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A splice variant of the *Neurospora crassa hex-1* transcript, which encodes the major protein of the Woronin body, is modulated by extracellular phosphate and pH changes

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ABSTRACT

The Woronin body, a septal pore-associated organelle specific to filamentous ascomycetes, is crucial for preventing cytoplasmic bleeding after hyphal injury. In this study, we show that T1*hex-1* transcript and a variant splicing T2*hex-1* transcript are up-regulated at alkaline pH. We also show that both *hex-1* transcripts are overexpressed in the *preg*^{c}, *nuc-1*^{RIP}, and *pacC*^{ko} mutant strains of *Neurospora crassa* grown under conditions of phosphate shortage at alkaline pH, suggesting that *hex-1* transcription may be coregulated by these genes. In addition, we present evidence that *N. crassa* PacC also has metabolic functions at acidic pH.

1. Introduction

Maintaining the supply of phosphorus, a crucial nutrient in energy transduction, genetic information, photosynthesis, and cell growth, is a key step in the survival of all living organisms. In *Neurospora crassa*, the availability of phosphate (Pi) is sensed by the *nuc-2* gene, the transcription of which is regulated in response to extracellular Pi changes [1,2]. NUC-2, an ankyrin repeat protein, transmits a metabolic signal downstream of the hierarchical regulatory pathway, thereby inhibiting the functioning of the PREG-PGOV complex under conditions of Pi shortage. This allows the translocation of the transcriptional regulator NUC-1 into the nucleus [3]. PREG and PGOV are a cyclin-like and a mitogen-activated protein kinase, respectively, whereas NUC-1 is a member of the basic helix–loop–helix (bHLH) family of proteins [4,5], which includes a large number of transcriptional regulators [6]. This dosage titration and hierarchical regulatory network activates the de-repression of nucleases, phosphatases, and transporters necessary for fulfilling the cell's Pi requirements [1,7–9]. In this study, we uncovered novel genes modulated by the transcription factor NUC-1. These genes are involved in various cellular processes such as protein biosynthesis, cell cycle and cell rescue, and defense and virulence. One of these (*hex-1*) encodes the major component of the Woronin body. Interestingly, variant splicing of the HEX-1 mRNA is modulated by extracellular Pi and pH changes, which increase the complexity of the molecular signaling process in response to Pi shortage in *N. crassa*.

2. Materials and methods

2.1. N. crassa strains, culture conditions, and RNA extraction

Wild-type *N. crassa* St.L.74-OR23-1VA (FGSC No 2489) and the strains carrying loss-of-function mutations in the *preg* (*preg*^{C}, FGSC No. 2532) and *pacC* (*pacC*^{ko}, FGSC No. 11397) genes were obtained from the Fungal Genetic Stock Center, University of Missouri, Kansas City, Missouri. The *pacC* gene encodes a Zn-finger transcription factor, which is a member of a conserved signal transduction pathway involved in the regulation of gene expression by pH [10]. The

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nuc-1^{RIP} strain was generated by the repeat-induced point (RIP) mutation procedure [11], and the mutant strain was selected by its inability to utilize RNA or DNA as the sole phosphorus source at pH 8.0. RIP mutations were identified by DNA sequencing of the *nuc-1*$^{+}$ and *nuc-1*^{RIP} alleles (Supplementary Figs. S1 and S2 and Supplementary methods).

2.2. Suppression subtractive hybridization (SSH) and screening of subtracted cDNA clones

SSH was performed on wild-type and *nuc-1*^{RIP} strains grown in low-Pi medium at pH 7.8 by using the PCR-Select^{TM} cDNA Subtraction Kit (Clontech Laboratories). For screening down- and up-regulated clones in the mutant strain, forward or reverse subtractions were performed, respectively, using the *nuc-1*^{RIP} strain as the driver or tester [12]. The PCR products of both the forward- and reverse-subtracted libraries were cloned into pGEM-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* Mos-Blue-competent cells. The cDNAs corresponding to differentially expressed sequences in the *nuc-1*^{RIP} strain were amplified by PCR, and the products were screened by reverse Northern hybridization, as described earlier [8].

2.3. DNA sequencing and validation of differentially expressed genes

The plasmids from arrayed clones that visually exhibited positive differential expression were purified and sequenced using the M13 forward primer, and the cDNA sequences were subjected to computational searches against the GenBank database [13]. The functional catalog FunCat created by MIPS was mined to associate functional categories with the identified *N. crassa* genes (http://mips.gsf.de/projects/fungi/neurospora.html).

For validating differential gene expression by Northern blotting, the subtracted cDNA clones were amplified by PCR, radioactively labeled with [α-^{32}P]dCTP, purified, and used as probes [8]. Differential expression of the *hex-1* gene was also validated by quantitative RT-PCR (qRT-PCR) amplification using the following oligonucleotides (5′–3′): CATCCGCATCTCCACCTC (hexF), GAGGACACGGTACTGCTTGA (hexR), CCTCACTCCCACCACTCTTC (T2 hexF), and GAACGATCAACCGGACCAAC (T2hexR). Melting curve analysis was performed using the Dissociation Curves Software version 1.0 (Applied Biosystems) to exclude primer dimers and unspecific PCR products. Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method [14] with *N. crassa* β-actin (NCU04173.3) as the endogenous reference gene amplified from the samples.

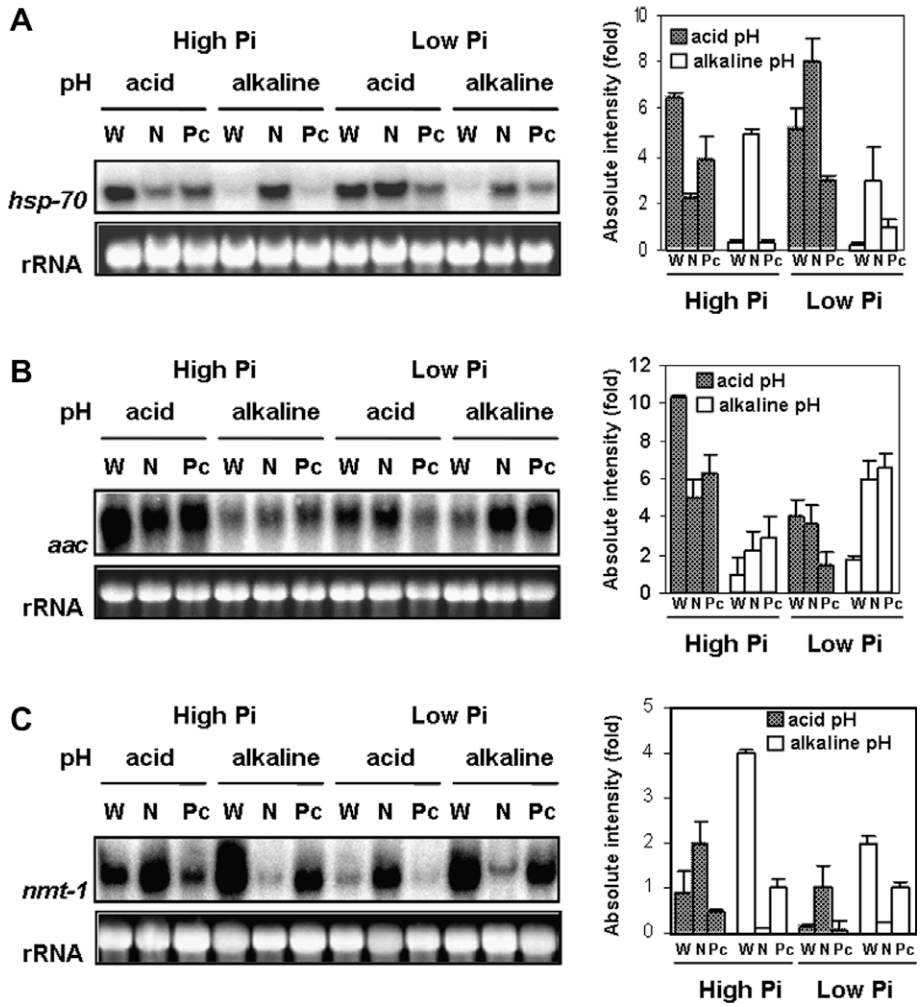

Fig. 1. Northern blot analysis of the *hsp-70* (A), *aac* (B), and *nmt-1* (C) transcripts in wild-type (W), *nuc-1*^{RIP} (N), and *pacC*^{ko} (Pc) strains of *N. crassa*. The conidia from each strain (approximately 10^6 cells mL^{-1}) were grown for 5 h at 30°C with shaking (200 rpm) in low- and high-Pi media (0.1 and 10 mM Pi, respectively). Both media were supplemented with 44 mM sucrose as the carbon source, and the pH was adjusted to 5.4 (buffered with 50 mM sodium citrate) or 7.8 (buffered with 50 mM Tris–HCl). Total RNA was extracted from the frozen ground mycelium (Supplementary material). The ethidium bromide-stained rRNA band is shown for comparison of the quantities of loaded RNA. The bars show the fold expression relative to the intensities of the Northern blots determined by densitometric analysis, and these represent the average values (±S.D.) obtained from three independent experiments.

3. Results and discussion

Following differential screening of the clones generated by both forward- and reverse-subtracted probes, 177 and 136 candidate clones were identified as being up- and down-regulated in the *nuc-1*^{RIP} strain, respectively, and these were isolated and sequenced. Functional classification of these genes led to the identification of putative proteins involved in diverse cellular processes. A high redundancy was also observed for some transcripts such as those encoding hexagonal peroxisome (HEX1) and thiamine biosynthesis (NMT-1) proteins (Supplementary Table S1). Upregulation of the *N. crassa* genes encoding HEX-1, HSP-70, and AAC (ADP/ATP carrier) and downregulation of the gene encoding NMT-1 were confirmed by Northern blotting. Furthermore, the expression of these genes was also assayed in the *pacC*^{ko} and *preg*^{C} strains with the purpose of unveiling possible interactions between the pH and Pi regulatory circuits (Figs. 1 and 2).

Heat shock proteins belong to a superfamily of chaperones that promote the folding and stabilization of many proteins in eukaryotic organisms [15]. *N. crassa hsp70* (NCU08693.3), a chaperone gene, was preferentially induced at acidic pH at 30°C, which are optimal culture conditions for fungal growth (Fig. 1A). Interestingly, transcription of the *hsp70* gene was down-regulated in the *pacC*^{ko} strain grown in both low- and high-Pi medium at pH 5.4, whereas it was up-regulated in this mutant strain grown under conditions of Pi shortage at alkaline pH (Fig. 1A). These results suggest that transcription of the *hsp70* gene is stimulated in a *pacC*$^{+}$ background at acidic pH but is inhibited under alkaline culture conditions. Transcription of *hsp70* can be modulated by PacC since its promoter region has two nucleotide consensus sequences for PacC binding (Supplementary Table S1). Moreover, transcription of the *hsp70* gene is up-regulated in the *nuc-1*^{RIP} strain, with the exception of the mutant grown in a high-Pi medium at acidic pH (Fig. 1A). These results suggest that the PREG/PGOV complex functions as a negative modulator of the *hsp70* gene at acidic pH, an effect not observed under alkaline growth conditions. Indeed, culture of the *nuc-1*^{RIP} strain at acidic pH under conditions of Pi shortage is highly stressful for the organism because both the PREG/PGOV complex and *nuc-1* gene are silenced [1,8,16].

The ADP/ATP carriers are gated channels through which ADP enters and ATP exits the mitochondrial matrix. In addition to the translocase activity, ADP/ATP carriers are components of the mito-

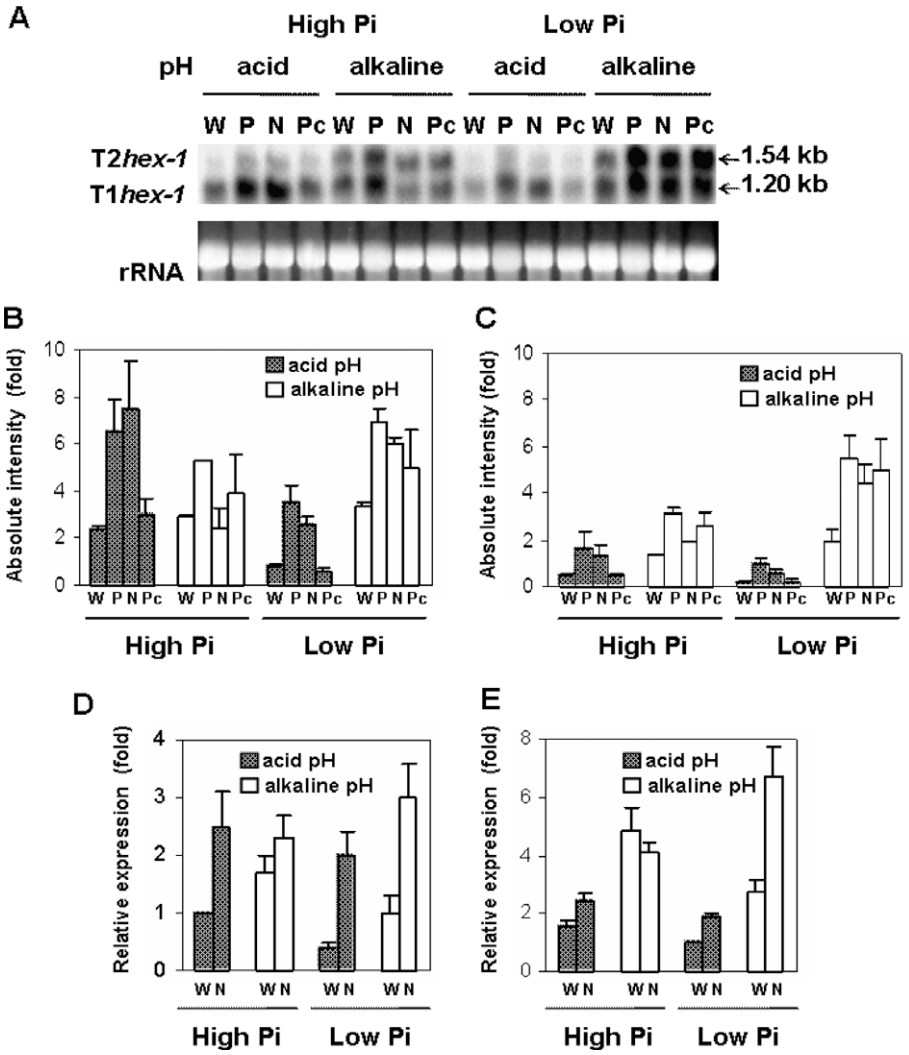

Fig. 2. Expression analyses of the *hex-1* gene. (A) Northern blot analysis of the *hex-1* gene in wild-type (W), *nuc-1*^{RIP} (N), *preg*^{C} (P), and *pacC*^{ko} (Pc) strains of *N. crassa*. The growth conditions were the same as those described in Fig. 1. The ethidium bromide-stained rRNA band is shown for comparison of loaded RNA. B and C show the expression profiles of T1*hex-1* and T2*hex-1*, respectively. The bars represent the fold expression relative to the intensities of the Northern blots determined by densitometric analysis. D and E show qRT-PCR validation of the T1*hex-1* + T2*hex-1* and T2*hex-1* expression profiles, respectively. Both Northern blots and qRT-PCR data are representative of the average values (±S.D.) obtained from three independent experiments.

chondrial permeability transition pore and have a crucial role in mitochondrial-mediated apoptosis and mtDNA maintenance [17,18]. The *aac* gene (NCU09477.3), which encodes an adenine nucleotide translocase, is also preferentially induced at acidic pH. Transcription of the *aac* gene in the *pacC*^{ko} strain also indicates that this gene is stimulated in a *pacC*$^{+}$ background at acidic pH but is inhibited in alkaline cultures (Fig. 1B). The promoter region of the *aac* gene has two nucleotide consensus sequences for PacC binding, as observed in the case of the *hsp70* gene (Supplementary Table S1). Moreover, transcription of the *aac* gene is up-regulated in the *nuc-1*^{RIP} strain cultured at alkaline pH (Fig. 1B). On the other hand, transcription of the *nmt-1* gene (no message in thiamine; NUC09345.3) is preferentially induced at alkaline pH. NMT-1 is involved in the biosynthesis of the pyrimidine moiety of thiamine (vitamin B1), an essential cofactor in intermediary metabolism. The *nmt-1* gene is highly expressed only under thiamine-depleted conditions in *Saccharomyces pombe* and other fungi [19]. However, NMT-1 is relatively abundant in *N. crassa* even in the presence of thiamine, suggesting that in addition to its role in thiamine supply, it has a role in growth. Thiamine synthesis in other organisms is induced in response to diverse stress conditions such as amino acid and sugar starvation [19,20]. Interestingly, transcription of the *nmt-1* gene is down- and up-regulated in the *nuc-1*^{RIP} strain cultured at alkaline and acidic pH, respectively. However, it is down-regulated in the *pacC*^{ko} strain regardless of the Pi levels and pH conditions during growth (Fig. 1C). Since the promoter region of the *nmt-1* gene does not have a nucleotide consensus sequence for PacC binding, regulation of the *nmt-1* gene is probably achieved *via* induction of the *nuc-1* gene by PacC at alkaline pH. The promoter region of the *nuc-1* gene, which is preferentially expressed at alkaline pH, has the nucleotide consensus sequence for PacC binding (Supplementary Table S1).

The Woronin body, a septal pore-associated organelle specific to filamentous ascomycetes, is crucial for preventing cytoplasmic bleeding after hyphal injury [21–25]. HEX-1 (NCU08332.3), its major component, together with a splice variant arising from the *hex-1* transcript, were previously described in *N. crassa*, where T1*hex-1* is the major transcript and T2*hex-1* is the minor transcript, both derived from vegetative hyphae grown in liquid medium at acidic pH [26]. However, both T1*hex-1* and T2*hex-1* transcripts accumulated in the apical hyphal compartment when the fungus was grown along a slab of solid medium [26], which may be correlated with a polarized cell growth defined by an alkaline pH gradient [27]. We also identified T1*hex-1* as the *hex-1* major transcript when the wild-type strain St.L.74A was grown in both low- and high-Pi media at pH 5.4. Moreover, T1*hex-1* and T2*hex-1* transcripts were up-regulated when the organism was grown in low-Pi liquid medium at pH 7.8. Thus, the *hex-1* splicing pattern observed in mycelia grown in liquid cultures at alkaline pH is similar to that observed in the vicinity of the hyphal apex [26], where the pH is also alkaline [27]. Interestingly, both T1*hex-1* and T2*hex-1* transcripts lead apparently to the same 19-kDa HEX-1 protein [26]. However, determining the possible role for this variant splicing will require additional work. To the best of our knowledge, the most unique example of a variant RNA splicing pattern controlled by extracellular pH is that described for human tenascin-C pre-mRNA [28]. Variant splicing observed in the case of the *hex-1* transcript may result from a variety of cellular stimuli and signals generated, for example, from nutritional stress, which could affect several enzymes involved in the regulation of this metabolic event. Changes in RNA stability and in the rate of RNA splicing or transport can also be modulated through posttranscriptional metabolic pathways [26,29–31]. Moreover, both T1*hex-1* and T2*hex-1* were overexpressed in the *preg*^{C}, *nuc-1*^{RIP}, and *pacC*^{ko} strains grown in low-Pi medium at pH 7.8 (Fig. 2A), suggesting that transcription of the *hex-1* gene may be modulated by these genes at alkaline pH. Nevertheless, transcription of both T1*hex-1* and T2*hex-1* was unaffected in the *pacC*^{ko} mutant grown at pH 5.4 (Fig. 2A), although transcription of T2*hex-1* was enhanced in medium containing sufficient Pi (Fig. 2B and C). Interestingly, the promoter region of the *hex-1* gene does not have the nucleotide consensus sequence for NUC-1 binding, whereas both the *hex-1* and *nuc-1* genes have the nucleotide consensus sequence for PacC binding [32] (Supplementary Table S1). The expression profiles of *hex-1* in the wild-type and *nuc-1*^{RIP} strains were also validated by qRT-PCR (Fig. 2D and E).

Briefly, SSH was successfully used to identify novel genes that were up- and down-regulated in the *nuc-1*^{RIP} strain of *N. crassa* grown under conditions of Pi shortage at pH 7.8. These genes are involved in diverse cellular processes such as cellular transport, cellular metabolism, protein biosynthesis, transcriptional regulation, development, and signal transduction. Interestingly, transcription of the *hsp70* and *aac* genes was stimulated at pH 5.4 in a *pacC*$^{+}$ background, whereas transcription of the *hex-1* and *nmt-1* genes was stimulated at pH 7.8. This suggests that in *N. crassa*, PacC has additional metabolic functions at acidic pH [33–35]. The identification of novel genes modulated by the transcription factors NUC-1 and PacC provides new insights into the metabolic interactions between extracellular Pi and pH sensing in *N. crassa*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.11.050.

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