On the role of protein synthesis in the circadian clock of *Neurospora crassa*

(protein turnover/temperature/compensation/rhythm)

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ABSTRACT Inhibitors of protein synthesis reset the biological clocks of many organisms. This has been interpreted to mean either that the synthesis *per se* of proteins is a step in the oscillatory feedback loop or merely that certain unstable protein(s) are required at certain times of the cycle to complete the feedback loop. We report here that *Neurospora* strains bearing the clock mutation *frq-7* are relatively insensitive to the resetting action of the protein-synthesis-inhibitor cycloheximide. Protein synthesis itself in this mutant is inhibited by the drug to the same extent as in wild type. Since the clock of *frq-7* continues to run relatively unimpeded even in the virtual absence of protein synthesis, it is unlikely that synthesis *per se* can be a part of the feedback cycle. Rather, we suggest that for normal operation of the *Neurospora* clock, certain protein(s) with a high turnover rate are required daily and, thus, must be resynthesized each day (at least) during discrete times in the cycle. The *frq-7* mutation simultaneously alters several distinct clock characteristics—period length, temperature compensation, and resetting by cycloheximide. A model is presented to unify these observations.

Circadian (daily) rhythms are controlled by an endogenous oscillator, or clock, which controls such diverse processes as cell division, photosynthesis, body temperature, and sleep/wake cycles. In the laboratory, circadian rhythms persist in continuous dark and constant temperature with a “free-running” period length of about 24 hr, can be synchronized to artificial light and temperature cycles, and exhibit a temperature-compensated period length—i.e., the period length is nearly constant over a wide range of physiological temperatures (1).

Although the biochemical basis of the timing mechanism underlying circadian rhythms is not known, in many organisms cytosolic (80S) protein synthesis appears to play an important role in circadian timing. This conclusion comes from experiments in which pulses of inhibitors such as puromycin or cycloheximide (CHX) reset, or phase-shift, the clock (2–7). In all organisms, the amount of resetting differs at different times of day—i.e., identical pulses causing the same inhibition of protein synthesis result in large phase shifts at certain times of day (sensitive phase) but small or no phase shifts at others (insensitive phase).

Despite the generality of these experimental results, the specific mechanism by which inhibition of protein synthesis affects circadian timing is unknown. There are two general hypotheses. In one (e.g., ref. 8), the periodic synthesis of one or more proteins is a required step in the cycle. Here, the process of synthesizing a protein or proteins is an indispensable part of the clock, and drug-induced resetting is due to interference with this process. Stated another way, the kinetics of the circadian oscillation here are directly

related to the amount of time required to make the protein(s); the kinetics of the clock reflect in some way the short-term rate of translation. A second type of model (e.g., ref. 9) suggests that proteins are needed to carry out an enzymatic reaction or transport process required for the clock, but synthesis *per se* of these proteins is not a step in the oscillation. Here, the short-term rate of protein synthesis is unimportant so long as a sufficient amount of the critical enzymatic activity has been made by the time it is needed. The critical proteins here are viewed as having a high turnover rate and, therefore, must be resynthesized each day during (but not necessarily only during) the time corresponding to the sensitive phase of the cycle.

The availability of strains of *Neurospora* with genetically altered clocks suggests a way of comparing and testing these two classes of models. An important prediction of the first class of models is that inhibition of the act of making proteins must phase-shift the clock. A prediction of the second class of model, but not of the first, is that one might find mutants in which either the stability or the steady-state level of the rapidly turning-over protein(s) has been increased. Since short-term inhibition of protein synthesis in such mutants might not lower the level of the stabilized or overproduced protein(s) below a critical threshold needed for clock function, the mutants would have a clock that could be relatively resistant to phase-shifting by inhibition of protein synthesis, but which nevertheless could complete the circadian cycle in a relatively normal manner.

In several organisms, single-gene clock mutants have been isolated that alter one or more clock parameters, including period length, temperature compensation, and light sensitivity (for a review, see ref. 10). *frq-7* is a clock mutant of the filamentous fungus *Neurospora crassa*, which has previously been shown to have a long (29 hr) circadian period and which has lost part of its temperature-compensating ability (11, 12). We report here that the clock of *frq-7* is largely refractory to phase-shifting by pulses of CHX at concentrations that maximally reset *frq*⁺ and other strains. This result supports the second model in which the enzymatic activity, not the rate of synthesis, of certain protein(s) is crucial for the oscillation. Furthermore, since *frq-7* also has altered temperature compensation, we suggest a connection between the mechanism of temperature compensation and this labile protein(s).

MATERIALS AND METHODS

Strains and Culture Conditions. All strains of *N. crassa* used in these experiments carry the band (*bd*) mutation, which allows for clear expression of the circadian conidiation rhythm but has no effect on phase or period length (13).

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Abbreviations: CHX, cycloheximide; CT, circadian time.

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Four clock mutants used in these experiments all map to a single locus on linkage group VII called *frq* and have period lengths at 25°C as follows: *frq-1*, 16.5 hr; *frq-3*, 24.0 hr; *frq-4*, 19.0 hr; and *frq-7*, 29.0 hr. The wild-type *frq*⁺ has a period length of 21.5 hr. Stocks were maintained at 25°C on Horowitz complete medium (14).

Assay of Rhythm. The circadian rhythm of conidiation in *Neurospora* was measured on race tubes as described (15). Procedures for determining phase-shifting of the *Neurospora* clock by CHX utilized a liquid culture method (16). In this procedure, conidia from 7- to 10-day-old slants were inoculated into Petri dishes containing 25 ml of Fries salts (14), 0.3% glucose, and 0.5% arginine hydrochloride (high glucose medium) and were grown under constant light at 25°C until a mycelial mat had formed (28–36 hr). Discs (11-mm diameter) were then cut with a cork borer and transferred in groups of seven to Petri dishes containing 25 ml of Fries salts with 0.03% glucose and 0.05% arginine hydrochloride (low-glucose medium). These dishes were then transferred from constant light to constant darkness at 25°C, with this light-to-dark transition setting the phase of the clock to circadian time (CT) 12. (CT 12 is equivalent to the end of the light period in a cycle of 12 hr of light/12 hr of dark.) From this point, the clock ran with its endogenous periodicity unless perturbed. Subsequent operations on cultures were performed under a red safelight (General Electric BCJ, 60 W) to which the *Neurospora* rhythm is insensitive. In different experiments, cultures were maintained in the dark, either with or without shaking, with no apparent effects on phase or period length. Occasionally, separate discs would grow together, and since these could not be separated without changing their phase, such fused discs were carried through subsequent operations as a single disc.

Three-hour pulses of CHX were administered in the low-glucose medium at various times, ranging from 12 to 40 hr after the light-to-dark transition. After incubation, discs were transferred to 25 ml of fresh low-glucose medium and washed free of exogenous CHX by gentle mixing, and each disc was transferred to a race tube (13) containing 0.15% glucose, 0.25% arginine hydrochloride, Fries salts, 5 µg of biotin per liter, and 2% agar. Phase and period length of the conidiation rhythm expressed by the cultures in the race tubes were determined as described (15) except that race tube data were collected and processed with a digitizer (Bit Pad, Summagraphics, Fairfield, CT) interfaced to a Northstar Horizon microcomputer.

Controls were of two types. In one, discs were transferred directly to race tubes at the time they were cut, just prior to the light-to-dark transition. In addition, for each set of discs treated with CHX, another set was treated only with an aliquot of fresh low-glucose medium. In all cases, the discs of a single Petri dish were treated identically and were considered as replicates whose results could be averaged. The phase of each culture was determined by regression analysis (15), and the average phase of six replicate tubes was calculated. Phase shifts were determined by the phase difference between CHX-pulsed cultures and their respective controls pulsed at the same time with medium.

Measurement of Inhibition of Protein Synthesis by CHX. At the same time that discs were prepared and used for phase-shifting experiments, parallel sets of seven discs in Petri dishes were prepared and treated with medium alone (controls) or with CHX. After the first 30 min of incubation, [³⁵S]methionine (0.06 µCi/ml final concentration; 1 Ci = 37 GBq) was added, and each dish was incubated for an additional 2 hr. Sets of seven discs were then transferred *en masse* into 5 ml of ice-cold 10% (wt/vol) CCl₃COOH, centrifuged at 2500 × *g* for 10 min, and resuspended and washed overnight in a second 5 ml of 10% CCl₃COOH to remove unincorporated isotope. The discs were then centri-

fuged, and, after aspirating off the CCl₃COOH, each set of discs was ground in a mortar and pestle with sea sand and 1 ml of cold 10% CCl₃COOH. This homogenate was centrifuged at 12,000 × *g* for 20 min, the supernatant was discarded, and the pellet was washed twice more in 7 ml of cold CCl₃COOH. The washed pellet was then suspended in 0.75 ml of 0.4 M NaOH and incubated overnight at room temperature with gentle mixing to solubilize the protein. The mixture was then centrifuged at 12,000 × *g* for 20 min, and the supernatant containing the dissolved protein was saved. The resulting pellet was extracted a second time, and the supernatant was combined with that from the previous step. Of these combined supernatants, 0.5 ml was neutralized with 0.1 ml of 2 M HCl and combined with 5 ml of scintillation fluid (3a70B, Research Products International, Mount Prospect, IL) for determination of the [³⁵S]methionine incorporated into protein; 0.1 ml was used to determine total protein by the dye-binding assay (17) with immunoglobulin as a standard. Inhibition of protein synthesis was determined after normalizing the incorporated cpm to the total protein. Previous studies utilizing strains whose ribosomes are resistant to CHX have shown this drug to have no effect on the uptake of methionine (18).

Chemicals. CHX was purchased from Sigma. [³⁵S]Methionine (1450 Ci/mmol) was obtained from Amersham.

RESULTS

Dose Dependence of CHX-Induced Phase Shifting. The phase-shifting effect of CHX on clock mutants at the *frq* locus is shown in Fig. 1A. The previous light/dark growth regimen was adjusted (see the legend) so that all strains were of approximately the same developmental age (52 ± 3 hr) and had been in low-glucose medium for >16 hr prior to the CHX pulse. For each strain, the beginning of the pulse occurred at CT 6 (middle of the subjective day during the first cycle in constant dark), a time previously reported to be just after the peak of CHX-induced phase-shifting for the wild-type (*frq*⁺) strain (7). All strains but one showed a threshold for phase-shifting at about 0.03 µg of CHX per ml and saturation at about 1 µg/ml. However, the clock of the long-period (29 hr) mutant *frq-7* appeared to be insensitive to the drug at this time at all but the highest concentration of CHX (30 µg/ml).

The lack of phase-shifting of *frq-7* by CHX was not due to a failure of CHX to inhibit protein synthesis in this strain. Protein synthesis in all strains, including *frq-7*, showed nearly identical sensitivity to CHX, exhibiting a threshold for inhibition at <0.01 µg of CHX per ml and >95% inhibition at 1 µg/ml (Fig. 1B). In view of the fact that CHX cannot directly inhibit organellar synthesis, the actual amount of inhibition of cytosolic protein synthesis here is even greater and is virtually complete at concentrations of 3 µg/ml and above.

Phase Dependence of Phase-Shifting by CHX. A second possible explanation for the lack of phase-shifting of *frq-7* was that the sensitive phase of the cycle occurred at a different time of day in this mutant. This was tested in two ways: (i) by determining dose-response curves for *frq*⁺ and *frq-7* at three additional times in the cycle, and (ii) by determining the phase-shifting effect of a normally saturating pulse (3 µg of CHX per ml) at all phases of the cycle for *frq-7*. Fig. 2 shows that at CT 22, CT 1, and CT 4, phase shifts induced in *frq-7* were quite small in comparison with those induced in *frq*⁺. The reduction was most striking at the time of maximum sensitivity, CT 1, where *frq*⁺ showed phase advances of >12 hr, while the maximum phase shift in *frq-7* was <3 hr. The delay phase shifts seen at CT 22 with CHX concentrations sufficient to saturate protein-synthesis inhibition (3 µg/ml) were similarly small in *frq-7* (1–2 hr) as compared with *frq*⁺ (5 hr).

FIG. 1. (A) Dose-response curves for phase-shifting by CHX at CT 6. Disks were cut from cultures grown 33 hr at 25°C as described and were transferred into low-glucose medium but kept in constant light. They were subsequently transferred to constant darkness after different lengths of time in low-glucose medium (*frq-7*, 0 hr; *frq-3*, 1 hr; *frq+*, 4.1 hr; *frq-4*, 3 hr; *frq-1*, 3.3 hr) and held for different durations in the dark prior to CHX treatment (*frq-1*, 12.8 hr; *frq-4*, 14.7 hr; *frq+*, 16.5 hr; *frq-3*, 18.1 hr; *frq-7*, 22.2 hr) so that strains with different period lengths were at approximately the same developmental stage and clock phase at the time of the pulse. Curves were fitted by eye; error bars represent ± 2 SDs and have been omitted for all data except *frq-7* for the sake of clarity. Δ , *frq+*; $\diamond$, *frq-1*; $\bullet$, *frq-3*; $\square$, *frq-4*; $\circ$, *frq-7*. (B) Dose-response curves for inhibition of protein synthesis by CHX. Cultures were prepared and treated as in A, and protein synthesis was determined as described.

To obtain a more detailed picture of the phase dependence of phase-shifting in *frq-7*, a phase-response curve was determined in which 3-hr pulses of CHX at either 1 μ g/ml or 3 μ g/ml were administered at 3-hr intervals throughout the entire circadian cycle. The results (Fig. 3) confirm that at no time in the cycle did *frq-7* show the large phase advances seen with these CHX treatments in either *frq+* or in any of the other *frq* mutants. Once again, inhibition of protein synthesis by CHX was the same at all times measured in both *frq+* and *frq-7* (data not shown).

Finally, it should be noted that in our experiments, maximum phase-shifting in *frq+* occurred at about CT 1–2, compared with a value of CT 5 previously reported (7). It is not known whether this difference was the result of 3- vs. 4-hr pulses or of minor differences in experimental protocol.

Effects of Temperature. Since the phase of maximal CHX-induced phase shifting in *Acetabularia* has been shown to be temperature dependent (9), we also examined the effects of temperature on CHX-induced resetting of both wild-type and *frq* mutant clocks. Fig. 4 shows that the dose-response curves for both phase-shifting and inhibition of protein synthesis at 30°C were similar to those determined at 25°C in all strains, including *frq-7*.

DISCUSSION

The principal finding of this paper is that inhibition of >95% of the cytosolic protein synthesis in the *Neurospora* clock

FIG. 2. Dose-response curves for phase-shifting by CHX at different phases. (A) *frq+*. (B) *frq-7*. Cultures were treated as described in Fig. 1A. For each strain, pulses were begun at approximately CT 22, CT 1, and CT 4.

mutant *frq-7* has only a small effect on the circadian clock in this strain. This is in striking contrast to the behavior of the wild-type strain or other mutants at the *frq* locus, in which inhibition of even 50% of cytosolic protein synthesis resets the circadian clock in a significant manner (Fig. 1 and ref. 7). It is also in contrast to results obtained with a wide variety of other organisms, including *Acetabularia* (2, 3), *Gonyaulax* (5, 6, 19), and *Aplysia* (4).

No drug can be assumed to be totally free of side effects. In earlier experiments (20), CHX-resistant strains of *Neurospora* were used to establish that the resetting behavior seen in *frq+* is due directly to the drug's effect on cytosolic protein synthesis. It is somewhat surprising then to note that, whereas 3 μ g of CHX per ml (sufficient to inhibit >95% of total protein synthesis in *frq-7*) results in minimal resetting, 30 μ g/ml (which results in only a few percent additional inhibition) has a 2- to 5-fold greater resetting effect [Figs. 1,

FIG. 3. Phase-response curves for 3-hr pulses of CHX at 25°C for *frq-7*. Mycelial disks were prepared as described. Cultures were pulsed between 15 and 45 hr after transfer to dark. $\bullet$, 1 μ g/ml; $\circ$, 3 μ g/ml. The dashed line indicates the phase-response curve for *frq+* for comparison (data from ref. 7).

FIG. 4. (A) Dose-response curves for phase-shifting by CHX at 30°C at CT 6. Cultures were treated as described in Fig. 1A. (B) Dose-response curves for inhibition of protein synthesis by CHX at 30°C. Samples were treated as described in Fig. 1B. $\blacktriangle$, *frq-1*; $\bullet$, *frq-3*; $\square$, *frq-4*; $\circ$, *frq-7*.

2 (CT 22), and 4]. While it is possible that the additional fractional percentage inhibited at the highest concentration represents just the fraction of protein synthesis needed for normal operation of the clock in *frq-7*, a much more likely explanation is that this anomalous resetting is due to a side effect of the drug. High concentrations of CHX are known to result in side effects (such as alterations in membrane permeability and transport) that are clearly independent of any effects on protein synthesis (21, 22), and in *Neurospora* it is known that both inhibitors of mitochondrial respiration (30) and membrane active agents (N. Koyama and J.F.F., unpublished data) can reset the clock when administered as short-duration pulses at times between CT 20 and CT 5. It is possible that this low level of side-effect-induced resetting represents a background that normally is obscured by the large effects directly attributable to protein synthesis inhibition but here is observed when the direct protein synthesis effect is attenuated. In any case, the surprising finding in this study is not that the *frq-7* clock can be slightly phase-shifted by exceptional concentrations of CHX, but rather that it is so little reset by drug concentrations that inhibit >95% of the cytosolic protein synthesis and that maximally reset not only *frq+* but also other strains bearing mutations at the *frq* locus.

The specific role of protein synthesis in circadian clock mechanisms has been the subject of several different types of models. In one type, the sequential synthesis of specific proteins is part of the basic oscillatory system (e.g., ref. 8). In two additional models (21, 23), the kinetics of at least one step in the circadian oscillator derive directly from the rate of synthesis of one or more proteins. A variation of this type of model is the suggestion that changes in the ribosome itself (i.e., its capacity or specificity for translation) is part of the oscillatory mechanism (22).

A prediction of these hypotheses is that inhibition of protein synthesis at least at some phase of the cycle must

reset the clock because synthesis *per se* of certain proteins is an essential step in the cycle. However, treatment of *frq-7* with CHX inhibits protein synthesis in this strain without major resetting of its clock, a result difficult to explain by using models of this type. This result suggests that, at least for *Neurospora*, the rate of protein synthesis explicitly is not reflected in the kinetics of the oscillation and, in that sense, that protein synthesis itself cannot be considered as a part of the clock mechanism.

Two factors mitigate the strength with which we can draw this conclusion. First, it is possible that as a result of its mutation, *frq-7* has bypassed the obligatory protein synthesis step altogether—i.e., that the oscillation in *frq-7* is altered in such a fundamental way that the feedback loop actually involves a different biochemical pathway. This seems unlikely, since phase-shifting by light (22, 24) and temperature (unpublished data) is essentially normal in *frq-7*. A second caution lies in the fact that the *frq-7* mutation has only reduced, not completely eliminated, the clock's sensitivity to CHX. While a part of this residual effect may be due to side effects of the drug, some part may still be due to protein synthesis inhibition. For instance, because of the long period of *frq-7*, it may be that larger phase shifts would only be seen with longer pulses of CHX. However, in *frq-3*, a mutant whose period length is also lengthened, there is no decrease in the sensitivity of the clock to CHX (Figs. 1 and 4; J.C.D., unpublished data).

A second type of explanation for the role of protein synthesis holds that certain unstable proteins are required during a part of the feedback cycle and that, because of their instability, they must be resynthesized or replenished daily at a time corresponding to the CHX-sensitive phase of the cycle. Their synthesis at other times in the cycle also is not precluded. In these models (e.g., refs. 9, 19, 20, and 25) phase-shifting by inhibition of protein synthesis results from the temporary decrease in the concentration (and hence in the activity) of a required protein to a level below a critical threshold at a time in the cycle when the activity is needed. This type of model is entirely consistent with the results obtained with *frq-7*. Indeed, several common types of mutation could result in the *frq-7* phenotype. For example, mutations resulting either in increased rates of synthesis or in reduced rates of turnover of the critical protein would render the system less sensitive to CHX-induced phase-shifting.

In addition to its reduced ability to be phase-shifted by CHX, *frq-7* has been shown previously to have two other changes in clock phenotype—it has a long period (29 hr at 25°C) and a partial loss in the temperature compensation of its period length (11, 12). As a basis for discussion, we have formulated a simple heuristic model concerning the nature of the *frq-7* mutation that accounts not only for the CHX data but also for the changes in period length and temperature compensation as well.

Theoretically, the constancy of circadian period length over a wide range of physiological temperatures [Q_{10} = period length at temp $T^\circ\text{C}$ /period length at temp $(T + 10^\circ\text{C})$ of the period length = 1] could result from either temperature "independence," in which the rate of the oscillation is dependent on a physical process, or temperature "compensation," in which a series of temperature-dependent events interact in such a way that the final output of the system does not vary with temperature. The notion of temperature compensation was first introduced (26) to account for the fact that temperature steps and pulses induce phase shifts and that the Q_{10} of the rhythm in many organisms is significantly greater than 1 at the low or high ends of the normal temperature range for the organism. In addition, it has also been possible to increase the Q_{10} of the *Neurospora* clock both by mutation (12, 27) and by changes in the composition

of the medium (28), and such results also strongly favor the notion of temperature compensation. A simple model for temperature compensation was presented by Hastings and Sweeney (29), which was based on two chemical reactions, $A \rightarrow B$ and $C \rightarrow D$, both of which are temperature dependent and are controlled by enzymes E_1 and E_2 , respectively. In this model the rate of the first reaction controls the length of the period, while D is an inhibitor of E_1 . As the temperature is increased, $A \rightarrow B$ initially goes faster, but D accumulates to a higher level as a result of an increase in the rate of $C \rightarrow D$, and eventually $A \rightarrow B$ returns to its original rate as a consequence of the increased inhibition of E_1 by D. Although not discussed in the original model, it is also likely that D can never fully inhibit E_1 —i.e., at maximum levels of inhibition, there remains a residual slow but temperature-dependent rate. In *Neurospora* this would be evidenced by the partial loss in temperature compensation above 30°C even in the wild-type strains.

One can interpret the *frq-7* mutation in terms of this model in at least two ways. One possibility is that the *frq-7* protein is involved in controlling the level of D; for example, it could be the enzyme E_2 , which controls the $C \rightarrow D$ reaction. If this protein had a high turnover rate, then CHX treatment in wild type would cause a decrease in the amount of D and a temporarily reduced inhibition (i.e., stimulation) of $A \rightarrow B$, resulting in a phase shift. If the *frq-7* mutation results in overproduction or stabilization of E_2 , the level of D would be significantly elevated at all temperatures, and the inhibition of $A \rightarrow B$ would also be saturated at all temperatures. There would be three consequences of this. (i) $A \rightarrow B$ would run slowly due to abnormally high levels of D, thus resulting in a long period. (ii) Because the inhibition of $A \rightarrow B$ is the same (i.e., maximal) at all temperatures, $A \rightarrow B$ would become temperature dependent, and the clock would no longer be temperature-compensated. (iii) The CHX-induced decrease of E_2 would not be sufficient to significantly reduce the level of D and relieve the saturated inhibition of $A \rightarrow B$. Therefore the reaction would run uninterrupted during the CHX pulse, and there would be little or no phase shift. A similar scenario can be generated if one assumes instead that the enzyme E_1 consists of two subunits, a regulatory subunit that binds the inhibitor D and a catalytic subunit, and that the *frq-7* mutation increases the level and/or stability of the regulatory subunit. In either case, simply by postulating (i) that the normal *frq-7* gene product is a labile protein that is involved in temperature compensation and (ii) that the *frq-7* mutation increases the level and/or stability of this product, this single mutation can account for all three disparate clock changes observed in the mutant—lengthened period, loss in temperature compensation, and loss in sensitivity to CHX.

Although the specific details of this simple scheme necessarily remain speculative, this does not lessen the validity of the more general conclusion concerning the basis for CHX-induced phase-shifting in *Neurospora* and the role of protein synthesis in the *Neurospora* clock. It seems likely that inhibition of protein synthesis causes phase shifts because it lowers the concentration of a labile protein or proteins needed at specific phases of the circadian cycle, not because the synthesis of such proteins is part of the basic feedback cycle. This conclusion is similar to that suggested for the

Acetabularia, *Gonyaulax*, and *Aplysia* clocks (9, 19, 25) and may turn out to be a general feature of circadian clock mechanisms. It also seems likely that the *frq-7* gene product is involved in the temperature compensation feature of the *Neurospora* clock.

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