GENETIC CONTROL OF THE SYNTHESIS OF REPRESSIBLE PHOSPHATASES IN *NEUROSPORA CRASSA*

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IN the genetic control of alkaline phosphatase synthesis in *Escherichia coli*, GAREN and ECHOLS (1962a,b) have presented evidence for the presence of two regulator genes, R1 and R2. It was proposed that R1 produces an inducer which has a positive function in controlling the synthesis of alkaline phosphatase and is converted into a repressor in the presence of the R2 product and high concentrations of inorganic phosphate. In *Neurospora crassa*, it has been shown that the synthesis of an alkaline phosphatase and an acid phosphatase is derepressed by lowering concentrations of inorganic phosphate in the culture medium (NYC, KADNER and CROCKEN 1966; NYC 1967). However, no genetic analysis on the regulation of synthesis of these enzymes has been reported.

In previous reports, we described the isolation and characterization of nuclease mutants in *N. crassa* (ISHIKAWA *et al.* 1967, 1968, 1969). During the recent study of the utilization of nucleic acid as a source of phosphate in *N. crassa*, we found that the nuclease mutations showed a pleiotropic effect on the formation of the repressible phosphatases. The present paper reports a relationship between the RNA utilization and a regulatory mechanism of phosphatase formation in *N. crassa*.

MATERIALS AND METHODS

Strains: The following strains of *Neurospora crassa* were used: wild type strain 74A (St. Lawrence); *nuc-1* mutants (A1, A2, A13, A24, A65); *nuc-2* mutants (B1, B2, B18, B21, B40); a phosphatase mutant *pho-1* (74A-T58-M98); *ad-2*, *ad-6* and *ad-9*. The isolation method and characteristics of the *nuc-1* and *nuc-2* mutants (nuclease mutants) have been described elsewhere (ISHIKAWA *et al.* 1969). Double mutants were constructed by the appropriate crosses of the single mutants.

Selection for phosphatase mutants: To isolate a phosphatase mutant lacking the activity of repressible alkaline or acid phosphatase and a constitutive phosphatase mutant producing repressible phosphatases constitutively in high-phosphate medium, a modification of the staining method according to DORN (1965) was employed. Wild-type conidia treated with 200 μg/ml N-methyl-N′-nitro-N-nitrosoguanidine for 4 hr at 25°C were plated on sorbose agar medium containing 20 μg/ml KH_2PO_4 to isolate mutants lacking a repressible phosphatase, or 1 mg/ml KH_2PO_4 to isolate mutants producing the repressible phosphatases constitutively. When colonies appeared (usually after 48 hr at 25°C) each plate was soaked with a staining solution containing

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0.01 M ethylenediamine tetraacetic acid (EDTA), 0.5 mg/ml α-naphthyl phosphate, 5 mg/ml naphthanyl diazo blue B, and 0.1 M veronal buffer, pH 9.0, in order to detect alkaline phosphatase activity or 0.05 M acetate buffer, pH 4.8, in order to detect acid phosphatase activity. After leaving for 30 min at room temperature, wild-type colonies stained dark purple on low-phosphate medium and pale red on high-phosphate medium. Colonies staining a different color from the wild type were isolated and assayed for phosphatase activity. Thus one mutant lacking the repressible alkaline phosphatase activity, *pho-1*, was isolated. Mutants lacking a repressible phosphatase or mutants producing the repressible phosphatases constitutively have not been found to date.

Media: Fries minimal medium was used as a high-phosphate medium. Phosphate-free medium was prepared by substituting KCl (1 g/l) in Fries minimal medium for KH_2PO_4. A low-phosphate medium was prepared by reducing the KH_2PO_4 concentration to 50 μg/ml, 1/20th that of Fries minimal medium. To prepare RNA medium, 100 μg/ml of RNA was supplemented in the phosphate-free medium as a sole source of phosphate.

Growth measurement: To test the growth yield, conidial suspensions of various strains were inoculated into 20 ml of liquid media in 100 ml Erlenmeyer flasks. Mycelial mats were harvested after 3 days, dried and weighed.

Preparation of crude extract: Mycelia were grown at 25°C or 35°C for the designated period in a 1000 ml Roux bottle containing 100 ml of the indicated medium. Mycelial pads were harvested on a Buchner funnel and the culture media were kept to assay for extracellular enzyme activities. The mycelia obtained were washed with deionized water and frozen quickly. Crude mycelial extract was prepared as described elsewhere (ISHIKAWA *et al.* 1969), except that 5 mM Tris-maleate buffer (pH 6.4) was used in place of phosphate buffer.

Determination of alkaline phosphatase activity: Alkaline phosphatase activity was measured by the method of TORRIANI (1960). The reaction mixture (2 ml) contained 0.05 M veronal buffer, pH 9.0, 0.5 mM p-nitrophenylphosphate (Daiichi Pure Chemicals) and 0.1 ml of enzyme solution. Five mM $MgSO_4$ was added to assay constitutive alkaline phosphatase activity, while 5 mM EDTA was added to assay repressible alkaline phosphatase activity. The reaction mixture was incubated at 37°C for 20 min, and the reaction was stopped by the addition of the same volume of 10% trichloroacetic acid. Insoluble material was removed by centrifugation and 2 ml of the supernatant obtained was mixed with saturated Na_2CO_3 solution. The absorbancy at 405 nm was then measured and converted to molar concentration of p-nitrophenol as calculated from a standard curve. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the conversion of 1 μmole of p-nitrophenylphosphate to p-nitrophenol per minute at pH 9.0 and 37°C.

Determination of acid phosphatase activity: Constitutive acid phosphatase activity was determined by the same procedure as described for the determination of alkaline phosphatase activity except that 0.05 M acetate buffer, pH 4.8, was used in place of the veronal buffer. The repressible acid phosphatase activity was assayed by the method of NYC (1967). The reaction mixture (2.0 ml) contained 0.05 M acetate buffer, pH 4.0, 0.1 ml of enzyme solution and 40 mM β-glycerophosphate (Merck) or 5 mM p-nitrophenylphosphate. The reaction was carried out as described for the alkaline phosphatase. The concentration of inorganic phosphate in the 5% trichloroacetic acid soluble fraction was determined according to FISKE and SUBBARROW (1925). Although the repressible acid phosphatase can hydrolyze only p-nitrophenylphosphate, the constitutive acid phosphatase can hydrolyze both p-nitrophenylphosphate and β-glycerophosphate; the former substrate was hydrolyzed less preferentially than the latter by the factor of 0.66 under the present assay conditions. Therefore, to measure repressible acid phosphatase activity in crude extract, a 0.66-fold amount of inorganic phosphate released from β-glycerophosphate was subtracted from the amount of inorganic phosphate released from p-nitrophenylphosphate. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the liberation of 1 μmole of inorganic phosphate from p-nitrophenylphosphate per minute at pH 4.0 and 37°C.

Determination of protein concentration: Protein concentration was determined according to LOWRY *et al.* (1951).

RESULTS

Phosphatases produced by Neurospora mycelia: Neurospora mycelia grown in high-phosphate medium produce a constitutive alkaline phosphatase (KUO and BLUMENTHAL 1961b) and two kinds of constitutive acid phosphatases (KUO and BLUMENTHAL 1961a; TOH-E unpublished). Mycelia grown in low-phosphate medium produce additional phosphatases; a repressible alkaline phosphatase (NYC *et al.* 1966) and a repressible acid phosphatase (NYC 1967). The repressible acid phosphatase was found in the culture medium as well as in the mycelial extract (NYC 1967). To differentiate the repressible phosphatase activity from the constitutive phosphatase activity in crude extracts, the particular assay procedures described in MATERIALS AND METHODS have been established. The repressible alkaline phosphatase was assayed in the presence of EDTA to differentiate it from the constitutive alkaline phosphatase, which is active only in the presence of $MgSO_4$ (NYC *et al.* 1966). Since the constitutive acid phosphatase was the only acid phosphatase of Neurospora hydrolyzing β-glycerophosphate as well as p-nitrophenylphosphate (NYC 1967; TOH-E unpublished), the activity of repressible acid phosphatase, which hydrolyzes only p-nitrophenylphosphate, was calculated by subtracting units of phosphatase activity assayed using β-glycerophosphate from those assayed using p-nitrophenylphosphate as substrate. The constitutive acid phosphatase II which hydrolyses only p-nitrophenylphosphate as substrate in the presence of divalent cation (TOH-E unpublished) was not detected by the present assay procedure, since the reaction mixture did not contain divalent cation.

The necessity of protein synthesis for the process of phosphatase derepression was tested as shown in Table 1. Although repressible phosphatase activities

TABLE 1

Effects of cycloheximide on repressible phosphatase activities

	Culture condition*			Assay condition†	
	Incubation period in P_i free medium (days)	No cycloheximide	5 μg/ml cycloheximide	No cycloheximide	5 μg/ml cycloheximide
Repressible alkaline phosphatase in extract	3	92.4	1.3	357.5	359.7
Repressible acid phosphatase in extract	3	68	0	—	—
Repressible acid phosphatase in medium	1	13	1	338	342
	2	213	2		
	3	377	8		

* Wild-type mycelia were grown in a 100 ml Erlenmeyer flask containing 20 ml high-phosphate medium at 25°C for 3 days without shaking. Mycelial pad was washed and put into phosphate-free medium (P_i-free medium) supplemented with or without 5 μg/ml cycloheximide and incubated at 25°C for another 1–3 days without shaking. Mycelial extract or culture medium was prepared and the activities of repressible phosphatases were assayed. Phosphatase activity in extract is expressed as milli-units per mg protein and that in medium as milli-units per ml culture medium.

† Mycelial extract or culture medium prepared from wild-type culture in the absence of cycloheximide as described above was used as the enzyme source. Phosphatase activity was assayed with or without 5 μg/ml cycloheximide. The activity in extract is expressed as milli-units per ml enzyme solution and that in medium as milli-units per ml culture medium.

TABLE 2

*Comparison of repressible phosphatase activities among wild type, nuclease mutants and phosphatase mutant**

Strain	Repressible alkaline phosphatase†		Repressible acid phosphatase			
			In extract†		In medium‡	
	High P_i	Low P_i	High P_i	Low P_i	High P_i	Low P_i
Wild type						
74A	0.9	324.8	0	286	1	123
nuc-1 mutants						
A1	0.9	0.4	0	0	2	1
A2	0.7	0.9	2	6	0	0
A13	0.9	0.9	4	0	0	0
A24	0.2	0.2	4	0	0	0
A65	0.7	0.7	6	2	0	0
nuc-2 mutants						
B1	0.9	1.1	0	3	1	1
B2	0.9	0.9	4	0	0	0
B18	0.9	1.1	0	0	0	0
B21	0.9	0.7	0	0	0	0
B40	0.7	0.9	8	0	0	0
Phosphatase mutant						
pho-1	0.9	10.5	6	339	2	81

* Mycelia were grown in high-phosphate medium (High P_i) or in low-phosphate medium (Low P_i) at 25°C for 3 days without shaking.
† Milli-units per mg protein.
‡ Milli-units per mg dry mycelia in culture.

appeared within 3 days of incubation in phosphate-free medium, cultures incubated with cycloheximide produced none or significantly reduced repressible phosphatase activities. Cycloheximide at this concentration did not inhibit repressible phosphatase activities. These results are compatible with a conclusion that the derepression of these enzymes requires *de novo* protein synthesis.

Phosphatase activities in wild type and nuclease mutants: The wild-type strain 74A and several nuclease mutants were grown in low-phosphate medium and in high-phosphate medium at 25°C for 3 or 7 days. The mycelia and culture media were harvested and the repressible and constitutive phosphatase activities were assayed. As shown in Table 2, the wild-type strain grown in low-phosphate medium produced significant amounts of repressible phosphatases but the synthesis of these enzymes was repressed in high-phosphate medium. All the nuclease mutants tested failed to produce both of the repressible phosphatases even under the derepressed condition. The wild-type strain grown in phosphate-free medium supplemented with 100 μg 3′-adenosine monophosphate produced significant amounts of repressible phosphatases as that grown in low-phosphate medium, but nuclease mutants grown in the same medium showed no activity of the repressible phosphatases. The constitutive phosphatase activities of cultures grown in high-phosphate medium were compared among wild type and nuclease mutants but no significant difference was found, as shown in Table 3.

A possibility that crude mycelial extracts of nuclease mutants contain an

TABLE 3

Comparison of constitutive phosphatase activities among wild type and nuclease mutants cultured in high-phosphate medium

Strain	Alkaline phosphatase in extract*	Acid phosphatase In extract*	Acid phosphatase In medium†
74A	71.1	106.2	9.4
A1	82.8	106.2	12.6
B1	99.0	119.9	8.9

* Milli-units per mg protein in mycelial extract obtained from 3 day old culture.
† Milli-units per mg dry mycelia obtained from 7 day old culture.

inhibitor for repressible phosphatases has been tested as follows: crude extract prepared from mycelia of the wild-type strain grown in low-phosphate medium for 3 days was mixed with crude extract prepared from nuclease mutants by the same procedure and the repressible alkaline phosphatase activity was assayed. No inhibition of the repressible alkaline phosphatase activity in extract of wild-type mycelia was observed in the presence of extracts of nuclease mutants. On the other hand, crude extracts of nuclease mutants were heated at 60°C or treated with ribonuclease A or subtilopeptidase A, but no increase of the repressible alkaline phosphatase activity was observed.

All the observations described above suggest that the *nuc-1* and *nuc-2* mutations result in the inability to produce the normal repressible alkaline and acid phosphatases.

Phosphatase activities of revertants from nuclease mutants: Since our data indicated that a single mutation occurring at the *nuc-1* or *nuc-2* locus resulted in the inability to produce normal repressible phosphatases in addition to the inability to utilize nucleic acid as a source of phosphate, we examined whether both abilities are restored by a single reversion. Many revertants which can grow on RNA medium were obtained from nuclease mutants, A1 and B1, by plating conidia of these mutants on RNA medium (Table 4). To test repressible phosphatase activities these revertants were grown in low-phosphate medium and in high-phosphate medium at 25°C for 3 days, and the mycelia and culture media were harvested. As shown in Table 4, all the revertants tested regained partially or fully the ability to produce the active repressible phosphatases. Some revertants obtained from B1 (e.g. B1R4 and B1R9) produced a significant amount of the repressible alkaline phosphatase even in high-phosphate medium, although repressible acid phosphatase activity could not be detected under the same condition. No such constitutive revertant was found among revertants obtained from A1.

Among the many revertants obtained from B1 a temperature-sensitive revertant, B1*ts-2*, was found. B1*ts-2* grew as the wild type in RNA medium at 25°C but not at 35°C, although this strain grew well in minimal medium at both temperatures (Table 5). To examine the ability to produce the repressible phosphatases, B1*ts-2* was grown in low-phosphate medium and in high-phosphate medium at 25°C and at 35°C for 3 days. The mycelia and culture media were

TABLE 4

*Growth characteristics and repressible phosphatase synthesis in revertants derived from nuclease mutants, A1 and B1**

Strain†	Growth (mg dry weight)		Repressible alkaline phosphatase		Repressible acid phosphatase in extract		Repressible acid phosphatase in medium	
	High P_i	RNA medium	High P_i	Low P_i	High P_i	Low P_i	High P_i	Low P_i
74A	78.9	33.3	0.9	324.8	0	286	1	123
A1	65.1	3.5	0.9	0.4	0	0	2	2
A1R4	63.7	4.3	0.9	48.2	7	86	3	21
A1R6	50.3	25.3	0.9	48.6	3	133	1	64
A1R8	60.6	5.8	0.4	31.4	0	148	0	11
A1R10	59.9	24.2	1.1	43.6	0	177	0	71
A1R11	53.1	23.7	1.3	189.7	11	194	0	81
A1R14	74.1	9.6	0.9	211.5	8	308	2	1
A1R15	66.1	18.4	0.9	37.9	0	120	1	4
A1R21	61.3	23.8	0.7	82.2	2	115	1	17
A1R29	61.8	19.5	0.2	41.9	0	35	0	32
A1R30	65.3	21.9	0.4	56.7	0	137	0	57
B1	76.6	2.9	0.9	1.1	0	3	1	1
B1R1	52.2	26.6	1.3	220.2	4	210	1	56
B1R3	57.5	27.1	1.1	252.9	3	156	1	37
B1R4	60.0	26.5	7.9	316.1	1	219	1	83
B1R6	57.0	30.9	1.5	279.0	0	202	1	53
B1R9	54.5	29.5	5.5	303.0	0	268	0	63
B1R10	67.0	32.6	2.0	222.4	0	34	1	32
B1R15	58.3	22.8	0.7	298.7	2	175	0	60
B1R19	53.5	28.2	0.9	281.2	4	292	0	46
B1R24	56.8	29.3	1.1	246.3	3	272	0	48
B1R30	65.7	28.7	0.9	355.3	0	133	0	80

* See Table 2 for the units of enzyme activity and for culture conditions.
† Revertants derived from A1 and B1 were indicated by strain numbers preceded with A1R and B1R, respectively. 74A is wild type.

harvested and repressible phosphatase activities were assayed. As shown in Table 5, B1*ts-2* partially restored the ability to produce the repressible alkaline phosphatase in low-phosphate medium at 25°C but not at 35°C. This strain

TABLE 5

*Growth characteristics and repressible alkaline phosphatase synthesis in B1*ts-2 *in comparison with the wild type and B1*

Strain	Growth (mg dry weight)				Repressible alkaline phosphatase			
	High P_i		RNA medium		High P_i		Low P_i	
	25°C	35°C	25°C	35°C	25°C	35°C	25°C	35°C
74A	78.9	77.6	33.3	31.4	0.9	0.9	324.8	505.8
B1	76.6	83.0	2.9	10.4	0.9	0.9	1.1	0.2
B1*ts-2*	78.8	78.6	33.2	3.4	5.2	0.4	38.8	1.3

Mycelia were grown in high-phosphate medium (High-P_i), in low-phosphate medium (Low-P_i) or in RNA medium at 25°C or 35°C for 3 days. See Table 2 for units of enzyme activity.

TABLE 6

Repressible phosphatase activities in a heterokaryon between A1 and B1 grown in low-phosphate medium

Strain	Repressible alkaline phosphatase	Repressible acid phosphatase in extract	Repressible acid phosphatase in medium
A1	1.3	0	2
B1	1.1	3	0
A1 + B1 (heterokaryon)	187.9	318	61

See Table 2 for units of enzyme activity.

produced a small amount of the repressible alkaline phosphatase at 25°C even in high-phosphate medium. However, this constitutivity of the repressible enzyme formation was not observed in the culture grown at 35°C. The preliminary genetic analysis of B1*ts-2* indicated that the extragenic suppressor mutation is probably not involved in this strain (ISHIKAWA unpublished).

Phosphatase activities of heterokaryon between nuclease mutants: As reported previously (ISHIKAWA *et al.* 1969), the nuclease mutants could be divided into two groups, A and B, by the complementation test. Group A and group B mutants were located at the *nuc-1* and *nuc-2* loci, respectively, by crossing analysis. Since heterokaryons made between group A and group B mutants restored the ability to utilize RNA as a source of phosphate, we examined whether the heterokaryons restored the ability to produce the repressible phosphatases. Heterokaryotic mycelia made between A1 and B1 were grown in low-phosphate medium at 25°C for 3 days. Mycelial extracts and culture media were prepared and repressible phosphatase activities were assayed. As shown in Table 6, heterokaryons were able to produce repressible phosphatase activities partially or fully equal to the wild-type strain. This result indicates that the *nuc-1* and *nuc-2* mutant alleles are recessive to their wild-type alleles in the ability to produce the repressible phosphatases.

Isolation and characterization of phosphatase mutant: Among the three thousand isolates tested by the staining method described in MATERIALS AND METHODS to isolate various kinds of phosphatase mutants, only one repressible alkaline phosphatase mutant, *pho-1*, has been isolated. Colonies of this mutant stained pale red on low-phosphate medium in contrast with wild-type colonies, which stained dark purple. This mutant was grown in low-phosphate medium and in high-phosphate medium at 25°C for 3 days. Mycelia and culture media were harvested, and phosphatase activities were assayed. The *pho-1* mutant grown in low-phosphate medium showed a significantly lower activity of repressible alkaline phosphatase than that of the wild type but approximately the same level of repressible acid phosphatase activity (Tables 2 and 7).

The *pho-1* mutant was crossed with the nuclease mutants, A1 and B1. To characterize isolates from the crosses, the cultures established from isolated ascospores were inoculated on sorbose agar medium containing 20 μg/ml inorganic

TABLE 7

Repressible phosphatase activities in the forced heterokaryons between pho-1 *and nuclease mutants grown in low-phosphate medium*

Strain	Repressible alkaline phosphatase	Repressible acid phosphatase in extract	in medium
pho-1 ad-2	19.0	292	89
A1 *ad-9*	0.9	33	0
B1 *ad-6*	0.9	26	2
pho-1 ad-2 + A1 *ad-9* (heterokaryon)	60.2	189	70
pho-1 ad-2 + B1 *ad-6* (heterokaryon)	128.4	187	77

See Table 2 for units of enzyme activity and for culture conditions.

phosphate. The activity of repressible alkaline phosphatase of each colony was tested by the staining method described in MATERIALS AND METHODS. A cross between *pho-1* and A1 gave approximately 25% wild-type segregants, but a cross between *pho-1* and B1 gave 20% wild-type segregants, indicating that the locus for the repressible alkaline phosphatase is widely separated from the *nuc-1* and *nuc-2* loci and that this locus may be located in linkage group II.

The complementation test for the repressible phosphatases was performed on agar plates containing 20 μg/ml inorganic phosphate. Conidia of both strains were mixed and inoculated on agar medium; colonies appearing were examined for repressible phosphatase activities by the staining method described in MATERIALS AND METHODS. The results indicated that heterokaryons made between *pho-1* and A1 or B1 showed repressible phosphatase activities. Heterokaryotic mycelia were prepared by mixing conidia of *pho-1 ad-2* and A1 *ad-9* or B1 *ad-6* in low-phosphate medium without supplements. As shown in Table 7, the forced heterokaryons produced the repressible alkaline phosphatase although the activity was less than that found in the wild type.

These results indicate that the mutant allele of the repressible alkaline phosphatase gene is recessive to the wild-type allele and that the normal repressible alkaline phosphatase gene is still present in the nuclease mutants.

DISCUSSION

It was found previously that a single mutation resulted in a loss of the ability to digest either RNA or DNA (ISHIKAWA *et al.* 1969). Two genes, *nuc-1* and *nuc-2*, were found to be responsible for such nucleic acid digestion. It was subsequently confirmed that these mutants showed a reduced activity of a nuclease species, nuclease N_3, which was suggested to be a complex consisting of nuclease N_3' and an inhibitor molecule of this enzyme. Recent studies on these mutants indicating that mutant proteins are structurally altered (HASUNUMA and ISHIKAWA unpublished), as well as preliminary data published (ISHIKAWA *et al.* 1969), strongly indicate that the *nuc-1* and *nuc-2* genes may be the structural genes for nuclease N_3' and the inhibitor molecule, respectively.

Wild-type mycelia of *N. crassa* produce a repressible alkaline phosphatase and a repressible acid phosphatase under the derepressed condition (NYC *et al.* 1966; NYC 1967). The repressible alkaline phosphatase, which has a molecular weight of 154,000 (KADNER, NYC and BROWN 1968), was clearly separated from nuclease N_3 and its components, nuclease N_3' and the inhibitor, on the elution profile of Sephadex G-100 filtration (ISHIKAWA *et al.* 1969). The repressible acid phosphatase, which has the molecular weight of approximately 50,000 (NYC 1967), was eluted slightly earlier than nuclease N_3 and clearly separated from nuclease N_3' and the inhibitor on the elution profile of Sephadex G-100 filtration. A significant amount of repressible acid phosphatase was found in the culture medium when the wild-type culture was grown under the derepressed condition, but no activity of nuclease N_3 was found in the same culture medium. These evidences suggest that the repressible alkaline phosphatase, repressible acid phosphatase, nuclease N_3 and its components, nuclease N_3' and the inhibitor, are protein species distinct from each other.

The nuclease mutants were unable to grow in RNA media but were able to grow in nucleotide media. However, it has been noticed that the growth of nuclease mutants was slightly less than that of the wild-type strain in nucleotide media (ISHIKAWA *et al.* 1969). Since nucleotide media are considered to be low-phosphate media, the activities of repressible phosphatases which are synthesized under the derepressed condition were compared among the wild-type strain and nuclease mutants. In the wild-type strain the synthesis of repressible phosphatases is dependent on the concentration of inorganic phosphate in the growth medium. The maximal synthesis is obtained when the concentration of inorganic phosphate is low; when the concentration is raised, the synthesis is repressed. The effect of a low concentration of inorganic phosphate on phosphatase formation is designated as induction and that of a high concentration of inorganic phosphate as repression (GAREN and ECHOLS 1962b).

Evidence has been presented in the present study that both the *nuc-1* and *nuc-2* mutants, which were selected on the basis of lack of RNA utilization, could not produce the repressible alkaline and acid phosphatases. By a single reversion, both abilities of RNA utilization and of production of two repressible phosphatases were restored. Furthermore, a temperature-sensitive revertant with regard to RNA utilization was also temperature sensitive in the production of two repressible phosphatases. All these results indicate that induction of these enzymes is under control of the *nuc-1* and *nuc-2* genes. Since it has been strongly suggested that the *nuc-1* and *nuc-2* loci may be the structural genes for nuclease N_3' and the inhibitor molecule, respectively, it may be indicated that these genes are regulatory genes for the formation of these enzymes. Temperature-sensitive revertants were also found in the regulator gene *araC* of the L-arabinose system in *E. coli* (IRR and ENGLESBERG 1970).

Heterokaryotic mycelia made between *nuc-1* and *nuc-2* mutants were able to produce two repressible phosphatases in low-phosphate medium. This result indicates that the *nuc-1* and *nuc-2* mutant alleles are recessive to the wild-type alleles of the genes responsible, respectively, for the induction and repression of these enzymes, and that a cytoplasmic factor may participate in this phenomenon.

A mutant strain *pho-1* which is able to utilize nucleic acid but which produces a significantly reduced level of the repressible alkaline phosphatase was isolated. This mutant gene is located on a part of the chromosome widely separated from the *nuc-1* and *nuc-2* loci. Since a mutant defective in repressible acid phosphatase activity has not been isolated to date, it cannot be said yet whether the genes for the two repressible phosphatases are closely linked or not. Heterokaryons made between a nuclease mutant and the repressible alkaline phosphatase mutant restored the activity of repressible enzymes. The result indicates that a normally functioning cistron of the repressible alkaline phosphatase is present in the nuclease mutants.

Some revertants, including a temperature-sensitive revertant from a *nuc-2* mutant, produced a significant amount of the repressible alkaline phosphatase under the repressed condition indicating that repression is partially blocked by these reversions. No fully constitutive mutant or revertant which produces a high level of the repressible phosphatases under the repressed condition was found to date. The effects of the mutations and reversions at the *nuc-1* and *nuc-2* loci on induction and repression of alkaline and acid phosphatases are summarized in Table 8 after GAREN and ECHOLS (1962b). This table indicates that a mutation in the nuclease gene can block completely or partially the induction of enzyme synthesis, partially block the repression of enzyme synthesis or do both. From this analysis, it may be suggested that the nuclease genes control two regulatory functions, those of induction and repression of acid and alkaline phosphatase syntheses, and that the induction of these enzymes is under the positive control of these nuclease genes.

There are several genetic systems which show positive control of enzyme synthesis. In the alkaline phosphatase system in *E. coli*, GAREN and ECHOLS (1962a, b) have presented evidence for the presence of two regulatory genes, R1 and R2. They postulated that R1 produces an inducer which is converted into a repressor in the presence of high concentrations of inorganic phosphate by the action of the second regulatory gene R2. In the control of L-arabinose metabolism in *E. coli*, the positive control by a single regulatory gene *araC* has been described (ENGLESBERG *et al.* 1965, 1968, 1969; SHEPPARD and ENGLESBERG 1966, 1967). In the control of L-rhamnose metabolism in *E. coli*, the presence of a positive controlling element was also reported (POWER 1967). In Saccharomyces, a muta-

TABLE 8

Phenotypic characteristics of nuclease mutants and revertants

Strain and condition	Induction of phosphatase synthesis	Repression of phosphatase synthesis
Wild type	Normal	Normal
nuc-1 and *nuc-2* mutants	Defective	Normal
Most *nuc-1* and *nuc-2* revertants	Normal or partially defective	Normal
Some *nuc-2* revertants (e.g. B1R4)	Normal or partially defective	Partially defective
B1*ts-2* at 25°C	Partially defective	Partially defective
B1*ts-2* at 35°C	Defective	Normal

tion of a similar regulatory gene has been described in the galactose system (DOUGLAS and HAWTHORNE 1964). The existence of the positive controlling elements which facilitate the initiation of transcription of various specific templates by RNA polymerase was recently suggested by the discovery of the RNA polymerase sigma factor (BURGESS *et al.* 1969; BAUTZ, BAUTZ and DUNN 1969). The cyclic AMP receptor protein (EMMER *et al.* 1970) or catabolite gene activation protein (ZUBAY, SCHWARTZ and BECKWITH 1970) may be one of these.

In the present study, if one believes that the *nuc-1* and *nuc-2* genes are the structural genes for nuclease N_3, a simple mechanism postulated for the control of repressible phosphatases may be that the reaction products formed by the action of nuclease N_3 play a role of an inducer and are transformed to the repressor in the presence of inorganic phosphate. This mechanism may well explain why no induction of phosphatases was observed in nuclease mutants which produce no such reaction products. However, there is evidence that the addition of one of the reaction products, nucleotide, did not induce the phosphatases in nuclease mutants; furthermore, it is difficult to postulate that the reaction products, such as nucleotides or fragments of nucleic acid, are in most cases transformed to the repressors but in some cases (e.g. in some *nuc-2* revertants) fail to be transformed to the repressors in the presence of inorganic phosphate. Therefore, we submit the following model for the positive control of repressible phosphatases based on that proposed for the alkaline phosphatase in *E. coli* by GAREN and ECHOLS (1962a, b): Nuclease N_3 may be an inducer which has at least three active sites; for hydrolysis of nucleic acid, for attachment to the chromosome region to initiate synthesis of repressible enzymes, and for inorganic phosphate. By an allosteric transition the inducer may be converted to the repressor in the presence of inorganic phosphate. Nuclease-negative alleles may produce a defective nuclease N_3 that has no nuclease activity and no inducer activity. It should be realized that this proposed model may have no more than a limited hypothetical utility, and the final evidence should be worked out from the direct analysis of the role of nuclease N_3 in the enzyme synthesis.

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SUMMARY

The *nuc-1* and *nuc-2* mutants of *Neurospora crassa* which were isolated on the basis of inability to utilize nucleic acid as a sole source of phosphorus were unable to produce the repressible alkaline and acid phosphatases under the derepressed condition. A single reversion resulted in the simultaneous restoration of abilities to utilize RNA and to produce two repressible phosphatases. A temperature-sensitive revertant with regard to RNA utilization also showed temperature sensitivity in the repressible phosphate synthesis. Some revertants produced a significant amount of repressible alkaline phosphatase constitutively under

the repressed condition, indicating that the nuclease genes control both the induction and repression of these enzymes. In heterokaryon tests, the *nuc-1* and *nuc-2* alleles were recessive to the wild-type alleles in induction of repressible phosphatases. A phosphatase mutant *pho-1*, which has significantly low activity of repressible alkaline phosphatase, was found and its gene located at a locus distinct from the nuclease loci. Heterokaryon tests between nuclease mutants and *pho-1* indicated that nuclease mutants may have a normal phosphatase gene.— These studies support a positive control model in which the *nuc-1* and *nuc-2* genes are responsible for a substance essential for the induction and repression of phosphatase synthesis.

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