CELL DIFFERENTIATION AND FLAGELLAR ELONGATION

IN NAEGLERIA GRUBERI

Dependence on Transcription and Translation

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ABSTRACT

This paper presents evidence that the phenotypic transformation of Naegleria gruberi from amebae to flagellates that occurs when cells are placed in a nutrient-free aqueous environment is dependent on transcription and translation. RNA and protein are synthesized during the hour-long differentiation. Actinomycin D and daunomycin selectively inhibit RNA synthesis, and cycloheximide selectively inhibits protein synthesis, throughout the time required for differentiation. These inhibitors prevent differentiation if added soon after the cells are transferred to nonnutrient buffer but cease to block specific differentiation events at subsequent, reproducible times, the transition points. After each transition point, morphogenesis can occur in the presence of the inhibitor and in the virtual absence of transcription or translation. A map of the transition points indicates that RNA synthesis is required until halfway through the temporal process from initiation to flagellum assembly, and that protein synthesis is required until three-fourths of the way through. Even when flagellum outgrowth can occur in the presence of cycloheximide, the length of the flagella formed is determined by the extent of synthesis of an unknown “limiting precursor.” The transition points for formation of flagella and for formation of the streamlined flagellate body shape are temporally separate. These results indicate that differentiation in Naegleria involves a redirection of cell metabolism to produce new RNA and protein molecules that are essential for morphogenesis.

The differentiation of Naegleria amebae to flagellates is a remarkable transformation of cell phenotype (10). It involves a change in cell shape and behavior from the constantly changing form of crawling amebae to the streamlined, asymmetric contour of swimming flagellates. The differentiation also involves a rearrangement of internal organelles, including, for example, the nucleus, which rolls about in amebae but is immobilized toward the anterior end of flagellates. It involves formation of all the organelles of the flagellar apparatus, including basal bodies, flagella, flagellar rootlet, and associated microtubular structures. The differentiation is transitory; after a time the flagellates change back to amebae, and only amebae are known to reproduce.
Equally remarkable are the properties of the differentiation system. All members of a clonal cell population can be caused to differentiate synchronously, and with reproducible kinetics, within an hour after initiation (12). Amebas can be grown either on bacteria (usually on a dilute nutrient agar), where they have a doubling time of 1.7 h (12), or in a soluble, partially defined axenic medium, where the doubling time is 8 h (9). At any time during thousands of generations, whether the amebae are growing or in early stationary phase, if the amebae are transferred from their growth environment to nonnutrient buffer, they differentiate with kinetics that are dependent on the growth conditions but independent of the precise position in the growth cycle. Placing the cells in nonnutrient buffer initiates differentiation (8), and the cells (in suspension) respond independent of one another and of cell population density (12). The change in environment causes each ameba to direct its efforts toward a developmental program that leads, through an orderly sequence of morphological changes, to expression of the flagellate phenotype.

Inhibitors offer a useful, although imperfect, means of probing the events of differentiation. The likelihood that differentiation of Naegleria depends on transcription and translation is supported by numerous reports that either actinomycin D or cycloheximide prevents the differentiation (1, 7, 16, 31, 32, 35, 45, 49). Notwithstanding these many reports, no one has used these inhibitors to define the biosynthetic requirements for differentiation more precisely. In this study, we have determined the suitable experimental concentration of each inhibitor, examined the effects of each inhibitor on RNA and protein synthesis, and measured the times at which each inhibitor ceases to affect various measurable events during differentiation. These events—the formation of flagella, the elongation of flagella to full length, and the change in cell shape—appear to depend on transcription and translation until specific, separate times during differentiation.

MATERIALS AND METHODS

Cell Culture and Differentiation

Amebas of Naegleria gruberi strain NEG (7) were grown on NM agar in association with Klebsiella pneumoniae strain BS (10) at 34°C as previously described (7, 12). Axenic strain NEG-M was cultivated in suspension in medium M or M7 at 32°C (9).

Conditions for differentiation have been described in detail (7, 9, 10, 12). Late-log or early stationary phase amebae were harvested and suspended in nonnutrient buffer at time zero. T buffer (2 mM Tris-HCl in demineralized water, pH 7.2, at 25°C) was used for bacteria-grown cells, and TK buffer (T buffer that contains 10 mM KCl) for axenic cells. A rapid centrifugation procedure was used so harvesting and washing could be completed in 3-5 min (7). The washed cell suspensions were incubated in a reciprocating shaker water bath, where the temperature was controlled within ±0.2°C.

Evaluation of Differentiation

We evaluated differentiation by fixing small aliquots of cell suspension in Lugol’s iodine and counting the number of cells with a given trait among 100 cells, using phase-contrast optics at × 640-800 (12). “Flagellates” are defined as cells with flagella, regardless of body shape.

We measured the length of flagella on the fixed cells at the magnification mentioned above, using a calibrated ocular micrometer and visually estimating the length of the apparent “longest” flagellum on each flagellate. These simple visual estimates of length are imperfect because of parallax and because of the curvature of the flagella, but estimates of average length based on measurements of 25 or 50 flagellates were reproducible, and different observers obtained similar measurements.

Most of the major differentiation experiments, and especially those that involved measurements of flagellum length, were evaluated “double blind.” After the samples were fixed, they were coded in random order by one investigator, counted and measured by another, and, finally, decoded. The double-blind experiments include those described in Figs. 2-4 and 11-13.

Inhibitors

Solutions of inhibitors were prepared in T or TK buffer. Cycloheximide (Actidine, crystalline, Upjohn Co., Kalamazoo, Mich.) was prepared as a 10 mg/ml solution. Actinomycin D, a gift from Merck, Sharp & Dohme International (Rhay. N.J.), was prepared as a 300-μg/ml solution and was allowed to dissolve overnight at 4°C before use. Daunomycin hydrochloride, from the Division of Cancer Treatment of the National Cancer Institute, National Institutes of Health, was stored as a 2-μg/ml solution. In experiments with actinomycin D and daunomycin, the solutions were protected from light.

RNA and Protein Measurements

RNA was measured by a modification of the method of Munro and Fleck (28, 46), and protein was determined by the method of Lowry et al. (26), using bovine serum albumin as standard.

We measured RNA synthesis using [2-3H]uracil (56.3 Ci/mole; New England Nuclear, Boston, Mass.) as described previously (46). We measured protein synthesis using [14C]lysine (G, 32 Ci/mmol; New England Nuclear). Because the amount of lysine incorporated was found to be proportional to the exogenous concentration, we used a high concentration at relatively low specific activity (see legend to Fig. 5). We measured incorporation by precipitating samples with ice 6.6% trichloroacetic acid–10 mM L-lysine, washing the precipitate twice by centrifugation and resuspension in 5% trichloroacetic acid–10 mM L-lysine, heating for 20 min at 100°C, cooling, and then rinsing onto 25-mm glass-fiber filters. The filters were rinsed with 5% trichloroacetic acid, then with 30 ml of 80% ethanol at 45°–50°C, and finally with 10 ml of ethanol/ether (1:1). The filters were then dried, and radioactivity was measured by scintillation count-
RESULTS

Differentiation without Net Synthesis of RNA or Protein

Fig. 1A shows the time course of differentiation of axenically grown cells at 25°C. Flagellates are first seen at 65 min, and more than 90% of the cells have flagella by 90 min. The curve, which approximates a summed normal distribution, indicates the population heterogeneity in time of differentiation (12). Half the cells have flagella at 78 min; this time is called the T₅₀. The T₅₀ for axenic cells at 25°C usually falls between 76 and 83 min; fifty cultures differentiated under these conditions had an average T₅₀ of 78.2 min with a standard deviation of 1.7 min (9). Cells grown on bacteria differentiate faster, more synchronously, and at least as reproducibly, with a T₅₀ at 25°C of 61 min.

Differentiation takes place without any change in cell number (Fig. 1B and reference 12). Not surprisingly, since differentiation occurs in non-nutrient buffer, there is no net accumulation of either RNA or protein (Fig. 1C and D). Any synthesis that occurs is balanced by degradation.

Requirements for Flagellum Formation

EFFECT OF INHIBITORS ON DIFFERENTIATION: The addition of either actinomycin D or cycloheximide to a cell suspension near the beginning of differentiation prevents the cells from undergoing any of the morphological changes associated with differentiation. Even over the course of a full day, flagellates never appear. The amebae tend to become somewhat rounded after about 2 h in cycloheximide but remain actively ameboid in actinomycin D. Amebae retain their viability in actinomycin D or cycloheximide for at least 3 h, as determined by measuring the efficiency of plating (7). The action of the inhibitors is not, however, reversible in an experimentally useful manner. Amebae incubated in actinomycin D and then washed free of external inhibitor did not differentiate (<5% flagellates). Amebae removed from cycloheximide did differentiate, but differentiation was delayed and asynchronous. Similar irreversibility of inhibition by actinomycin D and delayed reversibility after cycloheximide have been found in other organisms (for examples, see references 3, 22, and 30).

Although actinomycin D and cycloheximide prevent differentiation when added early, if either inhibitor is added just as flagellates begin to appear (e.g., at 60 min [Fig. 1]), cells form flagella in the presence of the inhibitor. Thus, these inhibitors do not prevent the morphological changes but only the events that prepare the cells for morphogenesis.

If either inhibitor is added at intervals between the initiation of differentiation and the time when flagellates appear, a transition interval is found during which some cells can continue to differentiate in the presence of the inhibitor and others cannot. Such an experiment for actinomycin D is shown in Fig. 2. Axenic cells were incubated at 25°C in a reservoir flask. At each time shown,
samples were removed and added to flasks containing actinomycin D to give a final concentration of 120 µg/ml. If samples were placed in actinomycin D before 30 min, no flagellates appeared. By 55 min, transfer of samples to the inhibitor was without effect on the formation of flagellates. Among samples transferred to the inhibitor between 25 and 55 min, progressively fewer cells were inhibited. By 30 min, about 2% of the cells were able to form flagella in actinomycin D; by 40 min, 50%; and by 50 min, 91%. Each individual cell passes through some quantal change, before which it is unable to form flagella in the presence of actinomycin D, and after which this phenotypic change is insensitive to the inhibitor. Such a quantal change defines, for each cell, a moment in the continuum of events during differentiation (12).

The successive increase in the proportion of cells that have accomplished this quantal change, i.e., that can differentiate in the presence of the inhibitor, indicates the population heterogeneity in the time that individual cells pass through the change. The plateau values indicate the percent of cells that had undergone the quantal change at the time they were transferred to the inhibitor. In the experiment shown in Fig. 2, half the cells have passed through this change at 40 min.

A similar experiment for cycloheximide is shown in Fig. 3. It will be noted that although the time of differentiation of the reservoir cells is comparable in Figs. 2 and 3, with a T50 of 79 min in both cases, differentiation becomes insensitive to actinomycin D before it becomes insensitive to cycloheximide.

The transition intervals for the cell population can be evaluated by plotting the percent of cells able to form flagella as a function of the time of transfer to the inhibitor. In Fig. 4, this is done for the experiments of Figs. 2 and 3. The resulting curves are parallel to each other and to the curve for flagella formation, indicating that cells become insensitive to the two inhibitors and form flagella with similar population heterogeneity. The time at which 50% of the cells in a population has become insensitive to an inhibitor is defined as the "transition point" (TP) (6). By 40 min, half the cells are insensitive to actinomycin D (TPAMD); by 60 min, half are insensitive to cycloheximide (TPCH); and by 79 min, half the cells have formed flagella (T50).

The accuracy of the measurements of TPAMD and TPCH depend on the use of suitable inhibitor concentrations. The concentrations used, 120 µg/ml of actinomycin D (10^-5 M) and 100 µg/ml of cycloheximide (3.6 x 10^-4 M), are comparable to those needed for cellular and acellular slime molds (5, 36, 43). Lower concentrations do not immediately arrest differentiation, and higher concentrations are not more effective. Actinomycin D, at a concentration of 30 µg/ml, added at the beginning of differentiation allows a few flagellates to appear;
FIGURE 3  Effect of cycloheximide on differentiation. Axenically grown cells were allowed to differentiate in TK buffer at 25°C at 3.5 × 10⁶ cells/ml (○). At the times indicated by the arrows, samples were placed in cycloheximide (100 µg/ml), and incubation was continued. No flagellates appeared in the samples transferred before 50 min, and the samples transferred after 70 min differentiated to an extent equal to or greater than the 70-min sample.

actinomycin D at 180 µg/ml does not arrest faster than a concentration of 120 µg/ml (e.g., samples transferred to actinomycin D at 120 and 180 µg/ml at 35 min gave 36 and 34% flagellates, respectively). Similarly, cycloheximide at 10 µg/ml delays differentiation but allows some flagellates to appear; a concentration of 40 µg/ml prevents differentiation if added at the beginning but does not arrest it immediately; and a concentration of 200 µg/ml does not have a greater effect than 100 µg/ml (e.g., samples transferred to cycloheximide at concentrations of 100 and 200 µg/ml at 60 min gave 46 and 56% flagellates, respectively). Thus the concentrations of actinomycin D and cycloheximide used in Figs. 2 and 3 are sufficient for rapid arrest of differentiation and give the best estimates attainable of the TP₄MD and the TP­₄H. Previous studies of the effects of inhibitors of transcription and translation on differentiation of Naegleria (1, 18, 31, 35, 45, 49) used inhibitor concentrations that were too low.

FIGURE 4  Temporal analysis of the inhibition of differentiation by actinomycin D and cycloheximide. This figure includes data from the experiments shown in Figs. 2 and 3. The curve for cells with flagella in the control flasks is the same as those shown in Figs. 2 (○) and 3 (△). For samples transferred to an inhibitor, the proportion of cells able to form flagella is measured as the "plateau percent flagellates," which is the average of three or more successively fixed samples. These plateau values are shown in Figs. 2 and 3. The plateau percent flagellates is then plotted as a function of the time the samples were transferred to actinomycin D (○) or to cycloheximide (△).

BIOCHEMICAL ASSAYS OF INHIBITOR EFFECTS: The obvious interpretation of these inhibitor experiments is tenable only if each drug is selectively inhibiting the expected synthesis, and if it remains an effective inhibitor of biosynthesis after it no longer inhibits differentiation. Cycloheximide at a concentration of 100 µg/ml rapidly and effectively inhibits protein synthesis in Naegleria. When cycloheximide was added together with [³H]lysine, only 1.6% of the control level of radioactivity was incorporated into protein after 120 min (Fig. 5), even though uptake of the [³H]lysine was not inhibited (data not shown). When the inhibitor was added at 60 min, after it no longer inhibited differentiation of all the cells, no further incorporation was observed, and the radioactivity in protein, in fact, declined 13% during the ensuing hour, indicating degradation of the protein labeled during the preceding 40 min (Fig. 5). At 60 min, incorporation of lysine into protein was rising at a rate of 47 cpm per minute per 10⁴ cells. Since incorporation immediately ceased when cycloheximide was added, without a
FIGURE 5 Effect of cycloheximide on incorporation of lysine into protein. O, control; V, cycloheximide added at 20 min; Δ, cycloheximide added at 60 min. Amoebae grown to stationary phase in medium M were sedimented, suspended in TK buffer at time zero, washed, and incubated at 5.5 x 10⁶ cells/ml in TK buffer at 25°C. [³H]Lysine was added (to 10 μCi/ml and 0.1 mM) at 20 min, and aliquots were transferred to flasks containing [³H]lysine with cycloheximide (final concentration, 100 μg/ml) at 20 and 60 min. Samples were precipitated with trichloroacetic acid and analyzed for radioactivity incorporated into protein. The total uptake of [³H]lysine, measured at 120 min, was similar in all three samples (data not shown). None of the cells placed in cycloheximide at 20 min differentiated, whereas 95% of the control culture differentiated, with a T₅₀ of 83 min, and formed flagella 14.3 μm long. Only 15% of the cells placed in cycloheximide at 60 min differentiated; the flagella on these cells reached a length of 6.8 μm.

rise of even 50 cpm, we conclude that cycloheximide at a concentration of 100 μg/ml is effective within a minute. Cycloheximide remains an effective inhibitor of protein synthesis after it ceases to be an effective inhibitor of differentiation (Fig. 5 and data not shown).

Actinomycin D at a concentration of 120 μg/ml rapidly but incompletely reduced incorporation of uracil into RNA (Fig. 6). On the basis of several experiments, inhibitor added together with isotope appears to take <5 min to become effective, and its effectiveness increases with time until by ~1 h after addition no further net incorporation occurs. Incorporation of uracil into RNA is inhibited 82–91% during the first hour after addition. Thereafter, inasmuch as little further incorporation occurs, inhibition reaches about 95% by 100–120 min. When actinomycin D is added at any time during differentiation, it inhibits RNA synthesis. For example, in the experiment shown in Fig. 6, when actinomycin D was added at 40 min, it reduced the rate of incorporation of uracil into RNA within 3 min, after which degradation of RNA labeled during the preceding 35 min exceeded any incorporation, resulting in a loss of 21% of the radioactivity from RNA by 100 min.

We have made a preliminary evaluation of the residual synthesis that occurs in the presence of actinomycin D. This synthesis is not the result of inhibitor concentration that is too low. Incorporation of uracil into RNA is inhibited 50% by actinomycin D at a concentration of ~6 μg/ml, but an increase in concentration from 120 to 180 μg/ml reduces residual incorporation by <25% (Fig. 7). The limited amount of RNA synthesized in actinomycin D is heterodisperse, with a broad peak centered around 15S, and this RNA is found in the polysomal fraction of cells, from which it is completely removed by treatment of the polysomal fraction with EDTA before sedimentation (data not shown). These observations, when compared with our earlier study of RNA synthesis in differentiating cells (46), suggest that the RNA synthe-
Effect of the concentration of actinomycin D on the incorporation of uracil into RNA. Actinomycin D at the indicated concentrations was added to samples of axenically grown cells in TK buffer together with [³⁵S]uracil (0.5 µCi/ml) at 7 min, and incorporation was measured at 100 min, at which time the control cells had incorporated 5,128 cpm/10⁷ cells.

Because actinomycin D did not completely inhibit RNA synthesis in these short-term experiments, we tested another inhibitor of transcription. In Dictyostelium, Firtel et al. (5) found that daunomycin, although itself a relatively ineffective inhibitor of RNA synthesis, gave nearly complete inhibition of RNA synthesis in combination with actinomycin D. We examined the effect of daunomycin, alone and in combination with actinomycin D, on differentiation and RNA synthesis in Naegleria.

Daunomycin, like actinomycin D, appears to intercalate between DNA base pairs and, thus, to inhibit transcription, but unlike actinomycin D, it does not appear to be specific to particular base pairs (20, 47). In Naegleria, daunomycin, even at a concentration of 250 µg/ml, proved to be only about half as effective in inhibiting incorporation of uracil into RNA as actinomycin D, and, even in combination, some residual synthesis remains—but only about half that seen in actinomycin D alone. In the experiment shown in Fig. 8, actinomycin D reduced incorporation to 5% of the control level by 110 min, daunomycin to 9%, and the combination of the two inhibitors to 2.6%. Daunomycin alone is more effective in Naegleria than it is in Dictyostelium, but there is less synergism between the two drugs.

Effect of several inhibitors on the incorporation of uracil into RNA. ○, control; △, actinomycin D (120 µg/ml); V, daunomycin (250 µg/ml); ●, both actinomycin D and daunomycin; x, cycloheximide (100 µg/ml). In B the ordinate is magnified 10 times. Late-log phase cells in medium M7 were sedimented, suspended in TK buffer at time zero, washed, and incubated in TK buffer at 25°C. At 3.5 min, samples of the cells were added to [³⁵S]uracil (3.3 µCi/ml) and the indicated inhibitor(s). Samples were precipitated with trichloroacetic acid and analyzed for radioactivity in RNA. 92% of the control cells differentiated, with a T₅₀ of 80 min; none of the cells in any inhibitor differentiated.

Daunomycin by itself or the combination of daunomycin and actinomycin D is an effective inhibitor of differentiation in Naegleria and remains effective slightly longer than actinomycin D. In the experiment shown in Fig. 9, actinomycin D inhibited differentiation until 42 min, daunomycin until 45 min, and the combination of the two inhibitors until 46 min. The difference between the time when actinomycin D ceases to be effective and that when daunomycin or the combination of the two inhibitors ceases to be effective is reproducibly <5 min. Daunomycin and the combination remain effective inhibitors of RNA synthesis when added after they no longer inhibit flagella formation (Table I).

The experiments described indicate that actinomycin D and daunomycin are effective inhibitors of RNA synthesis in Naegleria, and that cycloheximide is an effective inhibitor of protein synthesis. But are these inhibitors selective? Although it is impossible to determine whether the inhibitors affect only the expected synthesis, it is
FIGURE 9 Effect of actinomycin D (120 μg/ml), daunomycin (250 μg/ml), and a combination of the two inhibitors on differentiation, all measured in one experiment. The procedure used was the same as described in the legends of Figs. 2 and 4. The differentiation of samples transferred to the combination of actinomycin D and daunomycin at 40, 45, and 50 min are shown. The times at which 50% of the cells became insensitive to actinomycin D (△), daunomycin (▼), and actinomycin D with daunomycin (○) were 42, 45, and 46 min, respectively. The T50 for the appearance of flagellates in the control cells (●) was 78 min.

Axenically grown cells differentiating in TK buffer at 25°C were added at 45 min to flasks containing [14C]uracil (0.37 μCi/ml) and the indicated inhibitor(s). The control percent flagellates was determined as described in Fig. 4. The radioactivity incorporated into RNA was measured at 90 min.

In cycloheximide, the rate of incorporation of [14C]uracil into RNA is increased. For example, in the experiment shown in Fig. 8, in the presence of cycloheximide, incorporation occurred at 140% of the control rate. The cause of this increase is unknown, but the result indicates that in these short-term experiments nothing is affected in cycloheximide-inhibited cells that diminishes the rate of RNA synthesis.

Actinomycin D and daunomycin cause a decrease in the rate of incorporation of [3H]lysine or [35S]methionine into protein. In the experiment shown in Fig. 10, in the presence of actinomycin, lysine was incorporated at 80% of the control value (at 120 min); daunomycin reduced the incorporation to 71% of the control value, and the combination of both inhibitors reduced incorporation to 55%. In Dictyostelium, the combination of actinomycin and daunomycin reduced incorporation of exogenous amino acids into proteins more than did either inhibitor by itself (5). In Naegleria, the inhibition of precursor incorporation into protein by these inhibitors (20–45%, Fig. 10) is much less severe than the inhibition of incorporation into RNA (91–97%, Fig. 8). Actinomycin D, dauno-

### Table I

| Inhibitor(s)   | Plateau % flagellates | Incorporation (% of control) |
|---------------|-----------------------|-------------------------------|
| None          | 98                    | 100                           |
| Actinomycin D (120 μg/ml) | 77                | 9                             |
| Daunomycin (250 μg/ml)         | 70                | 24                            |
| Both          | 52                    | 4                             |

Possible to discover whether they produce nonspecific inhibition. A convenient way to do this is to assess the effect of the inhibitors on the reciprocal synthesis, the transcription inhibitor on protein synthesis, and vice versa. Because both RNA synthesis and protein synthesis depend on the function of several metabolic pathways, these syntheses are sensitive indicators of nonspecific inhibition.

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FIGURE 10 Effect of actinomycin D and daunomycin on the incorporation of lysine into protein. ○, control; △, actinomycin D at 120 μg/ml; ▼, daunomycin at 250 μg/ml; ●, actinomycin D plus daunomycin. Amebae grown to stationary phase in medium M7 were sedimented, suspended in TK buffer at time zero, washed, and incubated in TK buffer at 25°C. At 12 min, aliquots were transferred to flasks containing inhibitors with [3H]lysine (at 20 μCi/ml and 0.1 mM). Samples were precipitated with trichloroacetic acid and analyzed for radioactivity incorporated into protein. 91% of the control cells differentiated with a T50 of 80 min; none of the cells in the flasks with inhibitors differentiated.

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mycin, and the combination of the two had similar transition points, even though the inhibition of both RNA synthesis and protein synthesis varied considerably.

**Measurement of Transition Points:**
Within a single experiment, a transition point can be estimated with a precision of ±1 min (for examples, see Figs. 4 and 9). The reproducibility of transition points was evaluated by repeated measurements under similar conditions. For example, in nine experiments with actinomycin D in which axenically grown cells incubated in TK buffer at 25°C were used, the mean T50 was 79.0 ± 2.4 min, and the mean TPAMD was 40.9 ± 2.5 min. The two times are correlated, with slower differentiations (longer T50) taking a longer time to reach the TPAMD. The time between the TPAMD and the T50 averaged 38.1 ± 1.5 min, and showed less variation than the time from initiation (time zero) to the TPAMD, indicating that most of the variability in T50 occurs early in differentiation. Since the cells take 3-4 min longer to become insensitive to daunomycin or the combination of daunomycin and actinomycin D (Fig. 9), it seems reasonable to conclude that axenic cells differentiating at 25°C reach the RNA transition point 34-38 min before they form flagella. The data for cycloheximide are less extensive, but indicate that axenic cells differentiating at 25°C no longer require protein synthesis 16-20 min before flagella appear.

**Table II**

| Cells grown | Differentiation temperature | TPAMD | TPCH | T50 |
|-------------|-----------------------------|-------|------|-----|
| Axenically  | 25°C                        | 41    | 61   | 79  |
|             | (0.52)                      | (0.77)| (1.0)|     |
| Axenically  | 22°C                        | 53    | 83   | 102 |
|             | (0.52)                      | (0.81)| (1.0)|     |
| On bacteria | 25°C                        | 30    | 48   | 61  |
|             | (0.49)                      | (0.79)| (1.0)|     |
| On bacteria | 30°C                        | 26    | 42   | 48  |
|             | (0.54)                      | (0.88)| (1.0)|     |

These estimates of the transition points and T50 are based on repeated measurements. Actinomycin D was used at 120 μg/ml, and cycloheximide at 100 μg/ml. The numbers in parentheses indicate the fraction of the time to the T50.

The striking result of this comparison is that, when the time required for differentiation (the T50) varies more than twofold, the proportion of the time required for the two transition points remains relatively constant. With the exception of the TPCH for bacteria-grown cells differentiating at 30°C, the TPAMD occurs at 49-54% of the time to the T50, and the TPCH occurs at 77-81% of the time to T50. The regular proportions, in addition to allowing prediction of the TPAMD and TPCH under other conditions, also indicate that the relative rates of the processes leading to the transition points occupy a constant proportion of the differentiation time, regardless of the absolute time (as influenced by growth conditions and by temperature).

**Flagellar Elongation: Length-limiting Precursor**
If cells that are incubated in the presence of actinomycin D or cycloheximide during differentiation form flagella at all, the flagella are motile. The hope of isolating paralyzed "phenocopies" in which flagella are assembled lacking some component needed for motility has not been realized. Measurements of the length of flagella formed in the presence of these inhibitors have produced results of interest.

Data from cells having no flagella were not included because measuring "zeros" would introduce an unnatural bias inasmuch as the average length then depends on the percentage of cells with flagella. In control cells, the length "starts" at 2 to 5 μm (few definite flagella are seen that are <2 μm long), and increases "linearly" to a final length of 14-15 μm. The apparent rate of elongation of the flagella on untreated cells depends on the environmental conditions. The rate for axenically grown cells differentiated at 25°C is about 0.27 μm/min (Figs. 11 and 12), and for bacteria-grown cells at 30°C it is about 0.44 μm/min (data not shown). Although in these experiments flagella appear to elongate at a nearly constant rate, this is, in part, a result of population heterogeneity in the time that individual cells begin to grow flagella. An analysis of flagellar elongation (C. Fulton, unpublished) indicates that flagella actually elongate at a constantly decelerating rate, as has been observed in other organisms (cf. references 34 and 44).

In cycloheximide, the length of the flagella that...
cells are able to form is a function of the time at which cells are transferred to the inhibitor (Fig. 11 and Table III). Cells transferred late enough grow flagella as long as, or nearly as long as, those on control cells, whereas cells transferred early form short flagella that are only about half the length of the control flagella. This is a reproducible result. For example, in five independent measurements with axenic cells transferred to cycloheximide at either 55 or 60 min (depending on the experiment), the treated cells formed flagella only 46% of the length of the control flagella (6.6 vs. 14.7 μm; 5.9 vs. 14.8; 6.7 vs. 14.7; 7.6 vs. 14.6; and 6.8 vs. 14.3 μm). The longer one waits before transferring differentiating cells to cycloheximide, the longer are the flagella that form. Some process dependent on protein synthesis occurs in these cells that, even after flagella are able to form, limits the length to which the flagella can grow. Possibly, the accumulation of a flagellar protein, which, under these experimental conditions, is the "limiting precursor," determines the length the flagella can attain. The accumulation of length-limiting precursor can be visualized by plotting the length of the flagella that form as a function of the time of transfer to cycloheximide. In axenic cells differentiating at 25°C, limiting precursor accumulates roughly 30 min before the elongation of flagella (Fig. 11); in bacteria-grown cells at 30°C, roughly 12 min (data not shown). The limiting precursor accumulates to an extent that allows nearly complete elongation of flagella by the time flagella begin to grow.

The results obtained with actinomycin D are quite different. In this inhibitor, flagella, if they form at all, grow to nearly full length (Fig. 12). Only 12% of the cells transferred to actinomycin D at 35 min formed flagella, but these flagella grew to 91% of the length of the control flagella. However, when cells were transferred to actinomycin D shortly after they became able to form flagella, the flagella elongated slowly. For example, in the experiment shown in Fig. 12, the flagella on cells transferred at 35 min elongated at an apparent rate of 0.13 μm/min as compared with 0.27 μm/min for the control. In contrast, flagellar elongation was not markedly slowed in cells whose length is limited in cycloheximide, especially in relation to the final length (Fig. 11).

Cells transferred to actinomycin D after it ceases to inhibit the formation of flagella regularly form flagella about 10% longer than the control flagella (Fig. 12 and Table IV).

Transition Points for Body Shape

During differentiation, after the flagella form, the cells elongate to a specific, asymmetric body shape (11, 12). Does the change in cell shape also depend on transcription and translation? Conditions for the reproducible differentiation of amebae of N. gruberi NEG to flagellate body shape are more difficult to achieve than those for formation of flagella (11). Axenic cells differen-

**Figure 11** Effect of cycloheximide on flagellar elongation in axenically grown cells differentiating at 25°C. The length of flagella was measured in the samples fixed in the experiment described in Fig. 3. The control (○) and samples transferred to cycloheximide at 55, 60, 65, and 75 min are shown, as is the length attained as a function of time of transfer to cycloheximide (C). The reason why the flagella did not attain full length in cycloheximide is unknown; they did attain full length in other experiments (for example, see Table III).

**Table III**

| Time of transfer | Plateau % flagellates | Average length (μm) ± SEM |
|------------------|----------------------|--------------------------|
| Control          | 91                   | 13.90 ± 0.23             |
| 40 min           | 41                   | 8.38 ± 0.25              |
| 45 min           | 65                   | 11.65 ± 0.31             |
| 50 min           | 81                   | 14.18 ± 0.28             |

Bacteria-grown cells were allowed to differentiate at 30°C and samples were transferred to cycloheximide (100 μg/ml). The TP\(_{ch}\) was 42 min, and the T\(_{so}\) was 48 min. Average flagellar length is the mean ± standard error for 100 cells (25 each measured at 70, 80, 90, and 120 min).
tiate synchronously to cells with flagella, but only a small and variable proportion (averaging roughly 15% but ranging from 0 to 70%) form the flagellate body shape. All measurements of body shape changes were made with cells grown on bacteria, which regularly form the flagellate body contour.

The flagellate body shape does not form in the presence of actinomycin D or cycloheximide unless these inhibitors are added after the respective transition points for formation of flagella. It is possible to employ the same approach used for studying flagellum formation (Fig. 4) to measure the transition points for the body shape change. The ability to form the flagellate shape becomes insensitive to actinomycin D after the transition point for ability to form flagella but before flagella appear (Fig. 13A). In contrast, the ability to form the flagellate shape becomes insensitive to cycloheximide after flagella appear (Fig. 13B). The order is a simple consequence of the timing of events. The measured transition points for actinomycin D occur 31 min and 31 min, respectively, before the appearance of flagella and the change in body shape, and those for cycloheximide occur 13 min and 11 min before the changes. In each inhibitor, the transition points for body shape are temporally separate from those for flagellum formation.

DISCUSSION
At least two of the main events of cell differentiation in Naegleria—the formation of flagella and the formation of the flagellate body shape—appear (Fig. 13A). In contrast, the ability to form the flagellate shape becomes insensitive to cycloheximide after flagella appear (Fig. 13B). The order is a simple consequence of the timing of events. The measured transition points for actinomycin D occur 31 min and 31 min, respectively, before the appearance of flagella and the change in body shape, and those for cycloheximide occur 13 min and 11 min before the changes. In each inhibitor, the transition points for body shape are temporally separate from those for flagellum formation.
Figure 13 Temporal separation of the inhibition of two phenotypic changes by actinomycin D (A) and by cycloheximide (B). Amebae grown on bacteria were allowed to differentiate in T buffer at 25°C. The time of appearance of cells with flagella, independent of body shape (○), and of flagellate-shaped cells, independent of flagella (▲), are shown. The slight differences in population heterogeneity for these two phenotypic changes have not been observed regularly (cf. references 10 and 12). Samples of cells were transferred (A) to actinomycin D (120 μg/ml) or (B) to cycloheximide (100 μg/ml) at successive time intervals, and plateau values for percent of cells with flagella (O) and with flagellate body shape (▲) were determined.

It appears to depend on transcription and translation. In addition, elongation of flagella to full length depends on prior protein synthesis, and the rate of elongation appears to depend on prior RNA synthesis.

The inhibitors used in our experiments promptly and selectively inhibit the expected synthesis and continue to do so after they cease to prevent differentiation. After each transition point, the measured morphogenetic change occurs in the presence of inhibitor, without significant delay. These observations indicate that most RNA synthesis and protein synthesis cease to be essential after the transition points. The occurrence of some residual RNA synthesis in actinomycin or daunomycin leaves open the question of whether any RNA synthesized after the transition points is important for differentiation. In many organisms, as in Naegleria, inhibition of RNA synthesis by actinomycin is incomplete, at least in short-term experiments (for examples, see references 5, 30, 36, and 43). The combination of actinomycin and daunomycin gives a more severe inhibition of RNA synthesis in Naegleria, as it does in Dictyostelium (5), than does either drug alone, without having a major effect on the transition point. This strengthens our conviction that the transition points for RNA synthesis are meaningful but does not establish whether any part of the residual RNA synthesis after the transition points is essential for differentiation. Cycloheximide promptly arrests protein synthesis, as it does in other eukaryotes (for examples, see references 19, 34, and 40), so we are confident that, after the cycloheximide transition points, differentiation is independent of protein synthesis.

A useful and interesting consequence of these experiments is that we have been able to separate the events of synthesis from those of morphogenesis. For both the formation of flagella and the formation of the asymmetric body shape, the cycloheximide transition points occur before the phenotypic change, which suggests that once sufficient protein synthesis has occurred to “prepare” the cells, morphogenesis can occur, on schedule, in the absence of further synthesis.

The conclusion that RNA synthesis and protein synthesis before the transition points are essential is a reasonable extrapolation of these results. However, the use of inhibitors is a negative approach, subject to uncertainties due, especially, to possible side effects of the inhibitors on the change being measured; this is a limitation inherent in all experiments with inhibitors of transcription and translation. Both cycloheximide (27) and actinomycin (4, 17, 37–39) have been reported to have side effects. These inhibitors do not exert a generally adverse effect on cell metabolism in the experiments reported here, as indicated by the lack of serious inhibition of the reciprocal synthesis, by the continued motility of amebae in the presence of the inhibitors, and by the occurrence of mor-
phogenesis in the inhibitors. Nevertheless, it is always possible that the measured phenotypic change is prevented by an undetected side effect of the inhibitor. Indeed, in examining the reciprocal syntheses, we detected side effects similar to those described in some other systems. Cycloheximide increased the rate of incorporation of uracil into RNA (cf. references 21 and 42), and actinomycin and daunomycin reduced the rate of incorporation of lysine into protein (cf. references 16, 17, and 41). Although the causes of these side effects are not known, simple explanations are possible, as discussed in some of the cited papers (see also references 27, 29, 37, and 38).

The conclusion that transcription and translation are required for differentiation is supported by the observation that all of the proteins of the flagellate phenotype examined so far are synthesized de novo during differentiation. This has been reported for both subunits of the tubulin of the flagellar outer doublets (14, 23) and, more recently, has been extended to both the subunits of flagellar central pair tubulin, to dynein, and to flagellar rootlet (rhizoplast) protein (P. A. Simpson, D. Larson, J. Sellers, A. D. Dingle, and C. Fulton, manuscript in preparation). In the case of flagellar tubulin, translatable mRNA for this protein cannot be detected in amebae but is abundant in differentiating cells, and the time-course of the appearance and disappearance of translatable flagellar tubulin mRNA correlates well with the previously determined time-course of flagellar tubulin synthesis (24). Actinomycin D prevents the appearance of this translatable mRNA during differentiation (15).

At first glance, one might suspect that differentiation in Naegleria would primarily involve a rearrangement of components that preexisted in the amebae. The rapidity of the differentiation and the fact that it occurs under starvation conditions, without net synthesis of RNA or protein, would support this view. However, the finding that each measured morphogenetic event requires prior RNA synthesis and protein synthesis, together with the finding of specific syntheses, argues that macromolecular syntheses play a major role in this differentiation. A similar dependence of developmental events on transcription and translation has been found in many systems, from bacteriophage to embryos, but not in all (40).

In the cases of both flagellum formation and body shape change, the transition point for RNA synthesis occurs well before that for protein synthesis. One might suggest an orderly flow of events from environmental signal to selective gene activity to protein synthesis, but there is no reason to suppose it is that simple. Each transition point reveals only the completion of a sufficient amount of the last essential synthesis for the measured morphogenetic event. Any essential synthesis before the transition point is not revealed. Thus, protein synthesis, for example, may be required before and during, as well as after, the completion of essential RNA synthesis.

The transition points provoke speculation. One might propose, for example, that the last essential RNA measured by a TPAMD is the mRNA for the last essential protein measured by a subsequent TPCH. Ignorance of the specific RNA and protein species that might be responsible for the observed transition points provides a challenge for future research but no foundation for such speculations.

The existence of a limiting precursor for flagellum length that increases in amount as differentiation proceeds supports the idea that at least one protein component that determines the length of flagella is synthesized during differentiation. Rosenbaum et al. and other investigators (3, 25, 34) have obtained analogous results in their studies of flagellar regeneration in Chlamydomonas. In this organism the length of the flagella that form during regeneration is determined by the cycloheximide-sensitive accumulation of a precursor. In both organisms, the limiting precursor is unknown, although flagellar tubulin is an obvious candidate (23, 25, 48). It also is not known whether the accumulation of limiting precursor is distinct from the quantal change that enables a cell to form flagella in cycloheximide. Perhaps flagella are not formed at all until a sufficient amount of the limiting precursor has been synthesized.

The slow elongation of flagella on cells transferred to actinomycin D shortly after the TPAMD could be explained if, at the time of transfer, the cells have synthesized only a limited amount of a specific mRNA that is necessary for synthesis of a length-limiting flagellar protein. These cells would accumulate the length-limiting protein slowly because of limiting mRNA, and, thus, the flagella would elongate slowly. Even if this hypothesis is correct—and other explanations are possible—there is no reason to suppose that the limiting precursor that we observed by using cycloheximide is the same as the limiting precursor suggested by the actinomycin D experiments.

The increased length of flagella in actinomycin
D is reminiscent of many examples that have been reported of the synthesis of increased levels of enzymes and other proteins in the presence of actinomycin D (see references 29, 33, and 41). This phenomenon, known as "superinduction," suggests that a selective increase in the synthesis of some flagellar precursor(s) might lead to the formation of longer flagella. Possibly, the inhibition of transcription might lead to an increase in the rate of translation of available messenger RNA (cf. reference 29). Because we know almost nothing about what limits the length of flagella in any organism, this remains conjectural at present.

This study maps an orderly sequence of events in the differentiation of Naegleria (Fig. 14). The external morphological changes defined previously (12) are shown at the top of the figure. The amebae cease to move and round up to form spheres. At about the same time, the basal bodies, which are absent in amebae, are assembled and assume a position under the plasma membrane (13). There the flagella are assembled (2) and elongate to their final length. From early in their assembly, the flagella are active. As the cells swim, they elongate into the body shape characteristic of flagellates (11).

Before any of these morphological changes, macromolecular syntheses occur that prepare the cell for morphogenesis. Translatable mRNA for flagellar tubulin appears (24), and flagellar tubulin synthesis begins (23). Essential RNA synthesis for flagellum formation is completed about halfway through differentiation, while the cells are still amebae, and essential protein synthesis is completed as the cells round up. Essential transcription and translation for the body shape change are completed later than those for flagellum formation, but they precede the appearance of the flagellate shape itself. These observations indicate that, in response to the environmental changes that initiate differentiation (8), the cells redirect their metabolism to include a program of differentiation-specific syntheses.

The resulting schedule provides guidelines and challenges for the analysis of cell differentiation in Naegleria. The measured transition points provide a framework within which one can hope to define temporally the effects on the system of other perturbations. For example, if one finds a control T_50 of 60 min and an experimental T_50 of 80 min, one can use the transition points as a basis for asking whether the delayed differentiation of the experimental population is the result of a delay only in early events, before the TP_AMD, or of some later delay, before or after the TP_CH, or of a generalized retardation of differentiation. This approach revealed that differences in the T_50 of more than twofold due to growth conditions and temperature were distributed proportionally between three measured stages (Table II). The usefulness of this

![Figure 14 Temporal progression of events during cell differentiation in Naegleria. The events are placed on a relative time scale in which the T_50 for cells with flagella is defined as 1.0 to permit comparison of events measured under different conditions. The approximate validity of this method for some of the measurements is supported by the results shown in Table II. Below the time scale, the actual times are given for bacteria-grown cells incubated at 25°C. Measurement of the times for some of the changes have been published elsewhere: for body shape changes, see references 10 and 12 (see also Fig. 13); for assembly of basal bodies, see reference 13; and, for the beginning of flagellar tubulin synthesis, see reference 23. The approximate time at which flagella reach full length can be seen in Figs. 11-12.](image-url)
scheduling information is independent of the molecular basis of the transition points.

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