Molecular Analysis of *nuc-1*⁺, a Gene Controlling Phosphorus Acquisition in *Neurospora crassa*

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In response to phosphorus starvation, *Neurospora crassa* makes several enzymes that are undetectable or barely detectable in phosphate-sufficient cultures. The *nuc-1*⁺ gene, whose product regulates the synthesis of these enzymes, was cloned and sequenced. The *nuc-1*⁺ gene encodes a protein of 824 amino acids with a predicted molecular weight of 87,429. The amino acid sequence shows homology with two yeast proteins whose functions are analogous to that of the NUC-1 protein. Two *nuc-1*⁺ transcripts of 3.2 and 3.0 kilobases were detected; they were present in similar amounts during growth at low or high phosphate concentrations. The *nuc-2*⁺ gene encodes a product normally required for NUC-1 function, and yet a *nuc-2* mutation can be complemented by overexpression of the *nuc-1*⁺ gene. This implies physical interactions between NUC-1 protein and the negative regulatory factor(s) PREG and/or PGOV. Analysis of *nuc-2* and *nuc-1*; *nuc-2* strains transformed by the *nuc-1*⁺ gene suggests that phosphate directly affects the level or activity of the negative regulatory factor(s) controlling phosphorus acquisition.

All microorganisms have elaborate regulatory systems for sensing the availability of nutrients and modulating metabolic activities to achieve constancy of the internal environment. When the filamentous fungus *Neurospora crassa* is grown on medium containing limiting amounts of inorganic phosphate or a rate-limiting phosphorus source such as phosphoethanolamine, a number of enzymes are synthesized that function to make more phosphate available to the cell (12, 18, 27). When *N. crassa* is grown with an adequate supply of phosphate, these enzymes are synthesized at much reduced levels, if at all. These enzymes include repressible alkaline and acid phosphatases, one or more high-affinity phosphate permeases, a phosphoethanolamine permease, and a number of nucleases. The regulatory pathway for the synthesis of these enzymes, as revealed by genetic and metabolic analyses, consists of four genes: *nuc-2*⁺, *preg*⁺, *pgov*⁺, and *nuc-1*⁺. The working model based on the dominance and epistatic interactions of mutant alleles of these genes is shown in Fig. 1. The *nuc-1*⁺ product is necessary for the expression of the structural genes. The putative *preg*⁺ and *pgov*⁺ products act together to antagonize the action of the *nuc-1*⁺ product. The *nuc-2*⁺ product inhibits the function of the *preg*⁺ and/or *pgov*⁺ product(s), and phosphate or one of its derivatives opposes the action of *nuc-2*⁺. One of the structural genes, *pho-4*⁺, which encodes a high-affinity phosphate permease, has been cloned and characterized (20, 21). Northern (RNA) analysis clearly demonstrated that the transcription of the *pho-4*⁺ gene is controlled by the *nuc-1*⁺ product. The two transcripts of the *pho-4*⁺ gene are present only when cells are grown under conditions of phosphate limitation. These transcripts are absent from the *nuc-1* mutant strain regardless of the phosphate concentration (20). To determine how *nuc-1*⁺ functions at the molecular level, we cloned and sequenced the *nuc-1*⁺ gene. The predicted sequence of the NUC-1 protein shows homology with the PHO4 and PHO2 proteins which, together, control the expression of phosphorus acquisition enzymes in *Saccharomyces cerevisiae* (29). The putative DNA-binding domain of

the NUC-1 protein, showing strong homology with the DNA-binding domain of the yeast PHO4 protein (28), contains the amphipathic helix-loop-helix (A-HLH) motif identified in a number of DNA-binding proteins in higher eucaryotes (26). Three motifs typical of transcriptional activators can be identified in the NUC-1 protein. The two *nuc-1*⁺ transcripts were found to be expressed constitutively, regardless of the phosphate concentration. Our results also showed that the *nuc-2* mutation can be complemented by overexpression of the *nuc-1*⁺ gene.

MATERIALS AND METHODS

Strains and growth conditions. Strains of *N. crassa* used in this study are described in Table 1. *Escherichia coli* Q358 and NM522 were used for bacteriophage infection and plasmid propagation, respectively. Vogel medium (5) was used to grow *N. crassa* for preparing genomic DNA. RNA was extracted from mycelia grown in Fries medium (23). Ordinary Fries medium, which contains 7.35 mM potassium phosphate, was called high-phosphate medium. Fries low-phosphate medium contained 0.05 mM potassium phosphate, with the deficit of potassium ions being made up with the molar equivalent of KCl.

Transformation and repressible alkaline phosphatase assay. Spheroplasts for *Neurospora* transformation were prepared as described by Akins and Lambowitz (1) and transformed by the method of Vollmer and Yanofsky (36). Transformants were selected on high-pH medium (23) containing 1 mM phosphoethanolamine as the sole phosphorus source. To eliminate background growth, we cotransformed the host strains with the DNA of interest plus the vector pSV50, which carries a dominant selectable marker conferring benomyl resistance (36). Benomyl was added only to the bottom agar at a concentration of 0.5 µg/ml. Chloramphenicol was included in both top and bottom agars to prevent growth of any bacterial contaminants. All host strains contained an auxotrophic mutation (*inl*, inositol) to allow their transformants to be distinguished from any possible airborne *Neurospora* contaminants. Homokaryotic transformants were isolated by successive colony isolations from plated single conidia. Putative homokaryotic transformants were

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FIG. 1. Model for the control of synthesis of phosphorus acquisition enzymes (24). The phenotypes of various strains carrying one or more mutations can be calculated by multiplying the positive or negative signs of the regulatory products. Only those regulatory products that are connected to the structural genes by a sequence unbroken by mutation can be included in the logical sequence. For example, in a *nuc-2* mutant, only the final two regulatory steps are operative ($- \times +$) and such strains are $-$, i.e., the enzymes are not made. The *preg* and *pgov* loci are identified only by the occurrence of mutants (*preg^c* and *pgov^c*) that express the structural genes constitutively.

grown under nonselective conditions, and 20 colonies from each transformant were tested for growth under selective conditions. Growth of strains and enzyme assays for repressible alkaline phosphatase were performed as previously described (17).

Screening of *Neurospora* genomic DNA libraries. Screening of the cosmid library ordered in 32 microtiter plates was performed as described by Vollmer and Yanofsky (36). The lambda genomic DNA library (provided by R. F. Geever, University of Georgia) was screened by plaque hybridization according to the protocol of Maniatis et al. (19). Hybond-N membrane filters (Amersham Corp., Arlington Heights, Ill.) were used for plaque hybridization instead of nitrocellulose filters.

Nucleic acid analyses. *Neurospora* genomic DNA was prepared by the method of Metzzenberg and Baisch (*Neurospora* Newsl. 28:20–21, 1981). Bacteriophage lambda was purified and DNA was isolated as described by Maniatis et al. (19). *Neurospora* poly(A)⁺ RNA was isolated, and Northern blots were prepared and probed as described previously (21). Labeled probes for both Southern and Northern analyses were prepared by random priming (7). Southern blotting and hybridization were performed with Zetabind membrane as recommended by the manufacturer (AMF CUNO, Meriden, Conn.). DNA sequencing was done with ³⁵S-labeled deoxyadenosine 5'-thiol triphosphate and Sequenase (U.S.

Biochemicals, Cleveland, Ohio). Sequencing templates were prepared by inserting restriction fragments into pGem3Zf (+/–) vectors (Promega Biotec, Madison, Wis.). DNA was purified by CsCl centrifugation whenever double-stranded templates were used for sequencing. Computer analysis of the sequence was done with programs from the University of Wisconsin Genetics Computer Group (6).

Nucleotide sequence accession number. The *nuc-1* sequence has been registered with GenBank under the accession number M37700.

RESULTS

Cloning of *nuc-1*⁺ gene. Since the *nuc-1*⁺ gene is required for the expression of the phosphorus acquisition enzymes (34), and a low-affinity phosphate permease, which is constitutive, barely functions to support growth above pH 7 (18), a *nuc-1* mutant strain is unable to grow on low-phosphate medium at high pH. An initial attempt to clone the *nuc-1*⁺ gene from *Neurospora* genomic DNA libraries by complementing a *nuc-1* mutation was unsuccessful (20; this work) because, as we later discovered, the *nuc-1*⁺ gene was not present in these libraries (1, 36). Since the closely linked *his-2*⁺ gene had been cloned (36), we performed chromosome walking from this gene using overlapping phage and cosmid clones as shown in Fig. 2. Usually it is difficult to correlate the direction of chromosome walking with the conventional linkage map unless there is a gene or some marker than can be easily detected. Almost 200 chromosome translocations have been isolated and characterized in *N. crassa* (32). These translocations have been used for mapping genes, centromeres, and chromosome tips. We found that the translocations are useful as physical markers for molecular orientation in chromosome walking. The molecular orientation was correlated with the conventional linkage map by walking over the break point of two of these chromosome translocations, *T(I→VII;I;V;VII)AR173* and *T(I→V)AR190*, which have known map positions with respect to *his-2* (32). The appearance of anomalous restriction fragments in blots of genomic DNA from these strains when individual clones from the walk were used as probes indicated that the chromosome break point of the relevant translocation strain was within the sequences included in the

TABLE 1. *N. crassa* strains

Strain ^a	Mating type	Genotype	Source ^b
40-21	<i>a</i>	Wild type	FGSC 988
15-02	<i>A</i>	<i>T(I→VII;I;V;VII)AR173</i>	FGSC 2468
22-14	<i>A</i>	<i>T(I→V)AR190</i>	FGSC 1951
08-14	<i>A</i>	<i>met-10</i>	FGSC 2937
07-24	<i>A</i>	<i>nuc-1; inl</i>	RLM
26-01	<i>a</i>	<i>nuc-1^c</i>	RLM
02-29	<i>A</i>	<i>nuc-2; inl</i>	RLM
11-23	<i>a</i>	<i>nuc-1; nuc-2; inl</i>	RLM
	<i>a</i>	<i>nuc-1; nuc-2; preg^c; inl</i>	SK

^a Stock number in the *Neurospora* collection of R. L. Metzzenberg.

^b Abbreviations: FGSC, Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City); RLM, R. L. Metzzenberg; SK, S. Kang.

FIG. 2. Genetic map of part of *N. crassa* linkage group IR and chromosome walking strategy. The centromere is indicated by the open circle. Cosmid clones C1, C2, and C3 were from a cosmid library (36). Clones G4, G5, G6, and G7 were isolated from a lambda genomic DNA library. The positions of relevant genes and translocation breakpoints are noted (32).

probe. Hybridization of labeled inserts of the lambda clones G4 and G5 to genomic DNA of the *T(I→VII;I;V;VII)AR173* strain revealed two anomalous restriction fragments which were not present in genomic DNAs from a wild-type strain or from a strain carrying the chromosome translocation *T(I→V)AR190* (Fig. 3). The *nuc-1* mutant was transformed with DNA purified from each phage clone to test for complementation. DNA from the three overlapping lambda clones, G5, G6, and G7, was capable of complementing the

nuc-1 mutation, providing strong evidence that these clones carry the *nuc-1*⁺ gene. This was further supported by the ability of G7 to complement a mutation in the closely linked *met-10* gene, in agreement with the conventional genetic map (Fig. 2).

Subcloning and sequencing of *nuc-1*⁺ gene. Two overlapping restriction fragments, one from G5 and one present in both G6 and G7, were able to complement the *nuc-1* mutation. These fragments were subcloned into pUC19 to give pNUC-1A and pNUC-1B, respectively. The restriction map of the overlapping region is shown in Fig. 4. Since the inserts of pNUC-1A and pNUC-1B overlap by 4.3 kilobases (kb), all the sequences necessary for *nuc-1*⁺ gene function must be contained in this 4.3-kb region. The DNA sequence of this region was determined on both strands by the dideoxy-chain termination method. The DNA sequence between the *Xba*I and *Eco*RI sites is shown in Fig. 5. The largest open reading frame (ORF) begins with an AUG codon at positions 778 to 780 and ends with a UAA stop codon at positions 3247 to 3249. A consensus sequence for the poly(A)⁺ addition site of eucaryotic messages (11), AATAAA, is present 227 nucleotides downstream from the postulated stop codon. It has not yet been determined whether this sequence actually functions in this respect. The highly conserved sequence elements of typical *Neurospora* introns (11) are absent from the *nuc-1*⁺ ORF. In vitro transcription and translation of the *nuc-1*⁺ gene produced a protein product of approximately the expected size, suggesting that there are no out-of-frame introns in this ORF (data not shown). However, we have not ruled out the existence of an in-frame intron.

If there is no intron, this 2.4-kb ORF encodes a protein of 824 amino acids with a predicted molecular weight of 87,429. Rare codons are used rather frequently compared with other *Neurospora* genes, as is typical of genes with low levels of expression (11, 14). The NUC-1 protein contains no cysteine residues, and the most abundant amino acids are serine (10.8%) and proline (10.9%). According to a hydrophathy plot, the NUC-1 protein contains no hydrophobic stretches (data not shown). The distribution of several amino acids is obviously nonuniform, especially that of proline and glutamine. Regions rich in these amino acids are indicated in Fig. 5. Known DNA-binding motifs such as zinc fingers or homeo domains were not detected in the NUC-1 protein, nor was a leucine zipper.

A computer search of the sequence data base for proteins showing significant homology to NUC-1 protein identified PHO4 (16, 37) and PHO2 (33, 37) of *S. cerevisiae*. These proteins, taken together, have functions analogous to that of the NUC-1 protein (29, 38), so it is not surprising that they

FIG. 3. Southern analysis of *Neurospora* genomic DNA. Genomic DNA isolated from *T(I→V)AR190* (lanes A), wild type (lanes B), or *T(I→VII;I;V;VII)AR173* (lanes C) was digested with *Eco*RV or *Hind*III, separated on a 1% agarose gel, transferred to Zetabind membrane, and probed with labeled 7.5-kb *Eco*RI fragment from the clone G5. Arrows indicate restriction fragments generated by the chromosome translocation *T(I→VII;I;V;VII)AR173*.

FIG. 4. Restriction map of the 4.3-kb genomic DNA region containing the *nuc-1*⁺ gene. The position of the ORF is based on the DNA sequence shown in Fig. 5. The *EcoRI* sites at both ends originated from multiple cloning sites in the lambda cloning vector and do not exist in *Neurospora* genomic DNA.

have structural homology as well. Alignment of the PHO4 and NUC-1 sequences revealed approximately 32% identity. When conservative changes were also considered, the relatedness of the two proteins was almost 51%. Homology was most striking in two runs of amino acids (Fig. 6). A stretch of 95 amino acids of the NUC-1 protein (residues 468 to 562) showed 53% identity (when two gaps were allowed) with a segment of the PHO4 protein (residues 168 to 197). Another strong similarity was observed between the carboxy-terminal ends of the NUC-1 (residues 668 to 763) and PHO4 (residues 251 to 312) proteins. We found less impressive but still significant homology between NUC-1 and the yeast PHO2 protein (data not shown). Overall amino acid identity between the NUC-1 and PHO2 proteins was 21%, and if conservative substitutions were taken into account, the relatedness was 42%. Both the NUC-1 and PHO2 proteins have glutamine-rich regions near their amino-terminal ends. Functional implications of these homologous domains are discussed below.

Transcript analysis. To determine whether the *nuc-1*⁺ gene is transcriptionally regulated by the *preg*⁺ and *pgov*⁺ products and whether the NUC-1 protein regulates its own transcription, we measured the level of *nuc-1*⁺ mRNA in strains grown with different phosphate concentrations and in different genetic backgrounds. To assess the relative amounts of poly(A)⁺ RNA in each lane and to estimate the level of the *nuc-1*⁺ mRNA, we probed each Northern blot with labeled DNA corresponding to the *am*⁺ gene (15), which is expressed constitutively. Northern blots probed with labeled *nuc-1*⁺ DNA revealed that two transcripts of approximately 3.2 and 3.0 kb were present at very low levels. If there is control of *nuc-1*⁺ expression at the transcriptional level, we would expect to see an increased level of *nuc-1*⁺ mRNA in the wild-type strain under low-phosphate conditions (compared with that under high-phosphate conditions), but no such increase was seen (Fig. 7).

The possibility of autogenous regulation can be addressed with the *nuc-1*^c mutant. Unlike the wild type, the *nuc-1*^c mutant has active NUC-1 protein even under high-phosphate conditions (23, 24). RNA was isolated from wild-type and *nuc-1*^c strains grown under high-phosphate conditions. Northern analysis revealed no difference in the amount of *nuc-1*⁺ mRNA between wild-type and *nuc-1*^c strains (Fig. 7). This excluded the possibility of autogenous regulation at the transcriptional level. Since the level of *nuc-1*⁺ mRNA was the same in *nuc-1*^c and wild-type strains, the constitutive phenotype of the *nuc-1*^c mutant used in this study could not be the result of an increased level of the *nuc-1*⁺ transcript.

Complementation of *nuc-2* mutation by introduction of multiple copies of *nuc-1*⁺ gene. On the basis of the properties of the *nuc-1*^c mutant, it had been proposed that this constitutive mutant is an overproducer of qualitatively normal *nuc-1*⁺ product such that the usual level of the *preg*⁺ and/or *pgov*⁺ product(s) is insufficient to titrate out the *nuc-1*⁺ product (23, 24). Since the *nuc-2*⁺ product is necessary for

counteracting the functions of the *preg*⁺ and/or *pgov*⁺ product(s), *nuc-2* mutants (whose NUC-1 proteins are inactivated by the PREG and PGOV proteins under low-phosphate conditions) are phenotypically indistinguishable from *nuc-1* mutants.

To determine whether one can complement a *nuc-2* mutation by increasing the level of the NUC-1 protein to the point that high NUC-1 protein levels counteract the negative effect of the PREG and PGOV proteins, we transformed *nuc-2* strains with pNUC-1A. Many of the transformants were expected to have multiple copies of the *nuc-1*⁺ gene. In *N. crassa*, transformation usually results in nonhomologous, often multiple, integration of transforming DNA. Depending on where it integrates, transforming DNA may be expressed at various levels. Due to this position effect, levels of gene expression do not strictly correlate with copy number of the integrated gene. However, in general, mRNA level increases as copy number increases. If only those transformants of *nuc-2* that overexpress NUC-1 protein could grow, we would expect to see a relatively low transformation efficiency compared with that obtained when the *nuc-1* mutant was the host strain, because such transformants carrying multiple copies of the *nuc-1*⁺ gene constitute only a fraction of the total transformants. As a control for possible changes in the transformation efficiency due to differences in the genetic background, we used pBJ004 carrying the *pho-4*⁺ gene, since *pho-4*⁺, a gene encoding a phosphorus-repressible phosphate permease, complemented both the *nuc-1* and *nuc-2* mutations when it was introduced by transformation (20). The *pho-4*⁺ gene, which normally requires the *nuc-1*⁺ function for expression, no longer depends on *nuc-1*⁺ when introduced into a strain by transformation (20). Transformants were selected on high-pH medium containing phosphoethanolamine as the sole phosphorus source. Under these conditions, only those transformants expressing phosphorus acquisition enzymes were able to grow. The results are summarized in Table 2. The pNUC-1A and pBJ004 plasmids transformed the *nuc-1* mutant equally well. However, the transformation efficiency of the *nuc-2* mutant with pNUC-1A was about three times lower than that with same amount of pBJ004. The same magnitude of reduction in transformation efficiency with pNUC-1A was observed when a *nuc-1*; *nuc-2* double-mutant strain was transformed. In agreement with the hypothesis, a host with mutations in the *nuc-1*, *nuc-2*, and *preg* genes showed the same transformation efficiency with pBJ004 as with pNUC-1A.

Regardless of the genetic background, pBJ004-transformed strains made discrete colonies with sharp outlines (i.e., wild-type colonies). By contrast, pNUC-1A-transformed strains showed two distinct types of transformants, with the relative frequencies depending on the host and the amount of DNA used in the transformation. With a *nuc-2* or *nuc-1*; *nuc-2* host, almost all colonies arising from transformation with pNUC-1A were translucent with indistinct borders, which is diagnostic of low-level expression of the

phosphorus acquisition enzymes. As expected, these transformants grew more slowly than the wild type when they were transferred to low-phosphate liquid medium, and many of them conidiated poorly. However, the *nuc-1* and *nuc-1; nuc-2; preg*^c strains transformed with pNUC-1A formed mostly colonies of wild-type vigor and morphology. As the amount of transforming pNUC-1A DNA decreased, the transformation efficiencies with the *nuc-2* and *nuc-1; nuc-2* hosts dropped sharply, and the proportion of vigorous, sharp-edged colonies decreased (data not shown).

Expression of repressible alkaline phosphatase in transformants. As an arbitrary measure of activity of the NUC-1 protein in transformants, we scored repressible alkaline phosphatase activity in transformant colonies by staining or by growing individual cultures and assaying enzyme activity in crude extracts. The level of this enzyme was derepressed more than 1,000-fold by phosphate starvation (Table 3). The *nuc-1* and *nuc-1; nuc-2; preg*^c strains transformed by pNUC-1A and grown under low-phosphate conditions showed various levels of alkaline phosphatase activity ranging from near that of the untransformed *nuc-1* mutant to that of wild type (Table 3 and data not shown). Transformants of the *nuc-1; nuc-2; preg*^c mutant expressed repressible alkaline phosphatase even on high phosphate, which was expected since the *preg* gene is not functional in these transformants.

To estimate the number of functional *nuc-1*⁺ genes in the transformants, we digested genomic DNA samples with *Xba*I and *Eco*RI (Fig. 4) and analyzed the digests by Southern blotting as shown in Fig. 8. We saw a weak correlation between number of copies of the *nuc-1*⁺ gene and the level of repressible alkaline phosphatase in *nuc-1* or *nuc-1; nuc-2; preg*(con) transformants. Even among transformants with single copies of the *nuc-1*⁺ gene, enzyme activity varied significantly, presumably because of differences in expression at different integration sites (data not shown). Most of the transformants that formed translucent colonies with indistinct borders had very low levels of repressible alkaline phosphatase.

The modal number of integrated *nuc-1*⁺ genes in transformed *nuc-2* and *nuc-1; nuc-2* strains was higher than that in transformants of the *nuc-1* and *nuc-1; nuc-2; preg*^c strains (Fig. 8 and data not shown). This was expected on the basis of current models, because *nuc-1* and *nuc-1; nuc-2; preg*^c strains need only a marginal level of NUC-1 protein to activate the synthesis of enzymes involved in phosphorus acquisition, whereas *nuc-2* and *nuc-1; nuc-2* strains need enough NUC-1 protein to titrate out the PREG and/or PGOV protein(s), plus a marginal excess to activate the genes involved in the phosphorus acquisition pathway. Transformants with more copies of the *nuc-1*⁺ gene generally grew more rapidly under low-phosphate conditions and made more repressible alkaline phosphatase, although we observed some exceptions. We had initially expected that *nuc-2* and *nuc-1; nuc-2* strains transformed by pNUC-1A would make repressible alkaline phosphatase constitutively because, according to the existing model, *nuc-2*⁺ was thought to be the sole sensor of the phosphorus level, and therefore activities and amounts of PREG and PGOV proteins should be constant in a *nuc-2* mutant regardless of phosphate concentrations. Surprisingly, the synthesis of repressible alkaline phosphatase in *nuc-2* and *nuc-1; nuc-2* transformants was normally repressible (Table 3). This result indicates that activities or amounts of either the NUC-1 protein or the negative regulatory factor(s) (the PREG and/or

PGOV protein(s)) change as a function of phosphate concentrations.

DISCUSSION

Recent work on several regulatory systems in *N. crassa* has yielded information on various aspects of the mechanism of gene control at the molecular level (8–10). In this report, we present the cloning and molecular analysis of the positive regulatory gene, *nuc-1*⁺, of *N. crassa*. The NUC-1 protein is presumed to be a factor that activates the transcription of structural genes for phosphorus acquisition. Studies of transcriptional factors in eucaryotes have revealed that these proteins are generally composed of several functional domains (13, 25); included are (i) domain(s) required for transport into the nucleus, (ii) the DNA-binding domain, and (iii) transcriptional activator domain(s). Transcriptional activation depends on regions of 30 to 100 amino acids that are usually separable from the DNA-binding domain. Three apparently unrelated structural motifs have been inferred to have activator function. According to deduced amino acid sequences, the NUC-1 protein seems to have all three potential activator motifs: an acidic domain, a glutamine-rich domain, and a proline-rich domain. An acidic domain of 30 amino acids (residues 22 to 51) at the amino terminus of the NUC-1 protein has a net negative charge of −10; about one-half of the amino acids in the middle of this region are distributed in such a way that they could form an amphipathic α -helix. The two most potent activator domains of the transcription factor Sp1 contain about 25% glutamine residues and very few charged amino acids (4). In the NUC-1 protein, 35 of the 58 glutamine residues are contained in a 120-amino-acid stretch (residues 101 to 220), and charged amino acids are underrepresented in this region. A glutamine-rich domain also occurs in a number of other transcriptional factors, although its function as a transcriptional activator has not been demonstrated (25). A proline-rich domain of the transcription-replication factor CTF/NF1 activates transcription when linked to various DNA-binding domains (22). Proline-rich domains have also been observed in many other transcription factors (25). A 123-amino-acid region (residues 434 to 556) of the NUC-1 protein contains 31 proline residues. It remains to be seen whether these three potential activator domains of the NUC-1 protein actually function as transcriptional activators.

In *S. cerevisiae*, at least five regulatory genes control expression of the phosphorus acquisition enzymes (29, 38). Expression of the acid phosphatase structural gene *PHO5* is controlled by two positive transcription factors, *PHO2* and *PHO4*, whereas only *PHO4* is necessary for the derepression of the repressible alkaline phosphatase structural gene *PHO8*. In *N. crassa*, the NUC-1 protein seems to have functions corresponding to those of both the *PHO2* and *PHO4* proteins in controlling phosphorus acquisition, although we cannot rule out the possibility that some auxiliary transcriptional factor is also involved. Some of the properties of the inferred NUC-1 protein have interesting characteristics in relation to the *PHO2* and *PHO4* proteins. First, the NUC-1 protein has three potential activator motifs, whereas the yeast *PHO2* protein has two potential activator domains. One of the putative *PHO2* domains is a stretch of 18 glutamines interrupted by 11 amino acids, while the other has a high density of acidic amino acids (33, 37). A moderately proline-rich domain and a strongly acidic domain are present in the yeast *PHO4* protein (16, 37). It is likely that different types of activator domains interact with different

TCTAGACCTTTTCTCTGCCTTCTTTACACGCTGCGAGGGGACGTTGGGACGCCGCCCGGGGCTGGGAACCGCTGAGACTCTTCAATTGTAAAGACCG 100
AGTCCACATCGCTACATACTTCAATCCAACCGACAGAAAAGAGAAAGCATCGACGAATCCAGTATCTCTATATTCCTGTCATACGCGTCCATTCAAAGG 200
GTCTCTGGCGAGCTACCAATCAGTAGCGCACATCTGGCGAGCTACCAATCAGTAGCGCACATCTGACAACTCTTCGCCGGGTCGCGCAGACCTGATCGC 300
GAGCCCGAACAGTTTCGTCGTCGGAATAATCTCGGGTCTCGTAGGTACCGTTAAATTACGGAGTCACCATAGACGGGACTGGCCCGGCCAAGACTCG 400
AAGCCCGTCGAGGTTATCCGGCCCTTTAAGCGGTATCCGTATCGCATTGTGATCAATAATCAAGTGCAAGGCGACTTGCAGAGCCGCTGAGAAAGT 500
CCTAAATCAAAGCCGAGTCCCCAACCGCCTGGCCTTGGCCTCAACGTCTTCGACTCAACTGATACCCAACAGCCGGTGTCAAAATTACTGGACTGCCT 600
CCGCCCAAAGCGCCGTACCCGTTTCCGTACCCGCACCCGCACCCGCACCCGCACCCGCACCCGTGCCCGCACCCGTGCCCGCACCCGTGCCGGA 700
CAGCCGGCCGTCCCCGAACCATCTTCAATCCGACTTTGGACAAAGCCTCCCCAGGAGGGCCTAGCCGGCGGGCGTGATGAACTCTCATCGTGGAGTGC 800
M N S S S W S A
CCCGGACCACAGTCACCACCGGCCCTACACACCGCAGCCGATGATGACTTCCACCAGTACTTGGATATAAACGACATGGGCGATCTATCTGACGCCCTTG 900
P D H S H H P A L H T A A D D D F H Q Y L D I N D M G D L S D A L
GACTTTGACTTTCGCGACTTTGGGGCTGATCACCATACTGCCCCCATGCTGGCCGCGATGCCGCGACCATCTGCTACATCCGTCCGGGGGAGAGCATC 1000
D F D F R D F G A D H H T A P H A G R H A A D H L L H P S G G E H L
TGGACACCCCATGACTGGTACCGACATGTCCATGATCTATCGCCGTAGACCATGCCATGCTCCGACATGGGGTGCAACAGCAACAGCACCACAGCA 1100
D T P M T G T D M S M I L S P V D H A M L R H G V Q Q Q Q H H Q Q
ACAGCAACACCAGCAACATCAGATGCCACCATACACGACTGCGCCATATCAAAACGCCCGACGGCCTTGATACAGCCATCTACACCTCAGAGGCC 1200
Q Q H Q Q H Q M P T I T T T A P Y Q N A P T A L I Q P S T P S E A
GTCGTCAACACCATCGATGCCAAATCCAGTTCTCCAGCAGCAGAAGTTGCATGCTCAGCACCAGCAACTGCAAGAGCAACAGGCCGCTTCTTCGCTT 1300
V V N T I D A Q I Q F L Q Q Q K L H A Q H Q O L Q E Q Q A A F F A S
CTCAACAGAACCACATAGTTCTCTACACCTCAGAGCCTGGAACCTACTGCTGGATCTAGTCAGAATTACTATGCCAGTCGACACTATCTGATCAACA 1400
Q Q N H I V P P T P Q S L E L T A G S S Q N Y Y A Q S T L S D Q H
CCACTCCGGGCCACAGAAACAGCAGCAGCCGAACAGGCCATCGACTACAGATATACAAGGATCAAGACCAGCATGATGTAGGTCTACAAACGTCTGCT 1500
H S G P Q K Q Q Q P Q Q A I D Y R Y T R I K D Q H D V G L Q T S A
CCGTCCAAGTCAAGAAAAAATATCACTAACCAACCATGGCAGATGTCTTCACCCCGTTGGTATCACCGGCTGTGACCCCGCTGGAGACCCATTTTC 1600
P S K S R K K N I T N Q P W Q M S F T P L V S P A V T P L E T H F P
CCATCGATACTCCGTTTGCCGTCCTTGGAGCGTACTTCAGCCCCCTTACCTCGCCAGCCTTGACCGCGCAAAATGACGCTTTGGGTATCATTGACCAGAG 1700
I D T P F A V P G A Y F S P L T S P A L H A Q N D A L G I I D Q R
GCTCGGGATGATGAGCGGGAGCTCGCCAAGGAGATGGAACCTCGAACCACCTGCCATGTCTCAAGCGAGCGTTAGCCAGGCGATTGGCTAGGAAGACG 1800
L G M M S G S S P R E M E L E P P A M S Q A S V S P G D L A R K T
CGCAAGAACGAGTGAAGGCCAGGGCAAAGAGCGGAGTGGCATCAAACAGTCTCCCATCTCGAAGCCGATACGTAGAAAGACGGCCACCACCCGATGC 1900
R K N A V K A R A K S G S G I K Q S P I S K P I R R K T A T T P M L
TCAACCCCAAGCTTTGAACCAACTGGTTGAGAATGCAGACCAAGTCAAGAACGCCAACAACTCTTACGCCGCTAATACCGACCTCGTCTTCGTCCAC 2000
N P Q A L N Q L V E N A A P S Q E R Q Q P L T P L I P T S S S S T
AGCTGGTGTAAGTACTGACTCTGAGAATGGGTCCATCTCCCCGAAAATCTCAACGACGTTGTCTTGCCGGTTGAGATGCCTCCGCCACCACTTCCGAAACCT 2100
A G V T D S E N G S I S P E N L N D V V L P V E M P P P P L P K P
CGCTCCGCCAAACCGTCCCCTTTTCTGGCACCTCAAGCGAGCGGTAGTCTGTGCTTATTAATCTCCAGCCGGGGCGGCCTGGAATTGCCTCTCTGCAA 2200
R S A K P S P F L A P Q A S G S A V P I N L Q P G R P G I A S P A T
CACCAGCATCACTGATGAACTAAGCTCACCAGGAATCGCAACCCCTTCAGTGGTAGGAACCGGCTCTCATGACCCAATGGATCCCGACCACATTGAGAA 2300
P A S L M K L S S P S N R N P S V V G T G S H D P M D P D H I E N
CTTGTAGCTCCCGACTCCATCAACTTCTCGTCTGCACCAAAACCGCCCTATAATTACCACTCTTGAACACCGGCTCTCGATCTCTCAAAAGGCC 2400
F E L P D S I N F S S A P K P A P I I T T L G T P A L D P L Q K A

GCCGCGCTCTACAAACGCCAGGCCTCCACACACCTTCTCCTGCGGTGGCCAAGCCTCTGGCACTTCCTTCGCCGCTCTTCCAGTCCACAACCTGA 2500
 A A P L O T P G L P P P S P A V A K P L A L P S A A L S S P Q L K
 AGCCCGATTCCGCTCATAGCCTGAAACGGACACCCAGCTTGACCCATGGGAAGAAGCAGCAAAAAACGTGCAAGCGTGACCTCCATCCAGATGTCCCC 2600
 P D S A H S L K R T P Q L A P M G R S S K K R A S V T S I Q M S P
 GGCCTTCGACCCAAGATCTCGCCAGCATCAAGCCTCTGCTACCAGGAGGTTCTGCGGGTCCGAAGACGAGCATCGATCTTGCTCGCCACCAATCC 2700
 A L R P K I S P S I K P L L P G G S A G A E D A A S I L L A T K S
 AACTACCAACGCATCCTCGAGGGCAACACAGTTCCCGCGGTTTCGTATCCAGCGAGCTTCTACGAATCTCACATCGAAGCGGACGTCGCACAAGATCG 2800
 N Y Q R I L E G N T V P G V S Y P S E L S T N L T S K R T S H K I A
 CGGAGCAAGGCAGGAGGAACAGAATCAACTCCGCGCTGCAGGAGATCGCCACGCTTCTTCCCAAGCTCCTGCGAAGGAGGGGGAGATGGTGATGGAGA 2900
 E Q G R R N R I N S A L Q E I A T L L P K A P A K E G G D G D G D
 CGGTCTAGTAGTAGCGGTGGGGCGGTGGCAGCGGGGTGCTGACAGGGAAGATAAGAGGGAGAAGGATAAGGACAAGGCTGGAGGGGGGATACCCAAT 3000
 G H S S S G G G G G S G G A D R E D K R E K D K D K A G G G I P N
 AGCAAGGCGAGCACGGTGGAGATGGCAATTGAGTATATCAAGCAGCTGCAGAAGGAGGTGGCTGACGCGAACAAGAGGGCGGAAGAGGCAGAGGAAGT 3100
 S K A S T V E M A I E Y I K Q L Q K E V A D A N K R A E E A E R K L
 TGGTGGAGATGAAGATGACGGGAGGCGCCGACGGGTCCGGGTATCAGTTGGTGATGCTGGCGATTGGGTACTCCAACCACGGAGGCTAATCCGGT 3200
 V E M K M Q G G A A T G S G S S V G D A G D L G T P T T E A N P V
 CGTGGATGAGGACCTGAAGTCTGGCGGTGGTACGCGATGGATGAATAAGATATCAAGATCGGATTGAAGAGCCTCCACAAACGCACGGCGTTCTTCTT 3300
 V D E D L K S G G G D A M D E End
 AACACACGTGTCACTAATCTGGCGTGTACAAACAGGCGAGTCGGGTGTATAAGTGGGCAAAAAGAGAAAGGGTTGGTTGACTTTCGGGTATAATTATGC 3400
 GGGTGTTCAGAGTGGGCGAGATCTTTGCTTTTATCATACAAGGTAAACATCTCCCGCGGTGGATTATGGGGTCAAAATAACATGCAGGCGGTGGTGT 3500
 GGATATACCCCTCCTCTTGAAATAGGAGTACTCAAATAGAAATAATAAGGGAGTCATGATGAACGGGATGATGGCGCTTGACGGGTGAGGTAACACGAA 3600
 ATTTAGGATC

FIG. 5. DNA sequence of the *nuc-1*⁺ gene and its flanking regions. The numbers on the right indicate nucleotide number starting from the *Xba*I site. Deduced amino acid sequence of the NUC-1 protein is shown below the DNA sequence, and the regions rich in glutamine, proline, or the highly acidic amino acids are underlined. The consensus sequence for transcription termination-poly(A)⁺ addition is marked with bold letters. Pyrimidine-rich sequences in the 5' upstream region are underlined.

general transcription factors or different parts of RNA polymerase II to activate transcription. The combinatorial action of different activator domains may also provide a way of regulating gene expression. Yeast may use two transcriptional factors for the expression of the *PHO5* gene simply because neither of them alone can provide the combination of activator domains necessary for full induction of the *PHO5* gene. The combined amino acid sequences of the PHO4 and PHO2 proteins (312 residues and 519 residues, respectively) are roughly equal in length to that of the NUC-1 protein (824 residues).

Since the yeast *PHO2* gene is involved in the regulation of both the adenine and histidine pathways as well as phosphorus acquisition (2), we thought it possible that the *nuc-1*⁺ gene product might participate in other metabolic pathways in *N. crassa*. *Neurospora* amino acid auxotrophs that also have a mutation in the *cpc-1* gene (an analog of the yeast *gcn4* mutant) cannot be derepressed in the synthesis of amino acid biosynthetic enzymes when starved of the required amino acid (3). However, the double mutant *cpc-1*; *nuc-1* did not show any kind of amino acid auxotrophic phenotype of the sort observed in *pho2 gcn4* double mutants in yeasts (2).

Recently, it has been shown that the yeast PHO2 and PHO4 proteins bind specifically to the *PHO5* promoter region (35). It is not yet known which domain(s) of the PHO2 protein is responsible for DNA binding. A homeo domain of the yeast PHO2 protein could have DNA-binding activity.

Ogawa and Oshima (28) showed that the carboxy-terminal 85 amino acids (residues 228 to the end) of the PHO4 protein are necessary for DNA binding. This region has significant homology with a number of other DNA-binding proteins (26). It has been proposed that these homologous regions have the potential to form two amphipathic helices separated by an intervening loop (26). Within this group of proteins, the amino acid residues are highly conserved in their helical regions and the basic motifs preceding helix I. The intervening loop regions are extremely variable in their lengths and amino acid compositions (26). This A-HLH motif is crucial for DNA binding by the PHO4 protein (28). The NUC-1 protein shows strong homology with an A-HLH motif of the PHO4 protein (Fig. 6B). The loop in the NUC-1 protein is much larger than that in the PHO4 protein. The presence of the potential A-HLH motif suggests that the DNA-binding domain is located at the carboxy-terminal region of the NUC-1 protein. Evidence supporting this supposition comes from the analysis of mutants; about one-half of the characterized *nuc-1* mutations (nine alleles) have amino acid changes in this A-HLH motif, and all the rest are frameshift mutations (S. Kang and R. L. Metzenberg, unpublished data).

It has been observed in several regulatory systems of *N. crassa* that positive regulatory genes are autogenously regulated (30, 31). Northern analysis showing that the level of *nuc-1*⁺ mRNA is constant rules out the possibility of autogenous regulation in the expression of the *nuc-1*⁺ gene.

FIG. 6. Amino acid sequence alignment of regions of similarity between the *N. crassa* NUC-1 and *S. cerevisiae* PHO4 proteins based on programs from the University of Wisconsin Genetics Computer Group (6). Identical amino acids are denoted by a slash, and conservative changes are marked by dots. (A) Comparison of the amino acid sequences of the putative regions interacting with the negative regulatory factor. (B) Regions of NUC-1 and PHO4 carrying the A-HLH motif.

Based on genetic evidence, a dosage titration model had been proposed some years ago to explain the interactions among regulatory genes of the phosphorus acquisition pathway in *N. crassa* (24). According to this model, the amount of the *nuc-1*⁺ product is constant and the negative regulatory factors, *preg*⁺ and *pgov*⁺, act by inactivating or complexing *nuc-1*⁺ product unless they are counteracted by the *nuc-2*⁺ product. In the present work, the finding of constitutive expression of *nuc-1*⁺ mRNA and complementation of the *nuc-2* mutation by the introduction of multiple copies of the *nuc-1*⁺ gene support this model. Although we cannot completely rule out the possibility of translational control by negative regulatory factors, two points of evidence argue against such control. We were not able to find any unusual features on 5' untranslated sequences, and most importantly, all *nuc-1*^c mutations map in the coding sequences of the *nuc-1*⁺ gene (Kang and Metzenberg, unpublished data). This implies physical interactions between NUC-1 and the negative regulatory factor(s). It has been proposed that in yeasts, the PHO80 protein (the negative regulatory factor)

interacts with the PHO4 protein and prevents the transcriptional activation of the structural genes by PHO4 under high-phosphate conditions (38). Forty amino acid residues (163 to 202) of the PHO4 protein are necessary for the interaction with the PHO80 protein (28). This region of the PHO4 protein shows strong homology with the NUC-1 protein (Fig. 6A), implying that the NUC-1 protein is also likely to interact with its negative regulatory factor(s) through this well-conserved region.

We believe that complementation of the *nuc-2* mutation by exogenous copies of the *nuc-1*⁺ gene results from the overexpression of the NUC-1 protein, although we do not yet know levels of either the *nuc-1*⁺ mRNA or NUC-1 protein in transformants. The presence of multiple copies of the *nuc-1*⁺ sequences in transformants of the *nuc-2* and *nuc-1*; *nuc-2* hosts strongly argues that only those transformants with multiple copies of the *nuc-1*⁺ gene could produce enough NUC-1 protein to titrate out a negative regulatory factor(s) and so be able to grow under selective conditions. This would explain why, when the *nuc-2* and *nuc-1*; *nuc-2* mutant strains were used as the hosts, the transformation efficiencies decreased sharply as the amount of pNUC-1A used in the transformations decreased. Those transformants with too few copies of the *nuc-1*⁺ gene probably could not grow because a negative regulatory factor(s) titrated out all the NUC-1 protein. However, in *nuc-1* and *nuc-1*; *nuc-2*; *preg*^c hosts, one exogenous copy of functional *nuc-1*⁺ gene was sufficient for growth under selective conditions since negative regulatory factors were not functional (because they were counteracted by the *nuc-2*⁺ product in a *nuc-1* strain, or because the *preg* gene had been mutated).

FIG. 7. Northern analysis of *nuc-1*⁺ gene expression in wild-type and *nuc-1*^c strains. (A) RNA from wild-type cells grown in high-phosphate (lanes 1) or low-phosphate (lanes 2) Fries medium was probed with labeled 2.2-kb *Sall*-*Eco*RI fragment from the *nuc-1*⁺ gene and 2.7-kb *Bam*HI fragment from the *am*⁺ gene (15). The size of the *am*⁺ transcript is 1.7 kb. The two panels differ only by the exposure time. The chemical amount of the *am* probe was ten times lower than that of the *nuc-1* probe. The specific activities of the two probes were about the same. (B) RNA from wild-type cells (lane 1) or *nuc-1*^c (lane 2) grown in Fries high-phosphate medium was hybridized with the *nuc-1* probe. The blot was washed and then rehybridized with the *am* probe to compare the amounts of RNA in the two lanes.

TABLE 2. Comparison of transformation efficiencies

Host for transformation	No. of transformants ^a with:	
	pNUC-1A	pBJ004
<i>nuc-1</i>	590	640
<i>nuc-2</i>	220	600
<i>nuc-1</i> ; <i>nuc-2</i>	200	610
<i>nuc-1</i> ; <i>nuc-2</i> ; <i>preg</i> ^c	610	630

^a Spheroplasts (1.3×10^7) were transformed with 1.5 μ g of pNUC-1A or pBJ004 DNA plus 1 μ g of pSV50, and benomyl-resistant transformants capable of growing on low-phosphate medium at high pH were selected. Sizes of the pNUC-1A and pBJ004 plasmids are roughly equal.

TABLE 3. Levels of repressible alkaline phosphatase in transformants

Strain ^a	Transformation host	Intensity of staining ^b		Sp act ^c (nmol/min/mg of protein)	
		High P _i	Low P _i	High P _i	Low P _i
Wild type	Untransformed	—	++++	3	5,324
<i>nuc-1</i>		—	—	2	13
<i>nuc-1</i> ^c		+++	++++	1,961	2,688
2	<i>nuc-2</i>	—	±	ND ^d	ND
3		—	±	4	45
4		—	±	ND	ND
5		—	—	7	25
6		—	+	ND	ND
7	<i>nuc-1; nuc-2</i>	—	++	5	600
8		—	±	ND	ND
9		—	±	2	62
10		—	±	ND	ND
11		—	+	ND	ND
12	<i>nuc-1; nuc-2; preg</i> ^c	++	+++	703	1,204
13		—	±	ND	ND
14		+++	++++	745	2,642
16		+++	++++	1,105	2,835
2-B ^e	<i>nuc-2</i>	—	++	3	671

^a Strain numbers correspond to those in Fig. 8.^b Strains were individually spotted onto high- or low-phosphate Fries medium plates. Colonies were stained by the method of Toh-E and Ishikawa (34). The symbols used range from — (no staining; no repressible alkaline phosphatase) to ++++ (very strong staining; high level of repressible alkaline phosphatase).^c Assays were done on crude extracts from mycelia grown in high- or low-phosphate Fries medium.^d ND, Not determined.^e The *nuc-2* mutant transformed by pNUC-1B. It has more than 10 copies of the *nuc-1*⁺ gene.

Repression of the synthesis of repressible alkaline phosphatase under high-phosphate conditions in *nuc-2* and *nuc-1*; *nuc-2* transformants by pNUC-1A suggests that the regulatory mechanisms of the phosphorus acquisition pathway must be more complex than was previously thought. Since the level of the *nuc-1*⁺ mRNA, and probably of the NUC-1

protein, is constant, such repression most likely results from increased synthesis or enhanced activity of the negative regulatory factor(s) under high-phosphate conditions. This suggests the presence of a novel mechanism controlling phosphorus acquisition by the cell. Both the *preg*⁺ and *pgov*⁺ genes have recently been cloned (Kang and Metzen-

FIG. 8. Southern analysis of genomic DNA isolated from cells transformed by pNUC-1A. Genomic DNA was digested with *Eco*RI and *Xba*I to estimate *nuc-1*⁺ gene copy number. The Southern blot was probed with labeled 1.8-kb *Sal*I-*Eco*RV fragment (Fig. 4). The resident *nuc-1* gene was present on a 7.9-kb band (lane 1), whereas inserted copies of the *nuc-1*⁺ gene appeared as a 3.7-kb band when the integration into the chromosome did not disrupt the sequences between *Eco*RI and *Xba*I. The size difference resulted from the presence of an *Eco*RI site present in the multiple cloning site in the lambda cloning vector. Both bands are marked by arrows. The genotype of the host strain is shown above the isolate numbers indicating each lane. The presence of multiple faint bands in most of the lanes with transformant DNA was not the result of partial digestion of genomic DNA, because reprobing of the same blot with labeled DNA carrying the *am*⁺ gene showed that all digestions were complete (data not shown). wt, Wild type.

berg, unpublished data), so it is now possible to test whether synthesis of these negative factors changes as a function of phosphate concentration and to relate their expression to the presence or absence of a functional *nuc-2⁺* gene.

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LITERATURE CITED

- Akins, R. A., and A. M. Lambowitz. 1985. General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. *Mol. Cell. Biol.* 5:2272-2278.
- Arndt, K. T., C. Styles, and G. R. Fink. 1987. Multiple global regulators control *HIS4* transcription in yeast. *Science* 237:874-880.
- Barthelmess, I. B. 1982. Mutants affecting amino acid cross-pathway control in *Neurospora crassa*. *Genet. Res.* 39:169-185.
- Courey, A., and R. Tjian. 1988. Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55:887-898.
- Davis, R. H., and F. J. DeSerres. 1970. Genetic and microbiological techniques for *Neurospora crassa*. *Methods Enzymol.* 17A:79-143.
- Devereux, J. R., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387-395.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Fu, Y.-H., and G. A. Marzluf. 1987. Characterization of *nit-2*, the major nitrogen regulatory gene of *Neurospora crassa*. *Mol. Cell. Biol.* 7:1691-1696.
- Fu, Y.-H., J. V. Palletta, D. G. Mannix, and G. A. Marzluf. 1989. *cys-3*, the positive-acting sulfur regulatory gene of *Neurospora crassa*, encodes a protein with a putative leucine zipper DNA-binding element. *Mol. Cell. Biol.* 9:1120-1127.
- Geever, R. F., L. Huiet, J. A. Baum, B. M. Tyler, V. B. Patel, B. J. Rutledge, M. E. Case, and N. H. Giles. 1989. DNA sequence, organization and regulation of the *qa* gene cluster of *Neurospora crassa*. *J. Mol. Biol.* 207:15-34.
- Gurr, S. J., S. E. Unkles, and J. R. Kinghorn. 1987. The structure and organization of nuclear genes of filamentous fungi, p. 93-139. In J. R. Kinghorn (ed.), *Gene structure in eukaryotic microbes*. IRL Press, Oxford.
- Hasunuma, K. 1973. Repressible extracellular nucleases in *Neurospora crassa*. *Biochim. Biophys. Acta* 319:288-293.
- Johnston, M. 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. *Microbiol. Rev.* 51:458-476.
- Kammerer, B., A. Guyonvarch, and J. C. Hubert. 1984. Yeast regulatory gene *PPRI*. I. Nucleotide sequence, restriction map, and codon usage. *J. Mol. Biol.* 180:239-250.
- Kinsey, J. A., and J. A. Rambosek. 1984. Transformation of *Neurospora crassa* with the cloned *am* (glutamate dehydrogenase) gene. *Mol. Cell. Biol.* 4:117-122.
- Legrain, M., M. D. Wilde, and F. Higler. 1986. Isolation, physical characterization and expression analysis of the *Saccharomyces cerevisiae* positive regulatory gene *PHO4*. *Nucleic Acids Res.* 14:3059-3071.
- Lehman, J. F., M. K. Gleason, S. K. Ahlgren, and R. L. Metzenberg. 1973. Regulation of phosphate metabolism in *Neurospora crassa*. Characterization of regulatory mutants. *Genetics* 75:61-73.
- Lowendorf, H. S., and C. W. Slayman. 1975. Genetic regulation of phosphate transport system II in *Neurospora*. *Biochim. Biophys. Acta* 413:95-103.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mann, B., R. A. Akins, A. M. Lambowitz, and R. L. Metzenberg. 1988. The structural gene for a phosphorus-repressible phosphate permease in *Neurospora crassa* can complement a mutation in positive regulatory gene *nuc-1*. *Mol. Cell. Biol.* 8:1376-1379.
- Mann, B., B. J. Bowman, J. Grotelueschen, and R. L. Metzenberg. 1989. Nucleotide sequence of *pho-4⁺*, encoding a phosphate-repressible phosphate permease of *Neurospora crassa*. *Gene* 83:281-289.
- Mermod, N., E. A. O'Neill, T. J. Kelly, and R. Tjian. 1989. The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. *Cell* 58:741-753.
- Metzenberg, R. L., and W. Chia. 1979. Genetic control of phosphorus assimilation in *Neurospora crassa*: dose-dependent dominance and recessiveness in constitutive mutants. *Genetics* 93:625-643.
- Metzenberg, R. L., and R. E. Nelson. 1977. Genetic control of phosphorus metabolism in *Neurospora*, p. 253-268. In G. Wilcox, J. Abelson, and C. F. Fox (ed.), *ICN-UCLA Conference Proceedings on Molecular Approaches to Eukaryotic Genetic Systems*, vol. 8. Academic Press, Inc., Orlando, Fla.
- Mitchell, P. J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245:371-378.
- Murre, C., P. S. McCaw, and D. Baltimore. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell* 56:777-783.
- Nyc, J. F., R. J. Kadner, and B. J. Crocken. 1966. A repressible alkaline phosphatase in *Neurospora crassa*. *J. Biol. Chem.* 241:1468-1472.
- Ogawa, N., and Y. Oshima. 1990. Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10:2224-2236.
- Oshima, Y. 1982. Regulatory circuits for gene expression: the metabolism of galactose and phosphate, p. 159-180. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), *The molecular biology of the yeast Saccharomyces: metabolism and gene expression*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Palletta, V. B., R. A. Akins, A. M. Lambowitz, and G. A. Marzluf. 1987. Molecular cloning and characterization of the *cys-3* regulatory gene of *Neurospora crassa*. *Mol. Cell. Biol.* 7:2506-2511.
- Patel, V. B., and N. H. Giles. 1985. Autogenous regulation of the positive regulatory *qa-1F* gene in *Neurospora crassa*. *Mol. Cell. Biol.* 5:3593-3599.
- Perkins, D. D., and E. G. Barry. 1977. The cytogenetics of *Neurospora*. *Adv. Genet.* 19:133-285.
- Sengstag, C., and A. Hinnen. 1987. The sequence of the *Saccharomyces cerevisiae* gene *PHO2* codes for a regulatory protein with unusual amino acid composition. *Nucleic Acids Res.* 15:233-246.
- Toh-E, A., and T. Ishikawa. 1971. Genetic control of synthesis of repressible phosphatases in *Neurospora crassa*. *Genetics* 69:339-351.
- Vogel, K., W. Hörz, and A. Hinnen. 1989. The two positively acting regulatory proteins PHO2 and PHO4 physically interact with *PHO5* upstream activation regions. *Mol. Cell. Biol.* 9:2050-2057.
- Vollmer, S. J., and C. Yanofsky. 1986. Efficient cloning of genes of *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* 83:4869-4873.
- Yoshida, K., Z. Kuromitsu, N. Ogawa, and Y. Oshima. 1989. Mode of expression of the positive regulatory genes *PHO2* and *PHO4* of the phosphatase regulon in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 217:31-39.
- Yoshida, K., N. Ogawa, and Y. Oshima. 1989. Function of the *PHO* regulatory genes for repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 217:40-46.