REGULATION OF PHOSPHATE METABOLISM IN *NEUROSPORA CRASSA*: IDENTIFICATION OF THE STRUCTURAL GENE FOR REPRESSIBLE ALKALINE PHOSPHATASE

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ABSTRACT

Five additional mutants of *Neurospora crassa* have been isolated that lack the repressible alkaline phosphatase. The mutations in these strains map at a previously assigned locus on Linkage Group V designated *pho-2* (GLEASON and METZENBERG 1974). The five new mutants, as well as three previously isolated by GLEASON and METZENBERG (1974), were examined for the presence of cross-reacting material to antibody prepared against purified wild-type enzyme. Two of the mutants produced high levels of cross-reacting material, thus providing evidence that the *pho-2* locus includes the structural gene for the repressible alkaline phosphatase. Two revertants were obtained from one of the mutants that contained cross-reacting material. Neither revertant produced an enzyme that could be distinguished physicochemically from that of wild type. A method for measuring very low levels of repressible alkaline phosphatase in crude extracts is also described.

A number of phosphate-metabolizing enzymes in *Neurospora crassa* are highly derepressed during conditions of phosphate limitation. These include an alkaline phosphatase (NYC, KADNER and CROCKEN 1966; LEHMAN *et al.* 1973; BURTON and METZENBERG 1974), and acid phosphatase (NYC 1967; NELSON, LEHMAN and METZENBERG 1976), a high pH, high affinity phosphate permease (LOWENDORF and SLAYMAN 1970; LOWENDORF 1972; LEHMAN *et al.* 1973), an 0-phosphorylethanolamine permease (METZENBERG, unpublished results), and one or more nucleases (HASUNUMA 1973). These same enzymes are repressed during growth on high levels of phosphate. Several mutations that alter the regulation of the phosphate-metabolizing family have been discovered (TOH-E and ISHIKAWA 1971; LEHMAN *et al.* 1973; METZENBERG, GLEASON and LITTLEWOOD 1974; LITTLEWOOD, CHIA and METZENBERG 1975). These studies have been summarized in a model involving three regulatory genes (LITTLEWOOD, CHIA and METZENBERG 1975).

A full genetic analysis of this system requires the identification of one or more structural genes for the phosphate-metabolizing family of enzymes. GLEASON and METZENBERG (1974) isolated four mutants that were specifically deficient in the repressible alkaline phosphatase. The mutations in these strains all mapped at a locus designated *pho-2*. GLEASON and METZENBERG could not eliminate the

possibility that all of the mutants were defective in a regulatory function specific for alkaline phosphatase expression. One mutant, carrying the MKG-2 allele, was most simply interpreted in this way.

We describe here the isolation of additional mutants lacking repressible alkaline phosphatase. The mutations in these strains were all found to map on Linkage Group V at the *pho-2* locus. The new mutants, together with three of those isolated by GLEASON and METZENBERG, were examined for the presence of material immunologically related to alkaline phosphatase by the double-diffusion test of Ouchterlony (CROWLE 1973). Two of the mutants were found to contain high levels of cross-reacting material that had no significant enzymatic activity. In addition, the double-diffusion test revealed that the cross-reacting material from one of these mutants differed antigenically from wild-type enzyme. These results provide evidence that some *pho-2* mutations lie within the structural gene for this enzyme. On the basis of this evidence, it was possible to clarify further the nature of the MKG-2 allele.

Two revertants were obtained from one of the mutants that contained cross-reacting material. Alkaline phosphatase produced in these revertants did not appreciably differ from wild type in either heat stability or Arrhenius activation energy.

MATERIALS AND METHODS

Strains: The characteristics and sources of the mutated alleles used in this work are listed in Table 1. Strains carrying various combinations of these were constructed by standard genetic techniques, as described by DAVIS and DESERRES (1970). All *Neurospora crassa* strains were made heterokaryon-compatible and near to isogenic with the two Oak Ridge wild types, 74-OR8-1a and 74-OR23-1A, by several sequential crosses.

Media, growth of strains, enzyme extraction and assay, heat-inactivation of enzyme, determination of Arrhenius activation energy, mutagenesis, and screening for mutants: These procedures have been previously described (LEHMAN *et al.* 1973; GLEASON and METZENBERG 1974).

Detecting low levels of repressible alkaline phosphatase: To measure very low levels of this enzyme, it was necessary to physically separate it from the other (constitutive) phosphatases rather than merely to inhibit most of the activity of the latter as had been done previously (LEHMAN *et al.* 1973; GLEASON and METZENBERG 1974). Under those previous conditions of assay, a rather high background of activity is still observed in crude extracts of *pho-2* strains.

Mycelial pads from strains grown on low phosphate medium were extracted in 0.02 M sodium acetate buffer, pH 5.0, as described by GLEASON and METZENBERG (1974). Protein concentrations were adjusted to 1.5 mg/ml in the same buffer and 0.2 ml were applied to 5 mm diameter columns of DEAE cellulose (Sigma Chemical Co.) previously equilibrated at 4° with 0.02 M triethanolamine-HCl buffer, pH 7.0, and packed to a height of 2.7 cm under 0.067 atmosphere positive pressure. The initial 0.2 ml of eluate was discarded. Then 0.8 ml of 0.02 M triethanolamine-HCl buffer, pH 7.0, was added to each column, and this entire volume was collected. In the case of extracts with low levels of enzyme, 0.5 ml of eluate were assayed as described by LEHMAN *et al.* 1973. More than 70% of input activity could be recovered from wild-type extract under these conditions. Reconstruction experiments with mutant extract plus small amounts of wild-type extract demonstrated that amounts less than 10^{-4} times the normal derepressed level of wild-type enzyme could still be recovered and detected (data not shown).

Ouchterlony double-diffusion plate test: The general procedure followed is described by CROWLE (1973). Strains containing *preg*^{c} and *pho-2* were grown on high phosphate medium to provide sufficiently concentrated crude extracts. Lyophilized mycelial pads were extracted in 0.15 M sodium HEPES (N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid) buffer, pH 7.4,

TABLE 1

Mutated alleles used in this work

Genetic locus (allele designation)	Nutritional requirements or other characteristics	Reference or origin
preg^c (preg c-2)	Constitutive expression of the phosphate metabolizing enzymes	METZENBERG, GLEASON and LITTLEWOOD 1974
pan-2 (Y153M96)	Requires pantothenic acid (furnished at 2 μg/ml)	Fungal Genetics Stock Center
inl (89601)	Requires inositol (furnished at 50 μg/ml)	Fungal Genetics Stock Center
trp-3 (td37)	Requires tryptophan (furnished at 1 mg/ml)	Fungal Genetics Stock Center
cot-1 (C102(t))	Colonial growth at $T \geq 30°$	Fungal Genetics Stock Center
pho-2 (MKG-1)	Deficient in repressible alkaline phosphatase	GLEASON and METZENBERG 1974
pho-2 (MKG-2)	Deficient in repressible alkaline phosphatase	GLEASON and METZENBERG 1974
pho-2 (RLM-20)	Deficient in repressible alkaline phosphatase	GLEASON and METZENBERG 1974
pho-2 (RLM-72)	Deficient in repressible alkaline phosphatase	GLEASON and METZENBERG 1974
pho-2 (JFL-134)	Deficient in repressible alkaline phosphatase	This work
pho-2 (JFL-317)	Deficient in repressible alkaline phosphatase	This work
pho-2 (JFL-327)	Deficient in repressible alkaline phosphatase	This work
pho-2 (JFL-341)	Deficient in repressible alkaline phosphatase	This work
pho-2 (JFL-606)	Deficient in repressible alkaline phosphatase	This work

after they had been first ground to a powder in a mortar and pestle. Protein concentrations were adjusted to 20 mg/ml (or 32 mg/ml in the case of the *pho-2* (RLM-20) strain, which had been grown on low phosphate medium) in the same buffer and 20 microliter samples applied to precut agar wells on a microscope slide, also buffered with 0.15 M sodium HEPES, pH 7.4. Appropriate dilutions of antibody were applied to the center well. Slides were incubated 48 hours at 4° in a humidified chamber and then photographed. They were incubated another 24 hours at 4° and inspected for additional "spur" patterns before being discarded.

Detecting preg^c *in* pho-2 *strains*: The presence of the *preg^c* marker in strains lacking repressible alkaline phosphatase could be ascertained by a simple plate test for the repressible acid phosphatase (NELSON, LEHMAN and METZENBERG 1976).

RESULTS AND DISCUSSION

Isolation of new mutants deficient in repressible alkaline phosphatase: Conidia of a strain marked by *pan-2* and *preg^c* were irradiated with UV, plated to high phosphate medium with pantothenic acid, and colonies were examined by stain-

ing for repressible alkaline phosphatase, as described by GLEASON and METZENBERG (1974). Out of 50,000 colonies screened, five isolates were found to be deficient only in repressible alkaline phosphatase. They were outcrossed and progeny marked by *pan-2* and *preg*^{c} were isolated. These were then crossed to a representative strain deficient in repressible alkaline phosphatase (*pho-2* (MKG-1)). In each case, no wild-type recombinant was observed among 150–300 progeny tested.

Testing for repressible alkaline phosphatase activity in the mutants: The five new mutant strains, as well as three of those isolated by GLEASON and METZENBERG, were tested for residual levels of repressible alkaline phosphatase activity by a simple column chromatographic purification procedure that quantitatively removes the constitutive phosphatases (see MATERIALS AND METHODS). A *pho-2* (MKG-2) strain was not included because it had already been shown that this mutant contains about 1% the wild-type level of enzyme (GLEASON and METZENBERG 1974). The eight strains tested had less than 2.0×10^{-4} times the level of repressible alkaline phosphatase activity present in wild type grown under the same conditions.

In this same test, wild type grown under repressing conditions reproducibly showed less than 10^{-4} the level of activity seen under derepressing conditions. Thus the true derepression ratio for alkaline phosphatase is at least an order of magnitude greater than the 10^{3} fold we previously reported in crude extracts (LEHMAN *et al.* 1973). This degree of repressibility is the largest among the simple eukaryote systems so far studied (HOROWITZ and METZENBERG 1965; METZENBERG 1972). It approaches the quantitative scale of regulation observed in differentiated tissues of higher organisms (RUTTER *et al.* 1968).

Testing for cross-reacting material in the mutants: The same eight mutant strains were tested for cross-reacting material to antiserum prepared against purified wild-type repressible alkaline phosphatase. Figure 1 shows the results of a typical double-diffusion plate test. None of the five new mutants contained detectable cross-reacting material (data not shown) nor did the *pho-2*(MKG-1) strain (well 5 in Figure 1). Wild type grown under repressing conditions also contains no detectable cross-reacting material (well 2). This result is consistent with earlier suggestions that derepression involves *de novo* synthesis of enzyme (GLEASON 1973). Extract from the strain containing *pho-2*(RLM-72) (well 3) produced a precipitin band that fused with the band from wild-type extract (well 4). By this test, no difference in antigenic quality between *pho-2*(RLM-72) and wild-type enzyme is evident. In contrast, a "spur" from the wild-type precipitin band (well 1) was observed in conjunction with the precipitin band from the *pho-2*(RLM-20) strain (well 6). This precipitin pattern suggests the existence of some antigenic difference(s) between *pho-2*(RLM-20) enzyme and that of wild type.

The relative amounts of cross-reacting material are similar in the three extracts which produced precipitin bands. This is indicated by the similar location of the bands in relation to the antibody and antigen sources (CROWLE 1973). Thus a protein immunologically similar to wild-type alkaline phosphatase but without

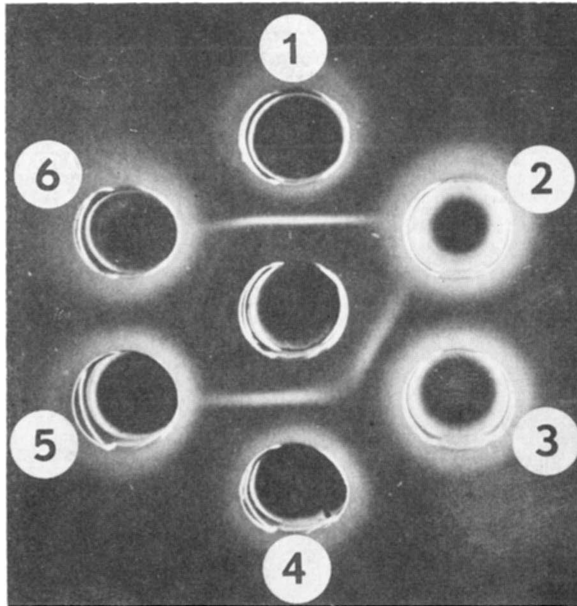

FIGURE 1.—Ouchterlony double-diffusion plate test. See MATERIALS AND METHODS for details. The center well contains a 1:8 dilution of ammonium sulfate-fractionated antiserum to purified wild-type repressible alkaline phosphatase (a gift from E. BURTON). The outer wells contain crude extract from (1) *pan-2;preg^c* (2) 74–OR8–1a wild type (repressed) (3) *pan-2;preg^c;pho-2* (RLM-72) (4) *pan-2;preg^c* (5) *pan-2;preg^c;inl, pho-2*(MKG-1) (6) *pan-2;pho-2*(RLM-20). Purified alkaline phosphatase (from E. BURTON) produced a single precipitin band in a similar experiment (data not shown).

enzymatic activity is present in *pho-2*(RLM-72) and *pho-2*(RLM-20) strains. Since some mutations which map at the *pho-2* locus result in the complete absence of cross-reacting material (MKG-1 for example), the simplest interpretation of these results is that the RLM-72 and RLM-20 alleles represent alterations in the primary coding sequence for the enzyme itself. Only amino acid sequence data can completely verify this interpretation.

Revertants of pho-2*(RLM 72)*: Conidia of a *pho-2*(RLM-72) strain marked with *pan-2* and *preg^c* were subjected to UV mutagenesis, and colonies derived from the survivors were screened for repressible alkaline phosphatase (GLEASON and METZENBERG 1974). Out of 2.0×10^6 colonies tested, two isolates were found which had regained enzymatic activity and were still *pan-2*; *preg^c*. The enzyme produced in these two strains was indistinguishable from that of wild type as judged by heat stability and Arrhenius activation energy.

The significance of the MKG-2 allele: GLEASON and METZENBERG (1974) found that the enzyme produced at low levels in a *pho-2*(MKG-2) strain was indistinguishable from that of wild type by a number of physicochemical criteria. In addition, they found that the level of cross-reacting antigen in this strain was reduced by an amount proportional to the reduction in specific activity. On the basis of these observations, they suggested that this mutant may be defective in

a regulatory element rather than in the structural gene itself. This regulatory element might be either *cis* or *trans* acting.

To distinguish between these two possibilities, a complementation test was performed between *pho-2*(MKG-2) and a mutation in the alkaline phosphatase structural gene. Table 2 records the results of such a test, using *pho-2*(RLM-72). By comparing lines 2 and 3 with lines 4 and 5, it is seen that both mutations are recessive to wild type. The result in line 1 clearly indicates that they fail to complement. This implies that the MKG-2 mutation either lies within the structural gene itself or within a *cis*-acting controlling element (or control site). Since the enzyme produced in an MKG-2 strain is indistinguishable from that of wild type, one interpretation of the complementation test is that *pho-2*(MKG-2) is an alteration in a control region adjacent to the structural gene. A leaky nonsense mutation within the structural gene or a mutation in the initiation codon and subsequent use of a secondary initiation site could also produce the same results.

To determine the genetic distance between the *pho-2*(MKG-2) and *pho-2* (RLM-72) alterations, a cross between *pan-2*; *preg*^{c} strains carrying these muta-

TABLE 2

Dominance and complementation tests involving pho-2*(MKG-2) and* pho-2*(RLM-72)*

Strain	Repressible alkaline phosphatase	Nuclear ratio
(*pan-2*;*preg*^{c};*pho-2*(RLM-72)) + (*inl*,*pho-2*(MKG-2)) heterokaryon	3.68	52% *pho-2*(MKG-2)
(*pan-2*;*preg*^{c};*pho-2*(RLM-72)) + (*inl*) heterokaryon	370	48% *pho-2*(RLM-72)
(*inl*,*pho-2*(MKG-2)) + (*pan-2*) heterokaryon	424	30% *pho-2*(MKG-2)
(*pan-2*;*preg*^{c};*pho-2*(RLM-72)) homokaryon	0.48	
(*inl*,*pho-2*(MKG-2)) homokaryon	6.22	
(*pan-2*) homokaryon	488	

Assays were performed at 25° on crude extracts from mycelia grown by sparging with sterile air for 17 hours at 25° in low phosphate medium. The numbers indicate specific activities (*n*moles *p*-nitrophenyl phosphate cleaved/min/mg protein). Nuclear ratios were determined by the method of ATWOOD and MUKAI (1955). The high residual activity in the *pho-2*(RLM-72) strain is due to constitutive phosphatases present in crude extracts. Similar results were obtained when heterokaryons were forced by *trp-3* and *pan-2* mutations (data not shown).

tions was performed. The MKG-2 parent was also marked by *inl*, located about 4 centimorgans distal to *pho-2* (GLEASON and METZENBERG 1974). Out of 6410 progeny tested, 3 isolates were found to be wild type for alkaline phosphatase expression. All three of the *pho-2*+ recombinants were *pan-2*; *preg*^{c}, indicating that they were probably not contaminants. They were also all *inl*. We cannot order the mutational sites in this interallelic cross due to the likelihood of high negative interference (STADLER and TOWE 1963).

Earlier results indicated that a functional product coded by the *nuc-1* gene is what is ultimately required for the expression of the genes for repressible alkaline phosphatase and other enzymes of the phosphate-metabolizing family (TOH-E and ISHIKAWA 1971; LITTLEWOOD, CHIA and METZENBERG 1975). It seems reasonable to suggest that the unlinked (see NELSON, LEHMAN and METZENBERG 1976) structural genes for these enzymes should have contiguous binding sites for the *nuc-1* product and that mutations in such sites would affect only the level of the enzyme in question, not the structure. Possibly *pho-2*(MKG-2) is such a mutation. Those mutations that completely eliminate all enzymatic activity and accumulate no detectable cross-reacting material (such as the MKG-1 allele and the five alleles isolated in the present work) might lie within the coding sequence for the enzyme and result in a drastic alteration of protein structure. Alternatively, they might lie within the site that normally binds *nuc-1* product and destroy all affinity for that product. It is difficult to distinguish experimentally between these two alternatives at present.

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