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Identification of a high molecular weight polypeptide that may be part of the circadian clockwork in *Acetabularia*

(biological clock/translation)

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ABSTRACT **In the chloroplast fraction of the unicellular and uninucleate green alga *Acetabularia*, we have detected a M_r ≈230,000 protein (p230) whose synthesis exhibits a pronounced endogenous diurnal rhythm. As judged by scanning densitometry of fluorographs of NaDodSO$_4$/polyacrylamide gels, the synthesis of other proteins in the same fraction was independent of the time in the cycle. The incorporation of [^{35}S]methionine into p230 was completely inhibited by cycloheximide, whereas chloramphenicol had no effect. This strongly suggests that p230 is translated on 80S ribosomes. Eight-hour periods of exposure to cycloheximide produced a shift in the phase of the oscillation of p230 synthesis. The results are consistent with the hypothesis that p230 is essential for expression of circadian rhythms in *Acetabularia*.**

Biological oscillations and, in particular, circadian rhythms (i.e., oscillations with periods of about 24 hr) increasingly attract attention because of their role in physiology, pathology, drug toxicity and efficiency, the effects of shift-work, and endogenous psychological disorders (1–3). Much effort has been made to elucidate the molecular mechanisms underlying circadian rhythms (for review, see ref. 4). These efforts have resulted in the postulation of a number of models (refs. 4–9; for further references see ref. 10). The coupled translation–membrane model has proven especially promising (9).

The coupled translation–membrane model explains the oscillations by a two-step mechanism. It postulates that in the first step, one (or a few) essential polypeptide is synthesized on 80S ribosomes. In the second step, this specific polypeptide is integrated into a membrane. This integration alters the properties of the membrane so that the synthesis of this essential polypeptide is suspended. By turnover, the amount of this essential polypeptide in the membrane falls below a critical threshold, thus restoring the synthesis of the essential protein and enabling a new cycle to begin. In accordance with this model, the synthesis of the essential protein should exhibit oscillations in the absence of any "Zeitgeber"—in other words, under constant conditions.

The coupled translation–membrane model is in agreement with a number of features of circadian rhythms, including the cycle-dependent, phase-shifting effect of cycloheximide that has been observed not only for the photosynthesis rhythm in the unicellular green alga *Acetabularia* (11, 12), but also for a membrane potential in the *Aplysia* eye (13), for a bioluminescence rhythm in *Gonyaulax* (14, 15), for a conidiation rhythm in *Neurospora* (16), for a chloroplast-migration rhythm in *Acetabularia* (unpublished data), and for a membrane-potential rhythm in the same organism (unpublished data). Results from genetic experiments in *Drosophila* (17–20) suggest that the *per* gene has a critical role in the expression of a circadian rhythm.

If the coupled translation–membrane model is correct, then the following conditions should be fulfilled. (*i*) There should be at least one polypeptide whose synthesis exhibits a characteristic diurnal oscillation under constant conditions. (*ii*) The synthesis of this polypeptide should be inhibited by cycloheximide. (*iii*) Periods of exposure to cycloheximide should result in a characteristic phaseshift in subsequent oscillations in synthesis.

We report the existence in *Acetabularia* of a polypeptide whose rate of synthesis oscillates under constant conditions.

MATERIALS AND METHODS

Treatment of Cells. *Acetabularia mediterranea* was grown in an artificial medium (21) under conditions described previously (22). Five days before the experiment was started, cells in the stage of cap formation were exposed to a 12-hr light/12-hr dark regimen at 20°C. After these 5 days, the cells were kept under constant light and temperature conditions. Cycloheximide treatments were given either immediately after the last dark period or 12 hr thereafter. Cells were incubated with 7 μM cycloheximide in artificial medium for 8 hr and then were washed three times with artificial medium to remove the drug.

At defined times after the last dark period, samples of 20 cells each were incubated for 30 min in 2.5 ml of artificial medium; 30 μCi of [^{35}S]methionine (Amersham Buchler; specific activity 1200 Ci/mmol; 1 Ci = 37 GBq) then was added and incubation was continued for 2 hr. Finally, the cells were incubated for 30 min in 200 ml of 0.1% Casamino acids (Difco).

Isolation of Cellular Contents. The cells were bundled at the rhizoid, the caps were cut off, and the cellular contents were separated from the cell walls by centrifugation for 20 sec at 2000 × *g* in isolation medium [0.33 M sorbitol/2 mM $NaNO_3$/2 mM EDTA/0.5 mM K_2HPO_4/20 mM NaCl/2.5% (wt/wt) Ficoll/50 mM Mes/1 mM $Mg(OAc)_2$/1 mM $MgCl_2$, adjusted to pH 6.1 with NaOH; ref. 23]. The sediment (chloroplast fraction) was suspended in 0.2 ml of isolation medium. The sample was frozen in liquid nitrogen and thawed twice and centrifuged at 10,000 × *g* (Eppendorf centrifuge) for 10 min. Trichloroacetic acid was added to the supernatant to a concentration of 6% (wt/vol). The sample was centrifuged for 10 min at 10,000 × *g*. The sediment was washed with 90% acetone, and 50 μl of electrophoresis sample buffer was added. The sample buffer contained 1 mM Na_2EDTA, 5% (vol/vol) glycerol, 0.025% (wt/vol) bromphenol blue, 1% (wt/vol) NaDodSO$_4$, and 0.05 M Tris Cl (pH 8.8). Dithiothreitol was added to a concentration of 5 mM, and the samples were heated for 2 min at 95°C. Finally, the samples were heated for 10 min at 56°C in the presence of 12.5 mM iodoacetamide (24).

NaDodSO$_4$/PAGE. Linear gradient gels (160 × 100 × 1.2 mm^3) were used. The gradient was established with acry-

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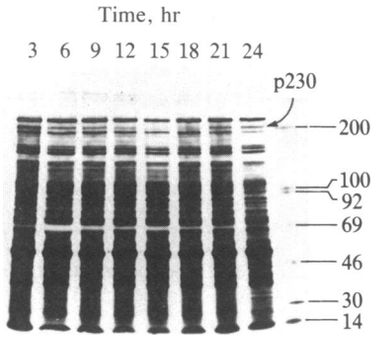

FIG. 1. Fluorograph of chloroplast-fraction proteins prepared from 20 cells that were labeled with [^{35}S]methionine for 2 hr at different times (hr) after the last dark period. The position of p230 is indicated. Lane at right represents molecular weight markers: myosin heavy chain (200,000), phosphorylase *b* (100,000; 92,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), lysozyme (14,000).

lamide concentrations from 5 to 10% (25). Approximately 50,000 cpm was applied to each lane. Gel electrophoresis and staining were performed as described (25). Fluorography was done by the method of Bonner and Laskey (26). The developed films were scanned with an LKB densitometer (2202 Ultroscan) and the peak areas were calculated with an Apple II computer system. To compensate for slight fluctuations in the radioactivity applied to the gel, the peak of the M_r 230,000 protein (p230) was related to a band lying directly above it. Similar results were obtained when p230 was related to other bands. The time series for control and phase-shifting experiments were analyzed by means of a least-squares (cosinor) method for period, phase (acrophase) percent rhythmicity, and statistical significance (27).

RESULTS

The coupled translation–membrane model (9) postulates that there is one (or a few) protein that is synthesized at different rates at different times of the day. To test this hypothesis, samples of 20 cells each were incubated with [^{35}S]methionine for 2 hr every 3–4 hr under continuous light conditions. The cellular contents were isolated and separated into supernatant and sediment. The sediment, which primarily contained chloroplasts (chloroplast fraction), was resuspended in isolation medium, subjected to two cycles of freezing and thawing, and then separated into sedimenting and nonsedimenting components by centrifugation. The nonsedimenting material, representing a portion of the membrane proteins released by the freezing–thawing cycles, was analyzed by one-dimensional NaDodSO$_4$/PAGE and fluorography (Fig. 1). Of the proteins resolved and analyzed in this manner, all but one (M_r 230,000) exhibited more or less constant rates of [^{35}S]methionine incorporation as revealed by densitometry (Fig. 2).

In all experiments, M_r 230,000 species (p230) was synthesized actively during the first hours after the last dark period but only very little between 10 and 18 hr. After 21 hr, the synthesis of this species increased (Fig. 3). Occasionally observed fluctuations in the rate of methionine incorporation into other bands did not show any regular dependence on the time of day. That only the p230 band showed such oscillations suggests that it represents one protein. This was corroborated by two-dimensional isoelectric focusing/NaDodSO$_4$/PAGE (unpublished data). The oscillatory behavior of the incorpo-

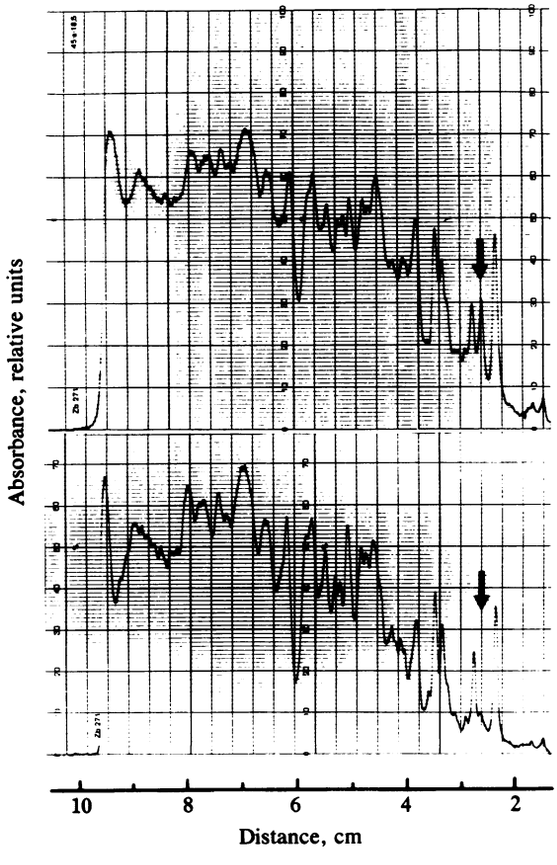

FIG. 2. Scanning-densitometry profiles of labeled chloroplast-fraction proteins (see Fig. 1) from cells that were labeled with [^{35}S]methionine 3 hr (upper profile) or 15 hr (lower profile) after the last dark period. Vertical arrow represents the position of p230.

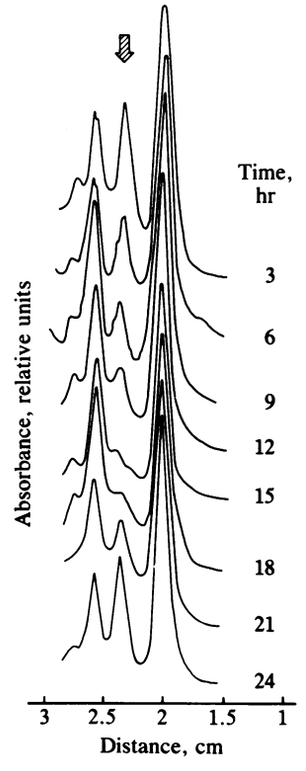

FIG. 3. Densitometric tracings of the eight lanes shown in Fig. 1, for the molecular weight range 190,000–260,000. The fluorograph was scanned at 663 nm. Vertical arrow indicates the position of p230.

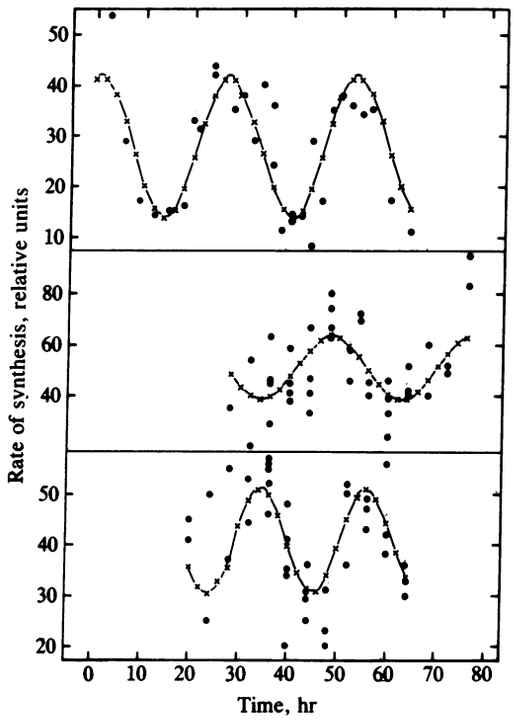

FIG. 4. Effect of cycloheximide pulses on the incorporation of [^{35}S]methionine into p230. Every 4 hr, 20 cells were incubated with [^{35}S]methionine for 1 hr. After gel electrophoresis and fluorography of the chloroplast-fraction proteins, the fluorographs were scanned and the areas under the peaks were calculated and plotted against time (●). The best fitting cosine curve (×—×) was calculated (27). (*Top*) Control cells. (*Middle*) Cells incubated with 7 μM cycloheximide for 8 hr after the last dark period and then washed several times. (*Bottom*) Cells treated with cycloheximide during the period 12–20 hr after the last dark period.

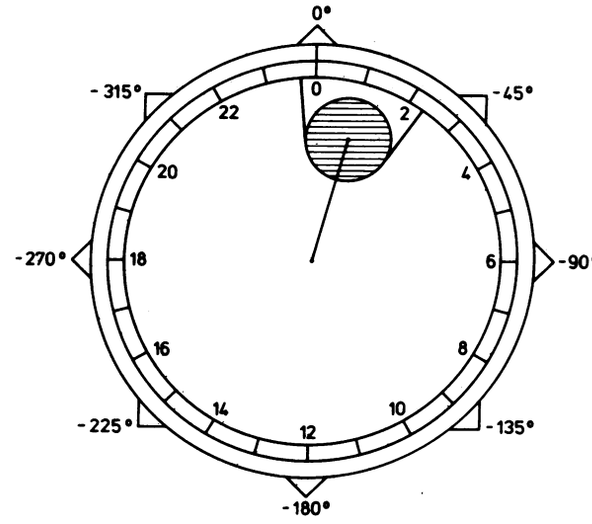

FIG. 5. Oscillation of [^{35}S]methionine incorporation into p230. Graphic presentation of the characteristics of the best-fitting cosine curve (27). The inner circle represents the cycle of 360° subdivided into circadian hours (hrc; 24 hrc = 26.0 hr). The hatched area corresponds to the 95% confidence area for acrophase and mesor (see Table 1). The 95% confidence limits for the acrophase are given in hrc.

ration rate could be observed for at least 64 hr after the last dark period (Fig. 4 *Top*). A statistical evaluation by means of a cosinor (least-squares) method (27) revealed that the data can be approximated by a cosine curve with a period of 26.0 hr and an acrophase (interval between light-on and the first maximum) of 1.0 hr. Under these conditions, the data for p230 represent 67% rhythmicity and the rhythm is highly significant ($P < 0.01$) (27) (Fig. 5; Table 1).

To investigate the location of p230 synthesis within the cell, the translation inhibitors chloramphenicol and cycloheximide were used. In the presence of 7 μM cycloheximide, synthesis of p230 was completely inhibited, whereas 0.3 mM chloramphenicol had no effect. This result strongly suggests that p230 is synthesized on 80S ribosomes.

Early experiments on individual cells of *Acetabularia* showed that periods of exposure to cycloheximide resulted in a characteristic phase-shift of the oxygen-evolution rhythm (11). In the present study, it was found that cycloheximide pulses affect the rhythm of the rate of p230 synthesis (Fig. 4 *Middle* and *Bottom*).

After cycloheximide treatment, rhythmicity is retained. Under these conditions percent rhythmicity of the data as judged from a cosinor evaluation is lower than in control cells (Table 1) but still statistically significant. A striking result is that cycloheximide pulses induce a change in period and phase. This suggests that the basic mechanism of the putative biological clock is affected and that, therefore, p230 may play an essential role in the oscillation. A rough estimation based on extrapolation indicates that these changes depend on when the cycloheximide is given during the cycle (Table 1).

DISCUSSION

We have found that there is one protein in *Acetabularia* whose synthesis oscillates under constant conditions. This protein, p230, fulfills three requirements that, according to the coupled translation–membrane model, are characteristic of an essential protein (9). The rate of its synthesis under constant light exhibits oscillations whose period is about 24 hr, its synthesis is inhibited by cycloheximide, and periods of treatment with cycloheximide result in characteristic phase shifts in its synthesis. In addition, our data indicate that cycloheximide pulses also affect the period of the p230 synthesis rhythm. Moreover, the coupled translation–membrane model suggests that there is a clear phase relationship between oxygen evolution and the synthesis of the essential

Table 1. Characteristics of best fitting cosine curves calculated by single cosinor method (27)

Hr of cycloheximide treatment	*n*	Period, hr	Mesor, relative units	Amplitude, relative units	Acrophase, hrc	*P*	% rhythmicity	Delay, degrees (hrc)
None	30	26.0	28	14	−1.0 (0.3 to −2.3)	<0.01	67	—
0–8	38	21.5	41	10	−14.4 (−12.3 to −16.3)	<0.01	45	135 (9)
12–20	37	28.5	51	12	−16.7 (−14.1 to −19.3)	<0.01	36	277 (18)

n, Number of estimations; mesor, average of calculated cosine curve; acrophase, time span between "light on" and first calculated maximum given in circadian hours (hrc) (95% confidence limits are given in parentheses); *P*, statistical significance of the rhythm. Rhythmicity is given in percentage of overall variability of data attributable to the rhythm. Delay is given in degrees (360° = 24 hrc) calculated on the basis of the time difference between the last minimum in the control experiment and the first minimum after termination of the cycloheximide treatment.

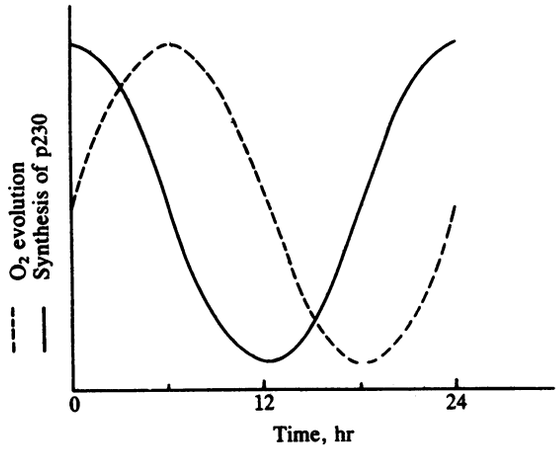

FIG. 6. Schematic presentation of the phase relationship between oxygen evolution and synthesis of p230. Based on data in ref. 9.

protein. The maximum of the synthesis of p230 should occur prior to oxygen evolution. This is in fact the case (Fig. 6). The phase difference is about 6 hr.

Under constant conditions, p230 is the only polypeptide in two-dimensional gel electrophoresis patterns (results not shown) whose rate of synthesis oscillates and which is synthesized during the early part of the cycle "daytime." Beside p230, two polypeptides with $M_r < 60,000$ were found whose syntheses also oscillate, but out of phase with that of p230 by roughly 180° (unpublished results). In this context, it should be mentioned that a polypeptide that occurs in the membrane fraction of *Acetabularia* chloroplasts (28, 29) is metabolically labeled in a fashion dependent on the time in the cycle and on temperature. In contrast to p230, this protein is synthesized in the presence of cycloheximide. Its role in the circadian clock and its relation to p230 is not clear. The same is true for the luciferase in *Gonyaulax* whose concentration oscillates in a circadian way (30).

That cycloheximide is capable of shifting the phase of the p230 synthesis rhythm is in agreement with the hypothesis that this polypeptide is involved in the central mechanism of the circadian rhythm (9). An effect of cycloheximide pulses on the period has been observed also in the case of the photosynthesis rhythm in *Acetabularia* (unpublished data) and of the conidiation rhythm in *Neurospora* (31). The effect on the period represents additional evidence for a central role of p230 synthesis in the circadian rhythm.

The oscillating behavior of the synthesis of p230 raises the question at what level does the circadian regulation of the synthesis occur. The observation that after removal of the nucleus, the rhythmic expression of p230 is retained (unpublished data) suggests that the regulation takes place at the translational level.

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