

# Cloning of a novel transcription factor-like gene amplified in human glioma including astrocytoma grade I

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Received November 6, 1996; Revised and Accepted July 9, 1997

**Gene amplification, which is generally considered to occur late in tumor development, is a common feature of high grade glioma. Up until now, there have been no reports on amplification in astrocytoma grade I. In this study, we report cloning and sequencing of a cDNA termed glioma-amplified sequence (*GAS41*) which was identified recently in a glioblastoma cell line by microdissection-mediated cDNA capture. This technique is tailored to isolate amplified genes from human tumors. An increased copy number of *GAS41* was found in glioblastoma multiforme and astrocytoma III, and at a high frequency in astrocytoma grades I and II. Sequence comparison indicates a high homology between the *GAS41* protein, the yeast and human AF-9 and the human ENL proteins. Both AF-9 and ENL belong to a new class of transcription factors, indicating that *GAS41* might also represent a transcription factor. With *GAS41* being the first gene found with increased copy number in low grade glioma, this study provides the first evidence that gene amplification can occur in early tumor development.**

## INTRODUCTION

Gene amplification occurs frequently in human tumors and was proposed to contribute to tumorigenesis. Specifically, there is ample evidence for a prevalent role of amplification in human gliomas. Astrocytic gliomas are the most common human tumors of the central nervous system. According to the World Health Organization (WHO), astrocytomas were divided into four groups of different grades: pilocytic astrocytoma WHO grade I, astrocytoma WHO grade II, anaplastic astrocytoma WHO grade III and glioblastoma multiforme (GBM) WHO grade IV. Southern blot analysis revealed amplification of several genes including *EGFR*, *TGF $\alpha$* , *PDGFR $\alpha$* , *GLI*, *MET*, *MDM2*, *SAS*, *CDK4* and *N-MYC* in human glioma (1–6). Comparative genomic

hybridization identified amplifications at chromosome regions 1q32.1, 5p15.1, 9p2, 9q12–13, 7q21, 12p and 22q12 (7–10).

As of yet, however, gene amplifications in gliomas seem to be restricted mostly to GBM and, at a lower frequency, to anaplastic astrocytoma. Independent studies on pilocytic astrocytoma and astrocytoma WHO grade II failed to reveal amplification of *EGFR*, *PDGFR $\alpha$*  and genes from chromosome region 12q13–15, including *MDM2*, *CDK4*, *SAS*, *GLI*, *A2MR*, *WNT1*, *GADD153* and *ERBB3* (1,2,5,11). Previously, we reported a single case of *MET* amplification in a grade II astrocytoma (12).

To gain further insight into the biology of amplifications in glioma, we recently identified several cDNAs from a homogeneously staining region (HSR) at 12q13–15 by microdissection-mediated cDNA capture (13). cDNA was generated from tumor cells, hybridized against metaphases of the tumor cells and recovered by microdissection of the HSR. Bound cDNAs were amplified by PCR and mapped to the amplified domain at 12q13–15.

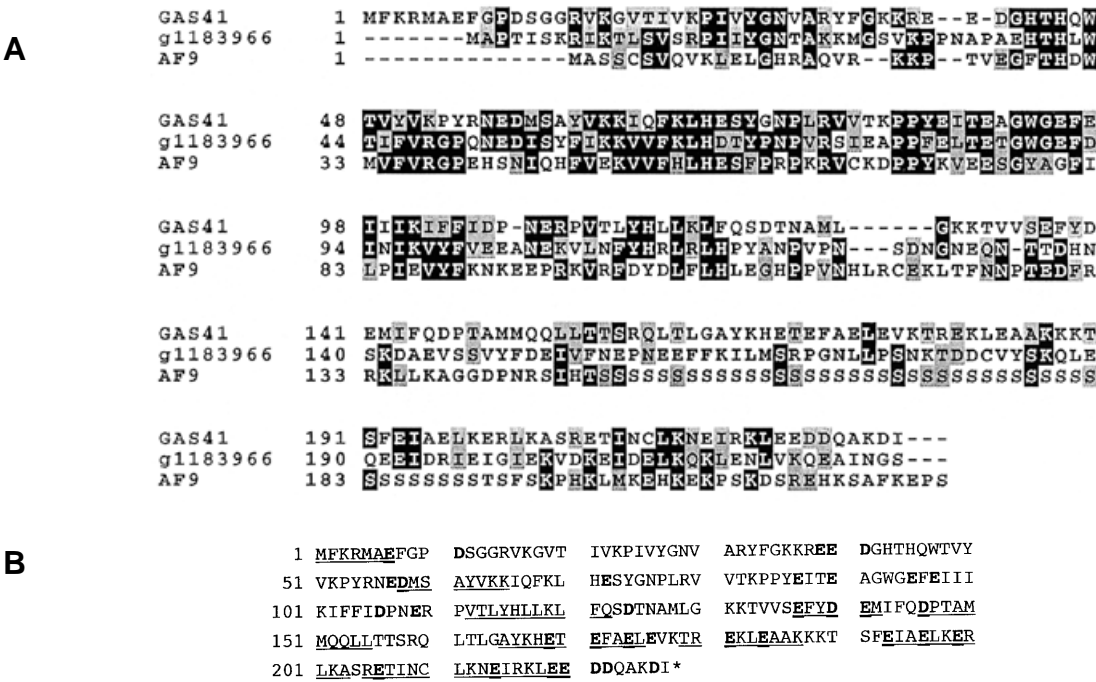
Here, we set out to isolate and characterize the corresponding cDNA (*GAS41*) of a microdissected cDNA fragment which maps within the amplicon at 12q13–15. Sequence comparison was performed and the gene *GAS41* was tested for amplification in high and low grade gliomas, including pilocytic astrocytoma.

## RESULTS

### Cloning and sequencing *GAS41* cDNA

Plasmid clone pGAS41 containing a 220 bp insert (GenBank accession No. U61384) was isolated previously from a homogeneously staining region of glioblastoma TX3868 (13). To isolate the corresponding cDNA, we established a  $\lambda$ ZAP Express (Stratagene) cDNA library from glioblastoma cell line TX3868. Using the partial cDNA fragment of pGAS41 (220 bp) as probe, positive phage clones (two positive clones per 10 000 p.f.u.) were identified and *in vivo* excised into plasmid clones. The newly isolated cDNAs were digested with *EcoRI* and *XhoI* and reconfirmed by Southern hybridization using the pGAS41 insert as probe. Sequencing of clone *GAS41* identified the 220 bp

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**Figure 1.** (A) Alignment of the putative protein sequence of GAS41 with homologous database sequences. Identical amino acids are inverted, similar amino acids are shaded. Gaps inserted by the sequence alignment software are indicated by '-'. GAS41, amino acid sequence of the putative protein deduced from the *GAS41* cDNA sequence; g1183966, amino acid sequence of a *Saccharomyces cerevisiae* protein similar to human AF-9 (GenBank accession No. gi1183966); AF9, amino acid sequence of the human AF-9 protein. (B) Predicted amino acid sequence of GAS41 with acidic amino acids shown in bold letters and regions with a high probability of forming  $\alpha$ -helical structures underlined.

sequence used for hybridization and revealed an open reading frame (ORF) coding for a protein of 227 amino acids.

**GenBank homology search and protein sequence analysis**

Homology searches of *GAS41* cDNA were performed using both BLASTX and BLASTN algorithms. BLASTN search revealed homology (62%) to the *Saccharomyces cerevisiae* AF-9 gene. BLASTX search revealed high homologies to several proteins including *S.cerevisiae* AF-9 protein, with 53% identity and 80% similarity, human AF-9 protein, with 39% identity and 63% similarity (Fig. 1A), and human ENL protein, with 39% identity and 63% similarity. AF-9 and ENL proteins were supposed to belong to a new class of transcription factors, suggesting a possible role for the newly isolated GAS41 (14) in transcription regulation.

The function of GAS41 as a transcription factor is supported by the finding of an enrichment of acidic amino acids (27%) found within a stretch of 60 amino acids in the C-terminal region. Protein structure-predicting programs indicate a high probability of this region forming  $\alpha$ -helices. Negatively charged  $\alpha$ -helical structures were the first defined activation domains in eukaryotic transcription factors. (15). The predicted amino acid sequence of GAS41 is shown in Figure 1B.

**Amplification analysis**

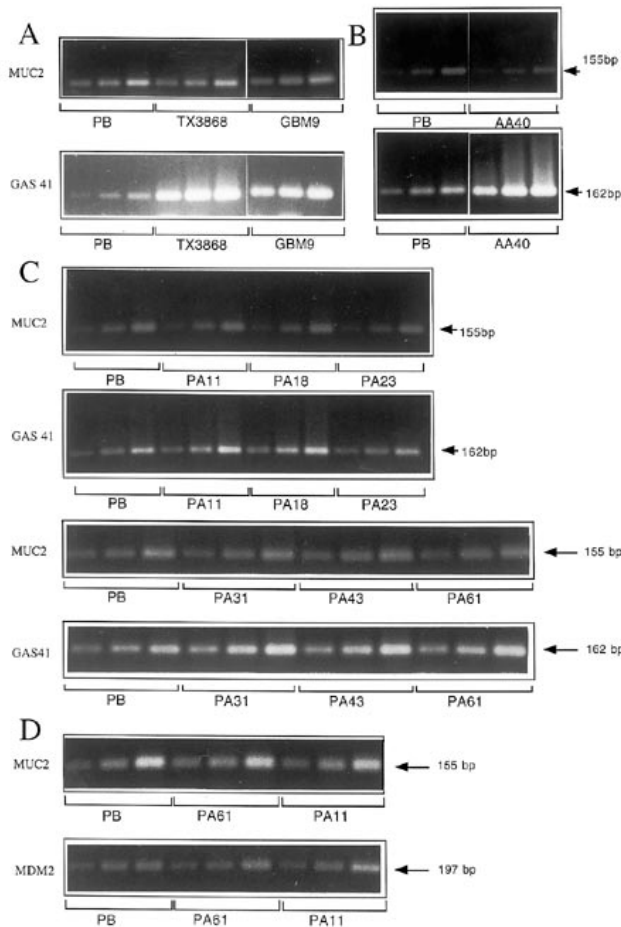
To gain further insight into the role of GAS41 amplifications in human glioma, we investigated 64 gliomas for gene amplification including 52 glioblastomas, four anaplastic grade III astrocytomas, two grade II astrocytomas and six pilocytic grade I astrocytomas. The tumors were analyzed preferentially by comparative PCR

(16) using primers located in the 5'-untranslated region of *GAS41* cDNA and *MUC2* primers (18) as a single copy control. To confirm the data obtained by comparative PCR, we analyzed selected tumors by Southern hybridization using *GAS41*, *SAS*, *CDK4* and *WNT1* as probes. In the majority of cases, however, the limited amounts of DNA were not sufficient for Southern blot analysis. Furthermore, probes for *GAS41* did not hybridize efficiently to single or low copy targets.

Representative amplification analysis by comparative PCR is shown in Figure 2. As positive control in our comparative PCR screening, we used DNA from glioblastoma TX3868 (Fig. 2A) which contains a *GAS41* amplification as previously demonstrated by Southern hybridization (13). Comparative PCR detected *GAS41* amplifications in gliomas of all grades, including glioblastoma as shown in Figure 2A, anaplastic astrocytoma as shown in Figure 2B and in pilocytic astrocytoma as shown in Figure 2C. Interestingly, the *MDM2* gene, which is also localized at 12q13-15, was not amplified in pilocytic astrocytoma as determined by comparative PCR (Fig. 2D).

As demonstrated for a subset of glioblastomas, the *GAS41* amplifications were also identifiable by Southern blot analysis. The same subset of glioblastomas showed co-amplification of the genes *SAS* and *CDK4* but did not reveal *WNT1* amplification. Examples of the amplification analysis by Southern blot hybridization are given in Figure 3.

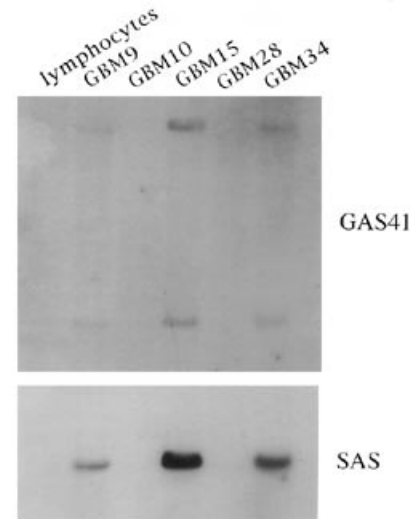
In total, our study revealed *GAS41* amplification in 20 of 64 tumors. Specifically, *GAS41* amplification was detected in 12 out of 52 glioblastomas (23%), two out of four grade III anaplastic astrocytomas (50%), one out of two grade II astrocytomas (50%) and five out of six (80%) pilocytic astrocytomas. Although comparative PCR does not allow a quantitative comparison of



**Figure 2.** Amplification analysis of *GAS41* in gliomas of different origin using comparative PCR. Three different concentrations of tumor and blood DNA were each amplified by PCR using primers specific for *GAS41* (lower panel) and *MUC2* (upper panel) as single copy control. PCR product sizes are indicated by arrows. (A) DNA from peripheral blood lymphocytes (PB), from glioblastoma multiforme (GBM) TX3868 with known amplification as positive control and from glioblastoma multiforme GBM9. Amplification of *GAS41* was detected in TX3868 and GBM9. (B) DNA from PB and from anaplastic astrocytoma AA40. Amplification of *GAS41* was detected in AA40. (C) DNA from PB and from pilocytic astrocytomas PA11, PA18, PA23, PA31, PA43 and PA61. Amplification of *GAS41* was detected in PA11, PA18, PA31, PA43 and PA61. PA23 shows no *GAS41* amplification. (D) Amplification analysis of *MDM2* in pilocytic astrocytomas. DNA from PB and from pilocytic astrocytomas PA61 and PA11 was amplified by PCR using primers specific for *MUC2* and *MDM2*. Amplification of *MDM2* was not detectable in PA11 and PA61.

amplification levels, our data indicate that the level of *GAS41* gene amplification is lower in pilocytic astrocytoma than in anaplastic astrocytoma and glioblastoma (Fig. 2). Results of the amplification analysis are summarized in Table 3.

Two pilocytic astrocytomas which were shown to harbor a *GAS41* amplification were selected for expression analysis by RT-PCR. Unfortunately, the amount of RNA was not sufficient for Northern hybridization. *GAS41* was found to be expressed in normal brain and in both pilocytic astrocytomas. For control purposes, we analyzed the expression of the gene for the heat shock factor 2 (*HSF2*) which is also located on chromosome 12. In comparison with the *HSF2* gene, the PCR products obtained for *GAS41* are stronger in pilocytic astrocytoma than in normal human brain (Fig. 4).



**Figure 3.** Amplification analysis by Southern hybridization using *GAS41* (upper panel) and *SAS* (lower panel) as hybridization probes. *GAS41* and *SAS* were found to be co-amplified in glioblastoma (GBM) cases 9, 15 and 34.

**Table 3.** *GAS41* amplification in gliomas of different grades

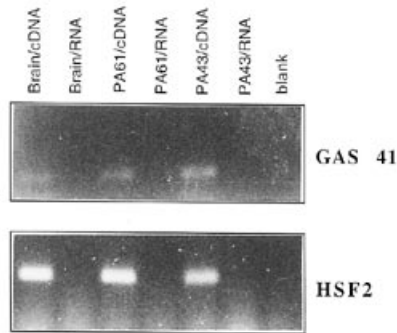
Tumor	Total no.	No. of amplified cases	Percentage
GBM	52	12	23
AIII	4	2	50
AII	2	1	50
PA	6	5	80
Total	64	20	31

## DISCUSSION

Sequence analysis and comparison of *GAS41* with known protein sequences revealed high homology of *GAS41* protein to yeast and human AF-9 protein and human ENL protein. The *AF-9* and *ENL* genes encode proteins which are involved frequently in translocation events and were suggested to belong to a new class of transcription factors (14). Both proteins share extensive sequence homologies in the N-terminal region and several common protein motifs including a proline-rich domain in the N-terminal overlapping sequence. Interestingly, the region of *GAS41* with the highest sequence homology corresponds to the N-terminal proline-rich regions of *AF-9* and *ENL*. This finding indicates a possible similar biological function of *GAS41* and the transcription factors *AF-9* and *ENL*.

The amino acid sequence of *GAS41* contains a much higher percentage (27%) of acidic amino acids in the C-terminal region than on average found in proteins (11.7%). Significant negatively charged acidic  $\alpha$ -helical structures were the first defined activation domains in eukaryotic transcription factors. Acidic activation domains were proposed to interact in a relatively non-specific manner with components of the initiation complex (15). In *GAS41*, a stretch of 40 amino acids starting at position 136 meets the criteria for an acidic activation domain. Besides 22% of acidic





**Figure 4.** Expression analysis of pilocytic astrocytomas with *GAS41* amplification using RT-PCR. DNA-free RNA from two pilocytic astrocytomas and normal brain was reverse transcribed and used for PCR with primers for *GAS41* (upper panel) and for *HSF2* (lower panel). PCR products specific for *GAS41* and *HSF2* transcripts were detected in normal brain, PA61 and PA43. Corresponding RNA samples which were not reverse transcribed did not yield PCR products.

amino acids, the region is negatively charged, and protein structure prediction programs indicate  $\alpha$ -helical structures.

In contrast to AF-9 and ENL, *GAS41* lacks a typical DNA-binding domain for transcriptional activation (15). It is conceivable that *GAS41* protein activates transcription in combination with a second protein containing a DNA-binding domain. Herpes simplex virus trans-activator VP16 is an example of transcription regulation by protein-protein interactions. The VP16 protein activates transcription of early viral genes by attachment to host-encoded proteins that recognize DNA sequences of viral promoters (17). Interestingly, VP16 also lacks a DNA-binding domain but contains a C-terminal acidic activation domain. These data are consistent with a function of *GAS41* as a transcriptional activator.

Using the complete sequence of *GAS41* presented above in *in situ* hybridization experiments, we were able to confirm the localization (data not shown) of *GAS41* within chromosome region 12q13–15, a region which frequently is involved in gene amplifications in gliomas. Recently, we demonstrated amplification of 12 different genes within a single amplified domain at 12q13–15 in glioblastoma (19). Reifemberger and co-workers showed frequent amplification of the genes *CDK4* and *SAS* in the 12q13–15 amplicon in human malignant glioma. However, these and additional studies failed to reveal 12q13–15 gene amplifications in low grade gliomas. Likewise, amplification of the *EGFR* gene has only been detected in glioblastoma and astrocytoma grade III. Here, we demonstrate the amplification of *GAS41* in pilocytic astrocytoma and astrocytoma grade II.

As for the amplification level, pilocytic astrocytomas contain a relatively high number of normal brain cells including oligodendrocytes, astrocytes and neurons, complicating the detection of amplification. This histological feature of low grade gliomas may account for low levels of amplification which were only detectable by comparative PCR but not by Southern blot analysis. An enrichment of tumor cells prior to amplification analysis will allow a more adequate assessment of the amplification level in the tumor cells. Alternatively, the amplification level in single cells could be analyzed by *in situ* hybridization.

The amplification frequencies of *GAS41* in glioblastoma and anaplastic astrocytoma are similar to those found for *CDK4* and *SAS*. While the amplification frequency of other genes appears to

increase during tumor progression, our data do not show a comparable correlation between tumor progression and *GAS41* amplification frequency. *GAS41* amplification frequency was detected in 23% of glioblastomas and 80% of grade I astrocytomas. Although a very limited number of low grade gliomas have been analyzed in this study, our results clearly indicate amplification of *GAS41* not only in late tumor progression but also in early tumor development. This finding is in contrast to a generally held belief of amplification as a late event in tumor progression. To verify further the idea of gene amplification as an early event in tumorigenesis, additional genes need to be tested for amplification in different tumor types. Specifically, genes which are localized in the vicinity of *GAS41* can be analyzed for co-amplification. As demonstrated for *SAS* and *CDK4*, both mapping at 12q13–15, co-amplification has been detected in several glioblastomas; however, no amplification of *MDM2*, also localized at 12q13–15, could be detected in pilocytic astrocytomas.

A final conclusion about the biological role of *GAS41* in early tumor development cannot be drawn unless all amplified genes in the vicinity of *GAS41* have been identified and analyzed in tumors of different grades. The directed identification and isolation of these genes will be facilitated greatly by methods such as microdissection-mediated cDNA capture. Up until now, the majority of amplified genes have been identified by screening known genes for amplification. The increasing use of methods tailored to identify amplified genes will help to analyze early amplification events and, in the long run, might alter our perspectives on the role of gene amplification in tumorigenesis.

In conclusion, *GAS41*, which most probably represents a transcription factor, is the first gene to be amplified in all grades of human glioma, including pilocytic astrocytoma.

## MATERIALS AND METHODS

### cDNA library construction and screening

cDNA was synthesized from mRNA of TX3868 glioblastoma cells using oligo(dT) primers. Second strand synthesis was performed according to the manufacturer's instructions (Stratagene). Following *EcoRI* adaptor ligation and digestion with *XhoI*, the cDNA fragments were ligated into ZAP Express™ vector. Packaging was done using Gigapack III Gold packaging extract from Stratagene. The library was amplified once prior to cDNA library screening. Phages were plated for screening at a density of 5000 p.f.u. per 132 mm plate. Phage lift hybridization was performed using <sup>32</sup>P-labeled *GAS41* insert DNA. Positive phage clones were *in vivo* excised into pBK-CMV phagemids according to the manufacturer's protocols (Stratagene), and plasmid DNA was digested with *EcoRI* and *XhoI* to release the insert DNA. In a second screening round, positive clones were identified by Southern hybridization. In brief, digested plasmid DNA was separated by gel electrophoresis and blotted onto nylon membrane. Subsequently, the membrane was hybridized with <sup>32</sup>P-labeled *GAS41* insert DNA.

### Sequencing and sequence analysis

Plasmid DNA of *GAS41* was isolated for sequencing using the Quiagen-Mini kit. Sequencing was performed using vector-specific primers close to the cloning site (T7 vector site: 5' ACC CGG GTG GAA AAT CGA TGG 3', T3 vector site: 5' ACA AAA GCT

GGA GCT CGC GCG 3'). Sequencing reactions were run on an ABI automated sequencer for 14 h.

Sequence comparison was performed using BLASTN and BLASTX algorithms. The predicted amino acid sequence was deferred using the MacVector program. The protein structure prediction program was SSP by V.V. Solovyev and A.A. Salamov (20) made available through the BCM launcher service.

### Southern blot analysis

Isolation of high molecular weight DNA from cell cultures and frozen tissue samples was performed according to standard protocols. In brief, DNA (5 µg) from peripheral blood lymphocytes and from tumor samples was digested with *EcoRI*, separated on a 0.8% agarose gel and blotted onto a nylon membrane (GeneScreen). Southern hybridization was carried out in a 500 mM phosphate buffer (pH 7.2) as described previously (9,10). Insert DNA (50 ng) was labeled with <sup>32</sup>P by the random primer method of Feinberg and Vogelstein (21).

### Comparative PCR

Concentrations of tumor DNA and peripheral blood DNA were determined by optical density measurement. A set of dilutions (0.5, 1.0 and 2 ng/µl) was made of each DNA, and 10 µl of each dilution were used for PCR amplification. For calibration purposes, PCR was performed with primers specific for the *MUC2* gene (17) which serves as a single copy control. PCR for the *MUC2* gene was for 27 cycles at 58°C annealing temperature with 1.5 mM MgCl<sub>2</sub>. The PCR products were separated in agarose gels and stained with Sybr Green I. In case of variations in signal intensity between blood DNA and corresponding tumor DNAs, the amount of the PCR templates was adjusted accordingly. For amplification analysis, calibrated amounts of tumor DNA and corresponding amounts of blood DNA were amplified with the *GAS41* primers. Enhanced signal intensities in all three dilutions of a given tumor DNA set indicate gene amplification. PCR for the *GAS41* gene was for 27 cycles at 60°C annealing temperature with 1.0 mM MgCl<sub>2</sub>. The primer sequences for *GAS41* are as follows: forward primer: 5' AGT TGA CGG TTA CTC ACC GC 3'; reverse primer: 5' ATT GTC CCA CGG AAG AAG C 3'. PCR for the *MDM2* gene was performed using the *MDM2* primer described by Elkahoul *et al.* (22) for 27 cycles at 60°C.

### RT-PCR

Prior to RT-PCR, 1.5 µg of RNA of each sample were treated with DNase I (RNase-free). After DNase I treatment, 50 ng of each sample were used for Alu-PCR to exclude any genomic DNA contamination. The remaining DNA-free RNA was divided into 450 ng of RNA used later in control experiments and 1 µg of RNA used for reverse transcription with the RT-PCR kit from Stratagene. Following reverse transcription, 1/10 of the total reaction volume (equal to 100 ng of RNA) was used for two separate PCR reactions, one using *GAS41* primers and one using *HSF2* gene primers (*HSF2*for: AGA ATT TGG AAT CCC TGC G, *HSF2*rev: AGT TGC CTC ACA AAG CTT GC). Both sets of PCR included several controls. One hundred ng of the corresponding RNAs were used to demonstrate that the RNA is DNA free, and the blank controls were run without any template. Both PCR reactions were run for 29 cycles on the same thermal cycler with

the identical PCR conditions as described for comparative PCR. The total volume of the PCR reaction was run on a 2% agarose gel and visualized by ethidium bromide staining.

### ACKNOWLEDGEMENTS

This work was supported by a Grant from the Deutsche Forschungsgemeinschaft (SFB 399, A1) and by a Grant from the Hedwig Stalter Stiftung.

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