*PERIODS OF GENETIC TRANSCRIPTION REQUIRED FOR THE SYNTHESIS OF THREE ENZYMES DURING CELLULAR SLIME MOLD DEVELOPMENT**

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Various aspects of mRNA synthesis and stability have been examined in bacteria by permitting transcription to occur over a known, usually brief, period and then determining how much of a specific protein subsequently accumulates in the absence of further RNA synthesis. Thus, bacterial cells have been exposed to an inducer for very brief periods during which RNA synthesis could proceed normally and were then permitted to synthesize the enzyme *de novo* in the absence of both the inducer and further transcription. The latter restriction was accomplished with base analogues,[1] uracil deprivation,[2] actinomycin D,[3, 4] and by infection with lytic viruses.[5] In an arginine- and uracil-requiring strain of *E. coli* infected with phage T6, protein and RNA synthesis were sequentially (and reversibly) restricted by successive precursor deprivations in order to study the accumulation of enzymes required for phage development.[2, 6] Under all of the above conditions, the amounts of enzymes synthesized were assumed to reflect in a simple fashion the over-all quantities and net concentrations of the corresponding mRNA species, and the subsequent decays of enzyme-forming capacity with time were assumed to reflect the manner in which the mRNA disappeared.

This approach has also been exploited to investigate the transcriptive and translative events required for accumulation and disappearance of the enzyme uridine diphosphate galactose:polysaccharide transferase during slime mold development. This enzyme, undetectable in the vegetative cells of *Dictyostelium discoideum*, appears at a specific morphogenetic stage, accumulates to a peak of specific activity, is preferentially released by the cells, and is then rapidly destroyed.[7, 8] The accumulation of the enzyme was shown to be sensitive to coincident inhibition of protein synthesis (by cycloheximide) and to prior inhibition of RNA synthesis (by actinomycin D).[9, 10]

By adding actinomycin at later and later times during the developmental sequence and then determining how much, if any, transferase activity subsequently accumulated, it was possible to define the limits of this transcriptive period and the temporal and quantitative relations between it and the translative period that followed. This was done both in *D. discoideum* wild type and in a temporally deranged mutant Fr-17.[10] This approach has now been extended to two other enzymes which accumulate at different times during cellular slime mold development, uridine diphosphate glucose (UDPG), pyrophosphorylase (EC 2.7.7.9)[11] and trehalose-6-phosphate synthetase (EC 2.3.1.15). The present communication describes the temporal and quantitative relations between the transcriptive and translative periods for the accumulation of all three enzymes. The results indicate that: (*a*) the three transcriptive periods, though many hours (7–9) in length, occupy relatively restricted portions of the total number of hours (24) required for morphogenesis to be completed under these conditions;

(*b*) they are initiated at different times during the developmental sequence; (*c*) during each transcriptive period, there is a linear or possibly sigmoid relation between the time over which transcription is permitted to occur (before addition of actinomycin) and the amount of the corresponding enzyme which subsequently accumulates; (*d*) the transcriptive periods begin, respectively, one, five, and seven hours prior to the appearances of the corresponding enzymes.

Materials and Methods.—Organism and culture conditions: *D. discoideum* strain NC-4 (haploid) was grown in association with *Aerobacter aerogenes*[12] to the beginning of the stationary phase. The cells were then harvested, washed free of the remaining bacteria, and aliquots of 1×10^8 cells were dispensed on 47-mm Millipore filters (AABP 047 00) resting on pads saturated with buffer–salts–streptomycin solution in 60-mm Petri dishes.[12] Under these conditions the cells aggregate and construct fruiting bodies over a 24-hr period with a high degree of synchrony.

Enzyme assays: For the UDP-gal transferase and trehalose-6-phosphate synthetase assays, the cells were harvested in 0.01 *M* Tris (tris-(hydroxymethyl)aminomethane), pH 7.4, with 0.005 *M* thioglycollate, or in 0.1 *M* tricine (N-tris (hydroxymethyl)methylglycine), pH 7.5, for the UDPG pyrophosphorylase assay and were stored at −20°C. The samples were then thawed, sonicated in a Branson sonifier for 1 min at a level of 2 amp, and assayed immediately.

UDP-gal transferase:[8] The reaction mixture contained 15 μmol dimethylglutarate, pH 7.4, 4 μmols KCl, 0.5 μmol $MgCl_2$, 36 mμmols UDP-galactose-(C^{14}) at a specific activity of 1.8 μc/μmol, purified mucopolysaccharide acceptor in a volume of 0.08 ml, and 0.2 ml of enzyme extract. The mixture was incubated 60 min at 30°C. Then 0.25 ml of 0.1 *M* HCl was added and the mixture was incubated 13 min at 100°C to hydrolyze the remaining UDP-gal. Three ml absolute ethanol were added to precipitate the acceptor. After 30 min in the cold, the precipitate was deposited on a Millipore filter (EHWPO2500), washed three times with 80% ethanol, cemented to a planchette, and counted. Acceptor-dependent enzyme activity was measured in mixtures containing and lacking a standard concentration of the acceptor (a 20-fold difference at peak activity) and expressed as cpm/hr/mg protein.

UDPG pyrophosphorylase:[11] The reaction mixture contained 2.5 μmols $MgCl_2$, 5 μmols uridine triphosphate (UTP), 2.5 μmols glucose (C^{14})-1-phosphate at a specific activity of 0.04 μc/μmol in a volume of 0.15 ml, and 0.35 ml of extract in 0.1 *M* Tricine pH 7.5 buffer. The mixture was incubated 10 min at 35°C and 1.5 min at 100°C. Alkaline phosphatase (25 μg of crystalline bacterial enzyme) was added and the mixture incubated 30 min at 45°C to destroy the remaining G-1-P. Then 0.01 ml of 5 *N* HNO_3, 0.3 ml of 14% mercuric acetate in 10% acetic acid, and 0.5 ml of 95% ethanol were added to precipitate the UDPG. After 10 min in the cold, the precipitate was deposited on a Millipore filter (EHWPO 2500), washed three times with 80% ethanol, cemented to a planchette, and counted. Activity was expressed as μmols UDPG produced/min/mg protein.

Trehalose-6-phosphate synthetase: This enzyme, previously demonstrated in *D. discoideum*,[13] catalyzes the reaction G6P + UDPG → trehalose-6-P + UDP.[14] The above stoichiometry was confirmed, and a quantitative enzyme assay based on the procedure of Cabib and Leloir[14] was devised[15] and has been employed in the present study. The reaction mixtures contained 15 μmols $MgCl_2$, 0.5 μmol ethylenediaminetetraacetate (EDTA), 100 μmols KCl, 8 μmols disodium glucose-6-phosphate, 2 μmols UDPG in 0.09 ml (pH 7.0), and 0.16 ml extract. The mixture was incubated 30 min at 37°C and the reaction was stopped by incubating 5 min at 100°C. The UDP that had been generated was assayed by adding 1.0 μmol neutralized phosphoenolpyruvate and 10 units of pyruvate kinase in 0.07 ml and incubating 60 min at 30°C. The pyruvate thereby produced was determined colorimetrically. Activity was expressed as μmols UDP (or trehalose-6-phosphate) produced/min/mg protein.

Actinomycin treatment: Actinomycin D was stored dry at 0°C in the dark and solutions were prepared a few hours before use. Washed cells, harvested from growth plates,

were deposited on the Millipore filters and allowed to develop for varying periods. The filters were then shifted to fresh pads saturated with buffer–salts–streptomycin solution containing 125 μg/ml actinomycin. All operations were carried out in dim light and the cells were incubated in light-proof containers.

Reagents: Labeled glucose-1-phosphate and UDP galactose were purchased from New England Nuclear Corp., and unlabeled UDPG, UDP-gal, and glucose-1-phosphate from Sigma. Phosphoenolpyruvate and pyruvate kinase were purchased from Boehringer. Alkaline phosphatase purified from *E. coli* was purchased from Worthington. Actinomycin D was graciously donated by Dr. Clement Stone of Merck, Sharp and Dohme, Inc.

Results.—The morphological features of the development of washed *D. discoideum* amoebae deposited on Millipore filters are schematically summarized in Figure 1. The initially smooth lawn of cells concentrates into conical, multicel-

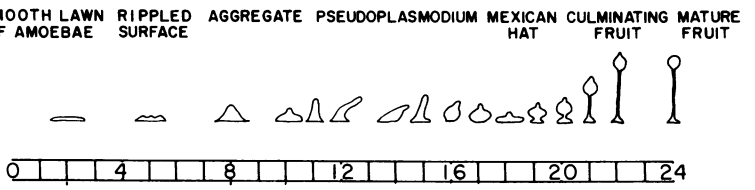
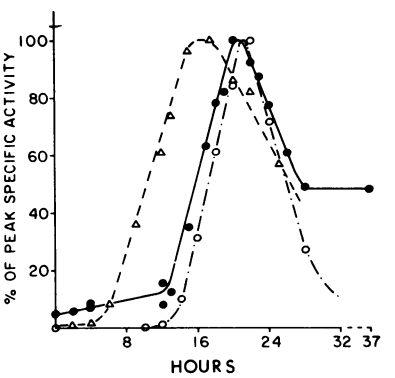


FIG. 1.—*D. discoideum* wild-type amoebae were harvested from growth plates, washed, and deposited on Millipore filters (see *Materials and Methods*). The various morphogenetic stages were attained at the times (hours after deposition on the Millipores) designated on the abscissa. Under routine conditions, this time sequence is precise within ±45 min.

lular aggregates, and these are transformed into organized fingerlike pseudoplasmodia. Subsequent morphogenetic movements result in the construction of fruiting bodies, each composed of a mass of spores surmounting a cellulose ensheathed stalk above a basal disk. The entire process requires 24 hours under these conditions. Figure 2 shows the changes in the specific activities of the three enzymes during the developmental sequence. In agreement with previously published results, trehalose-6-phosphate synthetase starts accumulating at 5 hours from a previously undetectable level, peaks at 16–17 hours, and then disappears.[15] UDPG pyrophosphorylase is initially present at low activity, remains relatively constant until 12 hours, and then increases rapidly to a peak

FIG. 2.—Developmental kinetics of the three enzymes. Cells were harvested from growth plates, washed, and deposited on Millipore filters as described in *Materials and Methods*. After incubation for the times indicated in the abscissa, the cells were harvested, frozen, sonicated, and assayed for three enzyme activities: trehalose-6-P synthetase (*triangles*); UDPG pyrophosphorylase (*closed circles*); UDP-gal:polysaccharide transferase (*open circles*). The specific enzyme activities thereby measured are expressed as percentages of their peak activities which were, respectively, 0.017 μmol trehalose-6-phosphate produced/min/mg protein; 0.25 μmol UDPG produced/min/mg protein; and 1500 cpm C^{14}-galactose transferred/hr/mg protein.



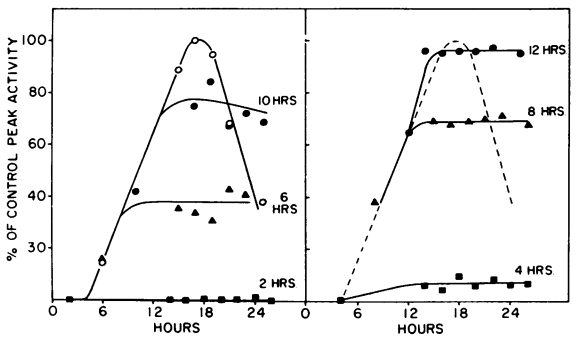


FIG. 3.—Effect of actinomycin on the accumulation and disappearance of trehalose-6-phosphate synthetase activity. Washed cells were deposited on Millipore filters at 0 time. At various times thereafter, one set of Millipores was shifted to new pads saturated with a solution containing actinomycin, 125 μg/ml of pad fluid, and a second (control) set was shifted to pads lacking actinomycin. The cells were collected at intervals thereafter for enzyme assays. The control curves varied only with respect to the absolute levels of peak enzyme activity (± 8%), and one of them is shown on the left (*open circles with solid line*) and is reproduced on the right (*dotted line*). All the curves were normalized to this one. Next to each experimental curve is the time after deposition of the cells on the Millipores at which they were exposed to actinomycin.

9 hours later. Part of this activity (the fraction associated with the stalk cells of the mature fruit) then disappears. The rest, associated with the spores, remains.[11] The accumulation and disappearance of UDP-gal transferase activity follow closely (30–60 min) behind those of the pyrophosphorylase.[8, 11]

The effect of actinomycin D on RNA and protein synthesis and on over-all morphogenesis in *D. discoideum* has been described in detail elsewhere.[16] The rate of uridine incorporation was depressed to *ca.* 20 per cent of the normal level and this residue was confined exclusively to the 4*S* region of sucrose gradient sedimentation profiles. In contrast, amino acid incorporation remained unaffected for periods of four to six hours after addition of the agent. Morphogenetic changes, both gross and cytological, were inhibited in a specific manner. Where further morphogenesis did occur after exposure to the drug, it was at the same rate as in untreated controls.[16] There was no indication of generalized damage to the cells nor of aberrations other than those stemming directly from the inhibition of RNA synthesis. Figures 3–6 show the effect of actinomycin D addition on the accumulation and disappearance of trehalose-6-phosphate

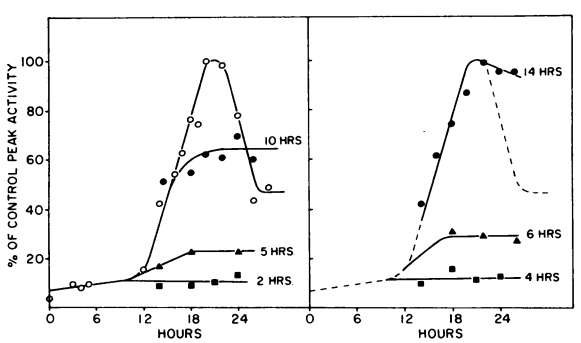


FIG. 4.—Effect of actinomycin on the accumulation and disappearance of UDPG pyrophosphorylase. The control curve (*open circles, solid line*) on the left, reproduced as a dotted line on the right, gives the combined data from two separate experiments and represents the time course of pyrophosphorylase accumulation and disappearance in the absence of actinomycin. Companion sets of Millipores were exposed to actinomycin at the times shown next to each of the experimental curves and were sampled at the times indicated in the abscissa for determinations of specific enzyme activity.

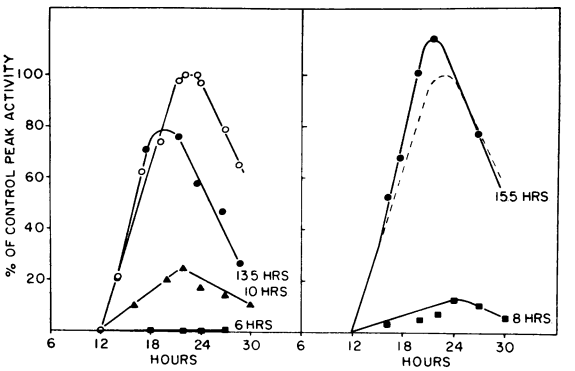


FIG. 5.—Effect of actinomycin on the accumulation and disappearance of UDP-gal polysaccharide transferase. See Fig. 4 for explanation. Two of the curves (*squares, triangles*) were published previously.[10]

synthetase, UDPG pyrophosphorylase, and UDP galactose:polysaccharide transferase, respectively. In the case of the synthetase, addition of actinomycin prior to 4 hours prevented significant synthesis of enzyme; addition after 13 hours permitted synthesis approximately at the normal rate and to a peak 100–105 per cent of that attained by the controls; addition betweentimes permitted intermediate amounts of enzyme to be synthesized, such that the later actinomycin was added, the more enzyme accumulated. Also, the disappearance of enzyme observed in the controls was prevented or interfered with by addition of actinomycin even as late as 12 hours. The same general pattern of inhibition was observed in the case of the pyrophosphorylase but over a different time period. Thus addition of actinomycin prior to 5 hours prevented synthesis of the enzyme; addition after 12 hours permitted synthesis at approximately the normal rate and to a peak 100–110 per cent of the control; addition between times permitted intermediate amounts to be synthesized. Addition of actinomycin even as late as 14 hours, though it did not interfere with accumulation of the pyrophosphorylase, did significantly affect its disappearance. In the case of the UDP-gal transferase, the pattern of inhibition is in agreement with previously published data.[10] Addition of actinomycin prior to 7.5 hours prevented any synthesis of enzyme; addition after 14–15 hours permitted synthesis at approximately the normal rate and to a level 100–110 per cent of the controls; addition at times in between permitted synthesis to intermediate levels. The precise relationships between the time of actinomycin addition and how much of each enzyme could subsequently be synthesized are shown in Figure 6 and are approximately linear

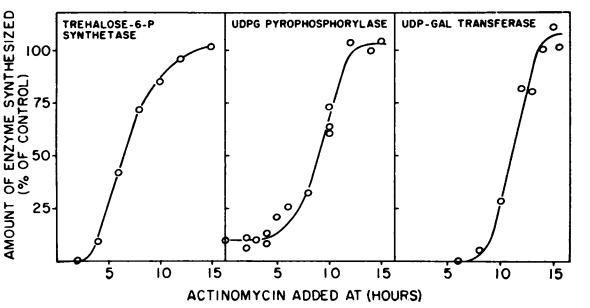


FIG. 6.—Relation between the times at which actinomycin was added and the peak level of enzyme activity that subsequently accumulated, expressed as per cent of the control peak. The points are calculated from curves shown in Figs. 3–5 and from others not shown. The data for UDP-gal transferase were published previously.[10]

or perhaps sigmoid. The data (i.e., peak levels of enzyme which accumulated) are taken from the curves in Figures 3–5 and from curves not shown.

Discussion.—The results described above indicate that during cellular slime mold morphogenesis, the accumulation of each of three enzymes requires a specific period of genetic transcription. The temporal relations of these periods with each other and with the ensuing translative events are summarized in Figure 7. In

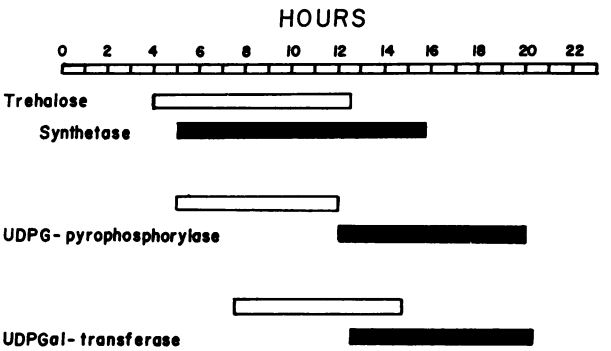


FIG. 7.—A schematic summary of the transcriptive and translative periods for accumulation of the three enzyme activities, derived from Figs. 3–6.

considering what kind of transcription is involved, it should be noted[17] that: (*a*) RNA synthesis goes on throughout the developmental sequence of both *D. discoideum* and a sister genus, *Polysphondylium pallidum;* (*b*) the RNA which is synthesized includes ribosomal and transfer RNA and material whose relative stability, association with polysomes, and size indicate it to be mRNA; (*c*) although considerable ribosomal turnover and resynthesis do occur during the developmental sequence, no differences could be detected between the ribosomes of vegetative amoebae and mature fruiting bodies with respect to protein composition or the sedimentation properties, base compositions, and specific hybridizability of the rRNA. While these data do not permit an unequivocal judgment, it is at least reasonable to suppose that the transcriptive products required for the accumulation of the three enzymes are the mRNA species which respectively dictate their primary structures.

It is noteworthy that these transcriptive periods apparently occupy restricted portions (*ca.* one third) of the time required for completion of the total morphogenetic sequence and of the time over which RNA synthesis occurs. This implies that not only the initiation of a transcriptive act but also its duration is under developmental control, a conclusion supported by the fact that in the temporally deranged mutant of *D. discoideum* (strain Fr-17) in which developmental events generally begin too soon and are completed too fast, the period of transcription required for the synthesis of UDP-gal transferase not only starts sooner than in the wild type but is of shorter duration (4 vs. 7–8 hr) in correspondence with the relative periods over which the enzyme accumulates in the two strains.[7, 10] Figure 6 indicates that, within each transcriptive period, there appears to be a

linear or perhaps a sigmoidal relation between the time over which transcription is allowed to proceed (before addition of actinomycin) and the amount of enzyme subsequently synthesized.

Figure 5 indicates time lags of one, seven, and five hours, respectively, between the initiation of each transcriptive period and the appearance of the corresponding enzyme. The possibility that they represent times needed for the combination of subunits into enzymatically active polymers, as observed in similar experiments with bacteria,[4] is ruled out by the fact that, in *D. discoideum*, addition of cycloheximide during the periods of enzyme accumulation froze the levels of all three enzyme activities immediately.[9, 11, 15] Thus, appreciable pools of preformed subunits do not appear to exist. The extents of the time lags (up to 7 hr) and the extreme differences (1–7 hr) among the three may imply the existence of specific translational controls or controls over the rate at which mRNA species might be conducted through the nuclear envelope to the site of translation.

The time lags also imply functional life spans of the RNA (up to 7 hr) greatly in excess of those encountered in bacteria and more comparable with values postulated for higher plants and animals.[18, 21] Unlike the situation obtaining in bacteria, the kinetics of enzyme synthesis either in untreated or actinomycin-poisoned cells display no hint of first-order decay. Furthermore, as seen in Figures 2–4, even when actinomycin was added early enough to limit significantly the extent of enzyme synthesis, the residue was synthesized at the same rate as in the controls. Finally, it should be noted that in the case of UDP-gal transferase, the five-hour time lag could be experimentally extended by at least three hours (by addition and then removal of cycloheximide) without significant detriment to the amount of enzyme subsequently synthesized.[22]

Figures 3 and 4 indicate that, for two of the enzymes at least, transcriptive periods are required for disappearance as well as accumulation of activity. Even in the case of the transferase, the requirement was demonstrable in mutant strain Fr-17[10] but not in the wild type where the two transcriptive periods apparently coincide. The meaning of these results is not clear since they could equally be due to genetically determined acts which specifically eliminate these enzymes or merely be the secondary consequences of the fact that actinomycin stopped morphogenesis short of the stages at which these enzymes normally do disappear.

Summary.—In the development of the cellular slime mold *Dictyostelium discoideum*, three different enzyme activities accumulate rapidly at specific morphogenetic stages and then disappear partly or completely. The enzymes are trehalose-6-phosphate synthetase, UDPG pyrophosphorylase, and UDP gal: polysaccharide transferase. The accumulations of all three enzymes are sensitive to prior inhibition of RNA synthesis by actinomycin D. The three transcriptive periods differ with respect to the times of their initiation and duration. They begin one, seven, and five hours before the appearances of the corresponding enzymes, thus indicating the existence of specific controls over the translation of mRNA into protein or over the migration of the former through the nuclear envelope to the site of translation.

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