Neurospora crassa heat shock factor 1 Is an Essential Gene; a Second Heat Shock Factor-Like Gene, *hsf2*, Is Required for Asexual Spore Formation[▽]

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Appropriate responses of organisms to heat stress are essential for their survival. In eukaryotes, adaptation to high temperatures is mediated by heat shock transcription factors (HSFs). HSFs regulate the expression of heat shock proteins, which function as molecular chaperones assisting in protein folding and stability. In many model organisms a great deal is known about the products of *hsf* genes. An important exception is the filamentous fungus and model eukaryote *Neurospora crassa*. Here we show that two *Neurospora crassa* genes whose protein products share similarity to known HSFs play different biological roles. We report that *heat shock factor 1* (*hsf1*) is an essential gene and that *hsf2* is required for asexual development. Conidiation may be blocked in the *hsf2* knockout (*hsf2^{KO}*) strain because HSF2 is an integral element of the conidiation pathway or because it affects the availability of protein chaperones. We report that genes expressed during conidiation, for example *fluffy*, *conidiation-10*, and *repressor of conidiation-1* show wild-type levels of expression in a *hsf2^{KO}* strain. However, consistent with the lack of macroconidium development, levels of *eas* are much reduced. Cultures of the *hsf2^{KO}* strain along with two other aconidial strains, the *fluffy* and *aconidial-2* strains, took longer than the wild type to recover from heat shock. Altered expression profiles of *hsp90* and a putative *hsp90*-associated protein in the *hsf2^{KO}* strain after exposure to heat shock may in part account for its reduced ability to cope with heat stress.

The heat shock response is a protective mechanism against sudden temperature change and other forms of physical and chemical stress. The response is brought into effect by heat shock proteins (HSPs), molecular chaperones that assist protein folding and stability (35). Several highly conserved families of HSPs, named according to their molecular masses, are expressed in response to heat shock and include HSP110, HSP100, HSP90, HSP70, HSP60, and a number of small HSPs. Some of the family members are expressed in non-heat shock conditions, but the expression of others is induced only upon exposure to stress due to the action of heat shock transcription factors (HSFs). HSFs are a functionally conserved group of molecules. They were first identified as regulators of the heat shock response (reviewed in reference 28), but it is now evident that certain HSFs also regulate developmental pathways (for a review, see reference 36).

Drosophila melanogaster and *Saccharomyces cerevisiae* each have one essential HSF (7, 48). In *S. cerevisiae*, while the induction of genes encoding chaperones and associated proteins is under the control of its HSF, a subset of genes induced by a variety of environmental stresses and encoding metabolic enzymes, components of the ubiquitin-proteosome degradation pathway, and antioxidant defense proteins are under the control of the transcription factors Msn2, Msn4, Yap1, and Skn7 (5, 17, 6, 25). In *Drosophila*, temperature-dependent alternative splicing of dHSF gives rise to four forms of dHSF

mRNA which encode HSF isoforms with different transcriptional activities (16). Although the functional significance of the HSF isoforms is not yet understood clearly, one possibility is that they activate different subsets of HSP or stress-responsive genes. Moreover, in species with multiple *hsf* genes, a functional specialization of certain HSFs toward developmental roles has occurred. For example, of the four vertebrate HSFs, HSF1 mediates heat shock gene expression, whereas HSF2 is reported to be unresponsive to temperature, while its activity changes throughout differentiation. In addition, alternative splicing of *hsf1*, *hsf2*, and *hsf4* transcripts generates a variety of HSF isoforms (13, 18, 45). Since these splice forms show tissue-specific expression, it is likely that they also have distinct roles.

In contrast, no *Neurospora* HSFs have yet been isolated, although HSPs are expressed in this organism during heat shock and cell differentiation (14, 19) suggesting that, as seen for other organisms, the *Neurospora* HSF(s) is required for development. *Neurospora crassa* undergoes both sexual and asexual developmental cycles. When nitrogen is limiting, female sexual development ensues, and when nuclei of opposite mating types fuse numerous asci are produced, each containing eight haploid spores (42). In addition, two asexual developmental pathways are described: macroconidiation and microconidiation. Macroconidial development in *Neurospora* can be induced by desiccation, nutrient deprivation (47), light (23), oxidative stress (29), and signals relayed from the organism's circadian clock (37). Once induced, aerial hyphae are formed which branch and eventually form chains of macroconidia, each containing between one and five nuclei (44). In a distinctly different developmental process, microconidia contain-

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ing one or rarely two nuclei are formed from smaller branched microconidiophores (30).

Several phases in the development of macroconidia have been recorded (31, 44). The first step is the production of branching aerial hyphae. Minor constrictions then develop, followed by the production of proconidia via budding from the terminal cells of each branch. Finally, proconidia are separated by major constrictions and mature into conidia, which are easily separated and dispersed by wind or water. A number of mutants affecting this developmental pathway are known. The *fluffyoid* (*fld*) and *aconidial-2* (*acon-2*) mutants are blocked before and the *fluffy* (*fl*) mutant is blocked after the formation of minor constrictions (31, 44). The *acon-3* mutant is blocked after the maturation of minor constrictions. *conidial separation-1* (*csp-1*) and *csp-2* are required for conidial separation (41). Genetic and molecular studies place *acon-2* upstream of *fl*, with *acon-3*, *csp-1*, and *csp-2* being downstream (9, 1).

We report here that *Neurospora hsf1* is an essential gene and that a deletion strain can be maintained only as a heterokaryon. In strains with *hsf2* deleted, no dramatic alteration of the heat shock response is detected. However, macroconidiation in *hsf2* is defective; aerial hyphae emerge but no macroconidia are produced. Consistent with this, differences are seen between the wild-type and *hsf2* knockout (*hsf2^{KO}*) strains in the expression of two conidiation-associated transcripts, *fl* and *eas*.

MATERIALS AND METHODS

Bioinformatic analysis. Genes were identified by searching the Broad Institute *Neurospora crassa* database. Protein prediction was performed using NCBI BLAST at http://www.ncbi.nlm.nih.gov/BLAST/ and COILS at http://www.ch.embnet.org/software/COILS_form.html. Sequence alignments were performed using ClustalW at http://www.ebi.ac.uk/clustalw/.

Strains and growth conditions. *mus-51a* (FGSC9718), *hsf2 a* (FGSC 11671), *acon-2 a* (FGSC3263), *fl a* (FGSC818), and the *hsp90a* (NCU01792), *hsp30* (NCU09364), *hsp78* (NCU02630), *hsp101* (NCU00104), *hsp70* (NCU08683), and *hsp90* (NCU04142) strains were obtained from the Fungal Genetics Stock Center (http://www.fgsc.net/) and crossed into either 30-7 *bd A* or 87-3 *bd a* to introduce the *bd* mutation. 87-3 *bd a* was obtained from Christian Heintzen (University of Manchester, United Kingdom). *fl,bd* was a kind gift from D. Bell-Pedersen (Texas A&M University). The *hsf1^{KO}* strain was made by amplifying 1-kbp regions 5′ and 3′ of the *hsf1* open reading frame (ORF) and fusing these to the *hygromycin phosphotransferase* (*hph*) gene. *mus-51a* was transformed with this fragment and transformants were selected on hygromycin-containing agar plates (8). Strains were maintained on minimal medium (1× Vogel's salts [10], 2% sucrose, 500 ng/ml biotin, 1.5% agar). Crossing medium contained 1× Westergaard's salts, 2% sucrose, 500 ng/ml biotin, and 1.5% agar (10). Spores were picked onto slants containing Vogel's minimal medium, heat shocked at 60°C for 1 h to induce germination, and then incubated at 30°C. Progeny were screened for hygromycin resistance on minimal medium containing 200 μg/ml of hygromycin B and/or for rhythmic conidiation on race tubes.

Race tube medium contained 1× Vogel's salts, 0.1% glucose, 0.17% arginine, 500 ng/ml biotin, and 1.5% agar. After inoculation with 5- to 10-day-old macroconidia, race tubes were placed in constant light (LL) at 25°C for 48 h and then transferred to constant darkness (DD). Growth fronts were marked daily. Heat shocks were given by immersing race tubes in a 50°C water bath for 1 h after 24 h in the dark at 25°C. The race tube cultures were then returned to 25°C to recover and examined daily for regrowth.

Microconidiation was induced according to the protocol of Ebbole and Sachs (11). Conidia were harvested by vortexing in 2 ml of sterile distilled water and filtered through 5-μm filters (Millipore) into sterile Eppendorf tubes. Microconidia were plated onto minimal (1× Vogel's salts, 1% sorbose, 0.005% fructose, 0.005% glucose, 1 M sorbitol) or minimal hygromycin-containing plates (200 μg/ml). Plates were incubated at 30°C until single colonies were detected (3 or 4 days later). Colonies were picked onto minimal agar slants, and this was followed by the transfer of conidia onto hygromycin-containing (200 μg/ml) slants.

Liquid culture medium for the growth of mycelial mats and shaking cultures contained 1× Vogel's salts, 2% glucose, and 500 ng/ml biotin. One hundred milliliters of liquid culture medium was inoculated with 2.3-cm-diameter discs cut from mycelial mats. These cultures were put into LL at 25°C with shaking at 150 rpm. After 24 h, cultures were placed into a shaking water bath at 45°C. Flasks were removed at regular intervals and their contents were immediately harvested by vacuum filtration onto filter paper and frozen in liquid nitrogen.

The experiment examining nitrogen starvation-induced conidial development was first described in the work of Bailey-Shrode and Ebbole (1). Strains cultured on Vogel's minimal medium slants (2% sucrose) were used to inoculate culture dishes of modified Vogel's minimal medium (1.5% sucrose) with 50 mM NH$_4$Cl in place of NH$_4$NO$_3$. After 3 days at 30°C, a cork borer was used to punch discs from the resulting mycelial mats. These discs were then transferred to 100-ml flasks containing 50 ml of modified Vogel's minimal medium and incubated for 22 h at 34°C in LL plus shaking at 150 rpm. The cultures were then washed in sterile distilled water and transferred to flasks containing Vogel's minimal medium lacking any nitrogen source. Tissue was harvested before and 1 h, 6 h, 12 h, and 24 h after transfer.

Induction of conidiation by exposure to air was carried out essentially as described in the work of Lee and Ebbole (26), with the following modifications. Disks were cut from mycelial mats and placed into Vogel's minimal medium with 2% glucose. After 24 h in DD at 25°C, disks were blotted dry and placed on agar blocks (Vogel's minimal medium, 2% glucose, 2% agar) in open petri dishes containing 15 ml of fresh culture medium.

RT-PCR. The RevertAid H$^-$ first-strand cDNA synthesis kit (Fermentas) was used for all reverse transcription-PCR (RT-PCR). Primers are listed in Table 1. For semiquantitative RT-PCR, 1 μg of total RNA was reverse transcribed using the poly(T) primer supplied with the kit, following the manufacturer's instructions. A limiting number of cycles were used to stop the subsequent PCRs during the exponential phase of amplification. After agarose gel electrophoresis, quantification was performed using Bio-Rad QuantityOne software and data were normalized against *actin* transcript levels.

Construction of the *hsf1* knockout strain. A *hsf1* deletion cassette was made containing the *hph* gene (confers resistance to hygromycin) flanked by approximately 1.6 kbp 5′ and 2 kbp 3′ of the NCU08512 ORF. One hundred microliters of a *mus-51* conidial cell suspension (2 × 10^9 conidia/ml) in a 2-mm gap cuvette (Bio-Rad) was electroporated with 0.5 μg of the knockout construct. A Bio-Rad gene pulser was used with the following settings: 12.5 kV/cm, 400 Ω, 25 μF. Transformants were selected on hygromycin plates (200 μg/ml) incubated at 30°C for 4 days. Transformants were crossed with 87-3 *bd a* to obtain homokaryons.

RNA extraction and Northern blotting. RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. Equal amounts of RNA were run through a 1% agarose formaldehyde gel and blotted onto Hybond N$^+$ nitrocellulose membrane (GE Healthcare). Blots were probed with randomly primed ^{32}P-labeled (Roche labeling kit; GE Healthcare radionucleotide) cDNA or ^{32}P-labeled RNA (MAXIscript kit; Ambion) and detected using phosphorimager screens (Fuji). Blots were prehybridized (NorthernMax; Ambion) and hybridized at 42°C for DNA probes and at 68°C for RNA probes. Blots were washed twice for 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS), once for 15 min in 1× SSC plus 0.1% SDS at room temperature, and once for 15 min in 0.1× SSC plus 0.1% SDS at either 42 or 68°C.

DNA extraction and Southern blotting. Genomic DNA was extracted from homogenized tissue by use of a cetyltrimethylammonium bromide protocol. DNA was digested with NdeI (*hsf2*) or NdeI and BamHI (*hsf1*), separated through 0.8% agarose gels, and transferred onto Hybond N$^+$ nitrocellulose membrane. The DNA was cross-linked to membranes with UV light. Blots were hybridized to digoxigenin (DIG)-labeled (Roche) probes against the 5′-flanking region of the knockout cassettes (http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html). Primers for the amplification of probe DNA are listed in Table 1. Each blot was incubated with prehybridization solution (NorthernMAX; Ambion) at 42°C for 1 h before the addition of 300 ng of denatured probe in 4 to 5 ml of fresh hybridization solution and incubation at 42°C overnight. After hybridization, the blots were washed according to the manufacturer's protocol, with the final washes carried out at 42°C. Detection of bound DIG-labeled probe was also carried out according to the manufacturer's protocol, and the blots were exposed to X-ray film for 5 to 25 min.

RESULTS

Identification of putative HSFs. Two putative *Neurospora* HSFs encoded by *hsf1* (NCU08512) and *hsf2* (NCU08480) were identified in a BLAST search of the *Neurospora* genome.

TABLE 1. Primers used

Gene	Use[a]	Primer[b]	
		Forward	Reverse
hsf1	RT/cloning/cDNA probe	CGGGATCCCG**CATGTCTTCTCCTAACC**	CGGGATCCCG**CGACAGAAGCAACAGAT**
	RNA probe/sqRT-PCR (1; 35)	CGGGATCCCG**CATGTCTTCTCCTAACC**	TAATACGACTCACTATAGGGA**CGTGA GGGCCTGCTGGAATAAGGTCG**
hsf2L	RT/cloning/cDNA probe	TAGGATCC**TACAGTGCAGATGGCTACC**	TAGGATCC**ACATAACGTCTCAAGATGC**
	RNA probe/sqRT-PCR (2; 30)	TAGGATCC**TACAGTGCAGATGGCTACC**	TAATACGACTCACTATAGGGAT**CCTC CATCTGGCGCTGAACCTGC**
hsf2S	RT/cloning	TAGGATCC**TACAGTGCAGATGGCTACC**	TAGGATCCC**AAGCTAACCAGGTCTCCG**
hsf2 5′ flank	Southern blot DNA probe	**GTAGCCTCTCCATACCTACC**	**GTAGAGTCCATTCTACCTCG**
hsp70	DNA probe	**GTCTCGACAAGAAGGTCG**	**TCTCGGAGGTGGACTTGG**
hsp90	DNA probe	**GGATCCTTGTTGCCGACAGAGTTA**	**GGATCCCATCAATGGGGTCGACAAGG**
NCU01792	DNA probe	**AGCGCTCTTCTGCTACCG**	**CATGGATCTTGGGTATGC**
fluffy	RNA probe/sqRT-PCR (1; 30)	**TGTGATGGCCAGATGCCATGC**	TAATACGACTCACTATAGGGA**CGAGT TATCGCTGCCCTCACG**
actin	sqRT-PCR (0.5; 20)	**GGCTGGCCGTGATCTTACCGACTA**	GAGACGAGGCGAGAATGGAACC
con-10	sqRT-PCR (1; 26)	**CGTCAACATGGCTGGCACTGGTAACG**	TTGTAATACGACTCACTATAGGGCAA ATTGTCAAAGCATTCAGTTGC
rco-1	sqRT-PCR (1; 35)	**GTACGAGGATGAGATTGCGCT**	TCTGATGAGCTTGTCCTCGGCTC

[a] For semiquantitative RT-PCR (sqRT-PCR), the numbers in parentheses indicate the amounts of cDNA in μl followed by the cycle numbers.

[b] Modifications are in lightface, and annealing portions are in boldface.

We cloned RNAs expressed from these genes and analyzed their sequences to determine the details of any introns. *hsf1* contains one intron and encodes a 787-amino-acid (aa) protein. Alternative splicing of *hsf2* RNA gives rise to at least three different transcripts encoding polypeptides of 613 aa, 374 aa, and 173 aa or of 579 aa, 340 aa, and 139 aa depending at which of the three possible in-frame ATGs translation is initiated (Fig. 1). HSFs from different species, though binding to similar DNA consensus sequences, are not highly conserved. However, all contain a DNA-binding domain at the amino terminus and an adjacent cluster of hydrophobic amino acids organized into heptad repeats required for oligomerization, and they often also contain a heptad repeat close to the carboxy terminus (49). In order to become functionally active, HSF protein monomers form homotrimers through the interaction of coiled-coil domains found toward the N terminus. HSF1 and the large HSF2 isoforms contain putative DNA-binding and trimerization domains. In addition, a carboxy heptad repeat is present in HSF1. In contrast, the 173-aa isoform of HSF2 contains only a partial DNA-binding domain (Fig. 1). Although the presence of these alternatively spliced transcripts in the five cDNAs we cloned and sequenced suggests that they are present at a high level relative to what is seen for the fully spliced transcript, further investigation revealed that in >99% of the transcripts, introns 1 to 3 have been removed (data not shown).

hsf1 is an essential gene; hsf2 is required for asexual development. To knock out *hsf1*, at least 1.5 kb of DNA 5′ and 3′ of the ORF was ligated to the *hygromycin phosphotransferase* gene (Fig. 2A). This construct was electroporated into conidia of a *mus-51*::*bar* strain which lacks an enzyme required for nonhomologous DNA joining. Due to the absence of *mus-51*, targeted transformation is almost 100% efficient (33). DNA from 20 transformants was extracted and recombination events at the *hsf1* gene were checked by Southern blot analysis (Fig. 2B). All transformants were heterokaryons harboring both nuclei with *hsf1* intact and nuclei with *hsf1* deleted. To obtain ho-

mokaryons, microconidiation was induced in two of the heterokaryons (11). Only five colonies derived from microconidia were hygromycin resistant, and once again these were heterokaryons (data not shown). In parallel, two of the transformants were backcrossed to the *bd* strain. Sixty-five spores derived from these crosses were selected and all 39 that germinated were inoculated onto hygromycin slants. None of these strains
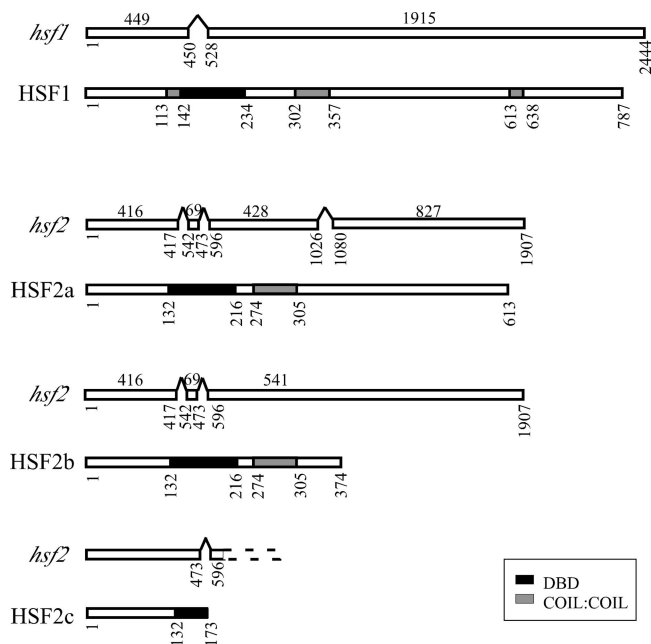

FIG. 1. RNA splice forms and protein domain structures of HSF1 and HSF2 isoforms. For each pair of diagrams the top schematic represents RNA and the bottom schematic represents protein. Open rectangles in mRNAs represent exons. Nucleotide numbers below transcripts start from the ATGs of the respective ORFs. Amino acid numbers are indicated below proteins. DBD, DNA-binding domain.
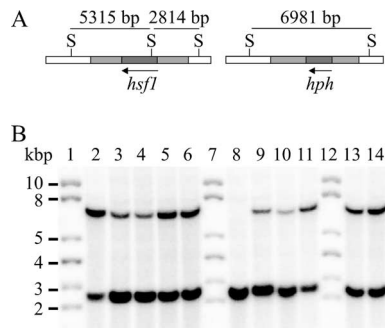
FIG. 2. Analysis of *mus-51::bar*, △*hsf1* transformants. (A) Schematic diagram showing the location of SacI restriction enzyme sites in the region of *hsf1*. (Left) Wild-type *hsf1* locus; (right) locus with the *hsf1* gene deleted and replaced with *hph*. (B) Southern blot of *mus-51::bar*, △*hsf1* strains. Genomic DNA digested with SacI and probed with DIG-labeled DNA homologous to a region 5′ of the *hsf1* ORF. Lanes: 1, 7, and 12, DNA ladder; 8, control parental *mus-51* genomic DNA; 2 to 6, 9 to 11, 13, and 14, DNA from different transformants.

were resistant to hygromycin, supporting our conclusion that *hsf1* is an essential gene.

A *hsf2* deletion strain (8) was obtained from the Fungal Genetics Stock Center (University of Missouri, Kansas City) and checked by Southern analysis (data not shown). The vegetative hyphal growth of the *hsf2* mutant appears normal, as does the growth rate, but the deletion strain has a striking phenotype; it produces very few macroconidia (Fig. 3). Only after several weeks are a few proconidial chains present, perhaps induced by starvation or desiccation.

The heat shock response in the *hsf2,bd* strain. We first assayed the phenotypic response of the *hsf2* mutant to heat shock by monitoring its growth along race tubes after exposure to high temperature. In order to assess the effect of heat shock on conidiation as well as on growth a *hsf2,bd* strain was initially

used in these experiments. The *bd* mutation allows conidiation to occur in the presence of the high carbon dioxide levels which accumulate in the enclosed race tube. In response to a 1-h, 50°C heat shock during the third day in DD at 25°C, growth of both *bd* and *hsf2,bd* strains stopped. On the second day after the heat shock 100% of the *bd* cultures had resumed growth, while only 4 days after the heat shock did 50% of the *hsf2,bd* cultures resume growth (Fig. 4A). We wondered if the delayed recovery was correlated with the aconidial state of the *hsf2,bd* strain, and we therefore repeated the experiment, this time including the *acon-2,bd* and *fl,bd* strains. Of note is that the *acon-2,bd* strain is a temperature-sensitive strain and no spores develop at 34°C or above. Therefore, all of the strains were cultured at either 25°C or 34°C before and after a 50°C heat shock. In both sets of conditions, the aconidial strains took longer to recover from heat shock than did the *bd* strain (Fig. 4B and C). These data suggest either that the germination of conidia that fall to the agar surface after heat shock accounts for the faster regrowth of the *bd* strain and/or that all the aconidial strains are inherently temperature sensitive.

If the latter of these possibilities is true, then these results implicate HSF2 in the heat shock response. Therefore, we followed levels of both *hsf1* and *hsf2* and of three HSP transcripts in control and heat-shocked cultures. In a *bd* strain, the levels of *hsf1* and *hsf2* mRNA declined during the first 45 min of a prolonged heat shock. In the *hsf2,bd* strain, *hsf1* transcription also decreased and, as expected, *hsf2* RNA was not expressed (Fig. 5A). In spite of this, the three HSP transcripts we assayed, namely, those for *hsp70* (NCU09602) and *hsp90* (NCU04142) and a transcript that arises from NCU01792 which we have named *heat shock protein 90-associated* (*hsp90a*), were upregulated in response to heat shock in both strains (Fig. 5B). Interestingly, two *hsp70* transcripts can be distinguished after transfer to high temperatures in both the wild-type and *hsf2,bd* strains. The lower-molecular-weight
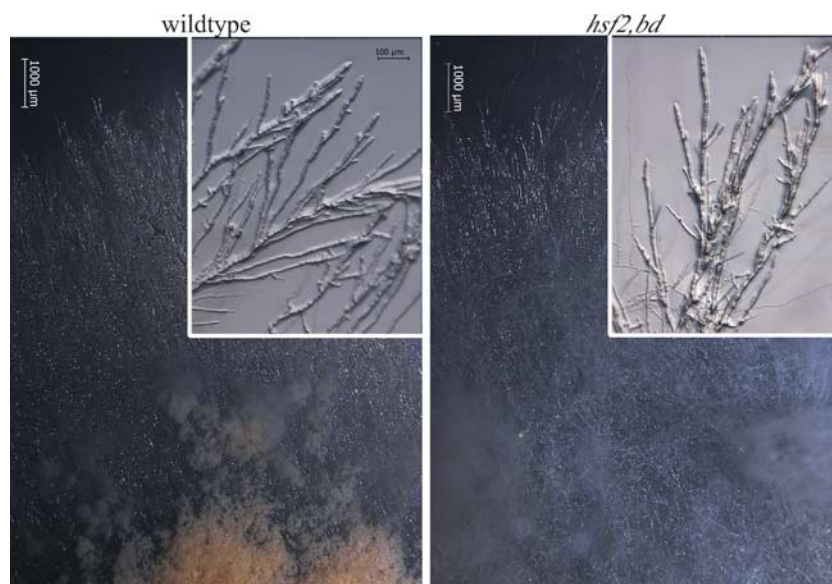

FIG. 3. The *hsf2*^KO strain is aconidial. Light microscopy showing growth of *bd* (left) and *hsf2*^KO,*bd* (right) strains on minimal agar plates (low magnification) and at higher magnification (inset; magnification, ×80) at the growth front. Macroconidia (asexual spores) are bright orange.
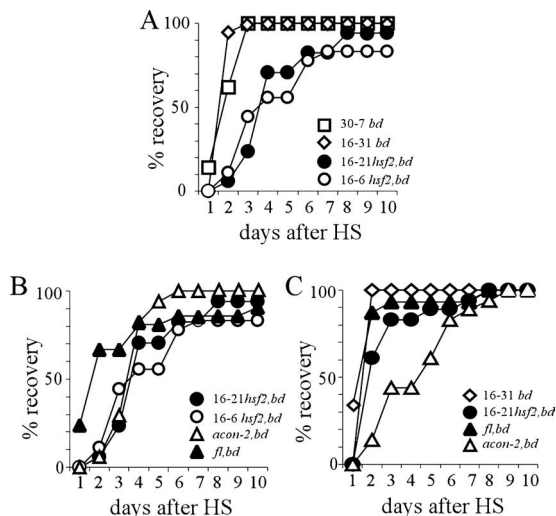
FIG. 4. Response of wild-type and aconidial strains to heat shock. Strains inoculated onto race tubes were germinated and grown in LL at 25°C (A and B) or 34°C (C) for 48 h before transfer to DD at 25°C (A and B) or 34°C (C). After 24 h in DD, cultures were exposed to a 1-h 50°C heat shock and then transferred back to DD at 25°C (A and B) or 34°C (C). Growth was monitored twice daily over the following 9 days. The graph shows the percentages of recovery for the following strains against time (h) after heat shock. (A) 30-7 *bd* (*n* = 21), 16-31 *bd* (*n* = 18), 16-21 *hsf2^{KO},bd* (*n* = 17), and 16-6 *hsf2^{KO},bd* (*n* = 18). Numbers preceding the strain names refer to the cross and progeny identifiers, e.g., 16-31 indicates cross number 16 and progeny number 31. (B) 16-21 *hsf2^{KO},bd* (*n* = 17), 16-6*hsf2^{KO},bd* (*n* = 18), *acon-2,bd* (*n* = 17), and *fl,bd* (*n* = 21). (C) 16-31 *bd* (*n* = 17), 16-21*hsf2^{KO},bd* (*n* = 18), *fl,bd* (*n* = 15), and *acon-2,bd* (*n* = 18). The average results of three independent experiments are plotted. HS, heat shock.

transcript is most likely another member of the *hsp70* family, *ssb1* (NCU02075), which shares 87% identity over 239 nucleotides to our 901-nucleotide *hsp70* probe. Of note is that *hsp90* transcripts were significantly more abundant 15 min after the onset of heat shock in the *hsf2,bd* strain than in the *bd* strain. One other significant difference was detected after 45 min of exposure to heat shock between levels of *hsp90a* RNA in the *bd* and *hsf2,bd* strains, with the levels being higher in the *bd* strain.

RNA levels of genes associated with conidiation are affected in the *hsf2* strain. To test where HSF2 acts in the known conidiation pathway, we investigated the expression of *hsf1*, *hsf2* (Fig. 6), *fl*, *conidiation-10* (*con-10*), *repressor of conidiation-1* (*rco-1*), and *eas* (Fig. 7) in the wild-type, *fl*, *acon-2*, and *hsf2* strains. We assayed gene expression upon the transfer of cultures to medium lacking nitrogen, conditions known to induce the conidiation pathway (1). Although there was a general increase in *hsf1* mRNA as development progressed, no significant difference in the levels of either *hsf1* or *hsf2* RNA was detected among the four strains. As previously reported, levels of *fl* and *con-10* RNA in the wild type increase from 6 hours after the transfer of cultures to medium lacking nitrogen (1). Among the aconidial strains, *fl* transcript levels are higher in the *acon-2* and *hsf2* strains at 6 h, whereas *con-10* transcripts show significantly increased levels of expression in the *hsf2* strain (similar to *con-10* levels in the wild-type) compared to those in the *acon-2* and *fl* strains at 0, 6, and 12 h. To our surprise, no differences in the transcript levels of *rco-1*, a re-
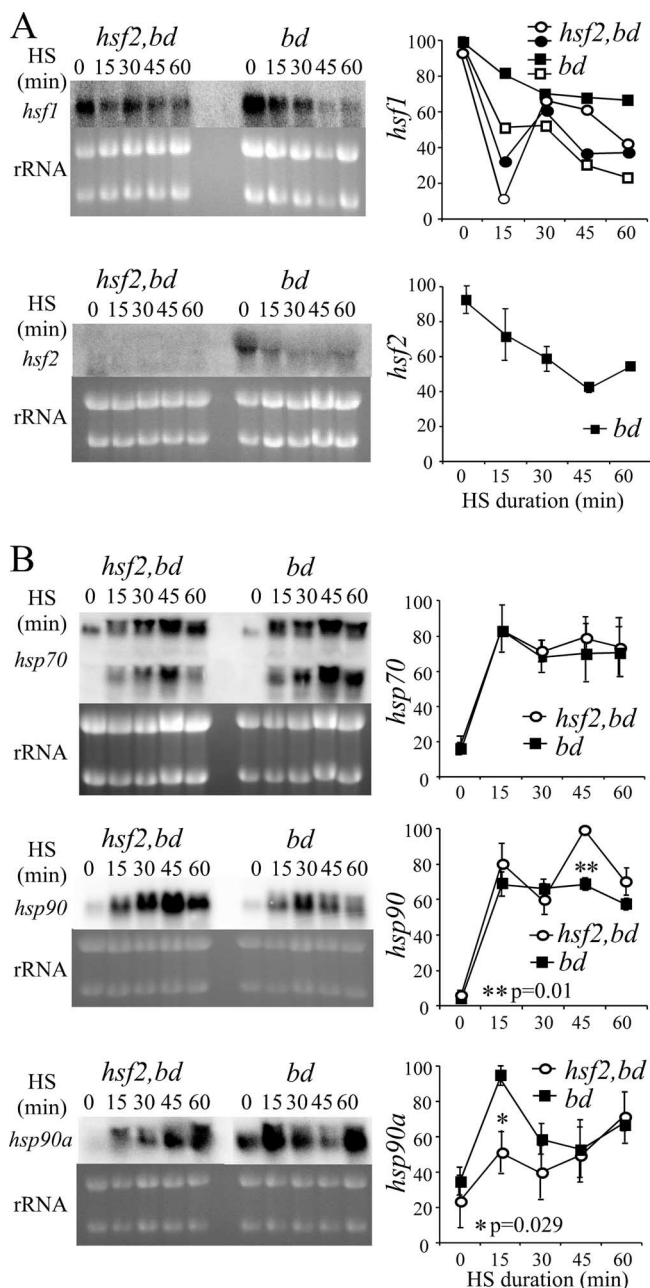

FIG. 5. Gene expression in wild-type and *hsf2,bd* knockout strains. Northern blots of *bd* and *hsf2^{KO},bd* RNA probed for *hsf1* and *hsf2* RNA (A) and *hsp70*, *hsp90*, and *hsp90a* RNA (B). Cultures transferred from LL at 25°C to LL at 45°C were harvested for RNA extraction after 0, 15, 30, 45, and 60 min. There are two sets of data for the *hsf1* transcript and three for all other transcripts. The *y* axes represent the relative amounts of each transcript (percentages of the maximum). HS, heat shock.

pressor of *con-10*, were detected between any of the strains at the time points sampled. In our study, by far the most striking difference between the aconidial strains and the wild type is the greatly reduced levels of *eas* in the *fl*, *acon-2*, and *hsf2* strains.

Conidiation can be induced by a number of environmental cues, for example, carbon and nitrogen deprivation and light (47). Synchronous conidiation can also be induced by the ex-
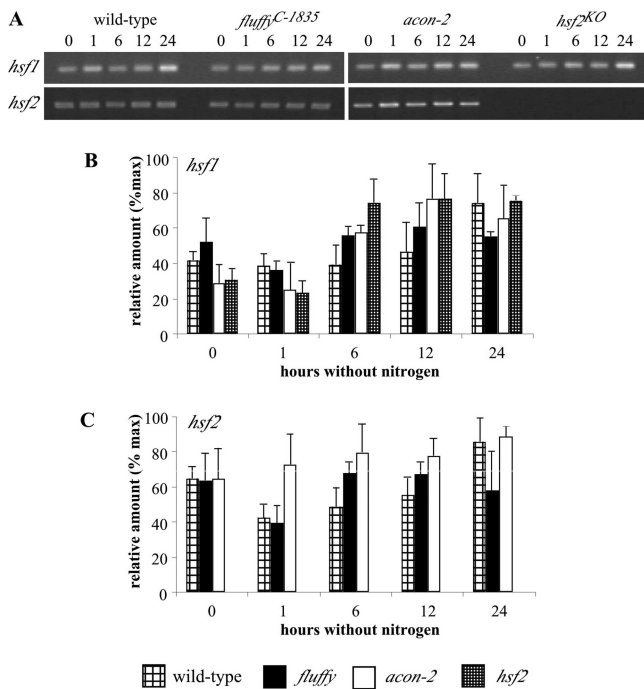
FIG. 6. Semiquantitative RT-PCR of *hsf* and *hsf2* RNA in wild-type, *fl*[C-1835], *acon-2*, and *hsf2*[KO] mutants. Cultures were harvested before and at various times after transfer to medium lacking nitrogen, conditions that initiate conidiation in the wild-type strain. (A) Representative gels showing RT-PCR products of each transcript. (B and C) Relative amounts of each transcript graphed after normalization against *actin*. % max, percentages of the maximum.

posure of mycelia to air (44). Interestingly, although developmentally induced genes are expressed at similar times in cultures induced to conidiate by either nitrogen starvation or exposure to air, evidence suggests that their sensitivity to other stresses, such as heat shock, may change. For example, it is reported that in air-exposed cultures, *con-10* is induced by heat shock, whereas in liquid cultures it is not (26). However, in our hands, no significant difference in the developmental induction of *con-10* expression between the wild-type and *hsf2* strains during air-induced development was revealed, and in neither strain was *con-10* induced by heat shock (Fig. 8). *con-10* has been shown to be regulated by numerous factors e.g., carbon and nitrogen deprivation and light, and it is probable that slight differences in experimental conditions account for the discrepancy between our results and those of Lee and Ebbole (26).

In an effort to determine whether development in the *hsf2* strain is indirectly affected through the misregulation of HSPs, we also assayed expression levels of the *hsp90* transcript. Again, no significant differences in expression were detected (Fig. 7B). Furthermore, strains with *hsp90a* (NCU01792), *hsp30* (NCU09364), *hsp78* (NCU02630), and *hsp101* (NCU00104) deleted showed no overt defect in conidiation (data not shown). While we can conclude that these HSPs are not essential for normal asexual development, *hsp70* (NCU08683) and *hsp90* (NCU04142) are essential genes, and thus, as for *hsf1*, their role in development could not be assessed by simple phenotypic observation of the respective gene knockout strains.

DISCUSSION

The following three genes encoding proteins with domains sharing similarity to known HSFs are present in *Neurospora* (4): *hsf1* and *hsf2*, which are discussed in this paper, and a third gene not included in our study, NCU02413, which encodes *response regulator-2* (*rrg-2*). *rrg-2* contains a truncated HSF DNA-binding domain and is involved in *Neurospora*'s response to oxidative stress (2). HSF1, HSF2a, and HSF2b all contain conserved HSF binding domains and coiled-coil regions that in other HSFs are required for trimerization (43). HSF binding domains recognize and bind to heat shock elements (HSE), consensus sequences found in the promoters of *hsp* genes (28). The basic HSE shows similarity across a wide range of organisms and is based around GAA repeats, the spacing and orientation of which vary and may influence which sites are recognized by different HSFs or differently phosphorylated HSFs (20). For *Neurospora*, HSE are not yet well defined, but nGAAn .. nTTCn motifs (where "n" can be any nucleotide) are present in the promoters of *hsp30* (38) and *hsp70* (21). Additionally, a factor in *Neurospora* protein extracts binds specifically to labeled oligonucleotides containing the classic yeast HSF binding sequence nTTCnnGAAnnTTCn (32). Band shift assays with purified *Neurospora* HSF(s) should aid in the identification of the discriminating consensus binding sites for HSF1 and HSF2.

Our finding that various splice forms of *hsf2* exist is consistent with recent publications from other laboratories. For example, alternative splicing of *hsf* RNA is developmental stage specific in *Schistosoma mansoni* (22), and in *Drosophila* different splice forms are made depending on the stress to which the organism is exposed (16). Moreover, in both *Drosophila* and humans, alternative splicing of *hsf* RNA produces either an activator or a repressor of transcription (45). The repressors retain the DNA-binding and trimerization domains but lack the C-terminal activation domain, similar to *Neurospora* HSF2b and HSF2c. Nonetheless, further work is required to elucidate the conditions under which the three splice variants of *Neurospora hsf2* are expressed and their purpose. Intriguingly, a possible function for HSF2b and HSF2c arises from a study reporting that in response to heat shock, proteins bind to putative HSE and/or stress elements in the *glycogen synthase* gene of *Neurospora* and repress transcription (15). Although we found only low levels of alternatively spliced *hsf* transcripts and observed that these did not correlate with reduced levels of glycogen synthase mRNA (data not shown), this does not rule out the possibility that forms of HSF2 protein change with time and in response to heat shock and/or development.

Whereas *hsf2*[KO] strains are viable but have an aconidial phenotype, we report that *hsf1* is an essential gene. No homokaryon *hsf1*[KO] transformants were obtained, and no viable *hsf1*[KO] progeny were obtained from crosses of *hsf1*[KO] and *hsf1*[+] heterokaryons with the wild type. The single *S. cerevisiae* and *Drosophila* HSF genes are also essential. In yeast, evidence suggests that HSF is required for the expression of both the constitutive and heat-inducible forms of Hsp90 (Hsc82 and Hsp82), and in the *S. cerevisiae hsf-82* mutant, in which Hsp82 is no longer induced upon heat shock, spindle pole body formation is affected and completion of the cell cycle at 37°C is blocked (51). Moreover, indirect effects of Hsp90 misexpression on cell wall integrity, for example, occurs via a reduction
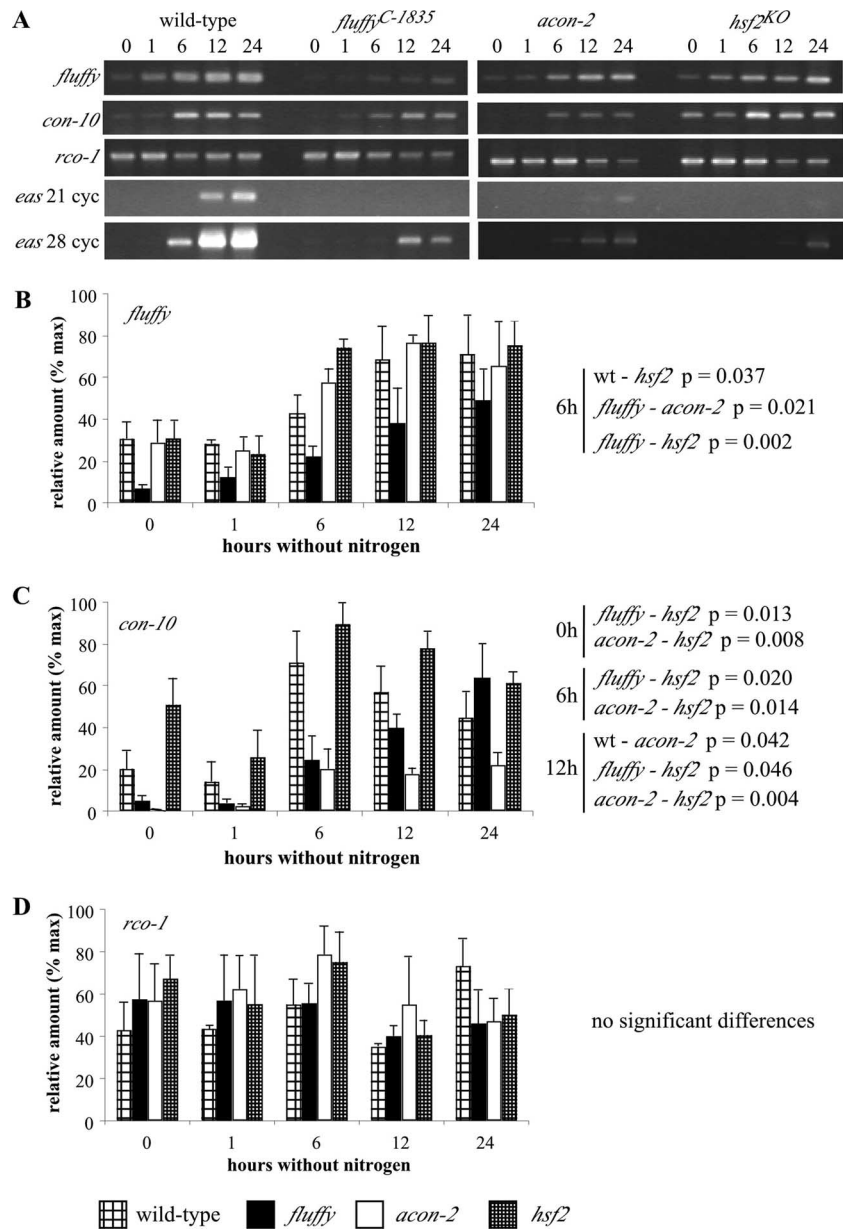
FIG. 7. Comparison of *fl*, *con-10*, *rco-1*, and *eas* expression in wild-type and conidiation-defective strains of *Neurospora*. Semiquantitative RT-PCR of *fl*, *con-10*, *rco-1*, and *eas* RNA in the wild type and the *fl*[C-1835], *acon-2*, and *hsf2*[KO] mutants. Cultures were harvested before and at various times after transfer to medium lacking nitrogen, conditions that initiate conidiation in the wild-type strain. (A) Representative gels showing RT-PCR products of each transcript. (B, C, and D) Relative amounts of each transcript graphed after normalization against *actin*. % max, percentages of the maximum; wt, wild type.

in Slt2 kinase activity and a consequent lack of transcriptional activation of a subset of cell wall genes (46). The essential nature of *Neurospora hsf1* would be easily explained if future experiments to elucidate its targets reveal a subset of genes that encode constitutive as well as heat shock-induced protein chaperones.

Our results suggest that HSF2 may have a role in regulating the level of HSP transcripts. We looked at the levels of *hsp70*, *hsp90*, and *hsp90a* specifically, because in other organisms the interaction of chaperones *hsp70* and *hsp90* with hormone receptors, kinases, and other signaling molecules is well known

(reviewed in reference 34) and the inhibition or overexpression of these chaperones is associated with defects in development in *Drosophila* and in yeast (40, 12, 51, 46). The putative protein encoded by NCU01792 contains a CS domain, a domain that binds to Hsp90 and which is found in p23, a Hsp90 cochaperone (27). In our experiments, there were no significant differences between *hsp70* transcript levels in the *bd* and *hsf2,bd* strains. However, immediately following heat shock, *hsp90a* RNA is significantly lower in the *hsf2,bd* strain compared to levels in the wild type, and *hsp90* transcript levels are higher than those seen for the wild type 45 min after exposure to heat
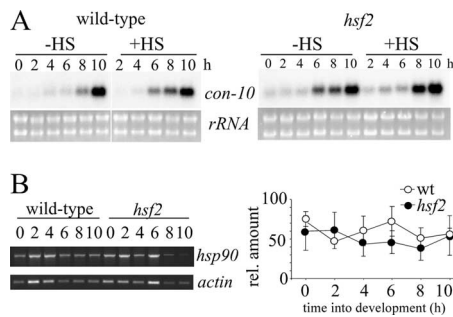
FIG. 8. con-10 and hsp90 expression during development in air-exposed cultures. (A) Northern blots of wild-type and hsf2 total RNA probed for con-10. Mycelial mats were taken out of liquid cultures and exposed to air at time zero. Control mats (no heat shock [−HS]) were harvested at 2-h intervals. Heat-shocked samples (+HS) were exposed to 50°C for 1 hour before being harvested. (B) (Left) Representative gel showing semiquantitative RT-PCR products of hsp90 and actin transcripts; (right) quantitation of hsp90 RNA from the same samples (n = 3). rel., relative; wt, wild type.

shock. Both of these departures from wild-type levels of gene expression could be directly due to the lack of HSF2.

Deleting hsf2 had a dramatic effect on asexual development; aerial hyphae developed but no conidial chains formed. The following six mutants blocked at different stages of conidial development have been placed in a conidiation pathway: the fld (linkage group IV [LGIV]), fl (LGII), acon-2 (LGIII), and acon-3 (LGIV) mutants and two conidial separation mutants, the csp-1 and csp-2 mutants. In addition, several recently generated knockout strains have been recorded as aconidial, including strains with mutations in vegetative growth and asexual development (the vad-2 [LGI] and vad-4 [LGIII] strains). hsf1 and hsf2 are both located on LGII, separated by approximately 100 kbp, and do not map to any of the previously described aconidial mutants. Bailey-Shrode and Ebbole place acon-2 upstream of fl and acon-3 in the pathway to conidiation (1). In this scheme, fl regulates acon-3 and eas, a gene encoding a hydrophobin found on the surfaces of macroconidia (3, 24), while acon-3 regulates con-6 and con-10. The rco-1 gene product represses the expression of eas, con-6, and con-10 (50, 26, 1). We were interested to find out if HSF2 is an integral element of the proposed pathway or whether it acts as a facilitator ensuring that the necessary chaperones are present to ensure the correct conformation of pathway components.

After initiation of development induced by the transfer of cultures to medium lacking nitrogen we found, in agreement with previous reports, that fl transcript levels in the wild type increase after 6 hours (1). fl is a transcriptional activator and a major regulator of conidiation in Neurospora (1, 39), whose expression has previously been reported to be low in the acon-2 strain (1). However, we found that fl was increased in all three aconidial strains, data which suggest that ACON2 regulates some posttranscriptional aspect of fl gene expression. In keeping with the lack of spore formation in the acon-2 and fluffy mutants, levels of con-10 are low in these strains. With the exception of eas, and the higher levels of fl at 6 h, all of the transcripts assayed throughout development showed similar levels of expression in the hsf2 and wild-type strains.

One scenario that could account for the aconidial phenotype

of the hsf2^{KO} strain is that the induction of HSPs that interact with components of the conidiation pathway are no longer sufficiently abundant for conidiation to proceed. Alternatively, HSF2 or a HSF2-induced protein is an integral component of the developmental pathway. We were unable in this study to distinguish conclusively between these possibilities. On the one hand, asexual development is normal in strains with different hsp genes deleted, and no significant differences in hsp90 expression over the course of development were detected. Moreover, levels of many of the transcripts we assayed show wild-type expression patterns in the hsf2 strain, which would be not be expected if protein folding is affected. On the other hand, some hsp genes are essential for survival, and their effect on asexual development could not be easily tested. However, if HSF2 or a HSF2-induced protein is indeed an integral part of the conidiation program, the gene expression data we have place HSF2 in a pathway parallel to that of fluffy. In support of this conclusion, wild-type fl and con-10 (repressed in a fluffy mutant) transcript levels are seen throughout development in the hsf2 strain. Hence, while acon-2 and fluffy can regulate the level of both con-10 and eas, the hsf2 strain must regulate levels of eas and other as yet unidentified genes essential for normal development via an independent pathway.

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