

# The tRNA-binding moiety in GCN2 contains a dimerization domain that interacts with the kinase domain and is required for tRNA binding and kinase activation

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**GCN2 stimulates translation of *GCN4* mRNA in amino acid-starved cells by phosphorylating translation initiation factor 2. GCN2 is activated by binding of uncharged tRNA to a domain related to histidyl-tRNA synthetase (HisRS). The HisRS-like region contains two dimerization domains (HisRS-N and HisRS-C) required for *GCN2* function *in vivo* but dispensable for dimerization by full-length GCN2. Residues corresponding to amino acids at the dimer interface of *Escherichia coli* HisRS were required for dimerization of recombinant HisRS-N and for tRNA binding by full-length GCN2, suggesting that HisRS-N dimerization promotes tRNA binding and kinase activation. HisRS-N also interacted with the protein kinase (PK) domain, and a deletion impairing this interaction destroyed *GCN2* function without reducing tRNA binding; thus, HisRS-N–PK interaction appears to stimulate PK function. The C-terminal domain of GCN2 (C-term) interacted with the PK domain in a manner disrupted by an activating PK mutation (*E803V*). These results suggest that the C-term is an autoinhibitory domain, counteracted by tRNA binding. We conclude that multiple domain interactions, positive and negative, mediate the activation of GCN2 by uncharged tRNA.**

**Keywords:** dimerization/eIF2 $\alpha$  kinase/GCN2/regulation/translation

## Introduction

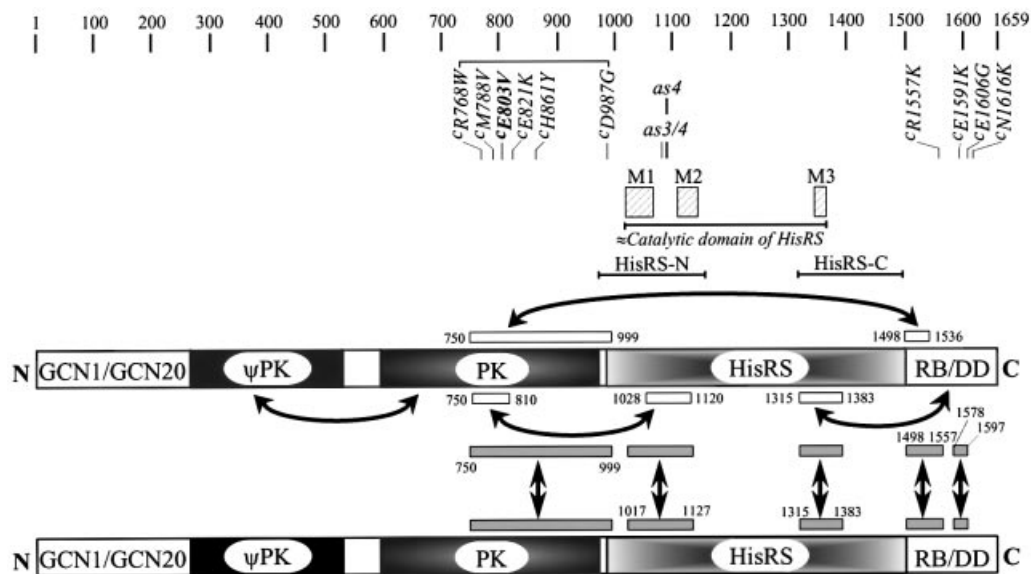
Yeast cells respond to amino acid or purine starvation by activating GCN2, a protein kinase (PK) that specifically phosphorylates the  $\alpha$  subunit of translation initiation factor 2 (eIF2 $\alpha$ ) on serine-51. Phosphorylation of eIF2 converts it from substrate to inhibitor of its guanine nucleotide exchange factor eIF2B, decreasing formation of the ternary complex (eIF2-GTP-Met-tRNA<sub>i</sub><sup>Met</sup>), which transfers Met-tRNA<sub>i</sub><sup>Met</sup> to the 40S ribosome. A decrease in ternary complex levels stimulates translation of *GCN4* mRNA, encoding a transcriptional activator of multiple amino acid biosynthetic enzymes. Four short open reading frames (uORFs) in the *GCN4* mRNA leader function in a specialized re-initiation mechanism that derepresses

*GCN4* translation in response to reductions in ternary complex levels that are too small to impede general protein synthesis. Dominant activating mutations in *GCN2* have been isolated that derepress *GCN4* under nonstarvation conditions, and the strongest of these *GCN2<sup>c</sup>* alleles also impairs general translation and cell growth due to a severe depletion of the ternary complex.

GCN2 is activated in amino acid-starved cells through binding of uncharged tRNA to a region homologous to histidyl-tRNA synthetase (HisRS), located C-terminal to the kinase domain (Wek *et al.*, 1989) (Figure 1). Mutations in the HisRS-like domain in conserved residues required for tRNA binding by class II aminoacyl-tRNA synthetases (the m2 motif) inactivated *GCN2* kinase function, and destroyed tRNA binding by the isolated HisRS domain (Wek *et al.*, 1995; Zhu *et al.*, 1996) and by full-length GCN2 (Dong *et al.*, 2000). It appears that starvation of yeast for any amino acid activates GCN2 and elicits derepression of *GCN4* translation (Hinnebusch, 1992; Wek *et al.*, 1995), implying that GCN2 can discriminate only between charged and uncharged forms of tRNA. Indeed, purified GCN2 bound various uncharged tRNAs with similar affinities, but interacted with charged tRNA<sup>Phe</sup> less strongly than with uncharged tRNA<sup>Phe</sup> (Dong *et al.*, 2000). GCN2 homologs have been identified in *Neurospora crassa* (Sattlegger *et al.*, 1998), *Drosophila melanogaster* (Santoyo *et al.*, 1997; Olsen *et al.*, 1998) and mouse (Berlenga *et al.*, 1998; Sood *et al.*, 2000), and the HisRS domain is conserved in all of these proteins; thus, activation by uncharged tRNA seems to be an evolutionarily conserved feature of the GCN2 family of PKs.

The extreme C-terminal segment of yeast GCN2 (C-term) is also essential for *GCN2* function *in vivo* (Wek *et al.*, 1990). Both the C-term and PK domains have dimerization activities, but only the C-term was required for dimerization by full-length GCN2 *in vivo* (Qiu *et al.*, 1998). The isolated C-term can bind double-stranded RNA *in vitro* and interact with ribosomes in cell extracts, suggesting that it mediates ribosome binding by GCN2 through interactions with double-stranded segments of rRNA (Zhu and Wek, 1998). It was shown recently that the C-term additionally promotes tRNA binding by the HisRS domain independently of its role in GCN2 dimerization (Dong *et al.*, 2000). Moreover, the C-term can physically interact with both the HisRS and PK domains, and the HisRS region can interact with the PK domain (Qiu *et al.*, 1998). Interestingly, tRNA antagonized interaction *in vitro* between the PK domain and a fragment containing the HisRS and C-term domains. Accordingly, we proposed that tRNA would activate GCN2 by impeding an inhibitory association between the PK and C-term domains (Dong *et al.*, 2000).

Given the central role of the HisRS region in activation of GCN2 by uncharged tRNA, it was imperative to map



**Fig. 1.** Summary of GCN2 dimerization and domain interactions. Two GCN2 polypeptides of 1659 residues [numbered at the very top from amino (N) to carboxyl (C) terminus] are depicted schematically as rectangular boxes subdivided into the following functional domains: the conserved N-terminal region required for interaction with GCN1 and GCN20 (GCN1/GCN20) (Garcia-Barrio *et al.*, 2000); a degenerate protein kinase domain ( $\psi$ PK); the protein kinase domain (PK); a histidyl-tRNA synthetase-related region (HisRS); and a C-terminal region required for ribosome binding and dimerization by GCN2 (RB/DD). The rectangular gray boxes located between the two GCN2 schematics that are linked by two-headed arrows depict the segments involved in self-interactions, with the amino acid positions of their end points indicated. The open rectangular boxes above or below the top GCN2 schematic indicate the segments required for the interactions between different functional domains indicated by two-headed arrows. The interactions involving the HisRS domain are based on results presented below (see text). The locations of the N-terminal and C-terminal segments of the HisRS region designated in the text as HisRS-N and HisRS-C, respectively, are indicated above the top schematic. The region that is similar in sequence to the catalytic domain of authentic HisRS is indicated immediately below the hatched boxes labeled M1–3, the latter indicating the positions of three sequence motifs conserved among class II aminoacyl-tRNA synthetases. Immediately below the sequence numbering are the locations and nature of various *GCN2<sup>c</sup>* mutations (designated with “*c*”) and the *as4* and *as3/4* mutations.

precisely the interactions between this region and the PK and C-term domains. In particular, contacts between the HisRS and PK domains may influence the ability of tRNA to promote an activated conformation of the PK domain. Considering that *Escherichia coli* HisRS is a dimer, it was also important to determine whether the HisRS-like domain in GCN2 dimerizes, and the consequences of dimerization in this region for tRNA binding and kinase activation. We show here that the GCN2 HisRS region has two dimerization domains, of which the N-terminal moiety most likely resembles the dimerization surface in the catalytic domain of authentic HisRS. Mutational analysis suggests that this region makes a crucial contribution to GCN2 function by promoting tRNA binding and through a stimulatory physical interaction with the kinase domain. We also obtained evidence for physical interaction between the C-term and PK domains that negatively regulates kinase activity in a manner overcome by uncharged tRNA in amino acid-starved cells.

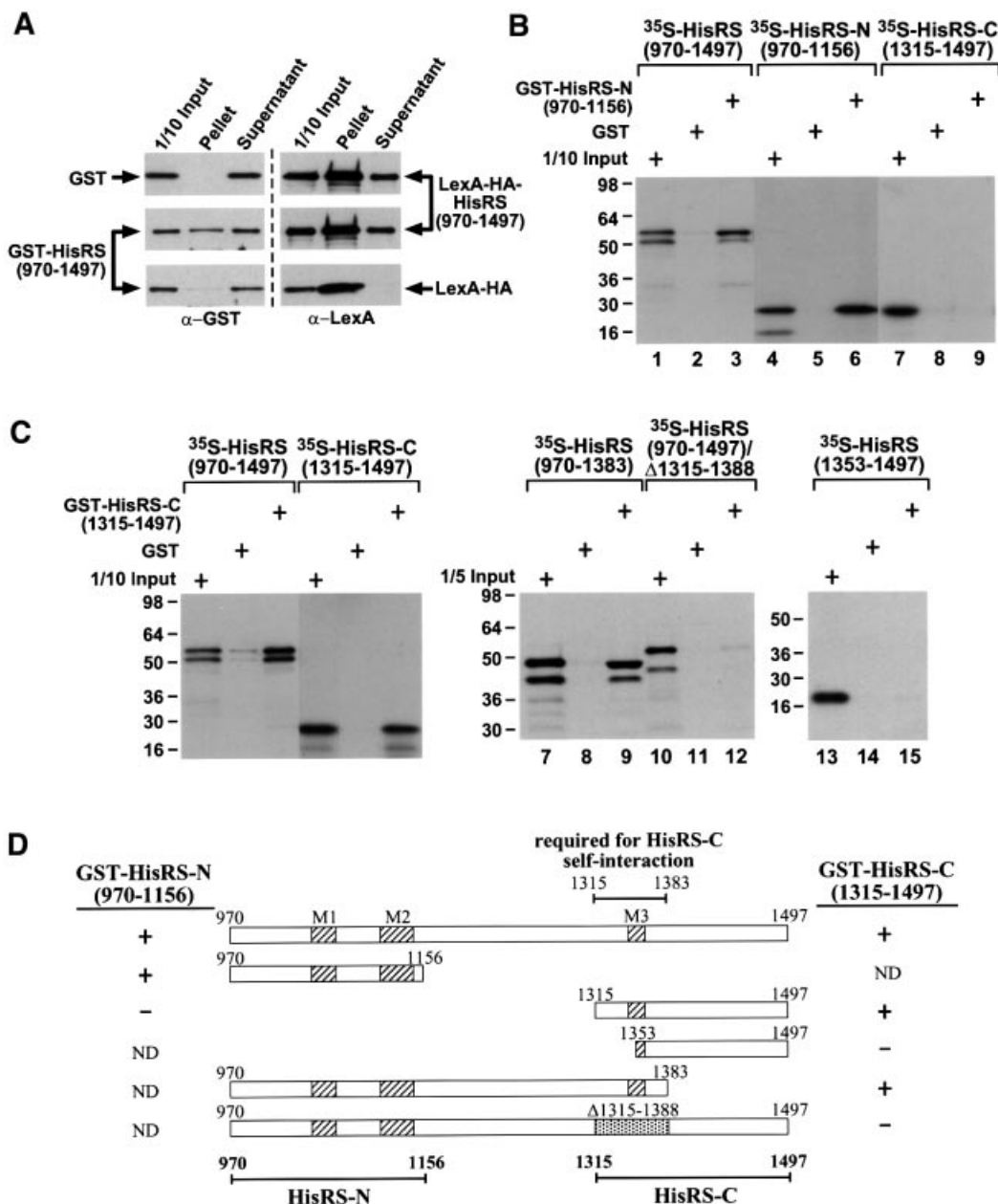
## Results

### **The HisRS-like region of GCN2 contains two independent dimerization domains**

Because the HisRS-related region of GCN2 contains sequence similarities to residues that comprise the dimer interface in authentic *E. coli* HisRS (Arnez *et al.*, 1995), we investigated whether the GCN2 HisRS-like domain can dimerize. In a first approach, we asked whether glutathione *S*-transferase (GST) and LexA-HA (hemagglutinin)

fusions to the HisRS-like segment (residues 970–1497), both expressed in yeast from the *ADHI* promoter, could be co-immunoprecipitated from cell extracts. As shown in Figure 2A, a fraction of GST-HisRS(970–1497) was co-immunoprecipitated by anti-HA antibodies along with LexA-HA-HisRS(970–1497), but not with LexA-HA alone; additionally, GST alone did not co-immunoprecipitate with LexA-HA-HisRS(970–1497). Western analysis using antibodies against GCN2 showed that GST-HisRS(970–1497) and LexA-HA-HisRS(970–1497) were expressed at similar amounts (data not shown). Based on the recovery of GST-HisRS(970–1497) in immune complexes, we estimated that ~1/4 of the total GST-HisRS(970–1497) was complexed with LexA-HA-HisRS(970–1497) in the extract. Given that the co-immunoprecipitation assay detects the heterodimers containing LexA- and GST-tagged HisRS fusions, but not the corresponding homodimers, our data suggest that a substantial proportion of the HisRS fusion proteins were dimerized *in vivo*.

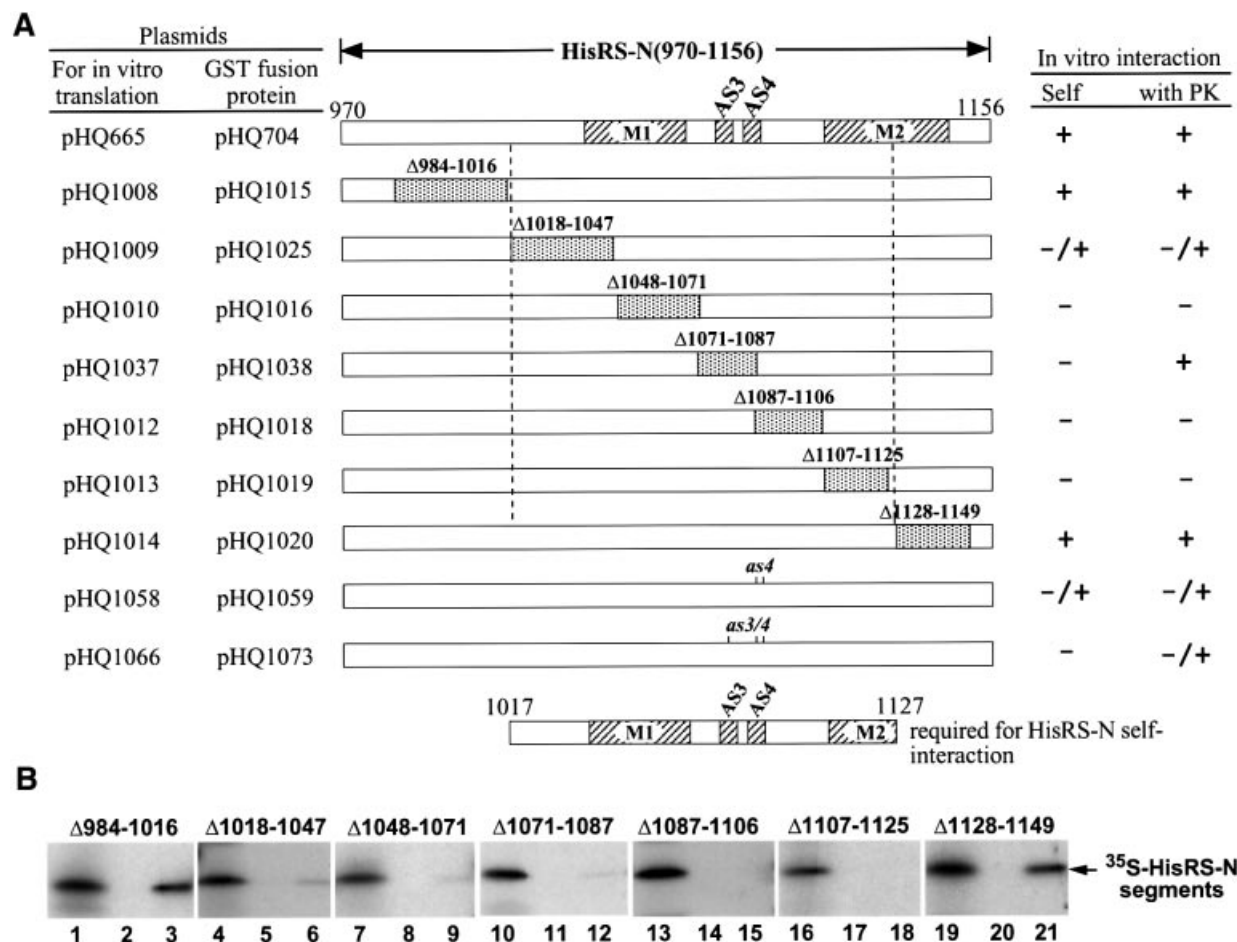
To map the interacting domain(s) within the HisRS segment, we carried out *in vitro* binding experiments using GST fusions expressed in *E. coli* containing the N- or C-terminus of the GCN2 HisRS-like region [residues 970–1156 (GST-HisRS-N) or 1315–1497 (GST-HisRS-C), respectively] and <sup>35</sup>S-labeled polypeptides synthesized *in vitro* containing the same GCN2 residues. As shown in Figure 2B (and summarized in Figure 2D), full-length and N-terminal [<sup>35</sup>S]HisRS fragments, but not the C-terminal [<sup>35</sup>S]HisRS fragment, bound to GST-HisRS-N but not to



**Fig. 2.** The HisRS-like region contains two independent dimerization domains. (A) Co-immunoprecipitation of GST-HisRS with LexA-HA-HisRS from yeast cell extracts. Transformants of *gcn2Δ* strain HQY132 (Qiu *et al.*, 1998) bearing *HIS3* plasmids encoding LexA-HA-HisRS(970-1497) (pHQ588) or LexA-HA (p2247), and *TRP1* plasmids encoding GST (pHQ242) or GST-HisRS(970-1497) (pHQ601), were grown in SC-His-Trp medium and whole-cell extracts were prepared. Aliquots of extracts containing 50 μg of protein were immunoprecipitated with anti-HA antibodies, and the immune complexes were resolved by SDS-PAGE and subjected to western blot analysis using anti-GST antibodies (left panels) or anti-LexA antibodies (right panels). The top panels show results from the transformant expressing GST and the LexA-HA-HisRS fusion; the middle panels derive from the transformant expressing the GST-HisRS fusion and LexA-HA. (B and C) The HisRS-like region dimerizes *in vitro* through the N- and C-terminal subregions. The full-length HisRS-like region (aa 970-1497) or truncated segments indicated across the top of each panel were translated *in vitro* in the presence of [<sup>35</sup>S]methionine and incubated with either GST, GST-HisRS(970-1156) (B) or GST-HisRS(1315-1497) (C) proteins, which were expressed in *E. coli* and immobilized on glutathione-Sepharose beads. After extensive washing of the beads, the bound proteins were resolved by SDS-PAGE and visualized by fluorography. Numbers to the left of each gel give the positions of size standards of the indicated molecular weight in kilodaltons. (D) Summary of results from the binding assays shown in (B) and (C). Rectangular boxes with the locations of conserved motifs M1-3 (indicated with diagonal hatching) represent the *in vitro* translated proteins with GCN2 residue numbers indicated at their N- and C-termini. The binding (+) or failure to bind (-) of these segments to GST-HisRS(970-1156) or GST-HisRS(1315-1497) is summarized to the right and left of the schematics, respectively. ND, not determined. The results of these assays indicate that dimerization by the C-terminal segment of the HisRS-like region is dependent on residues 1315-1383, as indicated by the bar above the boxes. Bars below the boxes represent HisRS-N and HisRS-C subregions, respectively.

GST alone. Thus, the GCN2 HisRS-N region can dimerize in the absence of other yeast proteins. The GST-HisRS-C fusion interacted with full-length (970-1497) and

C-terminal (1315-1497) segments of the HisRS-like domain (Figure 2C, lanes 1-6), indicating that an additional dimerization domain resides in the HisRS-C

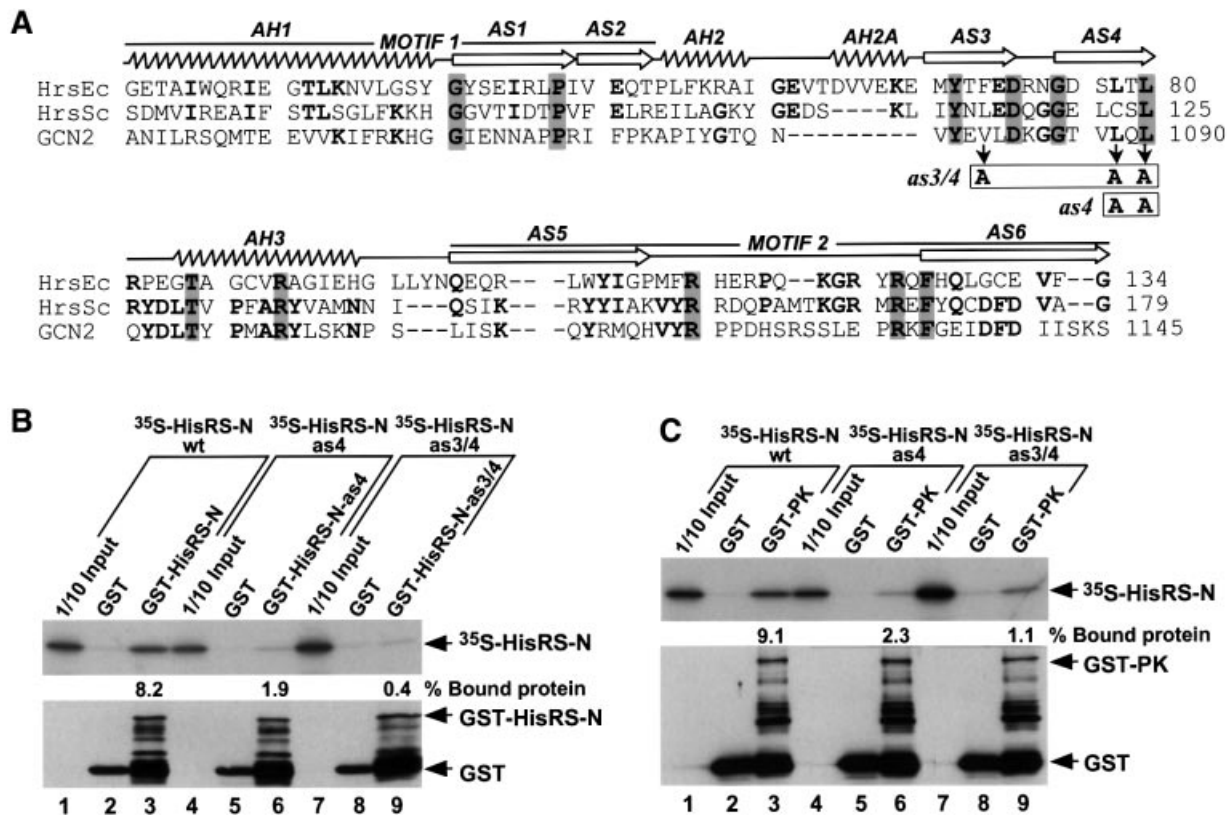


**Fig. 3.** The minimal segment required for dimerization of the HisRS-N region also mediates interaction with the PK domain *in vitro*. (A) Summary of *in vitro* protein binding assays measuring dimerization or binding to the PK domain by wild-type or mutant versions of the HisRS-N segment. Wild-type HisRS-N is shown schematically at the top with the locations of motifs M1, M2 and predicted  $\beta$ -strands AS3 and AS4 indicated with hatching. Beneath the wild-type HisRS-N segment are shown mutant derivatives containing the indicated internal deletions (shown by shading) or the *as4* or *as3/4* point mutations. Each was synthesized *in vitro* and labeled with [ $^{35}$ S]methionine, and also expressed as a GST fusion in *E. coli*, using the corresponding plasmids listed on the left. Each GST fusion protein was immobilized on glutathione–Sepharose beads and incubated with the  $^{35}$ S-labeled HisRS-N protein containing the same deletion or mutation to assay for self-interactions. The results of these assays shown in (B) are summarized to the right of the schematics in (A) under the column headed ‘Self’. The GST–PK(568–998) fusion immobilized on glutathione–Sepharose beads was also incubated with the same panel of  $^{35}$ S-labeled HisRS-N proteins and the results of these binding assays (data not shown) are summarized in (A) under the column headed ‘with PK’. The rectangular box at the bottom represents the minimal dimerization domain in the HisRS-N region deduced from these experiments. (B) The  $^{35}$ S-labeled HisRS-N segments containing the different deletions indicated above the gels were incubated with immobilized GST (lanes 2, 5, 8, 11, 14, 17 and 20) or GST–HisRS-N fusion proteins containing the same deletions present in the  $^{35}$ S-labeled HisRS-N segments (lanes 3, 6, 9, 12, 15, 18 and 21). After extensive washing, the bound proteins were resolved by SDS–PAGE and visualized by fluorography. Lanes 1, 4, 7, 10, 13, 16 and 19 contain 1/5 of the input radiolabeled proteins used in the binding experiments.

region. GST–HisRS-C also interacted with [ $^{35}$ S]HisRS(970–1383), but not with [ $^{35}$ S]HisRS(1353–1497) (Figure 2C, lanes 7–9 and 13–15, respectively), suggesting that the 1315–1383 interval is required for dimerization by HisRS-C (summarized in Figure 2D). This conclusion was supported by the finding that deletion of the 1315–1388 interval from full-length [ $^{35}$ S]HisRS abolished its binding to GST–HisRS-C (Figure 2C, lanes 10–11; see Figure 2D).

To localize the dimerization determinants in the HisRS-N domain, we carried out binding assays with GST–HisRS-N and derivatives of [ $^{35}$ S]HisRS-N bearing successive internal deletions of 16–32 residues. Deleting blocks of residues in the 108 amino acid interval between 1018–1125 severely impaired or eliminated dimerization by these proteins *in vitro* (Figure 3, *in vitro* interaction, self). Interestingly, this segment of GCN2 HisRS-N

corresponds to the dimeric interface between core catalytic domains in the crystal structure of *E. coli* HisRS (Arnez *et al.*, 1995), including conserved residues in motif 1,  $\beta$ -strands AS3 and AS4, and the N-terminal half of motif 2 (Figure 3). Accordingly, we made substitution mutations in the GCN2 HisRS-N region at positions corresponding to amino acids that make symmetric hydrophobic contacts between the catalytic domains of the *E. coli* HisRS dimer (Arnez *et al.*, 1995). Two residues in the predicted  $\beta$ -strand AS4, Leu1088 and Leu1090, were substituted simultaneously with alanines (*as4* mutation), and these mutations were also combined with an Ala substitution of Val1080 in the predicted  $\beta$ -strand AS3 (*as3/4* mutation) (see Figure 4A). Introduction of the *as4* or *as3/4* mutation into GST–HisRS-N and [ $^{35}$ S]HisRS-N weakened or abolished, respectively, complex formation between these proteins *in vitro* (Figure 4B; summarized in Figure 3A).



**Fig. 4.** Point mutations in the HisRS-N region abolish self-interaction and impair interaction with the PK domain *in vitro*. (A) Alignment of the HisRS-N region of GCN2 (amino acids 1036–1145) with segments from authentic histidyl-tRNA synthetases of *E. coli* (HrsEc) and *Saccharomyces cerevisiae* (HrsSc) encompassing motifs M1 and M2, the  $\alpha$ -helices (AH1–AH3) and  $\beta$ -strands (AS1–AS6) present in the crystal structure of *E. coli* HisRS (Arnez *et al.*, 1995), all indicated above the structure. The single or double mutations in  $\beta$ -strands AS3 (V1080A) and AS4 (L1088A, L1090A), designated *as4* and *as3/4*, are indicated with boxes beneath the GCN2 sequence. (B and C) *In vitro* binding of [<sup>35</sup>S]HisRS-N segments (aa 970–1156) synthesized *in vitro* containing mutations *as4* or *as3/4* to GST fusion proteins containing the same HisRS-N segments (B), or the PK domain (C). The upper panels display the fluorograms of <sup>35</sup>S-labeled proteins added to the reactions (1/10 Input) or bound to the GST or GST–HisRS-N proteins, as indicated. The percentages of the total <sup>35</sup>S-labeled proteins bound in each reaction were quantified by phosphoimaging analysis and are indicated below the relevant lanes. The lower panels show the results of immunoblot analysis using GST antibodies of the GST proteins bound to glutathione–Sepharose beads in each reaction. The positions of GST, GST–HisRS-N and GST–PK fusion proteins are indicated on the right.

These findings suggest that GCN2 contains a dimerization interface resembling that of authentic HisRS.

**Both dimerization domains in the HisRS-like region are required for GCN2 function *in vivo***

To determine whether dimerization of the HisRS-N region is required for GCN2 function *in vivo*, each of the internal deletions and point mutations shown in Figure 3A was generated in a plasmid-borne copy of GCN2, and the resulting *gcn2* alleles were tested for complementation of the 3-aminotriazole-sensitive phenotype (3AT<sup>S</sup>) of a *gcn2Δ* mutant. GCN2-mediated derepression of GCN4 translation, with attendant derepression of histidine biosynthetic genes, is required for growth in the presence of 3AT, an inhibitor of the HIS3 product. None of the *gcn2* alleles complemented the 3AT<sup>S</sup> phenotype of the *gcn2Δ* mutant. Western analysis of cell extracts with GCN2 antibodies indicated that all but three of the *gcn2* alleles were expressed at levels similar to that of wild-type GCN2 (Table I). Although *gcn2-Δ1018–1047*, *gcn2-as4* and *gcn2-as3/4* products were expressed at only 60–70% of the wild-type level, these alleles still failed to complement the *gcn2Δ* mutant when overexpressed from high copy-number plasmids (data not shown). Deletion of the

C-terminal dimerization domain in the HisRS-like region in *gcn2-Δ1315–1383* also destroyed GCN2 function *in vivo* (Table I). We conclude that the dimerization domains in the N-terminal and C-terminal halves of the HisRS-like region are both required for GCN2 function *in vivo*.

**Effects of mutations in the HisRS-N domain on tRNA binding and dimerization by full-length GCN2**

We showed previously that an internal deletion of all HisRS domain residues C-terminal to the HisRS-N domain had only a small effect on the ability of full-length GCN2 to dimerize with a full-length LexA-tagged GCN2 protein expressed in yeast (Qiu *et al.*, 1998). In that study, the *gcn2-Δ1161–1570* product formed heterodimers with LexA–GCN2 at ~75% of the level seen for wild-type GCN2. Similar results were obtained for a deletion of the PK domain, whereas deletion of the C-term domain reduced dimerization to only 8% of the wild-type level. Thus, dimerization by full-length GCN2 *in vivo* is more critically dependent on the C-term than on dimerization surfaces in the PK or HisRS-C domains. To evaluate the importance of the HisRS-N region for dimer formation by full-length GCN2,

**Table I.** Phenotypes and expression of plasmid-borne *gcn2* alleles with mutations in the HisRS-like domain<sup>a</sup>

Plasmid	<i>gcn2</i> allele	Growth on 3-AT	Relative GCN2 protein level
Vector	none	–	NA
pHQ644	<i>GCN2</i>	+	100
pHQ1041	<i>gcn2-Δ984–1016</i>	–	90
pHQ1039	<i>gcn2-Δ1018–1047</i>	–	60
pHQ1042	<i>gcn2-Δ1048–1071</i>	–	90
pHQ1047	<i>gcn2-Δ1071–1087</i>	–	120
pHQ1043	<i>gcn2-Δ1087–1106</i>	–	110
pHQ1040	<i>gcn2-Δ1107–1125</i>	–	130
pHQ1051	<i>gcn2-Δ1128–1149</i>	–	130
pHQ1092	<i>gcn2-Δ1315–1383</i>	–	110
pHQ1076	<i>gcn2-as4</i>	–	70
pHQ1077	<i>gcn2-as3/4</i>	–	60

<sup>a</sup>Wild-type *GCN2* and the indicated *gcn2* alleles were introduced on single-copy plasmids into *gcn2Δ* strain H1149 and the resulting transformants were tested for growth on synthetic minimal medium containing 30 mM 3AT. +, growth (Hinnebusch and Fink, 1983); –, no growth after 3 days at 30°C. The relative amount of GCN2 protein expressed from each allele was determined by subjecting whole-cell extracts from the same transformants to SDS-PAGE and immunoblot analysis using antibodies against GCN2.

we asked whether the products of *gcn2-Δ1018–1047*, *gcn2-Δ1048–1071*, *gcn2-as4* and *gcn2-as3/4* could be co-immunoprecipitated with LexA–GCN2 from cell extracts. As shown above, all of these *gcn2* mutations weakened or abolished dimerization of the isolated HisRS-N segment *in vitro* (Figure 3). In contrast, none of them reduced co-immunoprecipitation of full-length GCN2 with LexA–GCN2 from cell extracts (Figure 5A). The latter result was also obtained for the *gcn2-m2* product, which lacks two key residues in motif 2 of the HisRS-like region required for tRNA binding *in vitro* (Wek *et al.*, 1995). In agreement with previous findings (Qiu *et al.*, 1998), only the *gcn2-Δ1536–1659* product (lacking the C-term domain) failed to interact with LexA–GCN2 in these assays. We conclude that the HisRS-N and HisRS-C dimerization surfaces, and also the tRNA-binding activity of GCN2, are dispensable for dimerization by the full-length protein.

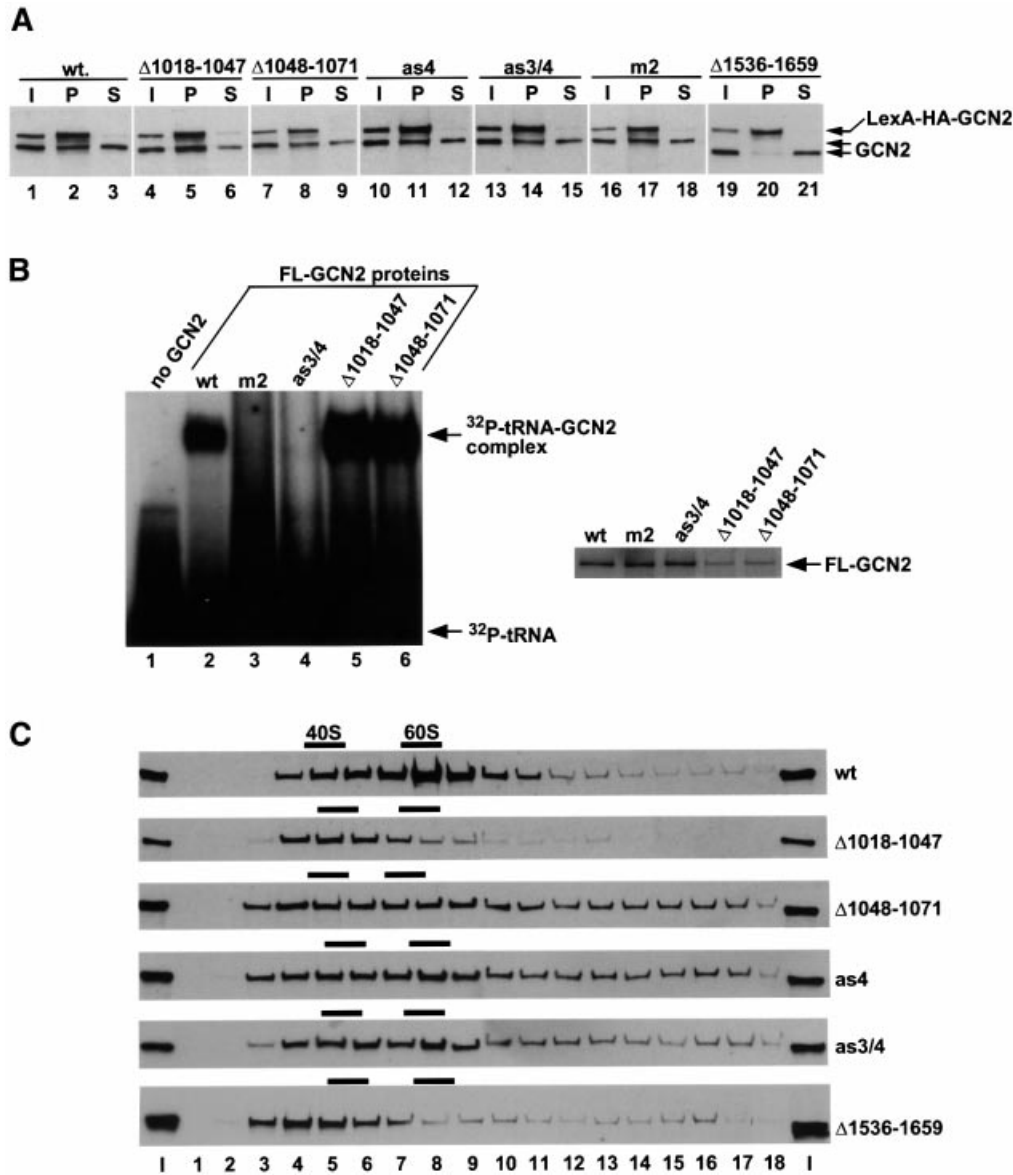
Although the HisRS domain is dispensable for GCN2 dimerization, the binding of uncharged tRNA could depend on dimerization of the HisRS domain within GCN2. To address this possibility, we purified full-length GCN2 proteins [tagged with Flag (FL) epitope] bearing mutations in the HisRS-N domain that abolish dimerization of this moiety, and assayed them for tRNA binding using a gel mobility shift assay (GMSA). In agreement with recent findings (Dong *et al.*, 2000), wild-type FL–GCN2 formed a complex with <sup>32</sup>P-labeled total yeast tRNA ([<sup>32</sup>P]tRNA), whereas the *gcn2-FL-m2* product (bearing motif 2 substitutions) did not (Figure 5B). The product of *gcn2-FL-as3/4* was also impaired for tRNA binding (Figure 5B), suggesting that the symmetric contacts in the dimeric interface of the HisRS-N region are required for tRNA binding by GCN2. Surprisingly, the *gcn2-FL-Δ1018–1047* and *gcn2-FL-Δ1048–1071* products lacking predicted structural elements in motif 1 (most of AH1; C-terminus of AH1, AS1, AS2 and AH2, respectively) reproducibly displayed a higher affinity than the

wild-type protein for tRNA (Figure 5B). The fact that these deletions destroy dimerization of the isolated HisRS-N domain *in vitro*, but do not impair tRNA binding by full-length GCN2, seems at odds with the idea that dimerization of the HisRS-N region is required for tRNA binding. This apparent contradiction is addressed below.

Having found that the *Δ1018–1047* and *Δ1048–1071* mutations in the HisRS-N domain did not impair dimerization or tRNA binding by full-length GCN2, we considered the possibility that they confer a Gcn<sup>–</sup> phenotype by disrupting ribosome binding by GCN2. We showed previously that removal of the C-term nearly abolished ribosome binding by GCN2, as judged by its failure to co-sediment with free 60S subunits in cell extracts resolved on sucrose gradients. In contrast, internal deletions in the HisRS-C region (residues 1161–1245 or 1401–1535) led to moderate reductions in ribosome binding (Ramirez *et al.*, 1991). The results in Figure 5C confirm our previous observations by showing that a large proportion of wild-type GCN2, but little or no *gcn2-Δ1536–1659* product (lacking the C-term), co-sedimented with 60S subunits (top and bottom panels). The *as4* and *as3/4* mutations had little or no effect on 60S subunit association, indicating that dimerization of the HisRS-N domain and tRNA binding are both dispensable for high-level ribosome association by GCN2. The *Δ1048–1071* mutation also seemed to have little effect on ribosome binding, whereas the *Δ1018–1047* deletion led to a strong reduction in 60S binding, comparable to that seen for deletion of the C-term (*Δ1536–1659*) (Figure 5C). Accordingly, the Gcn<sup>–</sup> phenotype of *gcn2-Δ1018–1047* may result from diminished ribosome association by the mutant product. This seems unlikely for the *gcn2-Δ1048–1071* product, however, implying that the HisRS-N region plays a role in stimulating PK function independently of tRNA binding or ribosome association by GCN2. As described below, this novel positive function may involve physical contact between the PK and HisRS-N regions.

#### **The N- and C-terminal subdomains of the HisRS region interact with the PK and C-term domains, respectively**

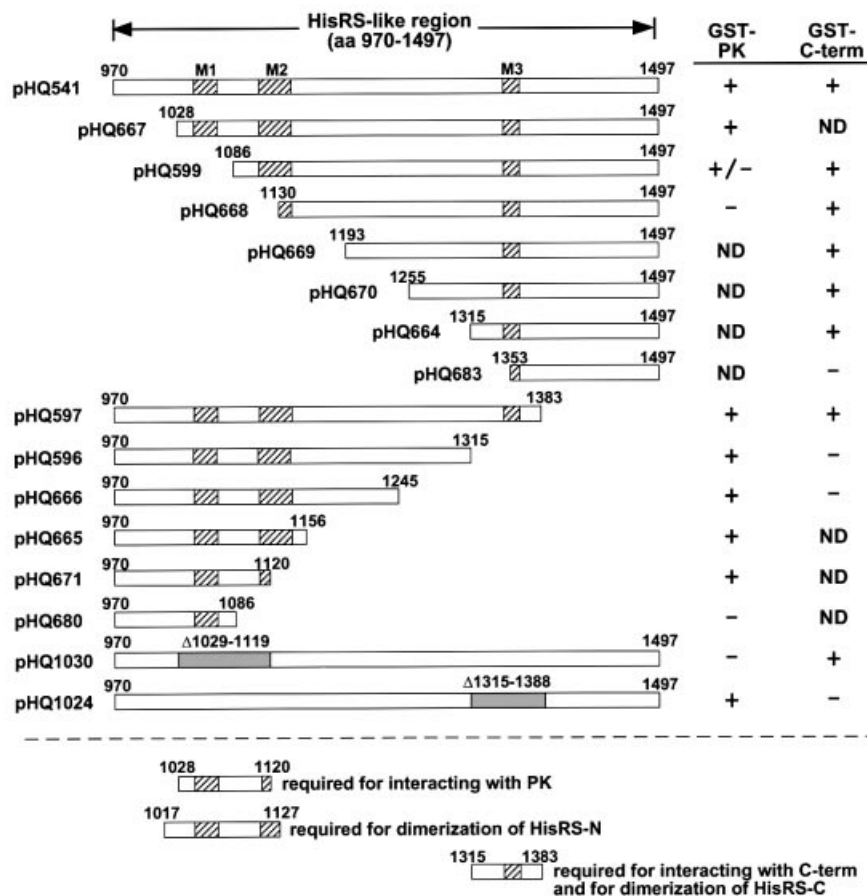
We showed previously that the isolated HisRS domain can interact strongly with the PK and C-term domains *in vitro* (Qiu *et al.*, 1998). To determine whether these interactions require HisRS residues involved in dimerization, we mapped the binding determinants for the PK and C-term domains in the HisRS segment. A panel of <sup>35</sup>S-labeled polypeptides containing the HisRS-like domain progressively truncated from the N- or C-terminus was tested for binding to GST fusions bearing PK residues 568–998 or C-term residues 1498–1659, using the same binding assay described in Figures 2–4. As summarized in Figure 6, residues 1028–1120 in the HisRS-N region were required for interaction with GST–PK(568–998), whereas residues 1315–1383 in the HisRS-C segment were needed for interaction with GST–C-term(1498–1659). It is interesting that the PK binding determinants are located in the PK-proximal portion of the HisRS-like domain, whereas the C-term binding determinants occur in the C-term-proximal section of the HisRS-like region.



**Fig. 5.** Effects of mutations in the HisRS-N region on dimerization, tRNA binding and ribosome association by full-length GCN2 *in vivo*. (A) Co-immunoprecipitation assay of dimerization by wild-type or mutant GCN2 proteins with LexA-HA-GCN2. Whole-cell extracts were prepared from transformants of *gcn2 $\Delta$*  strain HQY132 bearing high copy-number plasmid pHQ400 encoding LexA-HA-GCN2 and plasmids bearing wild-type GCN2 (p630), *gcn2- $\Delta 1018-1047$*  (pHQ1044), *gcn2- $\Delta 1048-1071$*  (pHQ1045), *gcn2-as4* (pHQ1081), *gcn2-as3/4* (pHQ1082), *gcn2-m2* (p332) or *gcn2- $\Delta 1536-1659$*  (p2461). Aliquots of extracts containing 50  $\mu\text{g}$  of protein were immunoprecipitated with anti-HA antibodies, and immune complexes were resolved by SDS-PAGE and subjected to immunoblot analysis with GCN2 antibodies, all as described previously (Qiu *et al.*, 1998). Lanes 1, 4, 7, 10, 13, 16 and 19: 1/10 of the input (I) amounts of extract used for immunoprecipitations; lanes 2, 5, 8, 11, 14, 17 and 20: 1/2 of the pellet (P) fractions from the immunoprecipitations; lanes 3, 6, 9, 12, 15, 18 and 21: 1/10 of the supernatant (S) fractions from the immunoprecipitations. (B) GMSA of GCN2-tRNA complexes. The following affinity-purified FL-tagged GCN2 proteins were incubated with  $^{32}\text{P}$ -labeled total yeast tRNA (0.5 pmol) in 20  $\mu\text{l}$  of RNA-binding buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 7.5 mM  $\text{MgCl}_2$ , 10% glycerol) at 30°C for 25 min: FL-GCN2 (2 pmol), FL-gcn2-m2 (2 pmol), FL-gcn2-as3/4 (2 pmol), FL-gcn2- $\Delta 1018-1047$  (~0.4 pmol) and FL-gcn2- $\Delta 1048-1071$  (~0.4 pmol). The [ $^{32}\text{P}$ ]tRNA-GCN2 complexes were resolved by electrophoresis in a 5% polyacrylamide gel in 0.5 $\times$  Tris-borate-EDTA buffer (3 h, 130 V), dried under vacuum and visualized by autoradiography, with the results shown in the upper panel. The same amounts of each protein used in the binding reactions were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue, with the results shown in the right panel. (C) Ribosome binding analysis. Cell extracts were prepared in a buffer lacking  $\text{Mg}^{2+}$  and cycloheximide (to dissociate polysomes and 80S ribosomes into 40S and 60S subunits) from transformants of yeast strain H1149 (Wek *et al.*, 1990) harboring high-copy plasmids containing wild-type GCN2 (p630), *gcn2- $\Delta 1018-1047$*  (pHQ1044), *gcn2- $\Delta 1048-1071$*  (pHQ1045), *gcn2-as4* (pHQ1081), *gcn2-as3/4* (pHQ1082) or *gcn2- $\Delta 1536-1659$*  (p2461). Extracts were resolved by velocity sedimentation in sucrose density gradients and fractions were collected while scanning for  $A_{254}$ , all as previously described (Ramirez *et al.*, 1991), and subjected to immunoblot analysis using antibodies against the N-terminus of GCN2 (Romano *et al.*, 1998). Fraction 1 is from the top of the gradient. The first and last lanes contain 1% of the input (I) amounts of extracts separated on the gradients. Positions of free 40S and 60S ribosomal subunits are indicated by bars above the gels.

It was also intriguing that the segments in the HisRS region required for interaction with the PK or C-term were essentially co-extensive with the dimerization domains in

the HisRS-N and HisRS-C regions, respectively (Figure 6, bottom). To explore whether PK binding and dimerization by the HisRS-N domain have identical sequence require-



**Fig. 6.** Mapping of amino acids in the HisRS-N and HisRS-C regions required for interaction with the PK and C-term domains *in vitro*. The rectangular boxes depict different segments of the HisRS-like region that were synthesized *in vitro* in the presence of [<sup>35</sup>S]methionine, using the plasmids listed to the left of the boxes. The gray segments in the pHQ1030 and pHQ1024 constructs depict deleted residues. <sup>35</sup>S-labeled proteins were incubated with GST, GST-PK(568–998) or GST-C-term(1498–1659) immobilized on glutathione–Sepharose beads. After extensive washing, the proteins bound to the beads were resolved by SDS–PAGE and visualized by fluorography, all as described above. None of these radiolabeled proteins bound to GST alone (data not shown). Binding (+) or failure to bind (–) to GST–PK or GST–C-term is indicated on the right under the columns headed ‘GST–PK’ or ‘GST–C-term’, respectively. ND, not determined. The rectangular boxes under the dashed line depict the GCN2 residues required for binding of HisRS-N to PK, for dimerization of HisRS-C and for its binding to the C-term, and for dimerization of HisRS-N, deduced from results in this figure and in Figures 2 and 3. The hatched boxes indicate the locations of conserved motifs M1–3.

ments, we carried out additional binding experiments using GST–PK(568–998) and the panel of [<sup>35</sup>S]HisRS-N polypeptides bearing internal deletions or substitutions described above. The results summarized in Figure 3A (column headed ‘with PK’) indicate that, with the exception of  $\Delta 1071$ –1087, all of the mutations that impaired self-interaction of HisRS-N similarly impaired its binding to GST–PK(568–998). Thus, there are nearly identical sequence requirements in the HisRS-N region for dimerization and binding to the PK domain.

#### Mapping residues in the PK domain required for interaction with the HisRS domain

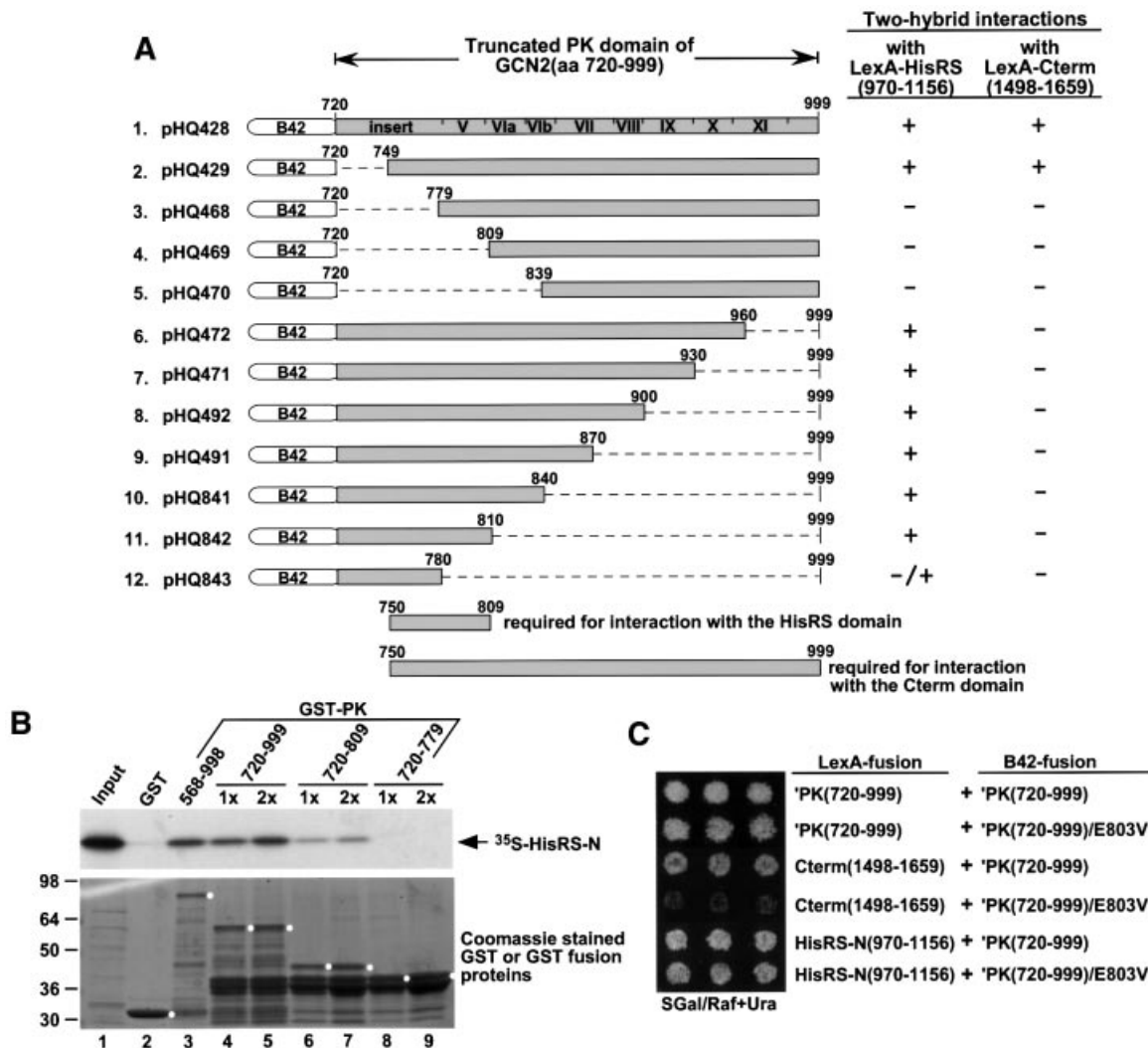
Given the role of the HisRS domain in binding tRNA and activating the kinase function of GCN2, it was important to identify the binding surface in the PK domain for the HisRS-N domain. Accordingly, we carried out two-hybrid assays using a LexA–HisRS-N construct and the panel of B42 activation domain fusions shown in Figure 7A containing different segments of the PK domain. These data showed that residues 750–809 of the PK domain are required for two-hybrid interaction with the HisRS-N

segment. The results of GST pull-down experiments using [<sup>35</sup>S]HisRS-N and GST–PK fusions containing different sections of the PK domain confirmed that PK residues 720–809 are sufficient for the PK–HisRS-N interaction, although the GST–PK(720–809) segment bound [<sup>35</sup>S]HisRS-N less tightly than the larger PK segment in GST–PK(720–999) (Figure 7B, lanes 4–7). Previously, we showed that a much larger segment of the PK domain, spanning residues 750–999, was required for interaction with the C-term segment of GCN2 in the two-hybrid assay (Qiu *et al.*, 1998). These last results are summarized in Figure 7A for comparison with the current findings on PK–HisRS-N interactions.

#### Evidence that PK–C-term interaction downregulates GCN2 kinase activity

*GCN2<sup>c</sup>* mutations conferring constitutive activation of GCN2 function *in vivo* map in the PK, HisRS and C-term domains, and the most potent of these mutations map in the C-term (Wek *et al.*, 1990; Ramirez *et al.*, 1992). Based on these observations, we speculated that the C-term might function as an autoinhibitory domain, and that binding of





**Fig. 7.** Mapping the binding determinants in the PK domain for the HisRS-N region and evidence that the *GCN2<sup>c</sup>-E803V* mutation impairs interaction between the PK and C-term domains *in vivo*. (A) Fusion proteins containing the truncated GCN2 PK domain (residues 720–999) or its deletion derivatives (depicted by gray rectangular boxes) and the B42 transcription activation domain were expressed from the plasmids listed on the left in transformants of yeast strain EGY48 coexpressing LexA–HisRS-N(970–1156) (from pHQ689) or LexA–C-term(1498–1659) (from pHQ311). Dashed lines represent the amino acids deleted from the PK domain, and the GCN2 residue numbers above the lines indicate the first and last residues deleted in each construct. The resulting transformants were tested for growth on SGal/Raf + Ura medium, indicative of a two-hybrid interaction that activates expression of the *lexAop-LEU2* reporter resident in the strains, as previously described (Qiu *et al.*, 1998). +, -/+ and – indicate strong, weak and no growth on the medium lacking leucine, respectively. (B) *In vitro* binding of [<sup>35</sup>S]HisRS-N (aa 970–1156) synthesized *in vitro* to GST fusion proteins containing different segments of the PK domain. The upper panel displays the amount of [<sup>35</sup>S]HisRS-N added to the reactions (1/10 Input) or bound to the GST or GST–PK fusion proteins as indicated above the panel. 1× and 2× represent two different relative concentrations of GST–PK fusion proteins used in the binding reactions. The lower panel shows the GST and GST–PK fusion proteins bound to the glutathione–Sepharose beads in the binding reactions stained with Coomassie Brilliant Blue. The full-length GST–PK fusion proteins are indicated by white dots next to the appropriate bands. (C) The *GCN2<sup>c</sup>-E803V* mutation impairs two-hybrid interaction between the PK and C-term domains *in vivo*. LexA fusion proteins containing truncated GCN2 PK(720–999) (encoded by pHQ433), C-term(1498–1659) (encoded by pHQ311) or HisRS-N(970–1156) (encoded by pHQ689) were expressed in transformants of EGY48 expressing B42 fusion proteins containing PK(720–999) (from pHQ428) or PK(720–999)/E803V (from pHQ823). The resulting transformants were tested for growth on SGal/Raf + Ura medium (lacking leucine), which is indicative of a two-hybrid interaction that activates expression of the *lexAop-LEU2* reporter.

tRNA to the HisRS region would overcome this inhibition. We reasoned that if this model is correct, one or more *GCN2<sup>c</sup>* mutations might weaken the interaction between the isolated PK and C-term domains. To explore this possibility, we introduced each of five *GCN2<sup>c</sup>* mutations mapping in the PK domain (*M788V*, *E803V*, *E821K*, *H861Y* and *R768W/D987G*) into the LexA–PK(720–999) and B42–PK(720–999) constructs used for two-hybrid analysis, and tested them for interaction with LexA and B42 fusions bearing the C-term. The PK fusions were also

tested for two-hybrid interactions with the appropriate constructs containing the wild-type 'PK(720–999) or HisRS-N segments to examine possible effects on PK dimerization or PK–HisRS interactions. Similarly, four *GCN2<sup>c</sup>* mutations mapping in the C-term (*R1557K*, *E1591K*, *E1606G* and *N1616K*) were introduced into the LexA and B42 fusions containing the C-term segment, and tested for interaction with the fusions bearing wild-type 'PK(720–999) or wild-type C-term. Interestingly, the *E803V* mutation in the kinase domain abolished inter-

action of the PK segment with the C-term, but had no effect on PK dimerization or PK interaction with the HisRS fragment (Figure 7C), and thus specifically impaired the PK–C-term interaction. None of the other *GCN2<sup>c</sup>* mutations affected any of the two-hybrid interactions (data not shown). These data suggest that the constitutive activation of GCN2 conferred by the *E803V* mutation involves the loss of an inhibitory physical interaction between the PK and C-term domains.

## Discussion

### **The HisRS-N domain functions in dimerization of the HisRS region, tRNA binding and stimulatory interactions with the PK domain of GCN2**

We have identified two dimerization domains in the N- and C-terminal halves of the HisRS-like segment of GCN2, located between residues 1017–1127 and 1315–1383, respectively (Figure 1). The HisRS-N dimerization domain represents a stretch of strong sequence similarity between GCN2 and authentic HisRSs (Ramirez *et al.*, 1992; Arnez *et al.*, 1995) that coincides with the dimerization interface in the catalytic domain of the *E.coli* enzyme (Arnez *et al.*, 1995). Conserved amino acids in  $\beta$ -strands AS3 and AS4 make symmetric hydrophobic contacts that stabilize a four-stranded  $\beta$ -sheet forming an arch over the dimeric interface in the catalytic domain of *E.coli* HisRS (Arnez *et al.*, 1995). Substituting the corresponding residues in GCN2 with alanines (the *as3/4* mutations) abolished dimerization of the GCN2 HisRS-N segment *in vitro*, suggesting that the dimerization interface of this segment is structurally similar to that present in authentic HisRS. The fact that these residues are required for GCN2 function *in vivo* strongly suggests that dimerization of HisRS-N is important for activation of GCN2 by uncharged tRNA. The HisRS-C dimerization domain coincides with the C-terminal end of the catalytic domain of *E.coli* HisRS, encompassing  $\beta$ AS9,  $\alpha$ H6 and motif 3 (Arnez *et al.*, 1995). This region is not present at the dimer interface of *E.coli* HisRS, indicating a structural divergence between the GCN2 HisRS-C region and authentic HisRS. Although the HisRS-C domain is required for GCN2 function *in vivo*, it is unclear whether the dimerization activity of this segment is important for GCN2 activation.

Internal deletions in the HisRS-N region that abolished dimerization of this isolated segment *in vitro* did not reduce the ability of full-length GCN2 to form heterodimers *in vivo* with a LexA–GCN2 fusion. The same result was obtained previously for a deletion that included the HisRS-C dimerization domain (Qiu *et al.*, 1998). These results can be explained by noting that GCN2 contains multiple dimerization domains (Figure 1) and that only the C-term appears to be critically required for dimerization by the full-length protein (Qiu *et al.*, 1998). The *m2* mutation that abolished tRNA binding did not impair dimerization between GCN2 and LexA–GCN2 (Figure 5A). This last finding implies that tRNA binding is not required for dimerization by GCN2. Accordingly, we propose that GCN2 occurs as a dimer under both non-starvation and starvation conditions. Rather than promoting dimerization, tRNA binding would trigger a

conformational change in GCN2 that activates the kinase domain.

The *as3/4* point mutations that impaired dimerization of the isolated HisRS-N domain (Figure 4) also destroyed tRNA binding by full-length GCN2 *in vitro* (Figure 5B). These results suggest that dimerization of the HisRS-N domain is required for tRNA binding by GCN2. In accordance with this conclusion, there is evidence that dimerization of class II aminoacyl-tRNA synthetases is required for proper folding of the catalytic domain, which encompasses the binding site for the acceptor stem of tRNA (Eriani *et al.*, 1993; Agou *et al.*, 1996). In contrast, a monomeric form of *E.coli* HisRS lacking the accessory C-terminal domain required for dimerization was active for tRNA binding, although its catalytic function was sharply impaired (Augustine and Francklyn, 1997). Given its considerable sequence divergence from authentic HisRS (Wek *et al.*, 1989), and the negative influence of the adjacent PK domain on tRNA binding (see below), it seems plausible that dimerization of the HisRS-N domain in GCN2 could be required for tRNA binding, as suggested by the effects of the *as3/4* mutation.

The *gcn2- $\Delta$ 1018–1047* mutation eliminates most of the predicted long helix of motif 1 (AH1) that forms part of the dimeric interface with its symmetrically related counterpart in the *E.coli* HisRS dimer (Arnez *et al.*, 1995). The adjacent  *$\Delta$ 1048–1071* mutation eliminates the remainder of motif 1 and the adjacent residues corresponding to Ile45 and Val46 in *E.coli* HisRS. The latter contribute hydrophobic contacts at the dimeric interface of the *E.coli* enzyme, although the fact that Ile45 is replaced by Arg1063 in wild-type GCN2 may signify a diminished role for motif 1 in dimerization of the GCN2 HisRS-N domain. The fact that these two deletions destroyed dimerization of the isolated HisRS-N segment *in vitro* supports the idea that motif 1 contributes to dimerization of HisRS-N in GCN2. If dimerization of HisRS-N is required for tRNA binding, how can we explain the fact that  *$\Delta$ 1018–1047* and  *$\Delta$ 1048–1071* did not impair tRNA binding by GCN2 (Figure 5B)? It is possible that dimerization by the PK domain upstream and the HisRS-C region downstream can compensate for the  *$\Delta$ 1018–1047* and  *$\Delta$ 1048–1071* mutations and maintain dimerization of the HisRS-N region in the absence of intact motif 1. In contrast, eliminating the critical dimerization contacts in  $\beta$ -strands AS3/AS4 by the *as3/4* mutations would not be suppressed by dimerization of the adjacent PK and HisRS-C domains, resulting in loss of HisRS-N dimerization and tRNA binding.

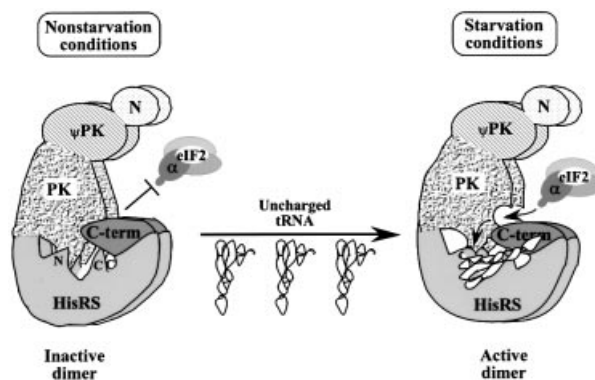
Given that the  *$\Delta$ 1048–1071* deletion did not impair tRNA binding or ribosome association by full-length GCN2, why is the *gcn2- $\Delta$ 1048–1071* product inactive *in vivo*? We found that HisRS-N residues 1028–1120 interact with the PK domain and that this interaction is impaired by the  *$\Delta$ 1048–1071* mutation (Figure 3). Hence, we suggest that the PK–HisRS-N interaction has a stimulatory effect on kinase activity that is disrupted by  *$\Delta$ 1048–1071*. The portion of the HisRS-N region that interacts with the PK domain corresponds to the ‘top’ of the catalytic domain of *E.coli* HisRS, which forms the dimeric interface, and also includes structures AH3 and AS5, which immediately precede motif 2 (Figure 4A) (Arnez *et al.*, 1995). By analogy with studies on Asp-RS

(Eiler *et al.*, 1999), tRNA binding to GCN2 may produce a conformational change in the HisRS-N domain that is transmitted to the PK domain and stimulates kinase activity (Figure 8). The segment of the PK domain that interacts with the HisRS-N region includes the C-terminal section of the large insert between kinase subdomains III and IV, portions of the ATP-binding and catalytic lobes, and the hinge region between the two lobes (Hanks and Quinn, 1991; Knighton *et al.*, 1991; Ramirez *et al.*, 1992). Thus, association of the HisRS-N region with this segment of the PK domain could enhance the correct orientation of the N- and C-terminal lobes of the PK domain (Johnson *et al.*, 1996). This part of the PK domain also contains three of the five *GCN2<sup>c</sup>* mutations described previously (including *E803V*), providing independent evidence that it plays an important role in kinase activation by uncharged tRNA.

It was striking that the *Δ1048–1071* mutation increased the affinity of GCN2 for tRNA. As this deletion should abolish association between the PK and HisRS-N domains (Figure 3), it appears that binding of the PK domain to the HisRS-N region interferes with tRNA binding. This conclusion may seem at odds with our suggestion that PK–HisRS-N interactions transmit the stimulatory effect of tRNA binding to the kinase domain; however, such opposing functions could be accommodated by proposing two different modes of PK–HisRS-N interaction in GCN2. One configuration would be incompatible with tRNA binding and would not stimulate PK function, whereas the other would allow tRNA binding and promote kinase activation (Figure 8). According to this hypothesis, the *Δ1048–1071* mutation eliminated the inhibitory effect of PK–HisRS-N interaction on tRNA binding, but since it also destroyed the crucial positive effect of HisRS-N on the kinase domain, there was a net loss of GCN2 function. This model envisions a less extreme structural reorganization of GCN2 elicited by tRNA binding than we suggested previously, wherein all contacts between the PK and HisRS domains were abolished in the tRNA-bound state (Dong *et al.*, 2000).

#### **Evidence that the C-term–PK interaction contributes to the latency of GCN2 kinase activity**

The isolated C-term domain of GCN2 can interact directly with the PK domain (Qiu *et al.*, 1998), and residues 1498–1535 in the C-term are required for this interaction (Qiu *et al.*, 1998). Previously, we showed that a large PK segment including residues 750–999 was required for binding to the C-term (Qiu *et al.*, 1998) (summarized in Figure 7A and Figure 1), suggesting that the C-term makes multiple contacts across the PK domain. Interestingly, we discovered here that the *GCN2<sup>c</sup>-E803V* mutation weakened binding between the C-term and PK segments. This mutation maps in the small segment of the PK domain (residues 750–810) required for the PK–HisRS-N interaction, and is encompassed by the larger PK domain required for C-term binding (see Figures 1 and 7). The *E803V* mutation did not affect the PK–HisRS-N interaction, indicating that it specifically disrupts PK–C-term association. These observations suggest that PK–C-term interaction is required for inhibition of kinase activity, and that its alteration by *E803V* leads to inappropriate activation of GCN2 under non-starvation conditions. In



**Fig. 8.** A model depicting allosteric alterations in domain interactions in GCN2 elicited by bound tRNA that result in kinase activation. The domains of GCN2 are labeled as follows: binding domain for GCN1–GCN20 complex at the N-terminus, N; pseudokinase domain,  $\psi$ PK; protein kinase domain, PK; HisRS-like region, HisRS; and C-term. The N- and C-terminal portions of the HisRS domain (labeled N and C in the HisRS region of the schematic on the left) interact with the PK and C-term segments, respectively. In the inactive form of GCN2 present in non-starvation conditions (left), productive association with the substrate eIF2 is prevented by binding of the C-term to the PK domain, and possibly also by HisRS–N–PK interactions (both inhibitory interactions are depicted by overlapped domains). The HisRS–N–PK interactions in this state are not compatible with tRNA binding. Under starvation conditions (right), uncharged tRNA binds to GCN2, making contacts with both the HisRS–N and C-term domains. The bound tRNA produces conformational changes in both domains that are transmitted to the PK domain. This opens up the substrate binding cleft in the PK domain and allows eIF2 binding and phosphorylation (the allosteric alteration in the PK domain is signified by the curved arrow in this domain.) The HisRS–N region remains engaged with the PK domain and contributes to its active conformation. The C-term also remains associated with the PK domain, but inhibitory C-term–PK contacts are lost upon tRNA binding. These inhibitory contacts are also weakened by the constitutively activating *E803V* mutation in the PK domain. Both inactive and active forms of GCN2 are depicted as dimers because GCN2 can dimerize in the absence of an intact HisRS domain and its tRNA binding activity. This model does not depict the potential ‘criss-cross’ interactions that could occur if the C-term of one subunit interacts with the HisRS–C region of the other subunit, as found in the crystal structure of authentic *E. coli* HisRS (see text for further details).

the wild-type protein, the autoinhibitory function of the C-term would be reversed by tRNA binding to the composite HisRS–C-term domain.

Recently, we found that tRNA binding to a recombinant HisRS–C-term segment weakened its association with the isolated PK domain (Dong *et al.*, 2000). This finding is consistent with the notion that tRNA binding disrupts an autoinhibitory interaction between the C-term and PK domains. Furthermore, the *E803V* mutation led to a large increase in the tRNA binding affinity of full-length GCN2 (Dong *et al.*, 2000). This last result, combined with our observation that PK–C-term interaction is impaired by the *E803V* mutation, supports the idea that tRNA binding stabilizes a conformation in which the C-term dissociates from the PK domain and becomes more accessible to tRNA. The *E803V* mutation would shift the equilibrium towards a conformation of GCN2 that can bind tRNA and become activated at low concentrations of uncharged tRNA present in non-starved cells.

The fact that other *GCN2<sup>c</sup>* mutations in the PK or C-term domains did not impair the PK–C-term two-hybrid interaction could be explained by proposing that these

**Table II.** Plasmids used in this study

Plasmid	Allele	Reference
Plasmids encoding GST fusion proteins		
pHQ242	GST under <i>ADH1</i> promoter in 2 $\mu$ <i>TRP1</i> plasmid pPM237 (L.Prakash)	this study
pHQ601	<i>GST-GCN2(970-1497)</i> in pHQ242 backbone	this study
pHQ529	<i>GST-GCN2(720-999)</i> in pGEX-5X-1 (Pharmacia Biotech.)	this study
pHQ531	<i>GST-GCN2(1498-1659)</i> in pGEX-5X-1	Qiu <i>et al.</i> (1998)
pHQ551	<i>GST-GCN2(568-998)</i> in pGEX-5X-1	Qiu <i>et al.</i> (1998)
pHQ701	<i>GST-GCN2(1315-1497)</i> in pGEX-5X-1	this study
pHQ704	<i>GST-GCN2(970-1156)</i> in pGEX-5X-1	this study
pHQ993	<i>GST-GCN2(720-809)</i> in pGEX-5X-1	this study
pHQ994	<i>GST-GCN2(720-779)</i> in pGEX-5X-1	this study
pHQ1015	<i>GST-GCN2(970-1156)/<math>\Delta</math>984-1016</i> in pGEX-5X-1	this study
pHQ1016	<i>GST-GCN2(970-1156)/<math>\Delta</math>1048-1071</i> in pGEX-5X-1	this study
pHQ1018	<i>GST-GCN2(970-1156)/<math>\Delta</math>1087-1106</i> in pGEX-5X-1	this study
pHQ1019	<i>GST-GCN2(970-1156)/<math>\Delta</math>1107-1125</i> in pGEX-5X-1	this study
pHQ1020	<i>GST-GCN2(970-1156)/<math>\Delta</math>1128-1149</i> in pGEX-5X-1	this study
pHQ1025	<i>GST-GCN2(970-1156)/<math>\Delta</math>1018-1047</i> in pGEX-5X-1	this study
pHQ1038	<i>GST-GCN2(970-1156)/<math>\Delta</math>1071-1087</i> in pGEX-5X-1	this study
pHQ1059	<i>GST-GCN2(970-1156)/<i>as4</i></i> in pGEX-5X-1	this study
pHQ1073	<i>GST-GCN2(970-1156)/<i>as3/4</i></i> in pGEX-5X-1	this study
Plasmids for <i>in vitro</i> translation of GCN2 segments		
pHQ541	<i>GCN2(970-1497)</i> under T7 promoter in pGEM-3Z (Promega)	Qiu <i>et al.</i> (1998)
pHQ596	<i>GCN2(970-1315)</i> in pGEM-3Z	this study
pHQ597	<i>GCN2(970-1383)</i> in pGEM-3Z	this study
pHQ599	<i>GCN2(1086-1497)</i> in pGEM-3Z	this study
pHQ664	<i>GCN2(1315-1497)</i> in pGEM-3Z	this study
pHQ665	<i>GCN2(970-1156)</i> in pGEM-3Z	this study
pHQ666	<i>GCN2(970-1245)</i> in pGEM-3Z	this study
pHQ667	<i>GCN2(1028-1497)</i> in pGEM-3Z	this study
pHQ668	<i>GCN2(1130-1497)</i> in pGEM-3Z	this study
pHQ669	<i>GCN2(1193-1497)</i> in pGEM-3Z	this study
pHQ670	<i>GCN2(1255-1497)</i> in pGEM-3Z	this study
pHQ671	<i>GCN2(970-1120)</i> in pGEM-3Z	this study
pHQ680	<i>GCN2(970-1086)</i> in pGEM-3Z	this study
pHQ683	<i>GCN2(1353-1497)</i> in pGEM-3Z	this study
pHQ1008	<i>GCN2(970-1156)/<math>\Delta</math>984-1016</i> in pGEM-3Z	this study
pHQ1009	<i>GCN2(970-1156)/<math>\Delta</math>1018-1047</i> in pGEM-3Z	this study
pHQ1010	<i>GCN2(970-1156)/<math>\Delta</math>1048-1071</i> in pGEM-3Z	this study
pHQ1012	<i>GCN2(970-1156)/<math>\Delta</math>1087-1106</i> in pGEM-3Z	this study
pHQ1013	<i>GCN2(970-1156)/<math>\Delta</math>1107-1125</i> in pGEM-3Z	this study
pHQ1014	<i>GCN2(970-1156)/<math>\Delta</math>1128-1149</i> in pGEM-3Z	this study
pHQ1024	<i>GCN2(970-1497)/<math>\Delta</math>1315-1388</i> in pGEM-3Z	this study
pHQ1030	<i>GCN2(970-1497)/<math>\Delta</math>1029-1119</i> in pGEM-3Z	this study
pHQ1037	<i>GCN2(970-1156)/<math>\Delta</math>1071-1087</i> in pGEM-3Z	this study
pHQ1058	<i>GCN2(970-1156)/<i>as4</i></i> in pGEM-3Z	this study
pHQ1066	<i>GCN2(970-1156)/<i>as3/4</i></i> in pGEM-3Z	this study
Plasmids encoding GCN2 and derivatives		
p332	<i>gcn2-m2</i> in p630 backbone	Wek <i>et al.</i> (1995)
p630	wild-type <i>GCN2</i> in in YE <sub>p</sub> 24 (Botstein <i>et al.</i> , 1979) 2 $\mu$ , <i>URA3</i>	Wek <i>et al.</i> (1990)
p2461	<i>gcn2-<math>\Delta</math>1536-1659</i> in p630 backbone	Qiu <i>et al.</i> (1998)
pHQ644	<i>GCN2</i> , <i>CEN6</i> , <i>ARSH4</i> , <i>URA3</i> , a derivative of p722 (Wek <i>et al.</i> , 1990)	this study
pHQ1039	<i>gcn2-<math>\Delta</math>1018-1047</i> in pHQ644 backbone	this study
pHQ1040	<i>gcn2-<math>\Delta</math>1107-1125</i> in pHQ644 backbone	this study
pHQ1041	<i>gcn2-<math>\Delta</math>984-1016</i> in pHQ644 backbone	this study
pHQ1042	<i>gcn2-<math>\Delta</math>1048-1071</i> in pHQ644 backbone	this study
pHQ1043	<i>gcn2-<math>\Delta</math>1087-1106</i> in pHQ644 backbone	this study
pHQ1044	<i>gcn2-<math>\Delta</math>1018-1047</i> in p630 backbone	this study
pHQ1045	<i>gcn2-<math>\Delta</math>1048-1071</i> in p630 backbone	this study
pHQ1047	<i>gcn2-<math>\Delta</math>1071-1087</i> in pHQ644 backbone	this study
pHQ1051	<i>gcn2-<math>\Delta</math>1128-1149</i> in pHQ644 backbone	this study
pHQ1076	<i>gcn2-<i>as4</i></i> in pHQ644 backbone	this study
pHQ1077	<i>gcn2-<i>as3/4</i></i> in pHQ644 backbone	this study
pHQ1081	<i>gcn2-<i>as4</i></i> in p630 backbone	this study
pHQ1082	<i>gcn2-<i>as3/4</i></i> in p630 backbone	this study
pHQ1092	<i>gcn2-<math>\Delta</math>1315-1383</i> in pHQ644 backbone	this study
pDH103	<i>GCN2-FL</i> in pEMBLyex4 (Cesareni and Murray, 1987) backbone	Dong <i>et al.</i> (2000)
pDH104	<i>gcn2-FL-m2</i> in pEMBLyex4 backbone	Dong <i>et al.</i> (2000)
pHQ1048	<i>gcn2-FL-<math>\Delta</math>1018-1047</i> in pEMBLyex4 backbone	this study
pHQ1049	<i>gcn2-FL-<math>\Delta</math>1048-1071</i> in pEMBLyex4 backbone	this study
pHQ1084	<i>gcn2-FL-<i>as3/4</i></i> in pEMBLyex4 backbone	this study

Table II. Continued

Plasmid	Allele	Reference
Plasmids for two-hybrid analysis of GCN2 segments		
p2247	<i>LexA-HA</i> in pEG202 (Golemis <i>et al.</i> , 1996) backbone	Zhang <i>et al.</i> (1997)
pHQ311	<i>LexA-GCN2(1498–1659)</i> in pEG202 backbone	Qiu <i>et al.</i> (1998)
pHQ400	<i>LexA-HA-GCN2(27–1659)</i> in p2247 backbone	Qiu <i>et al.</i> (1998)
pHQ433	<i>LexA-GCN2(720–999)</i> in pEG202 backbone	Qiu <i>et al.</i> (1998)
pHQ588	<i>LexA-HA-GCN2(970–1497)</i> in p2247 backbone	Qiu <i>et al.</i> (1998)
pHQ689	<i>LexA-GCN2(970–1156)</i> in pEG202 backbone	this study
pHQ428	<i>B42-GCN2(720–999)</i> in pJG4-5 (Golemis <i>et al.</i> , 1996) backbone	Qiu <i>et al.</i> (1998)
pHQ429	<i>B42-GCN2(750–999)</i> in pJG4-5 backbone	Qiu <i>et al.</i> (1998)
pHQ468	<i>B42-GCN2(780–999)</i> in pJG4-5 backbone	this study
pHQ469	<i>B42-GCN2(810–999)</i> in pJG4-5 backbone	this study
pHQ470	<i>B42-GCN2(740–999)</i> in pJG4-5 backbone	this study
pHQ471	<i>B42-GCN2(720–929)</i> in pJG4-5 backbone	this study
pHQ472	<i>B42-GCN2(720–959)</i> in pJG4-5 backbone	this study
pHQ491	<i>B42-GCN2(720–869)</i> in pJG4-5 backbone	this study
pHQ492	<i>B42-GCN2(720–899)</i> in pJG4-5 backbone	this study
pHQ823	<i>B42-GCN2(720–999)/E803V</i> in pJG4-5 backbone	this study
pHQ841	<i>B42-GCN2(720–839)</i> in pJG4-5 backbone	this study
pHQ842	<i>B42-GCN2(720–809)</i> in pJG4-5 backbone	this study
pHQ843	<i>B42-GCN2(720–779)</i> in pJG4-5 backbone	this study

mutations have more subtle effects than *E803V* on PK–C-term association, and overcome the inhibitory function of the C-term without disrupting the strongest contacts between these two domains. In this view, the C-term is not completely dissociated from the PK domain upon tRNA binding, and only the subset of interactions required for PK inhibition are replaced by C-term–tRNA interactions. This hypothesis is akin to the suggestion above that tRNA binding switches the PK–HisRS–N interaction between inhibitory and stimulatory modes rather than completely dissociating the two domains (Figure 8). Thus, several results obtained here imply that tRNA binding produces a more subtle rearrangement of domain interactions than previously imagined (Dong *et al.*, 2000).

Finally, what could be the role of interactions between the C-term domain and the HisRS–C region of GCN2? The authentic HisRS contains an accessory domain at its extreme C-terminus that interacts with the catalytic domain of the opposing subunit in the dimer (Arnez *et al.*, 1995). It is tempting to propose a similar ‘criss-cross’ interaction for GCN2 in which the C-term of one subunit interacts with the HisRS–C region of the other subunit in the dimer. In this way, dimerization at the C-terminus of GCN2 would be stabilized by HisRS–C–HisRS–C and HisRS–C–C-term interactions in addition to the C-term–C-term interactions described previously (Qiu *et al.*, 1998). This speculation is also in accordance with the proposed role of the accessory C-terminal domain of *E. coli* HisRS in tRNA binding (Arnez *et al.*, 1995) and our finding that tRNA binding by GCN2 requires both the C-term and HisRS domains (Dong *et al.*, 2000).

## Materials and methods

### Plasmids

Plasmids used in this study are listed in Table II. Details of their construction are available as Supplementary data at *The EMBO Journal* Online.

### Biochemical methods

Co-immunoprecipitation and immunoblotting were conducted as described previously (Qiu *et al.*, 1998) using GCN2 antibodies described

previously (Romano *et al.*, 1998) and GST antibodies purchased from Santa Cruz (1:2000 dilution). Preparation of GST and GST fusion proteins, *in vitro* translation of GCN2 fragments and GST pull-down assays were carried out as described previously (Qiu *et al.*, 1998). Preparation of FL–His<sub>6</sub>-tagged GCN2 proteins, <sup>32</sup>P-labeling of total yeast tRNA and GMSAs of GCN2 binding to <sup>32</sup>P-tRNA were all conducted as described previously (Dong *et al.*, 2000). Ribosome binding of GCN2 was analyzed as described previously (Ramirez *et al.*, 1991).

### Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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