

Papers for today:

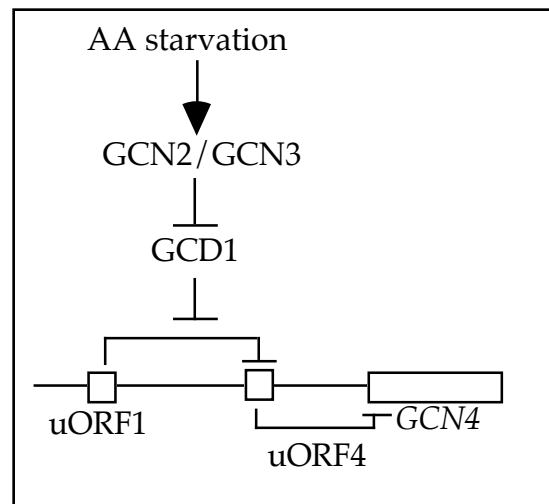
Preiss T, W Hentze M. Starting the protein synthesis machine: eukaryotic translation initiation. *Bioessays*. 2003 25:1201-1211.

Reprise

Last time we looked at two genetic systems for studying translation in yeast. The first was the work of Tom Donahue. He used the fact that cells normally do not recognize non-AUG codons efficiently as initiation codons to select mutations with relaxed specificity. Using a combined selection and screen he was able to identify mutations in a set of genes he called suppressor of initiation (SUI). These genes encoded subunits of three initiation factors: *SUI1*, eIF1; *SUI2*, 3 and 4, eIF2 α , β and γ ; and *SUI5*, eIF5. This experiment directly implicated these three factors as regulators of the accuracy of initiation.

We also began to look at a system which uses an unusual form of translational control, the amino acid general control system. In this system, the *GCN4* transcriptional activator turns on the transcription of dozens of genes encoding enzymes of amino acid biosynthesis. Expression of *GCN4* responds to the availability of amino acids; amino acid limitation turns on the expression of Gcn4 protein and that regulation occurs at the level of translation. We looked at experiments that identified a set of genes as regulators of *GCN4*. The *GCN* genes, when mutated, result in low constitutive expression of *GCN4*; the normal role of these genes must be to turn on Gcn4 in response to starvation. Mutation of the *GCD* genes results in high constitutive expression of *GCN4*, suggesting that their role is normally to repress expression.

Epistasis experiments suggested a model for the regulation of *GCN4* in which the Gcd1 protein directly represses expression and the Gcn2 and Gcn3 proteins antagonize that repression, indirectly stimulating expression. We also looked at the structure of the *GCN4* gene, noting the presence of four upstream open reading frames (uORFs). These uORFs are required for regulation since deleting them all results in high constitutive translation of *GCN4*. Analysis of mutations eliminating individual uORFs suggested a model in which uORF4 (and uORF3) repress translation of *GCN4* while uORF1 (and uORF2) antagonize that repression in response to amino acid starvation. Combined with the analysis of the *GCN* and *GCD* mutations suggested the model pictured above, with Gcd1 directly antagonizing the effect of uORF1 on uORF4.



Today we will look at the molecular mechanisms underlying this form of control.

What's special about uORF1?

What about uORF1 explains its ability to alter reading of the downstream uORFs?

Hypothesis 1: it is the position of the uORF in the mRNA that gives it its effect.

The idea is that the uORFs nearer the 5' end of the mRNA would be more likely to allow ribosomes to reinitiate scanning.

To test this uORF1 and 4 and the region surrounding them were exchanged. The result was that the uORF had the same effect regardless of its position invalidating the idea of the position of the uORF explaining its effect.

Hypothesis 2: the sequence of uORF1 causes it to antagonize uORF4

Mutation of the uORFs showed that their primary sequences are not required

Hypothesis 3: the sequence of the mRNA surrounding uORF1 cause it to antagonize uORF4

The region upstream or downstream of the uORF could alter some aspect of translation of uORF1. Since the ORF is rather short, the region of the ORF and sequences both upstream and downstream can be in contact with the ribosome at the same time. The neighboring sequences (context) could the process of initiation, elongation or termination.

Exchanging regions 5' of the uORFs had no effect, but exchanging the downstream region altered the behavior of the uORF.

In a second experiment the region downstream was replaced with random sequences, then constructs were selected that had the effect of uORF1. Only certain sequences could replace the uORF1 3' region, but almost any sequence could substitute for the region downstream of uORF4. This suggests that the region downstream of uORF1 makes it unusual, conferring its effect.

How does uORF1 antagonize uORF4?

The way the uORF1 and uORF4 affect downstream translation is exactly opposite.

- uORF4 has the classic effect described by Kozak—the presence of the ORF upstream of *GCN4* strongly interferes with expression.
- uORF1 does have the same effect when present by itself, though its inhibition is much weaker. However, when present in combination with uORF4 it has the effect of allowing increased expression of *GCN4* when the cell is starved for amino acids.

The unusual nature of the uORF1 sequence immediately downstream of the uORF suggested that something about the way a scanning ribosome releases from the uORF might explain its effect.

- Since ribosomes that ultimately translate either uORF4 or *GCN4* invariably read uORF1 first this system is explicitly controlled by reinitiation.
- Normally, reinitiation is rather inefficient but it appears to be quite efficient at uORF1. This suggests that the sequence context may allow more efficient reinitiation of scanning.
- In eukaryotes, the efficiency of reinitiation increases as the distance between the site of termination and the site of subsequent initiation increases. Reinitiation is expected to depend on the rate that the ribosome can reacquire initiation factors and initiator tRNA used up in initiation at uORF1. This may happen as the ribosome continues to scan downstream, putting a kinetic constraint on reinitiation (it has to happen rapidly enough to allow reading of uORF4 if *GCN4* translation is to be blocked).

What is the evidence that this is correct?

A rate-limiting component regulating *GCN4* reinitiation is eIF2

We now know that *translational reinitiation* regulates expression of *GCN4*. But what regulates reinitiation in response to amino acid starvation? Genetic analysis identified upstream genetic factors, both positive (*GCN*) and negative (*GCD*). How do these factors transmit the starvation signal?

Genetic analysis implies that eIF2 activity regulates the ability to reinitiate translation on uORF4:

Selection of more GCD mutations. Since these mutations are epistatic to the *gcn2* and *gcn3* mutations, and have the opposite phenotype, they can be selected as revertants of *gcn2 gcn3* double mutations.

The *gcn* mutants confer 3-AT sensitivity. Introducing a *gcd* mutation confers 3-AT resistance, which can be selected.

The *gcd* mutants also confer TS lethality. The wild-type versions were cloned by identifying plasmids which complemented the lethality (selection) and restored 3-AT sensitivity (screen)

The proteins encoded by these GCD genes are subunits of two important factors, eIF2 and eIF2B.

Three of them are allelic with the *SUI2*, *SUI3* and *SUI4* genes that encode eIF-2 α , eIF-2 β , and eIF-2 γ . We know that the *SUI* mutations reduce the activity of eIF2 which might be expected to reduce the ability of the scanning ribosome to recognize uORF4.

Others are subunits of eIF2B. These mutations reduce the efficiency of regenerating eIF-2•GTP and thus reduce the concentration of eIF-2•GTP•met-tRNA_f, which would also be expected to reduce the chance that uORF4 could be recognized.

This suggests that the Gcn4 regulation is regulated by the kinetics of eIF-2 action.

Phosphorylation of eIF2 stimulates GCN4 translation.

The fact that mutation of subunits of either eIF2 or eIF2B alters translational control of *GCN4* suggests that regulation depends on altering the kinetics of recycling of eIF2.

Gcn2 is a protein kinase. The *GCN2* gene was cloned and sequenced. The sequence shows evidence of a bipartite structure to the factor: the N-terminal domain is similar to a Ser/Thr protein kinase; the C-terminal domain is similar to histidyl-tRNA synthetase (HisRS).

A model for the function of Gcn2:

- Since tRNA synthetases bind to tRNAs and add amino acids to them, Gcn2 might bind to tRNAs lacking amino acids (deacylated)
- Deacylated tRNAs might only accumulate to significant amounts when the cell is starved for the corresponding amino acid
- So when Gcn2 binds a deacylated tRNA that could be used as a signal the cell is starving
- Binding the deacylated tRNA would activate the protein kinase domain of Gcn2, and it could then phosphorylate the α subunit of eIF2, indirectly titrating available eIF2B

Gcn2 includes a region similar to histidyl-tRNA synthetase. This region is required for activation.

There are special mutations of *GCN2*, called *GCN2^c*, which have the opposite phenotype of the original *gcn2* mutations, a Gcd⁻ phenotype (that is, they are constitutively derepressed).

- These map in the HisRS domain
- They could allow Gcn2 to phosphorylate eIF2 α independent of amino acid starvation.
- *GCN2^c* mutations alter amino acids conserved among synthetases

Gcn2 probably binds deacyl-tRNA and undergoes an allosteric change to activate the protein kinase domain. The Gcn⁻ mutations probably either preclude binding of

deacyl-tRNA or eliminate the allosteric change. The Gcn^c mutations probably shift the protein to the active conformation constitutively.

Regulation of *GCN4* translation involves eIF2 and eIF2B.

The conclusion is that translation of *GCN4* is regulated by the availability of the eIF2 ternary complex, and that depends on the availability of the recycling factor eIF2B.

Amino acid starvation activates Gcn2, which phosphorylates eIF2 α

The phosphorylated form binds more strongly to eIF2B, sequestering a proportion of the factor in an inactive complex

This leads to slower recycling of eIF2•GDP to eIF2•GTP and thus to reduced kinetics of reinitiation at *GCN4*

Since the ribosomes scanning along the mRNA after reading uORF1 only have so much time before they pass uORF4, the lower the availability of the ternary complex the lower the probability they will read uORF4 in preference to *GCN4*

This explains much of the system of translational regulation, but not all.

Why does this system regulate *GCN4* while not affecting cellular translation?

The *GCN* and *GCD* genes encode essential translation initiation factors. Deleting any of these genes is lethal. The mutations were selected to be both viable and to affect expression of the *GCN4* gene. In fact, the mutations have no discernable effect on bulk translation though they can cause a huge increase in expression of *GCN4* (think of the expression of *GCN4-lacZ* in wild type versus in a *GCD1* mutant background—the difference is almost 100-fold in repressing conditions)

Of course, as I emphasized early on, the fact that the mutations affect only *GCN4* is partly a result of the selection scheme which called for a viable strain that over expresses or under expresses *GCN4*. The meaning of the phenotype is deeper, though.

- Translation initiation occurs when a preformed 40S ribosomal subunit bound to multiple initiation factors and initiator tRNA collides with the eIF-4F subunit bound to the 5' end of an mRNA
- The rate of that process is dependent on the concentration of the 43S preinitiation complex (it has units of mol⁻¹ sec⁻¹). Decreasing the availability of bulk 43S complex would reduce the rate of bulk translation.
- Translational reinitiation at *GCN4* does not depend on the concentration of the 43S complex in solution. A 40S subunit that binds to the *GCN4* mRNA uses up its factors in translating uORF1. To reinitiate it must reacquire the necessary factors.
- Apparently, even without acquiring the factors the 40S subunit returns to scanning. This imposes a time limit on binding the factors; if the factors bind before the subunit gets to uORF4 it will initiate there and not at *GCN4* but if they bind after it passes uORF4 it may initiate at *GCN4*
- The *GCN* and *GCD* factors regulate the efficiency that the factors bind to the scanning 40S subunit. The forward rate of factors binding may change only slightly, but result in a large change in the proportion of ribosomes that reinitiate on uORF4. The concentration of 43S preinitiation complex probably changes very little even under the most severe circumstances, which is why bulk initiation is unaffected.

What is important about this system is that the *kinetics* of factor binding regulates the *GCN4* system.

Papers for next time:

Sonenberg N, Dever TE. Eukaryotic translation initiation factors and regulators. *Curr Opin Struct Biol.* 2003 13:56-63.