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SHORT COMMUNICATION

Transcription of the *Neurospora crassa* 70-kDa class heat shock protein genes is modulated in response to extracellular pH changes

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Abstract Heat shock proteins belong to a conserved superfamily of molecular chaperones found in prokaryotes and eukaryotes. These proteins are linked to a myriad of physiological functions. In this study, we show that the *N. crassa hsp70-1* (NCU09602.3) and *hsp70-2* (NCU08693.3) genes are preferentially expressed in an acidic milieu after 15 h of cell growth in sufficient phosphate at 30°C. No significant accumulation of these transcripts was detected at alkaline pH values. Both genes accumulated to a high level in mycelia that were incubated for 1 h at 45°C, regardless of the phosphate concentration and extracellular pH changes. Transcription of the *hsp70-1* and *hsp70-2* genes was dependent on the *pacC*$^{+}$ background in mycelia cultured under optimal growth conditions or at 45°C. The *pacC* gene encodes a Zn-finger transcription factor that is involved in the regulation of gene expression by pH. Heat shock induction of these two *hsp* genes in mycelia incubated in low-phosphate medium was almost not altered in the *nuc-1*$^{-}$ background under both acidic and alkaline pH conditions. The NUC-1 transcriptional regulator is involved in the derepression of nucleases, phosphatases, and transporters that are necessary for fulfilling the cell's phosphate requirements. Transcription of the *hsp70-3* (NCU01499.3) gene followed a different pattern of induction—the gene was depressed under insufficient phosphate conditions but was apparently unaffected by alkalinization of the culture medium. Moreover, this gene was not induced by heat shock. These results reveal novel aspects of the heat-sensing network of *N. crassa*.

Keywords *Neurospora crassa* · *hsp70* · Heat shock · Pi sensing · pH regulation · *nuc-1* · *pacC*

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Introduction

The heat shock response was first described in the early 1960s when induction of new RNA synthesis was observed in the chromosomes of *Drosophila melanogaster* salivary glands after a temperature shift (Ritossa 1962, 1996). The current model on the function of the highly conserved heat shock system is that in a wide range of organisms, from prokaryotes to eukaryotes, heat shock proteins (HSP) act as molecular chaperones in the renaturation or degradation of damaged proteins; folding, assembly, and membrane translocation of newly synthesized proteins; activation of regulatory protein systems; and autoregulation. Furthermore, their expression is linked to many other stress conditions such as osmotic and oxidative stress, indicating the physiological complexity of their regulation in response to cellular stimuli (Bukau et al. 2006; Christis et al. 2008; Daugaard et al. 2007; Ellis 2006; Hohmann 2002; Kalmar and Greensmith 2009; Li et al. 2009; Mayer and Bukau 2005; Nicchitta 2009; Steel et al. 2004; Tokuriki and

Tawfik 2009). However, certain related members of this conserved protein family are expressed in cell cultures under nonstress conditions (Hartl and Hayer-Hartl 2002; Leal et al. 2009).

Transcription of heat shock genes has been well documented in *N. crassa* (Britton and Kapoor 2002; Freitag et al. 1997; Kapoor et al. 1995; Plesofsky et al. 2008; Rensing et al. 1998; Tremmel et al. 2007; Ungermann et al. 1994) as well as in other filamentous fungi (Faircloth et al. 2009; Fox et al. 2007; Georg and Gomes 2007; Montero-Barrientos et al. 2008; Rezaie et al. 2000; Turkel et al. 2006; Xavier et al. 1999). However, little is known about the cell stress response to phosphate (Pi) deprivation and changes in extracellular pH. The molecular mechanism controlling the response to phosphorus deprivation in *N. crassa* consists of four regulatory genes—*nuc-2*, *preg*, *pgov*, and *nuc-1*, which are involved in a highly conserved hierarchical relationship (Metzenberg 1979). Pi shortage is sensed by the *nuc-2* gene, the product of which inhibits the function of the PREG–PGOV complex. This allows NUC-1 to translocate into the nucleus (Peleg et al. 1996a, b). NUC-1 is a basic helix-loop-helix transcriptional regulator involved in the derepression of nucleases, phosphatases, and transporters that are necessary for fulfilling the cell's Pi requirements (Kang 1993; Kang and Metzenberg 1990; Ogawa et al. 2000). Regulation of gene expression by pH in *N. crassa* and *Aspergillus nidulans*, among other filamentous fungi, involves the conserved PacC signal transduction pathway that mediates many metabolic events at either acidic or alkaline pH values (Caddick et al. 1986; Freitas et al. 2007; Gras et al. 2009; Leal et al. 2009; Nahas et al. 1982; Silva et al. 2008; Tilburn et al. 1995). The *pacC* gene encodes a Zn-finger transcription factor that is proteolytically activated to a 27-kDa form at alkaline pH values by a conserved signaling cascade composed of six *pal* genes (Tilburn et al. 1995). In *N. crassa* and *A. nidulans*, PacC, in addition to its other functions, is required for the development and glycosylation of the Pi-repressible acid phosphatase that is secreted in an acidic milieu (Nozawa et al. 2003a, b). Expression of the Pi-repressible acid phosphatase is modulated by Pi and pH changes, and its expression is dependent on both NUC-1 and PACC transcription factors, suggesting possible interactions between the pH and Pi regulatory circuits (Silva et al. 2008). Our study aimed to evaluate the transcriptional level of the structurally related *N. crassa* genes NCU09602.3, NCU08693.3, and NCU01499.3, which are designated here as *hsp70-1*, *hsp70-2*, and *hsp70-3*, respectively, under various culture conditions. The expression of these genes was also assayed in the $pacC^{ko}$ and $nuc\text{-}1^{RIP}$ strains. Transcription of the *hsp70-1* and *hsp70-2* genes was dependent on the $pacC^{+}$ background in mycelia cultured under optimal culture conditions or at 45°C, which suggested that in *N. crassa*, the expression of these genes is under the control of the pH regulatory circuit. Transcription of the *hsp70-3* gene followed a different pattern of induction—it was not induced by heat shock and was found to be independent of the $pacC^{+}$ background.

Materials and methods

Strains, culture conditions, and heat shock treatment

Wild-type (control) *N. crassa* St.L.74.OR23-1VA (FGSC No 2489) and a strain with a *pacC* loss-of-function mutation ($pacC^{KO}$, FGSC No 11397; Galagan et al. 2003) were obtained from the Fungal Genetic Stock Center, University of Missouri, Kansas City, MO (McCluskey 2003) and maintained on slants of Vogel's medium (1.5% agar). The $pacC^{KO}$ cultures were supplemented with hygromycin (450 μg/ml). The $nuc\text{-}1^{RIP}$ strain was generated by the repeat-induced point (RIP) mutation procedure (Selker and Garrett 1988) as previously described (Leal et al. 2009).

Conidia from each strain (about 10^6/ml cells) were grown for 15 h at 30°C in an orbital shaker (200 rpm), in both low- and high-Pi medium (0.1 or 10 mM Pi) adjusted to pH5.4 (buffered with 50 mM sodium citrate) or pH7.8 (buffered with 50 mM Tris-HCl), supplemented with 44 mM sucrose as the carbon source, and prepared as previously described (Rodrigues and Rossi 1985). To assay the effect of heat stress, mycelia from the strains grown for 15 h at 30°C were incubated in various culture conditions for 1 or 2 h at 45°C.

Northern analysis

To validate differential transcription of the *hsp70* genes by Northern blot during adaptation to Pi, pH, and temperature, DNA probes specific to each *hsp70* gene were obtained by PCR amplification using the following primers: 5′-TGGCTCCAACGACAACGA-3′ (forward) and 5′-CATGA ATGAATTGTCTTCATC-3′ (reverse) for NCU09602.3; 5′-AGCTTGAACCTCTTCGACAA-3′ (forward) and 5′-AGATTTTTTATTGTAAACCC-3′ (reverse) for NCU08693.3; and 5′-TTTAATCTGCCCATACTCCCG-3′ (forward) and 5′-TTCTTGACGTGCTCCTCAAA-3′ (reverse) for NCU01499.3. Mycelia of the strains cultivated for 15 h at 30°C or incubated for 1 or 2 h at 45°C were used for RNA preparation. Approximately 15 μg of total RNA, extracted with the TRIzol® reagent (Invitrogen, Carsbad, CA), was electrophoresed on 1.5% agarose gel containing formaldehyde, blotted onto Hybond-N+ membranes, and hybridized with purified DNA probes labeled with [α-^{32}P] dCTP using the Random Primers DNA Labeling System (Invitrogen). Unincorporated nucleotides were removed

with Sephadex G-50 Chromatography. Autoradiograms were scanned using a ScanJet 4C Scanner (Hewlett Packard) and analyzed using ImageQuant 5.1 software (Molecular Dynamics). Pixel intensities for each gene were quantified and normalized to a corresponding 28S rRNA blot.

Results and discussion

Transcription of 70-kDa class heat shock protein genes is associated with the folding and translocation of proteins across membranes as well as with cell division and developmental stage progression, acquisition of thermotolerance, cell rescue, and a myriad of other physiological processes. Moreover, genes encoding the HSP70 protein family exhibit complex patterns of expression that may respond to growth or stress conditions. In spite of this, heat induction of HSP70 genes in response to extracellular pH changes is poorly understood, and to the best of our knowledge, current understanding is restricted to *N. crassa* (Gras et al. 2009; Leal et al. 2009). Transcription of the *N. crassa hsp70-2* (NCU08693.3) gene was previously demonstrated in germinating conidia of the 74A strain incubated for 5 h at 30°C in either low- or high-Pi medium at pH 5.4. This transcript did not accumulate to significant levels at pH7.8 (Leal et al. 2009). Based on the identification of HSP70 protein signature motifs, the structurally related genes NCU09602.3 (*hsp70-1*), NCU08693.3 (*hsp70-2*), and NCU01499.3 (*hsp70-3*; Fig. 1) were chosen for transcriptional analysis. The HSP70-1 and HSP70-2 proteins are homologous to the heat shock proteins Ssa2 (cytosolic) and Ssc1 (mitochondrial), respectively, from *Saccharomyces cerevisiae* (Craig et al. 1989; Daugaard et al. 2007; Deocaris et al. 2006; Ellwood and Craig 1984; Hartl and Hayer-Hartl 2002; Kregel 2002; Mayer and Bukau 2005; Werner-Washburne et al. 1989). HSP70-3 is probably unique to ascomycetes and, thus, differs from the heat shock proteins HSP70-1 and HSP70-2, which are highly similar in eukaryotic organisms (Georg and Gomes 2007). However, the predicted intracellular localization of this heat shock protein is controversial because searches using the TargetP program indicated mitochondrial localization (http://bioinformatics.albany.edu/~ptarget/), whereas those by the BaCelLo and Wolf pSORT programs (http://gpcr.biocomp.unibo.it/bacello/pred.htm; http://wolfpsort.org/) suggested cytoplasmic localization.

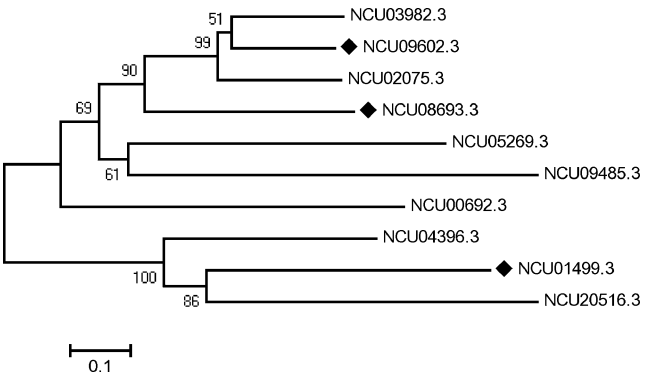

Fig. 1 Phylogenetic analysis of *Neurospora crassa hsp70* genes. Multiple alignments of 10 HSP70 amino acid sequences using the *N. crassa* genome database was performed using ClustalW (http://www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed from these alignments using the neighbor-joining method with the software package MEGA version 3.1 (http://www.megasoftware.net). Three of them were chosen for transcription analysis (*filled diamonds*). The *number at the nodes* represents the percentage of bootstrap values (1,000 replicates). The *scale bar* represents the phylogenetic distance of 0.1 amino acid substitution per site

Transcription of the *hsp70-1* and *hsp70-2* genes in the mycelia of *N. crassa* grown for 15 h in high-Pi medium at pH5.4 and 30°C, which are optimal conditions for fungal growth, was strongly reduced at alkaline pH values in either high- or low-Pi culture. However, alkaline pH had a strong effect on the heat shock induction of both these *hsp* genes, which accumulated to a higher level (at least 20-fold) and for a long duration in either sufficient or low-Pi medium (Fig. 2). Therefore, extracellular phosphate changes had a limited effect on heat shock induction at alkaline pH values. In contrast, transcription of the *hsp70-1* and *hsp70-2* genes in the mycelia of *N. crassa* grown for 15 h in high-Pi medium at pH5.4 and 30°C was strongly reduced in low-Pi cultures in an acidic milieu. Furthermore, although these two *hsp* genes revealed similar transcription patterns, the *hsp70-2* gene showed very poor induction upon heat shock (maximum of 2-fold) in either high- or low-Pi cultures at acidic pH values (Figs. 2 and 3). Thus, we hypothesize that the *hsp70*-2 gene is associated with conidial germination at 30°C under low-Pi and acidic pH conditions, which are conditions under which the secretion of Pi-repressible acid phosphatases occurs (Freitas et al. 2007; Silva et al. 2008). The *hsp70-3* gene follows a different pattern of induction. Transcription of this gene is depressed in mycelia cultured under low-Pi conditions at either acidic or alkaline pH values but is not induced by heat shock (Fig. 2).

Expression of the *hsp70-1* and *hsp70-2* genes in mycelia grown under high-Pi conditions and at acidic pH values as well as their induction by heat shock at either acidic or alkaline pH values is strongly reduced in a *pacC*$^{-}$ background. In other words, transcription of both these *hsp* genes is positively regulated by PACC at 30°C and 45°C (Fig. 4). In contrast, induction of the *hsp70-3* gene by heat shock occurs in the absence of PACC and only at alkaline pH values (Fig. 4). These results suggest that in *N. crassa*, the transcriptional regulator PACC has novel metabolic functions regardless of the extracellular pH.

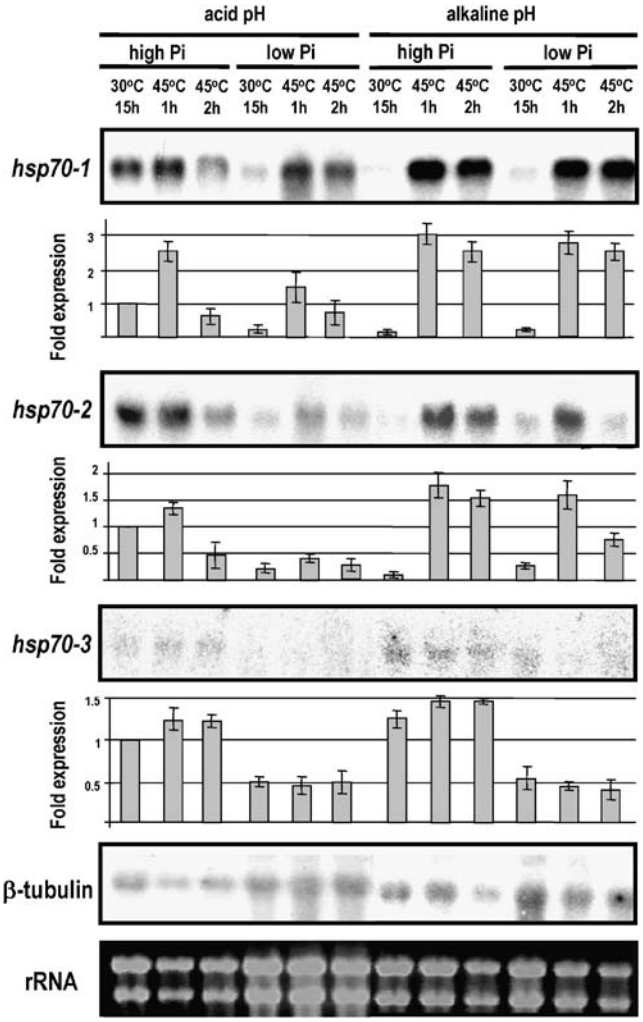

Fig. 2 Gene expression analysis. Northern blot analysis of *hsp70-1* (NCU09602.3), *hsp70-2* (NCU08693.3), and *hsp70-3* (NCU01499.3) transcripts using total RNA obtained from *N. crassa* mycelia. The wild-type strain was cultured for 15 h at 30°C in low- or high-Pi liquid medium (0.1 and 10 mM Pi, respectively) at pH5.4 or 7.8. The effect of heat shock was assayed in mycelia incubated for 1 or 2 h at 45°C. The ethidium bromide-stained rRNA band is shown as a loading control. The β-tubulin gene was hybridized as additional loading control on the northern blot. *Bars* show fold expression determined from the intensity measured by densitometric analysis. Data are average values ± standard deviation (SD) obtained from two independent experiments

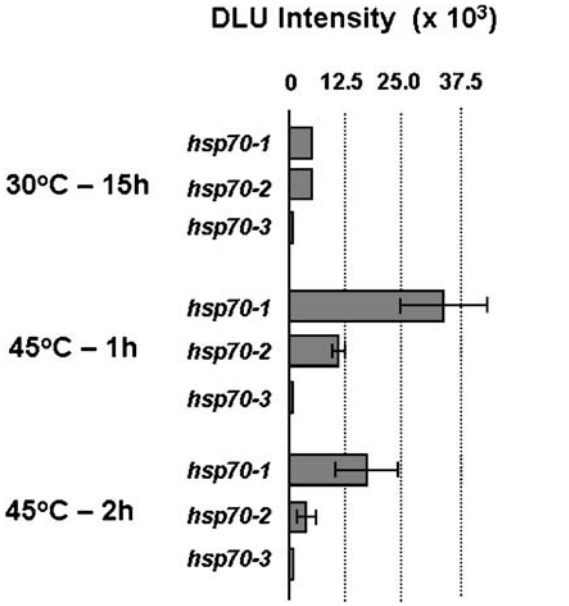

Fig. 3 Relative gene expression levels of the three *hsp70* gene family members in response to low-Pi cultures at pH5.4 (northern blots are shown in Fig. 2). The images were captured by the Cyclone Storage Phosphor System (Packard) at 600 DPI resolution and analyzed with the OptiQuant program version 3.0 (Packard). The signal intensities of these image areas were expressed in Digital Light Units (DLU) per mm and normalized for the screen background. We assumed identical DLU values for both *hsp70-1* and *hsp70-2* transcripts expressed in mycelia incubated for 15 h at 30°C. The *bars* indicate the fold expression determined from the intensities measured by densitometric analysis. Data are average values ± standard deviation (SD) obtained from two independent experiments

Heat shock induction of the *hsp70-1* and *hsp70-2* genes was also observed in the *nuc-1*^{RIP} strain cultured under low Pi conditions at acidic and alkaline pH values. Moreover, although the levels of the transcripts encoding the HSP70-3 protein were slightly elevated in the *nuc-1*^{RIP} strain, no induction by heat shock was observed (Fig. 5). It is worth noting that culturing the *nuc-1*^{RIP} strain in a low-Pi medium at acidic pH values is highly stressful to the cells because both the PREG/PGOV complex and the *nuc-1* gene are silenced (Gras et al. 2007, 2009; Leal et al. 2007, 2009; Metzenberg 1979). This suggests that the transcription of these *hsp70* genes is independent of the *nuc-1* gene. Therefore, the *nuc-1*^{RIP} analysis seems to be more useful as a control for the observed positive regulation by PACC.

In this study, we showed that the *hsp70-1* and *hsp70-2* genes of *N. crassa* are preferentially expressed in an acidic milieu but are induced by heat shock regardless of the extracellular pH. Furthermore, transcription of both these *hsp* genes is positively regulated by the transcription factor PACC, regardless of the pH, whereas heat shock induction of the *hsp70-3* gene occurs in a *pacC*$^{-}$ background and only at alkaline pH values. Together with previously published data (Freitas et al. 2007; Gras et al. 2009; Leal et al. 2009; Nozawa et al. 2003a), these results indicate that the sensing of alkaline pH by the conserved PACC-signaling cascade is not the sole function of this protein. The previously reported exclusive activation of the *A. nidulans* PacC protein at alkaline pH values was observed in cultures under nonphysiological conditions, i.e., when the culture medium was very complex and when both high Pi-repressible and salt-stress conditions existed in which the synthesis of Pi-repressible enzymes was fully repressed (Freitas et al. 2007; Peñas et al. 2007; Perez-Esteban et al. 1993). Thus, the physiological activation of PACC also

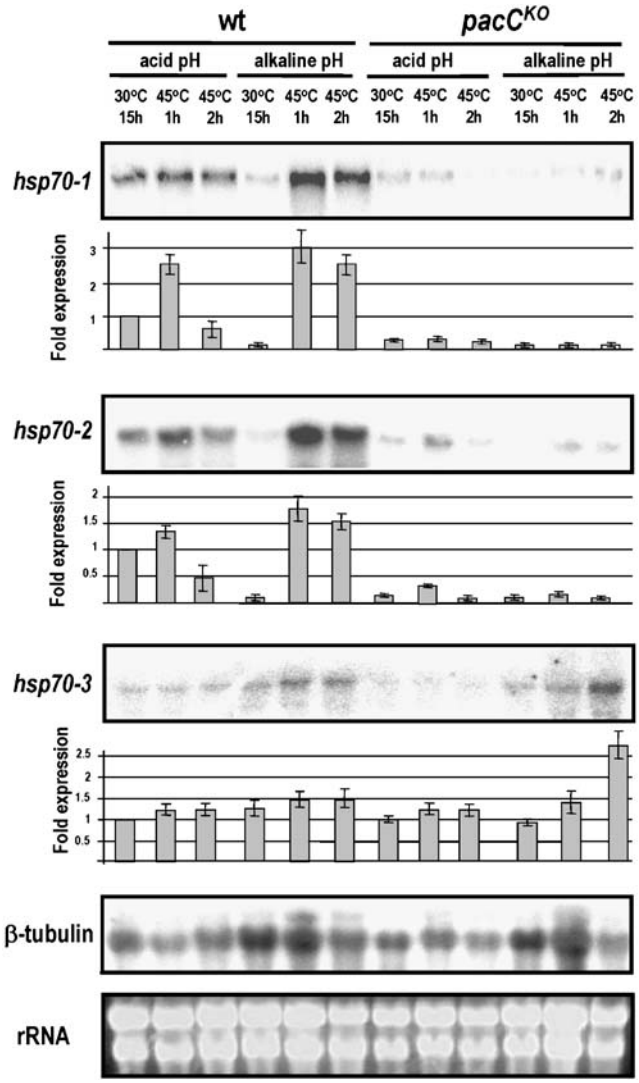

Fig. 4 Gene expression analysis. Northern blot analysis of *hsp70-1* (NCU09602.3), *hsp70-2* (NCU08693.3), and *hsp70-3* (NCU01499.3) transcripts using total RNA obtained from *N. crassa* mycelia. The wild-type (*wt*) and *pacC*^{KO} strains were cultured for 15 h at 30°C in high-Pi liquid medium (10 mM Pi) at pH5.4 or 7.8. The effect of heat shock was assayed in mycelia incubated for 1 or 2 h at 45°C. The ethidium bromide-stained rRNA band is a loading control. The β-tubulin gene acted as additional loading control for the northern blots. *Bars* show fold expression, determined from the intensity measured by densitometric analysis. Data are average values ± standard deviation (SD) obtained from two independent experiments

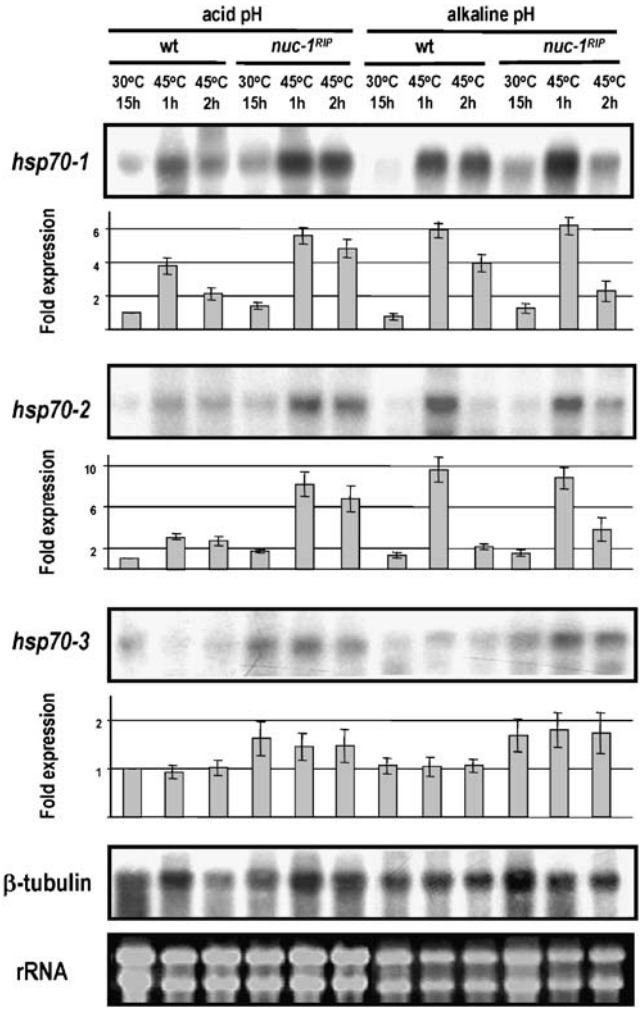

Fig. 5 Gene expression analysis. Northern blot analysis of *hsp70-1* (NCU09602.3), *hsp70-2* (NCU08693.3), and *hsp70-3* (NCU01499.3) transcripts using total RNA obtained from *N. crassa* mycelia. The wild-type (*wt*) and *nuc-1*^{RIP} strains were cultured for 15 h at 30°C in a low-Pi liquid medium (0.1 mM Pi) at pH5.4 or 7.8. The effect of heat shock was assayed in mycelia incubated for 1 or 2 h at 45°C. The ethidium bromide-stained rRNA band is a loading control. The β-tubulin gene acted as additional loading control for the northern blots. The *bars* show fold expression determined from the intensity measured by densitometric analysis. Data are average values ± standard deviation (SD) obtained from two independent experiments

occurs in minimal medium supplemented with glucose as the carbon source at pH5.4 (Silva et al. 2008). Moreover, transcription of these *hsp70* genes might be directly or indirectly modulated by the transcriptional regulator PACC. PACC is homologous to Rim101p (*Candida albicans* and *S. cerevisiae*) and PacC (*A. nidulans*), which are transcription factors that regulate pH-conditioned gene expression in these eukaryotic microorganisms (Lamb and Mitchell 2003; Ramon and Fonzi 2003; Tilburn et al. 1995). PacC binds to 5′-GCCARG-3′ sequences upstream of pH-conditioned genes and either activates or represses transcription. The initial guanine residue in this consensus sequence is critical for PacC binding (Tilburn et al. 1995). The Rim101p binding site is 5′-NCCAAG-3′, which is preferentially followed by A or C in the adjacent 3′ position (Ramon and Fonzi 2003). The presence of this pentanucleotide followed by A or C was identified in the sequences upstream of the *N. crassa hsp70-1*, *hsp70-2*, *hsp70-3*, *hsf2* (NCU08480.3), and *hsf3* (NCU02413.3) genes, whereas this binding consensus was absent in the sequence upstream of the *N. crassa hsf1* (NCU08512.3) gene. Thus, the *hsf1*

gene might not be under the direct control of PACC. Eukaryotic heat shock factors (HSFs) regulate constitutive and stress-inducible transcription of various genes, including the *hsp* genes and, thereby, play a central role in the regulation of numerous cellular reprogramming events (Hahn et al. 2006; Hashikawa et al. 2007; Sakurai and Takemori 2007; Thompson et al. 2008). HSF proteins recognize continuous and discontinuous repeats of 5′-nGAAn-3′ in target genes; these repeats are present in the sequences upstream of the *hsp70-1*, *hsp70-2*, and *hsp70-3* genes. Therefore, interactions between the PACC, HSF, and HSP proteins are of great complexity including their competition for the target genes. In conclusion, regulation of these three structurally related *hsp70* genes by the PACC protein depends upon specific culture conditions such as the incubation temperature and extracellular pH changes, which are novel aspects of the heat-sensing network of *N. crassa*.

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