed-back signal which could conceivably control the amount of transcription, or the amount of translation, or both. In addition it may be noted that the translation of the RNA, normally lagging 4.5 hr behind its transcription in the controls, could be made to lag 3 hr more by the application of cycloheximide. Thus, both the stability of the mRNA in this system and the times of utilization do not appear to be uniquely time-dependent but in fact may also depend upon concurrent metabolic events and the operation of the over-all developmental program.

The results summarized in Figure 6 and in Figures 4 and 5 suggest that cycloheximide may exert its effect by reducing the stability of the nascent RNA rather than interfering with its fabrication. It remains to be seen whether this activity is peculiar to cycloheximide or inherent in the interference with protein synthesis.

It is conceivable that the effect of cycloheximide on mRNA sta-

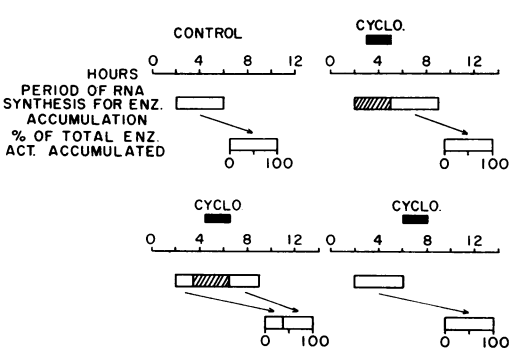

FIG. 6.—A schematic summary and interpretation of data from Figs. 1, 3, and 4. See text for details.

bility may be a reflection of its capacity to stop the synthesis of new ribosomal subunits.[10] Thus, the newly transcribed messages may ordinarily be stabilized by association with newly synthesized ribosomal subunits before migration through the nuclear envelope and may be rendered labile to nucleolytic attack when this association is prevented.

Summary.—During morphogenesis in *Dictyostelium discoideum* strain FR-17 there is a 4-hr period of RNA synthesis, delineated by actinomycin sensitivity, which is required for the appearance of the enzyme UDP-Galactose polysaccharide transferase. Interference with protein synthesis during this transcription disrupts the transcriptive process but does not affect the transferase-specific RNA previously fabricated. The cells appear to compensate for the disruption by initiating a second round of transcription after the resumption of protein synthesis. By these means the period of transferase-specific RNA synthesis can be extended considerably beyond its normally prescribed time limits.

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*ISOLATION OF HIGH-MOLECULAR-WEIGHT, P^{32}-LABELED INFLUENZA VIRUS RIBONUCLEIC ACID**

BY HARI O. AGRAWAL AND GEORGE BRUENING

VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

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Several investigators[1–7] have reported the isolation of ribonucleic acid (RNA) from influenza virus and influenza virus-infected tissues by methods which could result in the isolation of intact viral RNA. But the only indication of the size of the RNA molecules in any of these preparations, a sedimentation coefficient of 11*S*, was given by Sokol, Schramek, and Šponar.[5] Schäfer[8] has quoted a value of 17*S* for RNA isolated from fowl plague virus, a virus similar to influenza, and recently, RNA's having considerably larger sedimentation coefficients have been isolated from other larger, lipid-containing viruses.[9, 10]

A particle weight of between 200 and 400 $\times$ 10^6 may be calculated from the various estimates of influenza virus dimensions, density, and hydration.[11] Depending on the influenza strain and the analytical methods employed, RNA has been estimated to represent from 0.7 to 1.2 per cent of the dry weight of the virus in preparations having high infectivity.[12–17] Thus, if the RNA of influenza virus