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A Functional Module of Yeast Mediator That Governs the Dynamic Range of Heat-Shock Gene Expression

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ABSTRACT

We report the results of a genetic screen designed to identify transcriptional coregulators of yeast heat-shock factor (HSF). This sequence-specific activator is required to stimulate both basal and induced transcription; however, the identity of factors that collaborate with HSF in governing noninduced heat-shock gene expression is unknown. In an effort to identify these factors, we isolated spontaneous extragenic suppressors of *hsp82-ΔHSE1*, an allele of *HSP82* that bears a 32-bp deletion of its high-affinity HSF-binding site, yet retains its two low-affinity HSF sites. Nearly 200 suppressors of the null phenotype of *hsp82-ΔHSE1* were isolated and characterized, and they sorted into six *e*xpression *w*ithout heat-shock *e*lement (*EWE*) complementation groups. Strikingly, all six groups contain alleles of genes that encode subunits of Mediator. Three of the six subunits, Med7, Med10/Nut2, and Med21/Srb7, map to Mediator's middle domain; two subunits, Med14/Rgr1 and Med16/Sin4, to its tail domain; and one subunit, Med19/Rox3, to its head domain. Mutations in genes encoding these factors enhance not only the basal transcription of *hsp82-ΔHSE1*, but also that of wild-type heat-shock genes. In contrast to their effect on basal transcription, the more severe *ewe* mutations strongly reduce activated transcription, drastically diminishing the dynamic range of heat-shock gene expression. Notably, targeted deletion of other Mediator subunits, including the negative regulators Cdk8/Srb10, Med5/Nut1, and Med15/Gal11 fail to derepress *hsp82-ΔHSE1*. Taken together, our data suggest that the Ewe subunits constitute a distinct functional module within Mediator that modulates both basal and induced heat-shock gene transcription.

WHEN exposed to thermal or chemical stress, organisms respond by vigorously transcribing genes encoding heat-shock proteins (HSPs). HSPs function as molecular chaperones and protect the cell—along with ubiquitin, proteases, metallothioneins, and antioxidant enzymes—from damage caused by the expression of misfolded proteins. In the yeast *Saccharomyces cerevisiae*, the expression of heat-responsive genes is stimulated by the sequence-specific transcriptional activator heat-shock factor (HSF) Hsf1 (ScHSF) (Sorger and Pelham 1988; Nieto-Sotelo *et al.* 1990; Sorger 1990). In response to metabolic, oxidative, or osmotic stress, the transcription of a number of *HSP* genes is additionally enhanced by the gene-specific activators Msn2/Msn4 and Skn7 (Boy-Marcotte *et al.* 1998; Treger *et al.* 1998; Gasch *et al.* 2000; Raitt *et al.* 2000; Amoros and Estruch 2001; Kandror *et al.* 2004). Nonetheless, the only activator known to promote basal heat-shock gene transcription is HSF (McDaniel *et al.* 1989; Park and Craig 1989; Erkine *et al.* 1996). Whether this basal expression is an indirect consequence of HSF's role in establishing and maintaining a nucleosome-remodeled ("nucleosome-free") structure over the transcription start site (Gross *et al.* 1993; Erkine *et al.* 1996), or whether HSF plays a more direct role in recruiting transcriptional coactivators under noninducing conditions, is unknown.

HSF is of additional interest, given that it can activate its target genes in the absence of several key general transcription factors (GTFs). These include Taf9 ($TAF_{II}17$, constituent of both SAGA and TFIID), Med17/Srb4 and Med22/Srb6 (both subunits of Mediator), TFIIA, Kin28 (TFIIH kinase), and even the C-terminal domain (CTD) of the large subunit of RNA polymerase II (Apone *et al.* 1998; Lee and Lis 1998; McNeil *et al.* 1998; Moqtaderi *et al.* 1998; Chou *et al.* 1999). Moreover, activated HSF has been shown to mediate gene-wide histone displacement and can do so in the absence of prominent chromatin remodeling (Swi/Snf), histone modification (Set1, Gcn5), and transcriptional elongation (Paf1) complexes (Zhao *et al.* 2005). These observations suggest the possibility that HSF uses a novel route for transcriptional activation of its target genes, a notion

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supported by artificial recruitment experiments. The latter has led to the suggestion that HSF's C-terminal activation domain can activate transcription via the Rgr1 subcomplex of Mediator (Lee *et al.* 1999).

Mediator is an evolutionarily conserved transcriptional coregulator, composed of 25 subunits in yeast, that integrates signals from sequence-specific activators and repressors to the general transcriptional machinery (GTM) (reviewed in Malik and Roeder 2000; Myers and Kornberg 2000; Rachez and Freedman 2001; Boube *et al.* 2002). Originally described as an activity in fractionated yeast extracts that stimulated both basal and activator-dependent transcription (Kelleher *et al.* 1990; Flanagan *et al.* 1991), Mediator physically interacts with the pol II CTD and under certain conditions copurifies with the 12-subunit core pol II as a holoenzyme (Koleske and Young 1994). Its association with pol II is reversible: it binds tightly to the hypophosphorylated, recruitment-competent pol IIa isoform, but very weakly (if at all) to hyperphosphorylated, elongation-competent pol IIo (Svejstrup *et al.* 1997). This is consistent with findings, both *in vivo* and *in vitro*, that Mediator remains at the promoter following escape of pol II (Pokholok *et al.* 2002), where it may serve to facilitate reinitiation (Yudkovsky *et al.* 2000). In addition, at several yeast promoters, recruitment of Mediator has been found to precede that of pol II (Bhoite *et al.* 2001; Cosma *et al.* 2001; Bryant and Ptashne 2003), demonstrating that it can exist free of RNA polymerase *in vivo*. Mediator has been shown to associate exclusively with the UAS of *GAL* genes and does so independently of pol II and GTFs (Kuras *et al.* 2003).

Biochemical and electron microscopic analysis of yeast Mediator have indicated the presence of three discrete domains: the "head," composed of Med6, Med8, Med11, Med17/Srb4, Med18/Srb5, Med19/Rox3, Med20/Srb2, and Med22/Srb6; the "middle," composed of Med1, Med4, Med7, Med9, Med10/Nut2, Med21/Srb7, and Med31/Soh1; and the "tail," composed of Med2, Med3/Hrs1, Med5/Nut1, Med14/Rgr1, Med15/Gal11, and Med16/Sin4 (Boube *et al.* 2002; Beve *et al.* 2005). A kinase module, composed of Cdk8/Srb10, CycC/Srb11, Med12/Srb8, and Med13/Srb9, loosely associates with the 21-subunit core Mediator complex (Liu *et al.* 2001). Biochemical isolation of a stable Rgr1 subcomplex, consisting of subunits of the middle and tail domains, has also been described (Lee and Kim 1998).

Each domain of Mediator appears to be directly targeted by sequence-specific regulators. For example, the MED17/TRAP80 subunit of Drosophila Mediator, ortholog of the head domain subunit Srb4, has been shown to engage in direct interaction with Drosophila HSF (Park *et al.* 2001). Likewise, mammalian MED17/TRAP80 is specifically targeted by p53 and VP16 (Ito *et al.* 1999). On the other hand, the mammalian middle domain subunit, MED1/TRAP220, is targeted by nuclear receptors (Ito *et al.* 2000). Three subunits of the yeast tail domain, Gal11, Med2, and Hrs1 (constituting the "Gal11 module"), physically interact with the Gcn4 activation domain *in vitro* and contribute to the recruitment of Mediator at Gcn4-regulated promoters (Zhang *et al.* 2004). Other subunits may be targets of corepressors. Srb7 binds the global yeast repressor Tup1, both *in vivo* and *in vitro*, and an Srb7 mutation that obviates Srb7–Tup1 interactions derepresses Tup1-regulated genes in *S. cerevisiae* (Gromoller and Lehming 2000). Specific subunits within Mediator also may interact with GTFs such as TBP, TFIIB, TFIIE, or TFIIH (Sakurai and Fukasawa 2000; Kang *et al.* 2001).

Consistent with their physical interactions, genetic analysis suggests that individual Mediator subunits can act as either positive or negative regulators of transcription (reviewed in Carlson 1997). For example, the yeast Gal11 and Rox3 subunits are required for Gal4 activation of galactose-inducible genes (Suzuki *et al.* 1988; Brown *et al.* 1995), Nut2 for Gcn4-mediated activation of amino acid biosynthetic genes (Han *et al.* 1999), and Med11 for MFα2 transcriptional activation (Han *et al.* 1999). Similar activator-specific functions of individual subunits have been described for Drosophila Mediator, including a role for MED23 in heat-shock gene expression and MED16 in lipopolysaccharide gene expression (Kim *et al.* 2004). In addition, the tail subunits of yeast Mediator have been shown to repress transcription of specific genes, including *HO*, *SUC2*, *PHO5*, *GAL1*, and *IME1* (Stillman *et al.* 1994; Piruat *et al.* 1997; Tabtiang and Herskowitz 1998; Han *et al.* 2001; Nishizawa 2001). The kinase module has also been shown to negatively regulate transcription, either via Cdk8/Srb10 phosphorylating the pol II CTD prior to formation of the preinitiation complex (PIC) (Hengartner *et al.* 1998) or by its directly phosphorylating DNA-bound activators, thereby leading to their ubiquitin-mediated proteolysis or export from the nucleus (Chi *et al.* 2001). Subsequent to PIC formation, Mediator enhances Kin28 phosphorylation of the pol II CTD at Ser5 (Guidi *et al.* 2004), suggesting a way in which Mediator may positively regulate gene transcription. These and other observations point to a complex role for Mediator as an integrator of intracellular signals.

We report evidence that subunits found in all three structural domains of Mediator govern yeast heat-shock gene transcription. Genes encoding these subunits, *MED7*, *NUT2*, *RGR1*, *ROX3*, *SIN4*, and *SRB7*, were identified in a genetic screen for extragenic suppressors of a crippled heat-shock gene, *hsp82-ΔHSE1*, that retains only a pair of low-affinity HSF sites. Recessive mutations in each of these genes enhance the basal transcription not only of *hsp82-ΔHSE1*, but also of wild-type heat-shock genes. Furthermore, the more severe mutations diminish heat-shock-induced transcription, thereby revealing a functional module within Mediator that

TABLE 1

Yeast strains

Strain	Genotype	Source or reference
SLY101	*MAT*α *ade- can1-100 cyh2^r his3-11,15 leu2-3,112 trp1-1 ura3*	Lee and Gross (1993)
KEY102	SLY101; *hsp82-ΔHSE1*	Gross *et al.* (1993)
HDY1002	KEY102; *hsp82-ΔHSE1/HIS3::URA3, TRP1*$^+$	This study
HS3000	HDY1002; *hsp82-ΔHSE1/lacZ:: LEU2*	This study
HS1001	HS3000; *hsp82-ΔHSE1-HIS3::ura3Δ* (*KanMX* excised by Cre recombinase)	This study
HS1002	HS1001; *MAT***a**	This study
HS1003	HS3000; *leu2::hsp82- ΔHSE1-lacZ::leu2::KanMX*	This study
HS1004	HS1003; *MAT***a**	This study
HS1005	HS1001; *sin4Δ::KanMX*	This study
J79	HS1004; *sin4-1001*	This study
J1	HS1004; *rgr1-1001*	This study
S5	HS1004; *med7-1001*	This study
J121	HS1004; *med7-1002*	This study
S2	HS1004; *srb7-1001*	This study
J20	HS1004; *srb7-1002*	This study
J15	HS1004; *nut2-1001*	This study
J84	HS1004; *nut2-1002*	This study
J34	HS1004; *rox3-1001*	This study
B20	HS1004; *rox3-1002*	This study
RRG2	HS1001; *srb10Δ::KanMX*	This study
DAD2	HS1001; *srb2Δ::KanMX*	This study
DAD3	HS1001; *gal11Δ::KanMX*	This study
JHD1	HS1001; *nut1Δ::KanMX*	This study
SBK2000	J20; *LEU2*$^+$	This study
SBK2001	HS1001; *SRB7- KanMX*	This study
SBK2002	HS1001; *met16Δ::KanMX*	This study
SBK2003	HS1001; *pfk27Δ::KanMX*	This study
SBK2004	HS1001; *ybl094CΔ::KanMX*	This study
SBK2005	J121; *LEU2*$^+$	This study
SBK2006	S5; *LEU2*$^+$	This study
SBK2007	J15; *LEU2*$^+$	This study
SBK2008	J84; *LEU2*$^+$	This study
SBK2009	B20; *LEU2*$^+$	This study
SBK2010	J34; *LEU2*$^+$	This study
DY150	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	D. J. Stillman
DY2694	DY150; *MAT*α *rgr1-Δ2::LEU2*	D. J. Stillman
SBK501	*MAT*α *leu2::hsp82-ΔHSE1-lacZ::leu2::KanMX trp1 ura3 rgr1-Δ2::LEU2*	This study
S288C	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
HSF1-GFP	S288C; *HSF1-GFP*	Invitrogen

collaborates with HSF in governing the dynamic range of heat-shock gene expression.

MATERIALS AND METHODS

Yeast strain construction: *Strain employed in genetic screen:* To place *HIS3* under the regulation of the *hsp82-ΔHSE1* promoter, we used oligonucleotide-directed mutagenesis (Norrander *et al.* 1983; Gross *et al.* 1993) to introduce a synthetic *Xho*I site at position −6 relative to the ATG initiation codon of *hsp82*. The template was an *Eco*RI fragment of *hsp82-ΔHSE1* [spanning −1362–+1543 (wild-type coordinates)] cloned into M13mp18. The mutation was confirmed by sequencing and the *Eco*RI fragment was subcloned into the *URA3* integrating vector, pRS306. The *HIS3* ORF (+1–+663), PCR amplified and appended with *Xho*I sites, was then ligated into the *Xho*I-opened pRS306 construct, creating pHD102. The construct was linearized at the unique *Stu*I site within the *URA3* gene and targeted to the *ura3-1* locus of the *hsp82-ΔHSE1* recipient strain, KEY102, creating HDY1002 (see Table 1).

To fuse the *hsp82-ΔHSE1* promoter to *lacZ*, *hsp82-ΔHSE1* spanning nucleotides −806–+309 (relative to the initiating AUG codon) was PCR amplified and appended with *Sma*I and *Pst*I sites. This fragment was cloned into the integrating vector YIp366, fusing *hsp82-ΔHSE1* with the *lacZ* ORF at codon 8. The resultant *hsp82/lacZ* fusion was thus composed of 103 amino acids encoded by *HSP82*, 13 amino acids encoded by the multicloning site, and 1016 amino acids encoded by *lacZ*. The *hsp82/lacZ* construct, linearized at the unique *Afl*II site within the *LEU2* gene, was then transformed into HDY1002. Integration into the *leu2-3,112* locus was confirmed by genomic PCR, creating HS3000. Strains HS1001 and HS1003 were constructed by one-step gene disruptions in HS3000 of *URA3* and *LEU2*, respectively, using *KanMX-loxP* as described (Guldener *et al.* 1996). The *KanMX* (*Kan*^{r}) gene was excised in HS1001 by ectopically expressed Cre recombinase (Guldener *et al.*

1996). HS1002 and HS1004, *MAT***a** derivatives of HS1001 and HS1003, respectively, were generated by galactose-induced expression of HO endonuclease of parental strains harboring pJH132 (*GAL1/HO-URA3-CEN*; gift of James Haber, Brandeis University).

Other strains: To construct an *hsp82-ΔHSE1/lacZ rgr1-Δ2* double-mutant strain, we crossed DY2694 (generously provided by David Stillman, University of Utah) with HS1004. Diploids were selected on media lacking uracil and leucine and then sporulated. Tetrads were dissected and three spores harboring both *rgr1-Δ2* and *hsp82-ΔHSE1/lacZ* were identified. All three were assayed for *lacZ* expression using a liquid β-galactosidase (β-gal) assay (see below) and found to exhibit comparable expression levels. One of them, SBK501, was employed in this study. To construct a *sin4*Δ knockout of HS1001, we used the *KanMX-loxP* gene disruption method as described above, creating HS1005.

Selection of His$^+$ revertants: Independent 5-ml cultures of strain HS1004 were grown overnight at 30° to saturation in 1% yeast extract, 2% peptone, and 2% glucose supplemented with 0.04 mg/ml adenine (YPDA). A total of 100–200 μl of the saturated culture were spread onto synthetic medium lacking histidine and containing 1.5 mM 3-aminotriazole (3-AT) and incubated for 6–10 days at 30°. Use of 3-AT, a competitive inhibitor of IGP dehydratase (*HIS3* gene product), was necessary to prevent growth of the HS1004 parental strain in the absence of a suppressing mutation. Mutagens were avoided to minimize the possibility of generating multiple mutations. On some plates, a large number of colonies of varying sizes were observed while on other plates no colonies were present. Colonies of varying sizes were picked and restreaked on 1.5 mM 3-AT plates lacking histidine to confirm growth of revertants. Using this method, >1000 His$^+$ revertants were generated.

Screening for *lacZ* expression: Each of the revertants was screened for expression of the *hsp82-ΔHSE1/lacZ* gene to confirm that the mutation affected *hsp82-ΔHSE1* transcription and that it was acting in *trans*. Master plates of His$^+$ revertants were constructed and used in XGAL assays. On average, only one in five revertants turned blue relative to the parental strain [XGAL^{Blue} (XGAL^B) phenotype] and thus, by definition, harbored an extragenic suppressor mutation.

Scoring of secondary growth phenotypes: To facilitate cloning of the suppressor gene by plasmid complementation (see below), His$^+$ XGAL^B suppressors were evaluated for the presence of growth phenotypes. The following phenotypes were tested: slow growth at 30° on YPDA (Slg$^-$); inability to grow on YPDA at 37° (temperature sensitivity, Ts$^-$); inability to grow on YPDA at 15° (cold sensitivity); inability to grow on synthetic media lacking either inositol (Ino$^-$) or inorganic phosphate (Pho$^-$); sensitivity to 300 mM urea (Us$^-$), 6% ethanol (Es$^-$) 3% formamide (Fs$^-$), or 5 mM caffeine (Caf$^-$) (all added to YPDA); and inability to grow on YPG (1% yeast extract, 2% peptone, 3% glycerol) (Gly$^-$).

Determination of recessivity: His$^+$ XGAL^B mutants were backcrossed to HS1001, a *MAT*α Leu$^+$ Ura$^-$ counterpart of HS1004 (see Table 1). Diploids were selected on −His, −Leu synthetic medium and scored for their β-gal (XGAL) and secondary growth phenotypes. In all cases, both primary and secondary phenotypes were complemented, demonstrating recessivity.

Sorting of suppressors into complementation groups: Suppressors with selectable growth phenotypes were crossed to *MAT*α Leu$^+$ Ura$^-$ spores of each complementation group (obtained by dissection of sporulated diploids derived from HS1001 backcrosses of a representative member). Ura$^+$ Leu$^+$ diploids were scored for retention of the XGAL^B phenotype (and, when ambiguous, the growth phenotype as well); those that did were considered members of that particular complementation group.

Cloning of suppressors by plasmid complementation and confirmation by genetic linkage: *EWE1:* Spontaneous His$^+$ XGAL^B mutant J79, exhibiting a strong Ino$^-$ phenotype, was streaked on medium containing 1.0 mg/ml 5-fluoroorotic acid (FOA) to select a spontaneous FOA^R (*ura3*$^-$) mutant. The resultant strain, termed J79u, was transformed with a YCp50-based *S. cerevisiae* genomic DNA library (Rose *et al.* 1987). Ura$^+$, Ino$^+$ transformants were selected. Plasmids were then isolated from two transformants, and their inserts were sequenced (Iowa State University DNA Sequencing and Synthesis Facility) using YCp50-specific primers straddling the unique *Bam*HI site (forward primer: CTGCTCGCTTCGC TACTTGG; reverse primer: CGATATAGGCGCCAGCAACC). The genomic inserts consisted of overlapping fragments (5 kb in length) derived from chromosome XIV (spanning coordinates 204,917–210,464 and 204,993–210,339); both encompassed *SIN4*. To demonstrate that the complementing activity was conferred by *SIN4*, we PCR amplified the *SIN4* ORF and its flanking upstream region and cloned the resultant fragment into pRS316. The *SIN4* construct was then transformed into J79u and shown to complement both Ino$^-$ and XGAL^B phenotypes. The XGAL^B phenotype of two other members of this complementation group, J73 and G128, was also fully complemented by the *SIN4* plasmid. To confirm that *EWE1* is *SIN4*, we deleted *SIN4* and assayed the β-gal phenotype of the resultant strain, HS1005. It exhibited robust XGAL^B and liquid β-gal phenotypes (see Figure 3 below), thereby demonstrating that *EWE1* is *SIN4*.

EWE2: Spontaneous suppressor J1 was transformed with the YCp50-based genomic library as above and selected for Ts$^+$ transformants on −Ura. Three different plasmids, containing overlapping inserts (spanning coordinates 274,710–286,266, 274,104–~282,500, and 273,213–278,613 of chromosome XII) and encompassing *RGR1*, were isolated. To further strengthen the possibility that the mutation was in fact in *RGR1*, J1 and two other members of the *EWE2* complementation group, J14 and J22, were crossed to an *rgr1*-Δ2 strain (Li *et al.* 1995) (DY2694, gift of David J. Stillman, University of Utah). The resultant diploids retained the XGAL^B and Ts$^-$ phenotypes, indicating noncomplementation. In contrast, crossing of DY2694 to S5, S2, and J15, members of the complementation groups *EWE3*, *EWE4*, and *EWE5* (see below), as well as to the parental strain HS1004, resulted in complementation (XGAL^W). Confirmation that the *EWE2* is *RGR1* came from the observation that an engineered deletion of the C-terminal domain of Rgr1 (creating strain SBK501 as described above) conferred a strong β-gal$^+$ phenotype (see Figure 6A).

EWE3: Spontaneous suppressor S5 was transformed with the YCp50-based genomic library as above and selected for growth at 30° on synthetic complete medium lacking uracil and containing 300 mM urea (Us$^+$ phenotype). Transformants were screened for complementation of the XGAL phenotype (XGAL^W), and five candidates were identified. Restriction analysis of plasmids isolated from these indicated the presence of two distinct, but overlapping inserts. They were both sequenced and found to span coordinates 65,248–70,216 and 64,636–71,484 of chromosome XV, a locus containing *MED7*. *MED7* was therefore PCR amplified, cloned into pRS316, and transformed into S5. It was found to fully complement both XGAL^B and Us$^-$ phenotypes. To further strengthen the possibility that *EWE3* is *MED7*, we used homologous recombination to replace *PFK27* (located 622 bp away from *MED7*), with the *KanMX* marker in strain HS1001, creating strain SBK2003. SBK2003 was then crossed to SBK2005 and SBK2006, isogenic *LEU2*$^+$ (Kan^S) derivatives of spontaneous suppressors J121 and S5, respectively. Kan^R, Ura$^+$ diploids were selected, sporulated, and dissected. In all cases (of 44 four-spore viable tetrads assayed), kanamycin resistance segregated opposite Slg$^-$ and

$XGAL^B$ phenotypes, thereby demonstrating that the mutation is tightly linked to *MED7*.

EWE4: Spontaneous suppressor S2 was transformed with YCp50 library as described above, and Us^+ transformants selected. Seven were screened for their XGAL phenotype, and four showed complementation ($XGAL^W$). Three plasmids were isolated; restriction analysis indicated the inserts were related although not identical. One was sequenced and spanned coordinates 1,072,800–1,080,059 on chromosome IV. As this region encompassed *SRB7*, we amplified the gene and its regulatory region by PCR, cloned the product into pRS315, and transformed the resultant *SRB7* construct into S2. We observed that it fully complemented the Us^- phenotype and partially complemented the $XGAL^B$ phenotype. To strengthen the possibility that *EWE4* is *SRB7*, we used homologous recombination to target the *KanMX* marker 100 bp downstream of *SRB7* in strain HS1001, creating strain SBK2001. SBK2001 was then crossed to SBK2000, an isogenic *LEU2*$^+$ derivative of the spontaneous suppressor J20, and Kan^R, Ura^+ diploids were selected, sporulated and dissected. In all cases (47 four-spore viable tetrads assayed), kanamycin resistance segregated opposite Slg^- and $XGAL^B$ phenotypes, thereby demonstrating that the mutation is tightly linked to *SRB7*.

EWE5: As for *EWE3* and *EWE4*, spontaneous suppressor J15, bearing a Us^- growth phenotype, was transformed with the YCp50 genomic library and complementation of its Us^- phenotype was tested at 30°. A single transformant that could grow on synthetic complete medium lacking uracil and containing 300 mM urea was identified; its *lacZ* phenotype was similarly complemented ($XGAL^W$). Curing of the plasmid on FOA restored Us^- and $XGAL^B$ phenotypes. The plasmid was isolated and sequenced and found to contain an insert of 10.8 kb (coordinates 869,847–880,543 on chromosome XVI). As this region encompassed *NUT2*, we PCR amplified the gene and its 5′-flank and cloned it into pRS315. Leu^+ transformants were found to be Us^+ and $XGAL^W$, consistent with *EWE5* being *NUT2*. To further strengthen this possibility, we used homologous recombination to replace *MET16* (located 444 bp away from *NUT2*) with the *KanMX* marker in strain HS1001, creating strain SBK2002, and linkage analysis was conducted as described for *EWE3* (44 four-spore viable tetrads assayed). Kanamycin resistance segregated opposite Slg^- and $XGAL^B$ phenotypes, thereby demonstrating that the mutation is tightly linked to *NUT2*.

EWE6: Spontaneous His^+ $XGAL^B$ suppressor J34 (representative of a complementation group of 33 members) was transformed as above. Transformants capable of complementing the Ts^- phenotype of J34 were selected. Plasmids were isolated from four Ts^+ transformants; two distinct plasmids were isolated on the basis of their restriction digestion pattern. Their inserts were sequenced and found to be ~15 kb in size, overlapping and derived from chromosome II (spanning coordinates 32,706–47,972 and 31,877–46,717). Both encompassed *ROX3*. *ROX3* was therefore PCR amplified and cloned into pRS316, and the resultant construct was transformed into J34. It complemented both Ts^- and $XGAL^B$ phenotypes, implicating *EWE6* as being *ROX3*. To strengthen this possibility, *YBL094C* (located 491 bp away from *ROX3*) was replaced with *KanMX*, creating strain SBK2004, and this strain was crossed to SBK2009 and SBK2010, *LEU2*$^+$ derivatives of the spontaneous suppressors B20 and J34. Resultant diploids were selected, sporulated, and dissected (45 four-spore viable tetrads assayed). Kanamycin resistance segregated opposite Slg^- and $XGAL^B$ phenotypes, thereby demonstrating that the mutation is tightly linked to *ROX3*.

β-Galactosidase measurements: *XGAL plate assays:* To screen for suppressors and score dissected spores for *lacZ* expression, we used either a highly sensitive, nonlethal β-gal plate assay as described (Duttweiler 1996) or a nitrocellulose filter lift assay (Vojtek *et al.* 1993).

ONPG liquid assays: Cultures were grown at 30° in YPDA to an A_{600} of 0.3–0.7 and then split into two portions. One was maintained at 30° (noninduced) and the other was heat-shocked at 39° for 45 min. The latter was then returned to 30° for 20 min to permit efficient export and translation of *lacZ* mRNA. At this point, cells were harvested and the activity of β-gal was determined from clarified extracts using this equation: $\text{units} = (1000 \times A_{420})/(t \times V \times A_{600})$, where A_{420} is a measure of the absorbance of the ONPG reaction, A_{600} is a measure of cell density, t is time in min, and v is volume in milliliters (Ausubel *et al.* 1995).

Microscopy: GFP images were collected with an Olympus AX70 microscope using a 41001 filter set (Chroma Technology, Brattleboro, VT), a ×100 numerical aperture 1.25 Olympus objective equipped with a Bioptechs (Butler, PA) objective heater, and a CoolSNAP HQ charge-coupled device camera (Roper Scientific, Duluth, GA).

Northern blot hybridization: Northerns were performed as described (Erkine and Gross 2003). The *SSA4*-specific hybridization probe was generated by linear amplification of a PCR fragment generated using forward primer +1827–+1848 (relative to ATG) and reverse primer +1922–+1901. All other probes were as previously described (Erkine and Gross 2003; Zhao *et al.* 2005). Hybridization signals were detected on a Storm 860 PhosphorImager and quantified using ImageQuant 1.11 software.

Chromatin immunoprecipitation: Chromatin immunoprecipitation (ChIP) assays were performed as described previously (Sekinger and Gross 2001), employing a polyclonal (rabbit) antibody raised against recombinant GST–ScHSF (Erkine *et al.* 1996). Promoter-specific primers for *SSA4* (coordinates relative to ATG) were forward, −450–−425, and reverse, −65–−93. All other primer pairs were as previously described (Sekinger and Gross 2001; Erkine and Gross 2003).

RESULTS

Experimental rationale: The ability of yeast HSF to activate transcription in response to thermal stress, and the role of its activation and regulatory domains in this process, have been extensively investigated (*e.g.*, Nieto-Sotelo *et al.* 1990; Sorger 1990; Bonner *et al.* 1992; Bulman *et al.* 2001; Chen and Parker 2002; Erkine and Gross 2003; Hashikawa and Sakurai 2004). However, a poorly understood function of HSF is its ability to stimulate basal transcription. That this activity is central to its physiological role is suggested by several observations. First, the *HSF1* gene is essential for viability, even at low temperatures (Sorger and Pelham 1988). Second, as shown in Figure 1, HSF localizes to the yeast nucleus under non-heat-shock conditions, consonant with it having a nuclear function even in the absence of stress. This pattern of intracellular localization resembles that of Drosophila HSF (Westwood *et al.* 1991), but contrasts with mammalian HSFs, which are principally located in the cytoplasmic compartment in the absence of stress (Sarge *et al.* 1993; Sheldon and Kingston 1993). And third, yeast HSF binds high-affinity HSEs under noninducing conditions, both

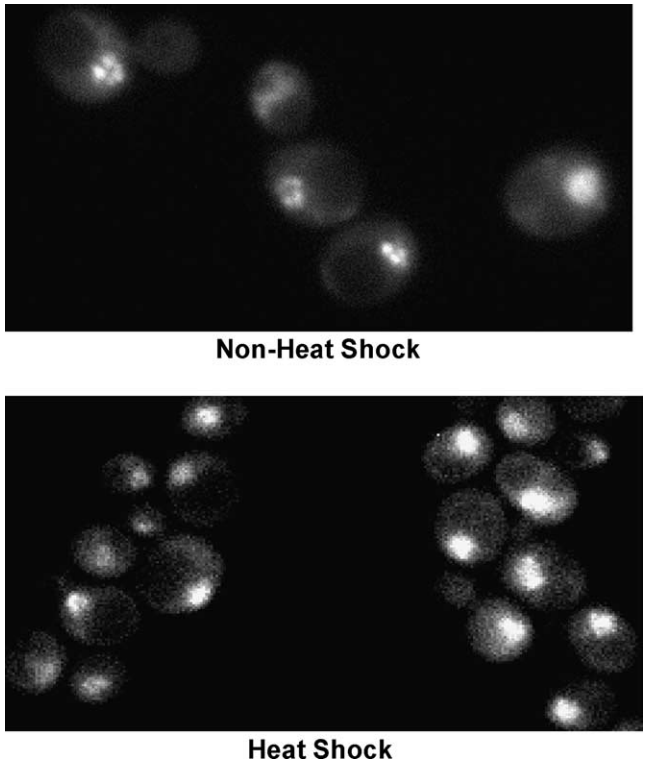

Figure 1.—HSF localizes to the yeast nucleus under both noninducing and heat-shock-activating conditions. Strain HSF1-GFP was cultivated and photographed under non-heat-shock (30°) or heat-shock (37° for 5 min) conditions as indicated.

in vitro and *in vivo* (Sorger *et al.* 1987; Jakobsen and Pelham 1988; Gross *et al.* 1990; Erkine *et al.* 1995, 1999; Giardina and Lis 1995; Sekinger and Gross 2001; Hahn *et al.* 2004) (see Table 3 below). In line with this, targeted mutagenesis of high-affinity HSEs severely reduces the basal transcription of *HSP82*, *HSC82*, and *SSA1* (McDaniel *et al.* 1989; Park and Craig 1989; Gross *et al.* 1993; Erkine *et al.* 1996).

Demonstration of the constitutive nuclear localization and DNA binding of yeast HSFs raises the question of what represses its potent N- and C-terminal activation domains in the absence of stress. Several "masking" domains have been identified. These include an N-terminal repressor domain (Sorger 1990), a region that overlaps the DNA-binding and trimerization domains (Nieto-Sotelo *et al.* 1990; Bonner *et al.* 1992; Chen and Parker 2002), and a heptapeptide sequence termed CE2 located adjacent to the C-terminal activation domain (Jakobsen and Pelham 1991). While these have been traditionally thought to physically interact with one or both activation domains, the possibility of alternative or additional mechanisms of repression, including the recruitment of corepressors, has not been ruled out. Recently, a role for protein kinase A in maintaining HSF in a repressed state, at least with respect to its regulation of *HSP12* and *HSP26*, has been reported (Ferguson *et al.* 2005).

To identify additional proteins that might collaborate with HSF in regulating noninduced heat-shock gene transcription, we devised a genetic screen to isolate extragenic suppressors that restored expression to an *hsp82* allele bearing a deletion of its high-affinity HSF site. This 32-bp mutation, termed ΔHSE1 (Gross *et al.* 1993), markedly diminishes affinity of the *hsp82* promoter for HSF both *in vitro* and *in vivo* (Erkine *et al.* 1999; Sekinger and Gross 2001). Notably, two low-affinity HSEs are retained at this allele that are weakly occupied by HSF (Sekinger and Gross 2001). Deletion of HSE1 also obviates formation of the DNase I hypersensitive (DH), nucleosome-free region characteristic of the wild-type promoter (Gross *et al.* 1993). In place of the DH site—which, despite its accessibility, is occupied by histones under noninducing conditions (Zhao *et al.* 2005)—are two stably positioned nucleosomes, one centered over the mutated UAS and the other centered over the core promoter (Nuc-2 and Nuc-1, respectively) (Gross *et al.* 1993; Venturi *et al.* 2000). Accompanying this structural transformation is a 100-fold drop in noninduced transcription (Gross *et al.* 1993). Promoter chromatin architecture of wild-type and mutant *hsp82* alleles is illustrated in Figure 2A.

Experimental strategy: To select for suppressors of the null expression phenotype of *hsp82-ΔHSE1*, we fused the mutant promoter to the *HIS3* coding region (in a nonrevertible *his3* background), integrated the chimeric gene into the genome, and selected for spontaneous His^+ revertants. In addition, to ensure the presence of an extragenic mutation, we integrated a second chromosomal reporter gene, *lacZ*, regulated by the same *hsp82-ΔHSE1* promoter, and screened His^+ revertants for β-gal expression. We also targeted the ΔHSE1 mutation to the gene's native locus using a similar integration strategy (summarized in Figure 2B), creating the strain termed HS1004 (see Table 1).

We selected >1000 independent His^+ revertants. Roughly 1 in 5 of these restored expression of the integrated *hsp82/lacZ* reporter (as indicated by an $XGAL^B$ phenotype in plate assays), consistent with the presence of an extragenic suppressing mutation. We scored suppressors for conditional growth phenotypes, including temperature sensitivity; cold sensitivity; inositol or phosphate auxotrophy; and sensitivity to ethanol, caffeine, and formamide. We also tested suppressors for enhanced sensitivity to urea (Us^-), which to our knowledge has not been previously examined (*S. cerevisiae* growth phenotypes reviewed in Hampsey 1997). We reasoned that protein-folding defects arising from spontaneous mutations might be exacerbated by exposure to low concentrations of urea, a protein denaturant that should readily enter cells, given its small size. Indeed, a number of suppressors displayed enhanced sensitivity to 300 mm urea. Interestingly, Us^- suppressors typically did not exhibit impaired growth on 3% formamide,

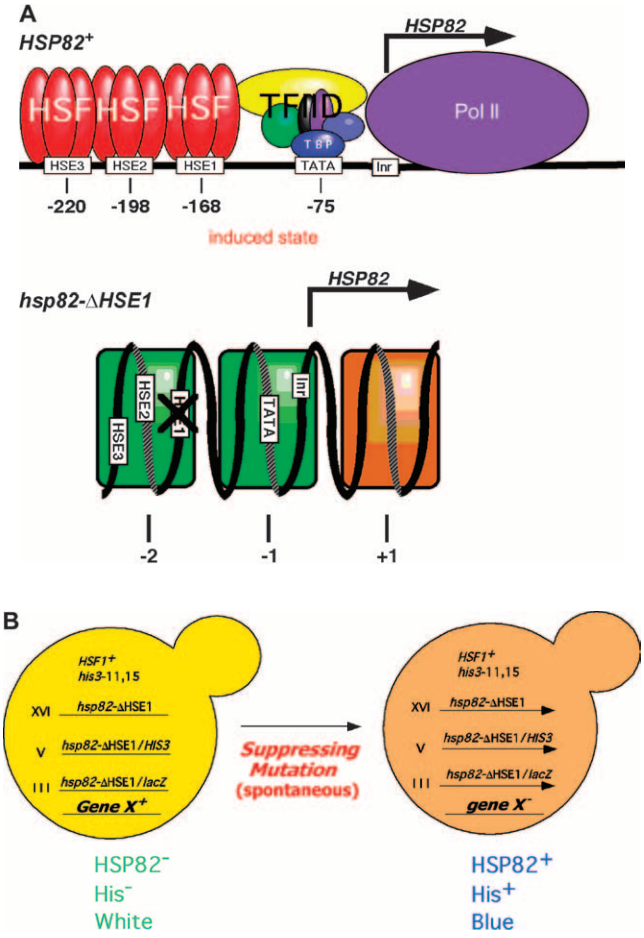

FIGURE 2.—Structural phenotypes of *hsp82* alleles and suppressor screen strategy. (A) Chromatin structure of the wild type and mutant *hsp82* promoter. At the wild-type allele, HSF occupies HSE1 prior to heat shock, and all three HSEs following it. Its promoter nucleosomes are highly remodeled (DNase I hypersensitive) under non-heat-shock conditions and displaced, along with ORF nucleosomes, upon heat shock (depicted) (ZHAO *et al.* 2005). At the *hsp82-ΔHSE1* allele, HSF occupancy is reduced 20-fold relative to *HSP82*$^+$ and its promoter nucleosomes (shaded green) are stable and precisely positioned. They are not detectably altered by heat shock. Nucleosome +1, located downstream of the initiation site, is also detectable at *HSP82*$^+$ under noninducing conditions. (B) Selection/screen strategy employed in this study. Depicted are the three *hsp82* alleles (all chromosomal integrants) present in strain HS1004 and the primary phenotypes of the parental strain and suppressor mutants.

sensitivity to which is thought to also result from folding defects (AGUILERA 1994).

Suppressors were backcrossed to an isogenic strain of the opposite mating type (HS1001); all were found to be recessive. They were then sorted into complementation groups, and a representative of each, chosen on the basis of the strength of its primary (β-gal) and secondary (growth) phenotypes, was used to clone the wild-type allele from a yeast genomic library by plasmid complementation. To confirm the assignment of each suppressor gene, we performed genetic linkage analysis or, alternatively, engineered a mutation directly into it and assayed the β-gal phenotype of the resultant strain (see MATERIALS AND METHODS).

Suppressors of the *hsp82-ΔHSE1* null transcription phenotype encode components of Mediator: All suppressors sorted into one of six complementation groups, termed *EWE1-6* (for *e*xpression *w*ithout heat-shock *e*lement). Strikingly, the genes represented by these complementation groups encode subunits of a single protein complex, pol II Mediator. Two of the six genes, *EWE1/SIN4* and *EWE2/RGR1*, encode subunits of Mediator's tail domain; three others, *EWE3/MED7*, *EWE4/SRB7*, and *EWE5/NUT2*, encode subunits of its middle domain; and the sixth, *EWE6/ROX3*, encodes a subunit in the head domain (summarized in Table 2). To quantify the effect of the suppressor mutations on *hsp82-ΔHSE1* expression, we measured β-gal levels of a representative allele from each complementation group. As expected, the suppressing mutations enhanced noninduced *hsp82-ΔHSE1* expression, and in most cases quite substantially (30- to 70-fold; Figure 3A, solid bars; summarized in Table 2). The mutations also increased heat-shock-induced β-gal levels, but far less markedly (1.8- to 3.5-fold; open bars). As a consequence, the suppressors reduced the dynamic range of expression of *hsp82-ΔHSE1* from 20-fold to, in most cases, <2-fold (Figure 3B).

Mutations in other subunits of Mediator fail to rescue the *hsp82-ΔHSE1* null transcription phenotype: Given the scope of the selection and the large size of several complementation groups (three containing >30 members each; Table 2), it seemed possible that we had saturated the screen. Yet our selection failed to pull out either *SRB10* or *GAL11*, which encode well-characterized negative regulatory subunits of Mediator. Indeed, Cdk8/Srb10, located in the loosely associated kinase module, negatively regulates transcription via its ability to phosphorylate both the CTD (HENGARTNER *et al.* 1998) and gene-specific activators (CHI *et al.* 2001), as discussed above. Additionally, Cdk8/Srb10 may play a role in Tup1-mediated repression (KUCHIN and CARLSON 1998). Gal11, located in the tail module, has been shown to negatively regulate transcription of genes such as *SUC2* and *SOL4* (HAN *et al.* 2001), as well as of artificial promoter fusions (NISHIZAWA 2001). Therefore, as Cdk8/Srb10 and Gal11 are encoded by nonessential genes, we constructed isogenic *srb10*Δ and *gal11*Δ derivatives of the parental strain. We also deleted *SRB2*, which encodes a nonessential subunit of the head domain, and *NUT1*, which encodes a tail domain subunit that negatively regulates the expression of oxidative phosphorylation genes (BEVE *et al.* 2005). However, as shown in Figure 4A, deletion of *SRB10*, *GAL11*, or *SRB2* fails to enhance the low basal expression of *hsp82-ΔHSE1*; its induced expression is likewise unaffected (Figure 4B). The *nut1*Δ mutation was found to have a modest (approximately twofold) stimulatory effect

TABLE 2

Summary of *EWE* suppressor screen

Complementation group	Gene	Growth phenotypes	No. of members	Noninduced expression (β-gal or mRNA levels)		
				hsp82-ΔHSE1	*HSP12*	*HSP104*
Wild type	—	None		1	1	1
EWE1	*MED16/SIN4*	Ts$^-$, Ino$^-$, Pho$^-$, Us$^-$, Fs$^-$, Gly$^-$	≥100	35	1	1
EWE2	*MED14/RGR1*	Ts$^-$, Slg$^-$	38	38	2	1.8
EWE3	*MED7*	Us$^-$, Slg$^-$	7	71	3.3	3.4
EWE4	*MED21/SRB7*	Us$^-$, Slg$^-$	7	31	4	1.4
EWE5	*MED10/NUT2*	Us$^-$, Ts$^-$, Slg$^-$	2	49	9	4.2
EWE6	*MED19/ROX3*	Ts$^-$, Us$^-$, Slg$^-$	33	13	21	1.5

His$^+$ revertants were selected on −His medium containing 1.5 mm 3-aminotriazole. Expression levels are provided for a representative member of each complementation group (see legend to Figure 5); for *EWE1*, strain HS1005 was used. mRNA levels are indicated for *HSP12* and *HSP104*; β-gal levels for *hsp82-ΔHSE1/lacZ*. Expression levels are relative to wild type and are summaries of data presented in Figures 3 and 5. Growth phenotypes are listed in approximate order of severity and are defined in MATERIALS AND METHODS.

on *hsp82-ΔHSE1* transcription (Figure 4C), considerably less than the spontaneous suppressors. Therefore, Gal11, Srb10, and Nut1, despite their ability to negatively regulate the transcription of many genes, are functionally distinct from Ewe subunits, as is the head subunit Srb2.

Mediator mutations derepress the basal transcription of wild-type *HSP* genes: We next investigated the effects of the suppressors on wild-type heat-shock gene expression. In addition to *HSP82*, the chaperone-encoding genes *HSP12*, *HSP26*, *HSP104*, and *SSA4* are regulated by HSF (HALLADAY and CRAIG 1995; LEE *et al.* 2002; FERGUSON *et al.* 2005). And, as summarized in Table 3, the promoter regions of these genes are occupied by HSF even under noninducing conditions (see also HAHN *et al.* 2004). Nonetheless, they exhibit interesting differences in their regulation (*e.g.*, see FERGUSON *et al.* 2005), and their transcription might be affected by the *ewe* suppressors in distinct ways. As shown in Figure 5, basal *HSP* mRNA levels generally increased in the presence of each *ewe* mutation. For instance, suppressor J34, harboring a spontaneous mutation in *rox3*, strongly enhanced the basal transcript levels of *HSP12* and *HSP26* (21-fold and 4.5-fold, respectively). J15, bearing a spontaneous *nut2* mutation, enhanced the noninduced expression of all four genes (3- to 9-fold), as did the *med7* suppressor S5 (2- to 3-fold). Furthermore, the *srb7* suppressor S2 derepressed *HSP12* basal transcription 4-fold.

Other suppressor/gene combinations were not as striking, such as those involving the *sin4* suppressor HS1005 and the *rgr1* suppressor J1 (Figure 5, Table 2, and data not shown). Nonetheless, *rgr1-Δ2*, encoding a C-terminal deletion of Rgr1 (JIANG *et al.* 1995; LI *et al.* 1995), not only strongly derepressed the *hsp82-ΔHSE1/lacZ* reporter (Figure 6A, solid bars), thereby demonstrating that *rgr1-Δ2* is a *bona fide ewe* suppressor, but also significantly enhanced the basal transcription of wild-type heat-shock genes (Figure 6C). As was true for the spontaneous *ewe* mutants, individual heat-shock genes respond somewhat differently to the *rgr1-Δ2* mutation: *HSP12*, moderately occupied by HSF (Table 3) and coregulated by Msn2/Msn4 (GASCH *et al.* 2000), is only weakly derepressed, whereas *HSP82*, strongly occupied by HSF, is strongly derepressed, as is *HSP104*, whose transcription is coregulated by HSF and Msn2/Msn4 (TREGER *et al.* 1998; GRABLY *et al.* 2002). Coupled with a dramatic reduction in induced transcription (Figure 6D), *HSP* genes exhibit a severely compromised dynamic range of expression in context of the *rgr1-Δ2* mutation (Figure 6E). This defect is also evident for the *hsp82-ΔHSE1/lacZ* reporter (Figure 6B). We note that the spontaneous *ewe* mutants have a comparatively weak effect on induced *HSP* mRNA levels (*e.g.*, compare *rgr1-1001* in Figure 5 with *rgr1-Δ2* in Figure 6).

***ewe* suppressors also derepress non-heat-shock gene expression:** Finally, to investigate whether the *ewe* mutations might also derepress the basal transcription of non-*HSP* genes, we assayed *PHO5* transcription under repressing (phosphate-rich medium) conditions. Like that of *hsp82-ΔHSE1*, the *PHO5* promoter is assembled into an array of sequence-positioned nucleosomes (ALMER and HORZ 1986). Under repressing conditions, its principal activator, Pho4, is phosphorylated by the Pho80/Pho85 cyclin–CDK complex, thereby sequestering it in the cytoplasm (KAFFMAN *et al.* 1998). Under these same conditions, the Pho2 transcriptional activator weakly associates with the *PHO5* promoter, although its presence does not lead to significant transcription (NOURANI *et al.* 2004). Thus, noninduced *PHO5* resembles *hsp82-ΔHSE1* in two ways: the nucleosomal state of its promoter and the constitutive presence of a gene-specific activator. And, as shown in Figure 7, spontaneous mutations in *srb7* and *med7* modestly derepress *PHO5* basal transcription (2.2- and 3.0-fold, respectively). The other suppressors had less of an effect. In other experiments, we observed that none of the suppressors detectably derepressed *ACT1* (data not shown;

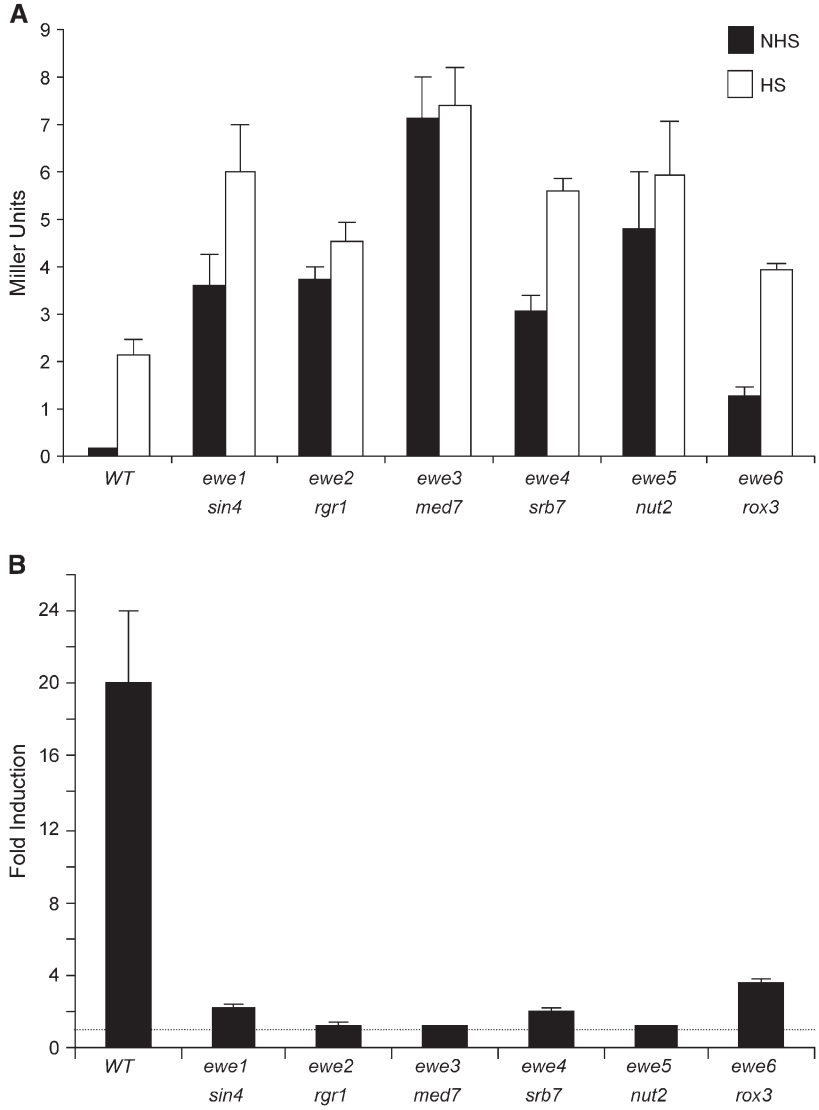

FIGURE 3.—*ewe* suppressors strongly activate noninduced transcription of the *hsp82/lacZ* reporter. (A) A representative suppressor of each complementation group was grown in rich medium at 30° to early log phase and then split into two aliquots, noninduced (NHS) and heat shock induced (HS). The latter culture was subjected to a 30° → 39° thermal upshift for 45 min and allowed to recover at 30° for 20 min; then both cultures were harvested, whole-cell extracts were isolated, and β-gal levels were determined as described in MATERIALS AND METHODS. Depicted are means ±SEM ($n = 3$). Strains used were HS1004 (WT), HS1005 (*ewe1/sin4*), J1 (*ewe2/rgr1*), S5 (*ewe3/med7*), S2 (*ewe4/srb7*), J15 (*ewe5/nut2*), and J34 (*ewe6/rox3*). (B) Fold inducibility of *hsp82-ΔHSE1* in the wild-type strain and spontaneous *ewe* mutants (derived from the data of A). Dashed line indicates a level of 1.0 (no induction).

see Figure 5 legend). Thus, although the derepressing effect of individual *ewe* mutations is not limited to heat-shock genes, the Ewe module does not globally restrict pol II transcription.

DISCUSSION

***EWE* genes encode subunits of the pol II Mediator:** In this study, we employed a genetic screen to identify coregulators of yeast heat-shock gene transcription. The screen, which pulled out nearly 200 extragenic suppressors, proved to be remarkably specific. All suppressors sorted into six complementation groups, each of which contains alleles of genes encoding subunits of the pol II Mediator. Mediator is a modular and dynamic complex that connects gene-specific activators to the GTM by acting as signal sensor, integrator, and processor (reviewed in BOUBE *et al.* 2002). We have termed these suppressors *EWE1-6*. Interestingly, three *EWE* complementation groups encode subunits—Med7, Srb7, and Nut2—that map to the middle module of Mediator, as defined by electron microscopy and *in vitro* reconstitution experiments (DOTSON *et al.* 2000; KANG *et al.* 2001). Two others encode subunits that map to its tail module (Rgr1 and Sin4), and one of the complementation groups encodes Rox3, which maps to its head module (BOUBE *et al.* 2002). All six proteins are highly conserved and have orthologs in human Mediator (SATO *et al.* 2004), and all but Sin4 are essential for viability. Relative locations of the Ewe subunits within Mediator are illustrated in Figure 8. Notably, Med7 and Srb7 physically contact Nut2 but not each other, and Sin4 contacts Rgr1. Rox3 does not appear to contact any one of the other five.

While other genetic screens have implicated Sin4 and Rgr1 (JIANG *et al.* 1995; WANG and MICHELS 2004); Nut2, Sin4, and Rox3 (TABTIANG and HERSKOWITZ 1998); or Sin4, Srb7, and Rox3 (LI *et al.* 2005) as participants in common regulatory pathways, to our knowledge, none has identified a unified regulatory

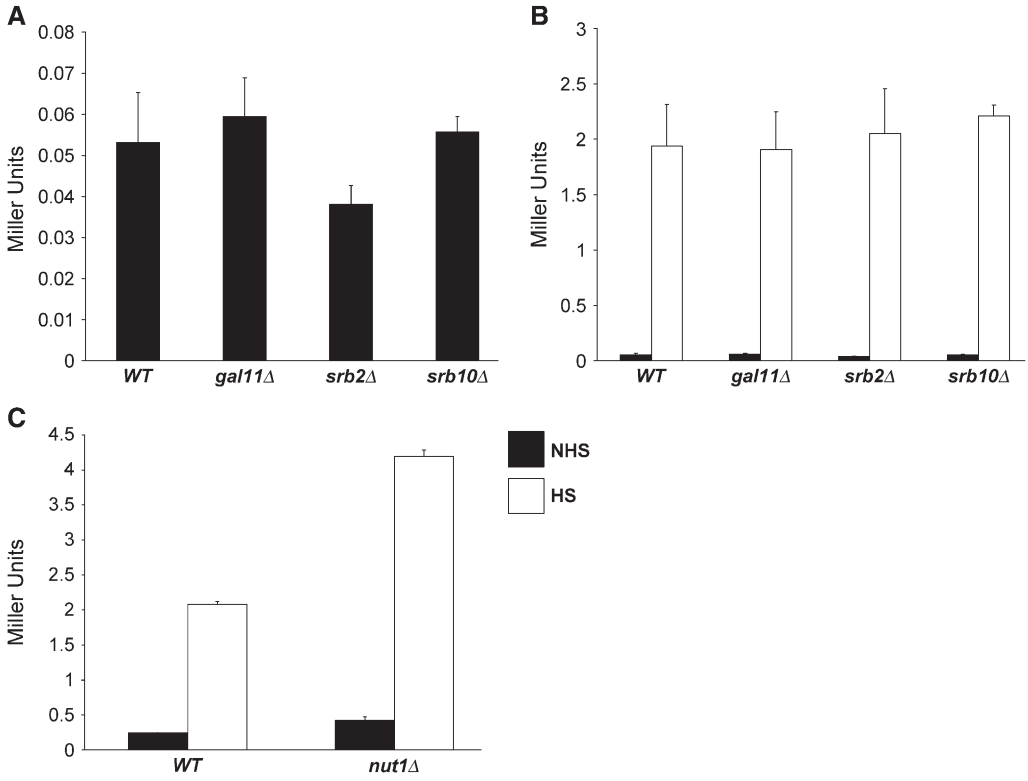

FIGURE 4.—Disruption of *GAL11*, *SRB2*, *SRB10*, or *NUT1* has little or no affect on *hsp82-ΔHSE1* expression. Wild-type (WT) and mutant strains were cultivated, split into NHS and HS aliquots, and assayed for β-gal activity as in Figure 3. (A) Noninduced *hsp82-ΔHSE1/lacZ* expression in WT (HS1001), *gal11Δ* (DAD3), *srb2Δ* (DAD2), and *srb10Δ* (RRG2) strains. (B) Heat-shock-induced expression of *hsp82-ΔHSE1/lacZ* (open bars) compared with nonshocked expression (solid bars) in the same strains. (C) *hsp82-ΔHSE1/lacZ* expression in WT and *nut1Δ* strains (HS1001 and JHD1, respectively) under NHS and HS conditions as indicated. (A–C) means ±SEM ($n = 3$).

role for all six proteins. This raises the possibility of a functional role for the Ewe subunits distinct from those previously ascribed to the Rgr1 or Gal11/Sin4 modules (reviewed in BJORKLUND and GUSTAFSSON 2004). Notably, we were aided in our identification of three of the complementation groups—*EWE3/MED7*, *EWE4/SRB7*, and *EWE5/NUT2*—by use of a novel conditional growth phenotype, enhanced sensitivity to 300 mM urea.

It is surprising that our screen failed to pull out genes encoding chromatin-associated proteins, especially since a previous screen, using a similar strategy (selection of bypass suppressors of a UAS deletion, in the earlier case of *SUC2*), isolated recessive mutations in several chromatin-associated proteins, including H2A, H2B, H3, Spt6, Spt10, and Spt16, in addition to three others: Bur1 and Bur2, which compose a cyclin/cyclin-dependent kinase heterodimer, and Bur6, a subunit of the heterodimeric NC2 negative general transcription factor (PRELICH and WINSTON 1993). The EWE screen isolated none of these. One explanation is that the repressive chromatin structure associated with the repressed *hsp82-ΔHSE1* promoter, illustrated in Figure 2A, is more stable than the chromatin assembling the *suc2ΔUAS* core promoter and thus not perturbed by recessive mutations in chromatin proteins. In addition, the *hsp82-ΔHSE1* promoter still retains binding sites for a gene-specific activator, whereas the *suc2ΔUAS* promoter retains none. It may be for this reason that the BUR screen failed to isolate any Mediator subunits, or it may be simply that *SUC2* and *HSP82* are regulated in a distinct manner.

TABLE 3

HSF occupancy levels

Gene	Noninduced	Induced
HSP12	0.7	0.7
HSP26	0.4	0.95
HSP82	1.0	2.1
HSC82	1.0	1.0
SSA3	0.2	0.65
SSA4	1.3	3.4

HSF ChIP signals at the indicated gene promoters were normalized to those of noninduced *HSP82* and represent the means of at least two independent experiments. Cells were heat-shock induced at 39° for 15 min. For these assays, *PHO5* served as a nonspecific ChIP control as previously described (SEKINGER and GROSS 2001).

Sin4 may be functionally distinct from the other five Ewe subunits: It is possible that, despite representing the largest complementation group, Sin4 functions differently from the other five suppressors. We suggest this for three reasons. First, recessive mutations in *sin4* frequently act as bypass suppressors of activator defects, particularly in the context of promoter/gene fusions such as used here (JIANG *et al.* 1995; TABTIANG and HERSKOWITZ 1998; MIZUNO and HARASHIMA 2000; WANG and MICHELS 2004; LI *et al.* 2005). Second, despite strongly derepressing the *hsp82-ΔHSE1/HIS3* and *hsp82-ΔHSE1/lacZ* fusion genes, loss-of-function *sin4* mutants fail to measurably enhance the basal transcript levels of nonchimeric *HSP* genes, including

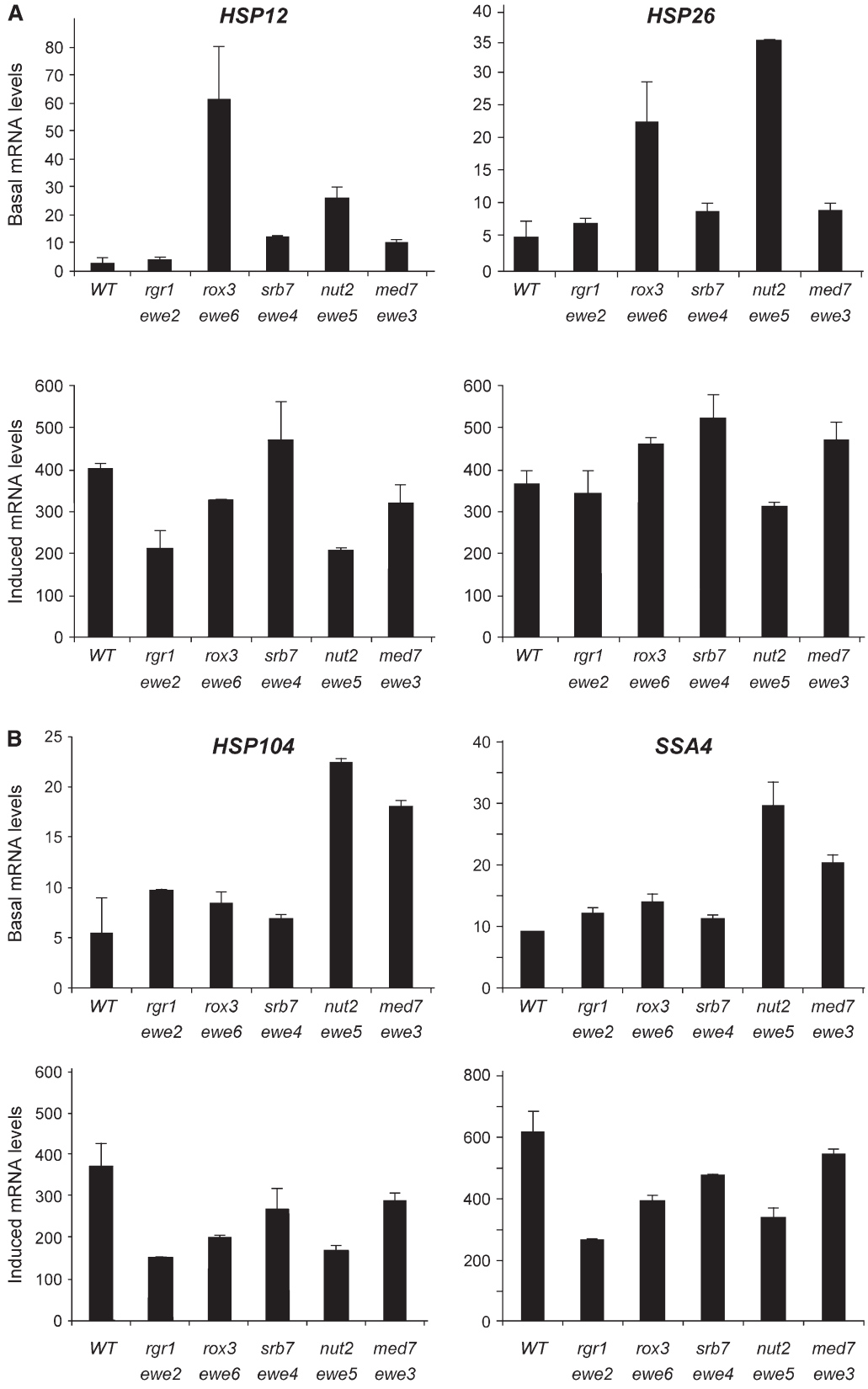

FIGURE 5.—*HSP* gene expression of *ewe* suppressors under control and heat-shock-inducing conditions. Northern analysis of wild-type (WT; HS1004), *rgr1* (J1), *rox3* (J34), *srb7* (S2), *nut2* (J15), and *med7* (S5) strains cultivated in rich medium to early log phase in 30°. Cultures were either maintained at 30° (basal) or shifted to 39° for 15 min (induced) prior to isolation of total cellular RNA. Steady-state *HSP* mRNA levels were detected using gene-specific probes; signals were normalized to those of *ACT1* (*y*-axis values are quotients: *HSP* mRNA/*ACT1* mRNA). *ACT1* expression, in turn, was independently quantified in each suppressor mutant relative to the abundance of the pol III transcript, *SCR1*, and found to be unaffected (S. B. KREMER and D. S. GROSS, data not shown). For all panels, means of two or three independent experiments ±SD are illustrated.

HSP12, *HSP26*, *HSP104*, *SSA4*, and *hsp82-ΔHSE1* itself (Table 2 ; H. SINGH, S. B. KREMER and D. S. GROSS, unpublished observations). This is consistent with previous findings that a *sin4* null mutation derepresses an *HO/lacZ* fusion gene but not *HO* itself (TABTIANG and HERSKOWITZ 1998); *sin4Δ* also derepresses *PHO5/lacZ* but not *PHO5* itself (discussed in TABTIANG and HERSKOWITZ 1998). Third, deletion of *SIN4* has little

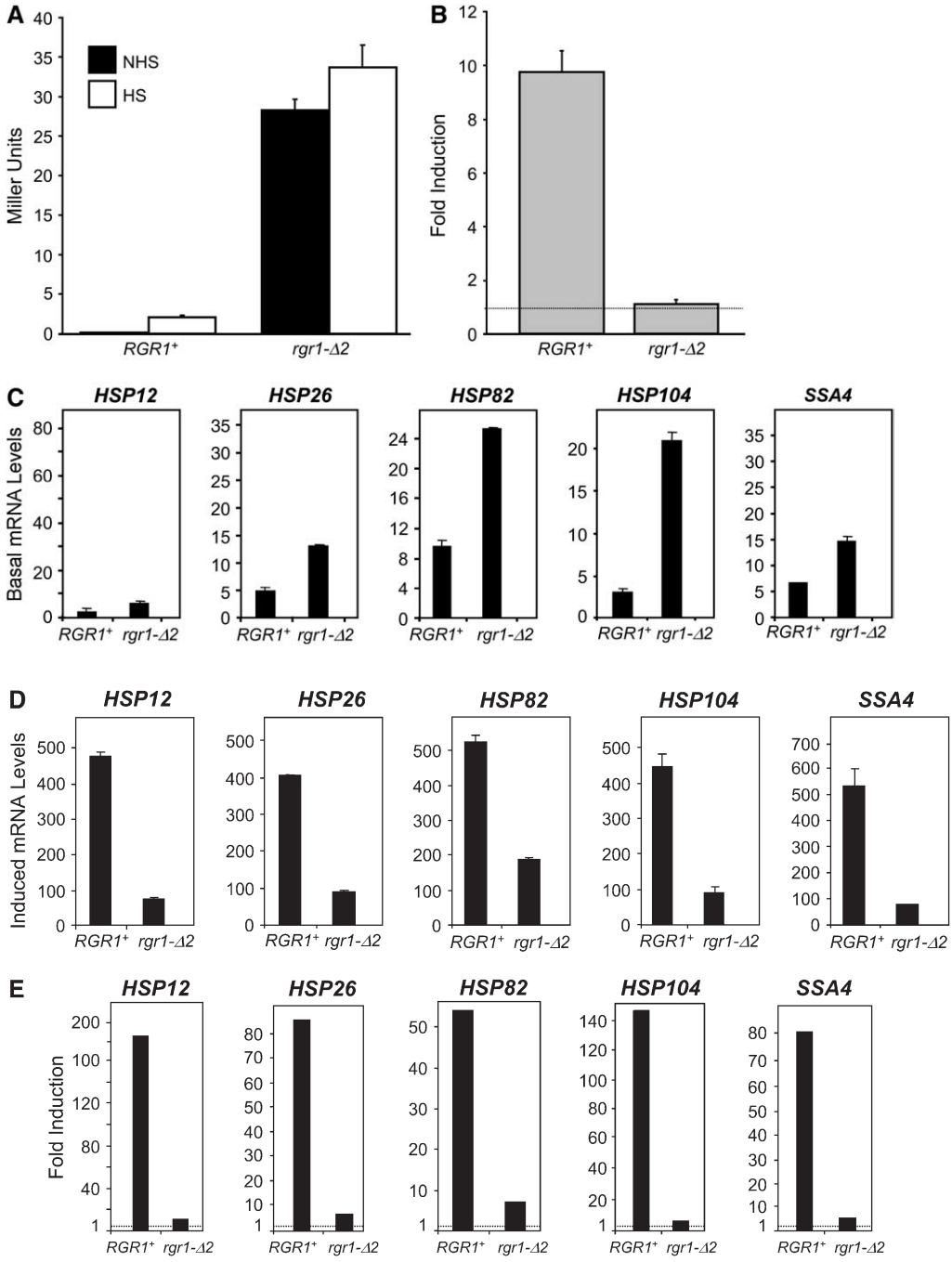

FIGURE 6.—The C-terminal truncation mutant *rgr1-Δ2* strongly enhances the basal transcription of both *hsp82-ΔHSE1* and wild-type *HSP* genes, while drastically reducing their induced transcription. (A) *hsp82-ΔHSE1/lacZ* (β-gal) expression levels in *RGR1*$^+$ (HS1004) and *rgr1-Δ2* (SBK501) strains under noninducing and heat-shock-inducing conditions were determined as in Figure 3. Shown are means ±SEM ($n = 4$). (B) Dynamic range of expression of the strains analyzed in A. Dashed line indicates a level of 1.0 (no induction). (C and D). Northern analyses of *RGR1*$^+$ (DY150) and *rgr1-Δ2* (DY2694) strains maintained at 30° (C) or subjected to a 15-min 39° heat shock (D) using *HSP*-specific probes as in Figure 5. Illustrated are means ±SD ($n =$ 2 or 3). (E) Dynamic range of heat-shock gene expression of *RGR1*$^+$ and *rgr1-Δ2* strains (data from C and D).

effect on induced *HSP12* and *HSP26* transcript levels (S. B. KREMER and D. S. GROSS, unpublished observations), in marked contrast to the dramatic reductions observed in the *rgr1-Δ2* mutant and also seen to some degree in the other spontaneous *ewe* mutants. Taken together, these observations argue against Sin4 being functionally equivalent to the other five Ewe subunits in regulating heat-shock gene expression.

Inactivating mutations in Ewe subunits likely increase the synthesis, rather than the stability, of *HSP* mRNAs: By the criteria of both liquid β-galactosidase and Northern hybridization assays, *ewe* mutations increase heat-shock gene expression. While it is formally possible that these mutations act by increasing *HSP* transcript stability, three lines of evidence argue that they act principally by enhancing *HSP* transcription. First, the abundance of both *HSP* gene transcripts and those of *hsp82/lacZ* increases in *ewe* mutants. As *HSP82* mRNA stability determinants map to its 3′-UTR (S. LINDQUIST, personal communication), and the 3′-UTR of the *hsp82/lacZ* transcript consists exclusively of *lacZ* sequence, the increase in *hsp82/lacZ* abundance is unlikely to stem from an *HSP* gene-specific enhancement of transcript stability. Second, a general increase in pol II transcript levels, while providing a possible explanation, is also unlikely, given that we measured

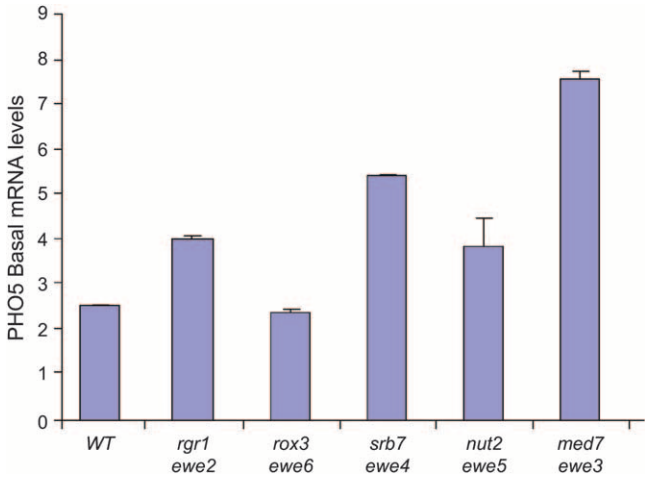

Figure 7.—Basal transcription of *PHO5* is elevated in the context of certain *ewe* mutations. Northern analysis of wild-type (WT; HS1004), *rgr1* (J1), *rox3* (J34), *srb7* (S2), *nut2* (J15), and *med7* (S5) strains cultivated in rich YPDA medium to early log phase in 30°. *PHO5* transcript levels were normalized to those of *ACT1*. Depicted are means of two independent experiments ±SD.

abundance of *HSP* mRNAs relative to that of *ACT1* mRNA; such an increase would probably be incompatible with cell viability as well. Third, heat-shock-induced *HSP* mRNA levels are not increased, and indeed are generally diminished, in *ewe* mutants. Thus, if *ewe* suppressors act to increase *HSP* mRNA stability, they would do so only under nonstressful conditions, which we believe is unlikely.

How might *ewe* suppressors act to negatively regulate heat-shock gene transcription? Repeated isolation of the Ewe subunits as suppressors of *hsp82-ΔHSE1* suggests that they compose a functionally distinct module within Mediator. Indeed, targeted deletion of *SRB2*, *SRB10*, or *GAL11* fails to suppress, even slightly, the null transcription phenotype of *hsp82-ΔHSE1*, and a *nut1Δ* mutation only weakly suppresses. Our findings are consistent with previous work showing that an *srb10Δ* mutation has little effect on the constitutive expression of *HSP82*, *HSC82*, and *SSA1* (Holstege *et al.* 1998). Therefore, an important functional role for the Ewe subunits is to negatively regulate noninduced *HSP* gene transcription. How might they accomplish this?

One possibility is that *ewe* mutations act indirectly to derepress *HSP* (and other) gene promoters. That is, the *ewe* mutations may impair the expression of gene(s) that themselves negatively regulate constitutive *HSP* transcription. However, it seems unlikely that an unbiased selection such as the one described here would isolate six subunits of Mediator yet fail to isolate a single gene whose protein product directly regulates the *hsp82-ΔHSE1* promoter, as well as the promoters of wild-type

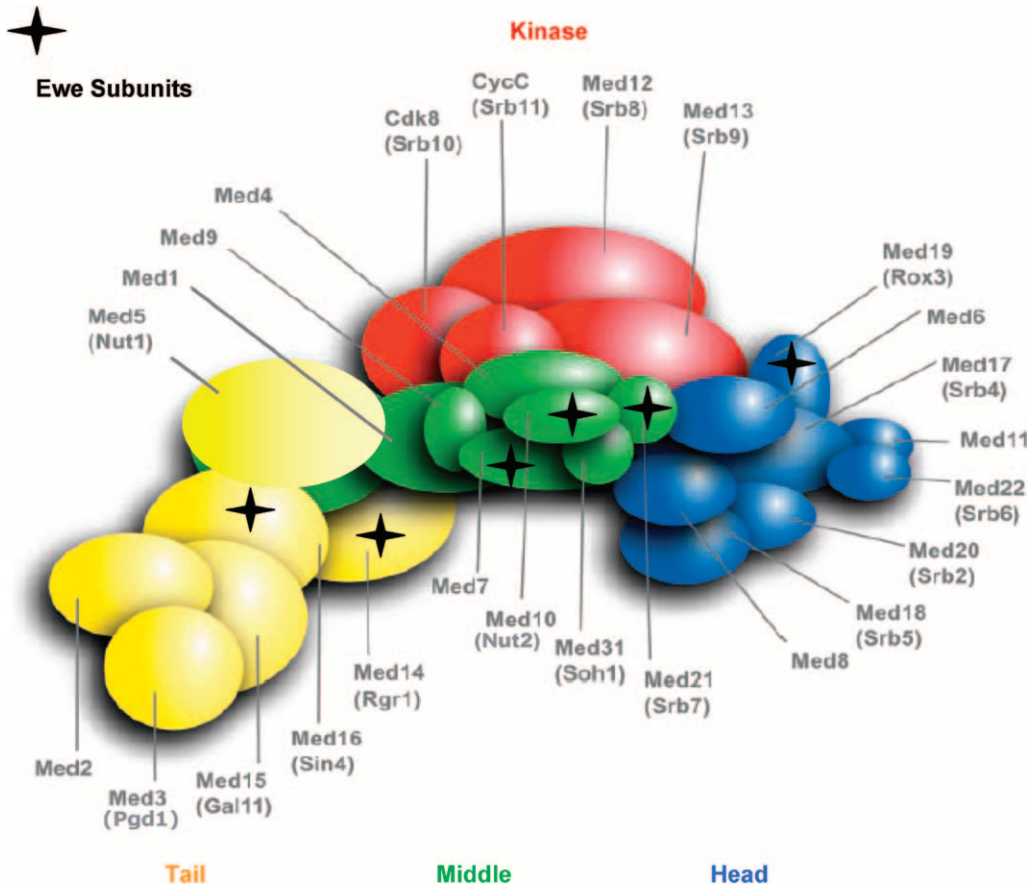

Figure 8.—Location of Ewe subunits within yeast pol II Mediator as depicted in an integrated interaction map. Subunit locations were deduced from the outcome of pairwise two-hybrid assays (Guglielmi *et al.* 2004 and references therein). Modified from Guglielmi *et al.* (2004) and presented here with permission.

HSP genes. Indeed, a direct role for Mediator in governing the noninduced expression of heat-shock genes is suggested by the fact that Mediator subunits have been detected within the upstream regions of several heat-shock genes, including *HSP12*, *HSP82*, *HSP104*, and *SSA4*, in cells grown under nonstressful conditions (30°, midlog growth phase) in a genomewide analysis (J.-C. Andrau and F. Holstege, personal communication). In this regard, Mediator, through its Ewe module, may act directly to repress basal heat-shock gene transcription. This could be achieved by impairing the recruitment of GTFs (including RNA pol II itself) and/or impairing their assembly into a functionally competent PIC. Other scenarios are also possible and are the subject of current investigation.

It is intriguing to consider that in repressing basal transcription, the Ewe module may serve a role similar to that of the TFIIB recognition element (BRE), mutation of which has been shown to result in a similar phenotype in mammalian systems: loss of the dynamic range of expression due to a loss of repression of basal transcription (Evans *et al.* 2001). Thus, both the Mediator components identified here and the BRE may function to regulate the transition of the PIC from an initiation-competent to an elongation-competent complex.

***ewe* mutations can severely impair induced transcription:** Evidence for a positive role of the Ewe module in activated transcription comes from expression assays of heat-shock-induced cells. These reveal a drastic reduction in induced transcript levels of wild-type heat-shock genes in the *rgr1-Δ2* mutant and, to a lesser extent, in the spontaneous *rgr1-1001* and *nut2-1001* mutants. Our data thus lend support to earlier speculation that HSF activates transcription of its target genes via a Mediator subcomplex containing Rgr1, Nut2, and Srb7 (Lee *et al.* 1999). Of note, the strong requirement for Rgr1 in HSF activation contrasts dramatically with the dispensability of other GTFs, including the Srb4 and Srb6 subunits of Mediator, the TFIIH kinase, and the pol II CTD (see the Introduction).

A special role for Mediator at promoters with constitutively bound activators: It is tempting to speculate that Mediator evolved to negatively regulate noninduced heat-shock gene transcription since HSF, unlike many inducible yeast activators, constitutively resides in the nucleus where it binds high-affinity target sequences. Thus, Mediator may be needed to prevent promiscuous heat-shock gene transcription. In the *ewe* mutants identified here, Mediator's negative regulatory role is compromised and basal *HSP* gene transcription is significantly elevated. It might be anticipated that other constitutively bound activators will exhibit a similar requirement, and indeed we find that *PHO5*, bearing a constitutively bound activator, is derepressed by *ewe* mutants. On the other hand, the nucleosomal nature of the noninduced *HSP82*$^{+}$ promoter (Sekinger and Gross 2001; Erkine and Gross 2003; Zhao *et al.* 2005), shared by other *HSP* gene promoters (Erkine *et al.* 1996) and greatly pronounced in mutants such as *hsp82-ΔHSE1* (Gross *et al.* 1993), might elicit a requirement for Mediator, although the reason for this is unclear. Clearly, additional work will be required to distinguish between these and other potential mechanisms by which the Ewe module of Mediator governs gene transcription.

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