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Genetic Determinants of Circadian Rhythmicity in *Neurospora*[1]

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Timex, a strain of *Neurospora crassa* which exhibits a circadian rhythm of conidia formation in growth-tube cultures, has been found to differ from wild-type strains by two genes. One gene, *inv*, is responsible for an invertase deficiency, whereas the second gene, *bd*, is of unknown function. Both genes map independently from other genes known to induce *Neurospora* rhythmicity. The *inv* gene is not essential for the timex phenotype because *bd* strains express that phenotype on certain media. Although *inv* strains do exhibit some rhythmicity of their own, the rhythmicity apparently is not a direct result of the invertase deficiency, since there is no correlation between invertase level and rhymicity in 29 strains tested. Of the 29 strains tested, 20 exhibited some rhythmicity in growth-tube cultures, suggesting that morphological manifestations of rhythmicity in *Neurospora* may result from the function or the loss of function of numerous genes, or both. There was no correlation in these strains between rhythmicity and (i) genetic background; (ii) geographical origin; or (iii) nutritional requirements.

The considerable physiological and behavioral research conducted on the circadian rhythms (biological clocks) of eucaryotic organisms (2, 6) has not yet settled the controversy as to whether the origin of these rhythms is endogenous or exogenous (1, 5). In addition, it has left unresolved the nature of the interactions between metabolic systems and the systems responsible for the rhythms, whether endogenous or exogenous. Experiments with inhibitors of nucleic acid and protein synthesis (9, 11, 13) and theoretical considerations (10, 12) have suggested that the biochemical-genetic systems which control metabolism may be organized in a manner that generates oscillations in the synthesis and activity of enzymes and nucleic acids. Oscillations in enzyme synthesis and activity would in turn be expected to produce rhythmicity in various physiological parameters, morphology, and behavior.

While developing a system with which to evaluate these concepts experimentally, we discovered a new strain (timex) of *Neurospora crassa* which exhibits a rhythm of conidia formation when growing over an agar surface. In all respects examined, the rhythm of timex has been found to be typical of the rhythms displayed by other eucaryotic organisms (21, 22); thus, the timex system is suitable for further studies on circadian rhythms at the biochemical level. *Neurospora* was chosen as the experimental organism because of its well-known biochemistry and genetics and because some strains, but not all, exhibit rhythmicity.

The experiments reported in this paper were designed (i) to analyze timex genetically; (ii) to investigate the roles of the two genes (*inv* and *bd*) responsible for the timex phenotype; and (iii) to determine how common the morphological manifestation of rhythmicity is in *Neurospora*. The accompanying paper (23) describes the characterization of the invertase deficiency associated with the *inv* gene.

MATERIALS AND METHODS

Strains. The several strains of *N. crassa* and three additional species of *Neurospora* used in this study are listed in Table 4. Three additional strains of *N. crassa*, DDP 9103 (*inos*, *pab*-2, *cot*, a), DDP 4026 (*tryp*-4, *pan*-1, a), and 74-OR8-1a, were obtained through the courtesy of D. D. Perkins. Gene symbols and most of the strain designations are those used by the Fungal Genetics Stock Center (3). Stocks were maintained on slants of Vogel's salts (28), 1.5% glucose, 1.5% agar (Difco), and appropriate supplements. Conidia were preserved by the silica-gel technique of Perkins (18).

Crossing and scoring. All crosses were made on slants of Westergaard and Mitchell's medium (29) at 25 C. The matings, ascus dissection, random asco-

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spore collection, and scoring for nutritional markers were done according to standard techniques (20). Scoring for growth rate and rhythmicity was done under standard conditions in tubes containing a growth medium (Casamino Acids, glucose-arginine, or minimal sucrose) described previously (22). Scoring of *bd* conidia and ascospores was done on plates containing Vogel's salts, 1.5% casein hydrolysate (Nutritional Biochemical Co.), 0.1% sorbose, and 2.0% agar. After incubation for 72 hr at 25 C, colonies of *bd* strains were considerably smaller (40 to 50%) than STA 4 wild-type colonies.

Invertase assays. Aerated, logarithmic cultures (method 2 of Davis, reference 7) were utilized for growth. The cultures were inoculated with 10^6 conidia/ml and were harvested at 1.0 mg (dry weight)/ml. The mycelial pads were washed with cold water and then were ground for 2 min with a glass homogenizer in buffer at *p*H 5 [0.05 M sodium acetate; 20 to 25 mg (dry weight)/5.0 ml of buffer]. The brei was assayed as such without centrifugation.

Assays for invertase activity (method modified from Metzenberg, reference 16) in these breis were conducted by (i) adding 4.4 μmoles of sucrose in 1.0 ml of water to 1.0 ml of suitably diluted brei in buffer at *p*H 5, (ii) incubating for 15 min at 37 C, (iii) stopping the reaction by adding 2.0 ml of the alkaline Somogyi reagent, and (iv) measuring the glucose and fructose produced by completing the Somogyi reducing-sugar assay (24). Protein determinations were done by the method of Lowry et al. (14) with bovine serum albumin as the protein standard. Specific activity is defined, according to Metzenberg (15), as the micromoles of sucrose hydrolyzed per milligram of protein in 15 min at 37 C.

RESULTS

Origin and genetic analysis of timex. A newly discovered *inv* strain (mutant allele responsible for an invertase deficiency; 23) was crossed to the STA 4 wild type to characterize the invertase deficiency. In that cross, two new phenotypes were discovered in one of the six asci dissected. Figure 1 illustrates the growth-tube phenotypes of the strains crossed and the meiotic products from this unusual ascus.

Timex (E3A), which is characterized by slow growth and a pronounced and persistent rhythm of conidia formation, is a double mutant, containing the *inv* mutation and a second mutation at a locus of unknown function designated band (*bd*). The presence of four distinct phenotypes in ascus E (*see* Table 3) suggested that timex was a double mutant, and this hypothesis was tested by crossing timex to a wild-type strain (74-OR8-1a) and by crossing an *inv* strain (MLS 2-28) to a *bd* strain (MLS 6-103). Random spores were collected from each cross and the resulting isolates were scored for sucrose utilization, growth rate, and rhythm expression (Table 1). The same four (and only four) phenotypes were found in approximately equal numbers from each cross, indicating that two unlinked genes are involved in the timex phenotypes. The deviations from 1:1:1:1 segregation were not significant at the 95% confidence level.

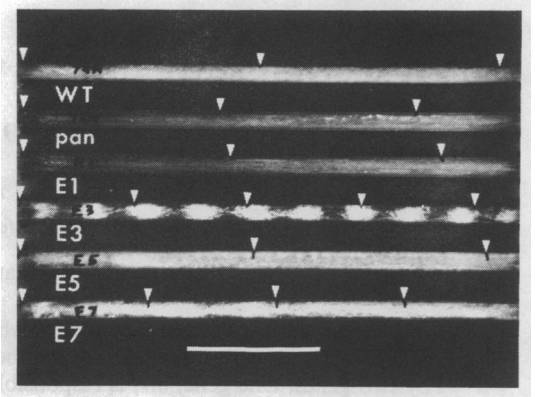

FIG. 1. *Growth-tube cultures of the strains crossed to give ascus E and the strains derived from that ascus. The strains were grown under standard conditions on the Casamino Acids medium. The arrows delimit 48-hr growth intervals; the white bar is 10-cm long. The strains illustrated are: WT, STA 4, wild-type; pan, pan 724-2a, inv; E1, E1A, inv, pan; E3, E3A, inv, bd; E5, E5a, pan; and E7, E7a, bd.*

TABLE 1. *Segregation and assortment of the inv and bd genes*

Cross	No. of isolates				Per cent germination
	inv$^+$, *bd*$^+$	*inv*$^+$, *bd*	*inv*, *bd*$^+$	*inv*, *bd*	
E3A (*inv*, *bd*) × 74-OR8-1a (*inv*$^+$, *bd*$^+$)	42	41	42	38	82.4
MLS 2-28 (*inv*, *bd*$^+$) × MLS 6-103 (*inv*$^+$, *bd*)	31	36	35	19	66.1

The two genes were mapped by first crossing the mutant strains to Perkins' alcoy linkage tester stock (18) and then by making three-point crosses with appropriate double mutants (Table 2). The *inv* gene is located 3.3 map units distal to *pab*-2 on linkage group VR, whereas the *bd* gene is located 1.5 map units distal to *pan*-1 on linkage group IVR. Both genes are genetically distinct from the patch gene (25) and the clock gene (8), the other genes known to elicit rhythmicity in *Neurospora*.

The *bd* gene discovered in ascus E was presumably the result of a spontaneous mutation in our STA 4 stock, since two strains allelic to and phenotypically identical to the standard *bd* strain (E7a) have been isolated from STA 4 conidia pre-

served on silica gel since the time of the original cross. Moreover, additional crosses of STA 4 to *pan* 724-2a (both strains purified by repeated plating and reisolation) have yielded no *bd* strains.

Genotype-medium interactions. As mentioned above, timex is characterized by slow growth and a pronounced and persistent rhythm. These characteristics pertain to growth on the Casamino Acids medium used in all previous investigations (21, 22) on the timex phenotype. On this medium, strains containing the *inv* gene alone (e.g., E1A) were characterized by rapid growth, thin conidial bands, and a rapidly damping rhythm (i.e., only a few cycles expressed); *bd* strains (e.g., E7a) were characterized by slow growth, dense conidial bands, and a rapidly damping rhythm (Fig. 1). The wild-type strain (STA 4) involved in this cross had a rapid rate of growth and did not form conidia or express a rhythm in these growth-tube cultures. The dense growth of STA 4 and E5 apparent in Fig. 1 is mycelial rather than conidial.

A defined glucose-arginine medium which allows the *bd* single mutant to express a "timex-like" phenotype has been recently developed. The *bd* strains growing on the glucose-arginine medium closely resembled (conidial bands are occasionally somewhat less dense or less defined) *inv-bd* strains growing on the Casamino Acids medium. For wild-type, *inv*, and *inv-bd* strains, there was no detectable difference between growth on the two media. These growth and rhythm characteristics (persistence, amplitude, and period) are summarized in Table 3. The experiments demonstrated that the *inv* gene is essential for the timex phenotype only on certain media.

Role of the enzyme invertase in Neurospora rhythmicity. Since the original *inv* strain (*pan* 724-2a) exhibited some rhythmicity, we examined the possibility that there is a cause and effect relationship between the invertaseless condition and rhythmicity in *Neurospora*. Four crosses (Table 1; *unpublished data*) demonstrated no segregation between these characteristics in 105 isolates tested. These results indicated that the partial rhythmicity and invertase deficiency are either due to the same gene, or to two tightly linked genes (less than 1 map unit apart).

The genetic correlation between rhythmicity and the invertaseless condition prompted us to survey a number of *Neurospora* strains to deter-

TABLE 2. *Linkage data for inv and bd*

Zygote genotype and recombination per cent	Parental combinations	Recombinations			Total, per cent germination, and linkage group	Marker isolation no.
		Singles region (1)	Singles region (2)	Doubles regions (1 and 2)		
+ + *inv*	84, 62	18, 11	4, 2	0, 0	181, 94%, V	37401, H 193, inv
inos *pab*-2 + (16.0, 3.3)						
+ + *bd*	87, 92	11, 6	2, 1	0, 0	199, 91%, IV	Y2198, 5531, bd
tryp-4 *pan*-1 + (8.5, 1.5)						

TABLE 3. *Ascus E isolates in growth-tube culture*

Medium	Strain	No. of bands	Density of bands[a]	Period of rhythm	Growth rate
				hr	*mm/day*
Casamino Acids	E1 (inv^-, bd^+)	4–5	+	Circadian[b]	82.5
	E3 (inv^-, bd^-)	>12	++++	22.1	38.8
	E5 (inv^+, bd^+)	0			80.0
	E7 (inv^+, bd^-)	4–5	++++	Circadian	47.9
Glucose-arginine	E1 (inv^-, bd^+)	4–5	+	Circadian	87.5
	E3 (inv^-, bd^-)	>12	++++	22.1	40.8
	E5 (inv^+, bd^+)	0			81.5
	E7 (inv^+, bd^-)	>12	++++	22.1	41.9

[a] Density of conidial bands ranged from no bands (−) to dense, well-defined bands (++++).
[b] After 20 to 24 hr.

mine whether a correlation existed between the level of invertase activity and the ability to express a rhythm of conidia formation. Preliminary experiments indicating a positive correlation (Sargent and Woodward, Federation Proc., p. 726, 1967) were biased by not adequately considering invertase level as a function of physiological age or of the large portion of invertase bound to the cell wall (23). These complications have been eliminated by measuring the activity of logarithmically grown cultures and by assaying whole homogenates rather than their supernatant fractions. More recent experiments (Table 4) indicated that no correlation exists between the two characters. The evidence demonstrated that rhythmicity is indeed common in *N. crassa*, but there are strains exhibiting rhythmicity which have a high level of invertase activity (e.g., Taiwan and 89601A) and there is at least one strain with a relatively low level of activity (Costa Rica) that exhibits no rhythmicity in growth-tube cultures.

TABLE 4. *Rhythmicity and invertase level in Neurospora*

Species	Source[a]	Rhythmicity on[b]		Invertase level (per cent of STA 4)[c]
		Minimal sucrose	Glucose-arginine	
N. intermedia (NITa)	AMS	−	−	311
N. sitophila (Eng Sit 21a)	AMS	−	−	159
N. tetrasperma	AS	−	−	151
N. crassa (STA 4)	FGSC	−	−	100
Strains of *N. crassa*				
Abbott 12a	DDP	−	+(>8)	295
SY4f8a	NHG	−		157
Lindegren 1A	RHD	−	+++(>7)	129
Em a	DDP	−	−	110
Taiwan (Tai II 17a)	AMS	+(4)	++(4)	900
Puerto Rico 18A	NHH	−	−	412
Liberia 4A	NHH	−	−	228
Singapore 2a	NHH	++(>5)	++(>7)	115
Philippine Islands (P.I. 42)	AMS	+(3)	+(>6)	44
Costa Rica (C.R. 20502A)	AMS	−	−	36
inos 89601A	AS	+(>5)	+++(>6)	410
ad-4 F6A		−	++(>7)	343
SF-26	HGG	−	+(>5)	226
L5D (lactose utilizer)		+(>6)	++(>6)	192
aur 17A	AS	−	+(>5)	179
arg-10 B317a	RHD	+(3)	+(>8)	149
4-121 (altered trehalase)	AS	−	+(>5)	147
arg-8 P50a	RHD	−	+++(>7)	118
inos 37401a	FGSC	−	+(>6)	117
bd E7a		++(>4)	++++(>14)	232
patch a	FGSC	+(>11)	+(>10)	137
inv 63 (partial mutant)		−	++(>8)	65
clock CL 11A	AS	++(>12)	+++(>20)	37
inv E1A		−	+(>4)	0
inv, bd E3A		+(5)	++++(>14)	0

[a] Abbreviations are FGSC, Fungal Genetics Stock Center; RHD, R. H. Davis; HGG, H. G. Gratzner; NHG, N. H. Giles, NHH, N. H. Horowitz; DDP, D. D. Perkins; AS, A. Sussman; AMS, A. M. Srb. All other strains were isolated in the authors' laboratories.

[b] The minimal sucrose medium contained Vogel's salts, 1.5% sucrose, and 1.5% agar. The glucose-arginine medium contained Vogel's salts, 0.3% glucose, 0.5% arginine, and 1.5% agar. Appropriate supplements were added when required. Density of conidial bands ranged from no bands (−) to dense, well-defined bands (++++). The number of conidial bands observed is enclosed within the parentheses following the band density designations; >12 means that 12 bands were produced before the growth front reached the end of the growth tube, thereby terminating the experiment.

[c] Specific activities of the mycelial brei from logarithmic cultures. Cultures were grown on the glucose-arginine medium minus agar.

DISCUSSION

This new strain of *N. crassa*, which exhibits a circadian rhythm of conidial differentiation that is pronounced, persistent, and typical of the rhythms in other eucaryotic organisms, should be helpful in determining the extent to which circadian rhythmicity is a manifestation of biochemical-genetic control systems. Both Pittendrigh's group (19) and Sussman's group (27) previously realized the value of *Neurospora* for such studies, but the only strains available for their use were patch (4), which has a relatively indistinct and damping rhythm, and clock, whose rhythm is not typically circadian (26).

Of the two genes involved in timex rhythmicity, it is evident that the *inv* gene is playing a less critical role. The *bd* gene is sufficient to produce a timex-like phenotype when the glucose-arginine medium is used to assay rhythmicity; the *inv* gene is only necessary when the Casamino Acids medium is employed.

Although the *inv* gene plays a less critical role in the timex phenotype, *inv* strains exhibit a weakly expressed and nonpersistent rhythm of their own. The lack of a correlation between invertase level and rhythmicity in the several strains tested suggests that the *inv* mutation induced rhythmicity through some indirect means. The indirect nature of any interaction is made more probable when it is recognized that a simple lack of invertase activity should have no serious consequences for growth on a medium containing glucose, the product of invertase activity; i.e., why should E1 (inv^-, bd^+) differ phenotypically from E5 (inv^+, bd^+) on the glucose-arginine medium?

Although the single mutation *bd* can induce a pronounced and persistent rhythm of conidial differentiation, it is not yet clear whether the *bd* mutation is involved with the generation or unmasking of rhythmicity; i.e., whether it affects the "clock" itself or merely the "hands." The widespread occurrence of rhythmicity in *Neurospora* (Table 4) suggests that the latter situation is more probable. The difficulty in distinguishing generation versus unmasking occurs not only in the comparison of strains, but also in the phenomenon of nonpersistence or damping of rhythmicity. Many strains exhibit a conidial rhythm for several days, but then become arhythmic although growth proceeds at a normal rate. We do not yet know whether the "clock" has stopped or whether conidia formation has become "uncoupled" from a driving oscillator. A later paper will consider the effects of medium components and inhibitors on the persistence of the *bd* rhythm.

The data in Table 4 demonstrate that the majority of *N. crassa* strains exhibit at least some rhythmicity of conidia formation in growth-tube cultures, but no correlation is evident between rhythmicity and (i) geographical location from which the wild-type strains were isolated; (ii) genetic background; or (iii) nature of the nutritional markers found in several of the strains. Although the work with patch, clock, and timex has shown that single-gene defects can induce rhythmicity in otherwise nonrhythmic wild-type strains, it is not yet clear how the rhythmic wild-type strains differ genetically from the nonrhythmic wild-type strains or from the single-gene rhythmic strains. More specifically, we would like to know whether there are single-gene differences responsible for the rhythmic wild-type strains.

If the rhythmic wild-type strains are rhythmic because of single-gene rather than multi-gene differences, it will be interesting to determine whether these wild-type strains carry the *bd* gene in different genetic backgrounds. If all rhythmic wild-type strains carry the *bd* gene, then a truly unique gene has been discovered. If several single genes induce rhythmicity, then it may be possible to identify the pertinent control systems by finding metabolic defects common to all rhythmic strains.

Finally, it should be noted that some strains approaching STA 4 wild type in growth rate, e.g., Lindegren 1A and 89601A, exhibit rhythmicity of conidia formation in growth-tube cultures. This finding suggests that metabolic disturbances sufficient to impede growth are not necessary for the expression of circadian rhythmicity. This presents a contrast to the requirements for the production of noncircadian rhythmicity by the clock strain of *Neurospora* (26).

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LITERATURE CITED

1. Aschoff, J. 1963. Comparative physiology: diurnal rhythms. Ann. Rev. Physiol. 25:581–600.
2. Aschoff, J. (ed.). 1965. Circadian clocks. North-Holland Publishing Co., Amsterdam.
3. Barratt, R. W., and W. N. Ogata. 1968. *Neurospora* stock list, 4th revision. *Neurospora* Newsletter 13:25–95.
4. Brandt, W. H. 1953. Zonation in a prolineless strain of *Neurospora*. Mycologia 45:194–208.
5. Brown, F. A., Jr. 1965. A unified theory for biological rhythms, p. 231–261. *In* J. Aschoff (ed.), Circadian clocks. North-Holland Publishing Co., Amsterdam.

6. Bünning, E. 1964. The physiological clock. Academic Press Inc., New York.
7. Davis, R. H. 1965. Growth, extraction, and assay methods for the enzymes ornithine transcarbamylase, aspartate transcarbamylase, and carbamyl phosphokinase from *Neurospora*. *Neurospora* Newsletter 8:12–14.
8. Durkee, T. L., A. S. Sussman, and R. J. Lowry. 1966. Genetic localization of the clock mutant and a gene modifying its band-size in *Neurospora*. Genetics 53:1167–1175.
9. Ehret, C. F. 1959. Induction of phase shift in cellular rhythmicity by far ultraviolet and its restoration by visible radiant energy, p. 541–550. *In* R. B. Withrow (ed.), Photoperiodism and related phenomena in plants and animals. American Association for the Advancement of Science, Washington, D.C.
10. Ehret, C. F., and E. Trucco. 1967. Molecular models for the circadian clock. I. The chronon concept. J. Theoret. Biol. 15:240–262.
11. Feldman, J. F. 1967. Lengthening the period of a biological clock in Euglena by cycloheximide, an inhibitor of protein synthesis. Proc. Natl. Acad. Sci. U.S. 57:1080–1087.
12. Goodwin, B. C. 1963. Temporal organization in cells. Academic Press Inc., New York.
13. Karakashian, M. W., and J. W. Hastings. 1963. The effects of inhibitors of macromolecular biosynthesis upon the persistent rhythm of luminescence in Gonyaulax. J. Gen. Physiol. 47:1–12.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
15. Metzenberg, R. L. 1962. A gene affecting the repression of invertase and trehalase in *Neurospora*. Arch. Biochem. Biophys. 96:468–474.
16. Metzenberg, R. L. 1963. The localization of β-fructofuranosidase in *Neurospora*. Biochim. Biophys. Acta 77:455–465.
17. Perkins, D. D. 1962. Preservation of *Neurospora* stock cultures with anhydrous silica gel. Can. J. Microbiol. 8:591–594.
18. Perkins, D. D. 1964. Multiple interchange stocks for linkage detection. *Neurospora* Newsletter 6:22.
19. Pittendrigh, C. S., V. G. Bruce, N. S. Rosensweig, and M. L. Rubin. 1959. A biological clock in *Neurospora*. Nature 184:169–170.
20. Ryan, F. J. 1950. Selected methods of *Neurospora* genetics. Methods Med. Res. 3:51–75.
21. Sargent, M. L., and W. R. Briggs. 1967. The effects of light on a circadian rhythm of conidiation in *Neurospora*. Plant Physiol. 42:1504–1510.
22. Sargent, M. L., W. R. Briggs, and D. O. Woodward. 1966. The circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. Plant Physiol. 41:1343–1349.
23. Sargent, M. L., and D. O. Woodward. 1969. Gene-enzyme relationships in *Neurospora* invertase. J. Bacteriol. 97:867–872.
24. Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. 195:19–23.
25. Stadler, D. R. 1959. Genetic control of a cyclic growth pattern in *Neurospora*. Nature 184:170–171.
26. Sussman, A. S., T. L. Durkee, and R. J. Lowry. 1965. A model for rhythmic and temperature independent growth in "clock" mutants of *Neurospora*. Mycopathol. Mycol. Appl. 25:381–396.
27. Sussman, A. S., R. J. Lowry, and T. Durkee. 1964. Morphology and genetics of a periodic colonial mutant of *Neurospora crassa*. Am. J. Botany 51:243–252.
28. Vogel, H. J. 1956. A convenient growth medium for *Neurospora* (medium N). Microbiol. Genet. Bull. 13:42–43.
29. Westergaard, M., and H. K. Mitchell. 1947. *Neurospora*. V. A synthetic medium favoring sexual reproduction. Am. J. Botany 34:573–577.