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11. Using data reported in (*10*), we have calculated $r = +.81$ ($P < .001$) between the reciprocal of the agonist concentration displacing 50 percent of bound GABA ($1/IC_{50}$) and the mean duration of agonist-induced channels. The $1/IC_{50}$ values (μM^{-1}) were as follows: muscimol, 25; 3APS, 14.3; isoguvacine, 6.25; GABA, 5; TAC, 2.32; THIP, 2.22; GABOB, 1.40; IAA, 1.11; BGP, 0.56; DAV, 0.2; and taurine, 0.02. Since the binding data reported in (*10*) were obtained, researchers in the same laboratory have repeated the study, and with the new data, $r = +.95$ (S. Lummis and R. W. Olsen, personal communication). GABA binding sites characterized in these cultured spinal neurons appear similar to those present on membranes extracted from intact spinal cord tissue [M. K. Ticku, A. Huang, J. L. Barker, *Brain Res.* **182**, 201 (1980)].
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14. We thank J. Mazzetta for the cultures; W. Vaughn for the computer programs; T. Smith for the voltage clamp; R. McBurney for collaborating on the fluctuation analysis protocol; H. Lecar, W. Klee, S. Snyder, and W. Vaughn for reading the manuscript; and M. A. Bragg for typing it.

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Genetic Evidence That Protein Synthesis Is Required for the Circadian Clock of *Neurospora*

Abstract. *Small doses of cycloheximide given at intervals (pulses) cause phase shifts of the circadian clock of* Neurospora. *The effects of this drug on the clock are mediated through its inhibition of protein synthesis, since two cycloheximide-resistant mutants whose 80S ribosomes are resistant to cycloheximide showed no phase shift after exposure to the drug.*

Metabolic inhibitors have been used to analyze molecular mechanisms underlying circadian rhythmicity in order to identify pathways of cellular biochemistry necessary for clock function. The rationale of this approach is as follows. If continuous application of the inhibitor alters the period length of the rhythm or if a pulse alters its phase, then the cellular target of the inhibitor is presumed to be important for the clock.

However, unequivocal identification of the target of clock alteration is difficult because most drugs affect multiple sites in the cell. For example, cycloheximide (CHX) inhibits cytosolic (80*S*) protein synthesis in eukaryotes; continuous application of this drug alters the period length of the clock in *Euglena* (*1*), and pulses cause phase shifts in *Acetabularia* (*2*), *Aplysia* (*3*), and *Gonyaulax* (*4, 5*). In addition, pulses of other inhibitors of protein synthesis, such as puromycin and anisomycin, cause phase shifts in *Acetabularia* (*6*) and *Aplysia* (*3, 7*). These results seem to suggest that protein synthesis in 80*S* ribosomes is necessary for clock function. However, under some conditions CHX inhibits mitochondrial function (*8*) and transport of amino acids and sugars (*9*), and it has been suggested (*4, 10*) that the effects of CHX on circadian rhythmicity are mediated through some mechanism other than inhibition of protein synthesis.

In one study in *Neurospora*, CHX did not alter the period length of the circadian conidiation rhythm in cultures growing on agar medium (*11*). However, CHX inhibited conidiation itself at such low concentrations that the clock could not be assayed under conditions where significant inhibition of protein synthesis occurred. For assaying the *Neurospora* clock, we have developed a liquid culture system that allows us to administer the drug in short pulses (*12, 13*) and have demonstrated that pulses of CHX cause phase shifts of the *Neurospora* clock (*14*).

In order to determine whether the phase-shifting effects of CHX are mediated through inhibition of protein synthesis, we examined the effects of the drug on two CHX-resistant mutants (*15*) in which the site of resistance is protein synthesis on 80*S* ribosomes (*16*). These mutants allow a direct test of whether CHX affects the *Neurospora* clock through its inhibition of protein synthesis or through some other unknown mechanism. If CHX acts through protein synthesis, then in these two CHX-resistant mutants the clock should also be resistant to CHX. If CHX acts through some other mechanism not affected by the mutations, then the clock of the mutants should show the same sensitivity to CHX as the wild-type strains. We now show that CHX alters the clock through its effects on protein synthesis.

The following strains of *Neurospora crassa* were used. The double mutant *bd, pan-2* (*17*) was crossed (*18*) to the cycloheximide-resistant mutants *cyh-1* and *cyh-2*. From these crosses, we isolated the triple mutants *bd, pan-2, cyh-1* and *bd, pan-2, cyh-2*. These two strains (referred to as the CHX-resistant strains) and *bd, pan-2* (referred to as the CHX-sensitive strain) were used for all experiments.

The strains were grown as described (*13*). Conidial inocula of the three strains were added to liquid glucose medium [0.3 percent glucose, Vogel's salts (*18*), 0.001 percent pantothenate]. All experiments were carried out at 25°C. The cultures were grown in constant light for 33 hours and formed mycelial mats. Disks (11 mm in diameter) were cut from the mats with a cork borer and washed in, and transferred to, liquid medium without pantothenate. At this time the disks were placed in constant darkness for the duration of the experiment. This light-to-dark (LD) transition sets the clock to a unique phase point from which it begins to "free run" (*12, 13*).

Beginning 15 hours after the onset of darkness, sets of six disks were treated with CHX (0.1 μg/ml) every 4 hours for the next 24 hours. At the end of each of these pulses, the disks were rinsed in drug-free medium and transferred to pantothenate-supplemented solid medium in race tubes, where they grew and formed conidial bands with circadian periodicity. Control disks to which CHX had not been added were also washed and transferred to race tubes at this time. The phase of the rhythm on the race tubes directly reflects the phase of the rhythm in liquid culture (*13*) and was calculated by linear regression analysis (*19*). The effect of the CHX pulse was determined by calculating the phase difference between a CHX-treated culture and a control culture transferred to race tubes at the same time.

The effects of CHX on protein synthesis were examined in the three strains as described (*14*). At 15 hours after the LD transition, circadian time 5 (CT 5), CHX was added to sets of six disks of each of the three strains at concentrations of 0, 0.1, or 0.5 μg/ml. After a 30-minute incubation period in CHX, L-[^{35}S]methionine (7.3 μCi; 1195 Ci/mmole; Amersham) was added, and the disks were incubated for 1 hour. The protein was precipitated with 10 percent trichloroacetic acid, and incorporation was measured by liquid scintillation counting. Protein was determined by the Lowry (*20*) method.

Figure 1 shows phase response curves for CHX pulses for the CHX-sensitive and CHX-resistant strains, in which the differences between control and experimental phases are plotted as a function of circadian time of the beginning of the CHX pulse (CT 12 is defined as the time of the LD transition). The wild-type

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Table 1. Inhibition of protein synthesis by cycloheximide in CHX-sensitive and CHX-resistant strains. Values shown are specific activities (10^3 count/min per milligram of protein).

Strain	Cycloheximide concentration		
	None	0.1 μg/ml	0.5 μg/ml
CHX-sensitive			
bd, pan-2	1005	345	92
CHX-resistant			
bd, pan-2, *cyh-1*	1082	1432	1018
bd, pan-2, *cyh-2*	996	952	905

phase response curve is similar to that reported earlier (*14*); namely, a CHX pulse at CT 5 gives a large phase advance, while at CT 19 there is little or no effect. The curves for the CHX-resistant mutants show a greatly reduced amplitude; at 10 of the 12 time points there is no phase-shifting effect of CHX at all, and for the other two points the observed 1-hour phase shift is within the limits of variability of the system and is probably not significant. Thus, the clock of the CHX-resistant mutants is essentially unaffected by pulses of CHX.

Protein synthesis in the mutants is resistant to CHX under the conditions of our experiments (Table 1). With CHX administered at 0.1 μg/ml, protein synthesis in the CHX-sensitive strain is inhibited about 65 percent, whereas there is little or no inhibition with the CHX-resistant strains. Even at 0.5 μg/ml, a concentration five times greater than that used for phase shifting, protein synthesis in the CHX-resistant strains is inhibited less than 10 percent.

Linear growth of the CHX-resistant strains is also inhibited much less than is growth of the wild type (Fig. 2). When the CHX dose is 0.5 μg/ml, the growth rates of the resistant strains are inhibited about 15 percent compared with a 70 percent inhibition for wild type.

Our results show that cycloheximide-induced phase shifting of the circadian clock of *Neurospora* is mediated through inhibition of protein synthesis rather than some other mechanism (Fig. 1) in that the clocks of two mutants whose cytosolic ribosomes are resistant to CHX are not phase shifted by pulses of CHX at concentrations that induce phase shifts in the wild-type (CHX-sensitive) strain. These data correlate directly with measurements of inhibition of protein synthesis by CHX in the CHX-sensitive and CHX-resistant strains (Table 1).

Although a second site, unrelated to that of protein synthesis, may also have been altered in these mutants, this possibility is unlikely for several reasons. (i) The same altered gene product would have to affect both sites. While this might be possible for one of the mutants, it is unlikely that both would behave in this manner, since the two CHX-resistance mutations are in different genes on separate chromosomes and therefore code for different gene products. (ii) A second gene, tightly linked to the CHX-resistance gene, might also have mutated and affect the second cellular target. Again, however, this cannot be the case, since such a gene might be linked to one of the CHX-resistance genes but not to both.

Jacklet (*21*) has concluded that another protein synthesis inhibitor, anisomycin, acts on the circadian clock of the isolated eye of *Aplysia* through its inhibition of protein synthesis rather than through some unknown side effect. This conclusion was based on the observation that anisomycin itself was effective in both phase shifting the rhythm and inhibiting protein synthesis, while closely related analogs did not affect either parameter.

That phase shifting by CHX in wild-type strains is directly proportional to the inhibition of protein synthesis (*14*) coupled with the present data supports the view that the synthesis of one or more proteins at specific phases of the circadian cycle is necessary for the normal functioning of the circadian clock of *Neurospora*.

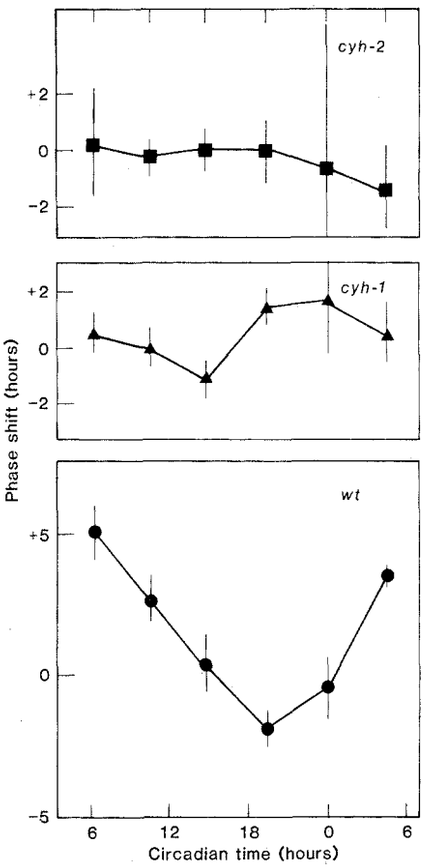

Fig. 1. Cycloheximide (0.1 μg/ml) phase response curves of CHX-resistant (*cyh-1* and *cyh-2*) and CHX-sensitive (*wt*) strains. Phase shift is control phase minus experimental phase.

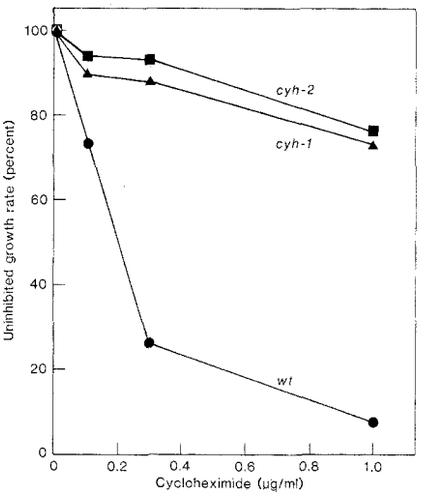

Fig. 2. Linear growth rates of CHX-resistant (*cyh-1* and *cyh-2*) and CHX-sensitive (*wt*) strains in the presence of CHX. Cultures were grown in constant darkness at 25°C on race tubes containing pantothenate-supplemented solid medium.

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