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Promotion of Met-tRNA$_i^{Met}$ Binding to Ribosomes by yIF2, a Bacterial IF2 Homolog in Yeast

Sang Ki Choi, Joon H. Lee, Wendy L. Zoll, William C. Merrick, Thomas E. Dever*

Delivery of the initiator methionine transfer RNA (Met-tRNA$_i^{Met}$) to the ribosome is a key step in the initiation of protein synthesis. Previous results have indicated that this step is catalyzed by the structurally dissimilar translation factors in prokaryotes and eukaryotes—initiation factor 2 (IF2) and eukaryotic initiation factor 2 (eIF2), respectively. A bacterial IF2 homolog has been identified in both eukaryotes and archaea. By using a combination of molecular genetic and biochemical studies, the *Saccharomyces cerevisiae* IF2 homolog is shown to function in general translation initiation by promoting Met-tRNA$_i^{Met}$ binding to ribosomes. Thus, the mechanism of protein synthesis in eukaryotes and prokaryotes is more similar than was previously realized.

A universally conserved step in gene expression is the initiation of protein synthesis at an AUG codon by using a specific initiator tRNA^{Met} (tRNA$_i^{Met}$). In prokaryotes, the methionine linked to the tRNA$_i^{Met}$ is formylated (fMet-tRNA$_i^{Met}$), and the translation factor IF2 facilitates the AUG codon-dependent binding of fMet-tRNA$_i^{Met}$ to the small (30S) ribosomal subunit (*1*). Selection of the correct AUG initiation codon is mediated by base-pairing interactions between 16S ribosomal RNA (rRNA) and the Shine–Dalgarno sequence of complementary nucleotides located 5′ of the start site. In eukaryotes, the heterotrimeric translation factor eIF2 forms a stable ternary complex with guanosine triphosphate (GTP) and Met-tRNA$_i^{Met}$, which can then stably bind to 40S ribosomal subunits (*2*). This 43S preinitiation complex binds near the 5′ end of an mRNA and then scans in a 3′ direction and selects the AUG start site through base-pairing interactions between the AUG codon of the mRNA and the anticodon of the tRNA$_i^{Met}$ (*3*). Several translation factors, including eIF2, are also thought to play an important role in AUG start site selection (*4*).

IF2 from *Escherichia coli* is a single polypeptide chain of 97.3 kD and contains a consensus GTP binding domain near the center of the protein. *Saccharomyces cerevisiae* has an open reading frame (ORF) on chromosome I (YAL035w) that is similar to prokaryotic IF2 proteins (*5*). We refer to the protein product of this gene, previously designated *FUN12* (*6*), as yIF2 for yeast IF2. The full-length 1002–amino acid yIF2 protein sequence shows 27% identity and 48% similarity with IF2 from *E. coli*. An IF2 homolog, as well as homologs of all three eIF2 subunits, were also identified in the archaeon *Methanococcus jannaschii* (*7*). The archaeal protein is truncated at its NH_2-terminus; however, the NH_2-terminal regions of *E. coli* IF2 and yIF2 are dispensable for cell viability (*8*, *9*). The discovery of IF2 homologs in all three kingdoms suggests a greater conservation in the mechanism of translation initiation than had been anticipated. We disrupted one copy of *FUN12* in a diploid yeast strain (*10*) and identified, by tetrad analysis, two spores with wild-type growth rates and two *fun12*Δ spores with a severe slow-growth phenotype on rich medium. Therefore, *FUN12* is critically important but nonessential for growth in yeast. Mitochondrial protein synthesis is very similar to prokaryotic translation, and null mutations in the yeast gene *IFM1* encoding mitochondrial IF2 cause a respiratory-deficient phenotype (*11*). In contrast, the slow-growing *fun12*Δ strains were able to grow on nonfermentable carbon sources (*9*), demonstrating that *FUN12* is not required for mitochondrial protein synthesis. To determine whether the slow-growth phenotype in the *fun12*Δ strains resulted from a defect in cellular translation initiation, we analyzed polysome profiles by velocity sedimentation of cell extracts in sucrose gradients (*12*). As expected, distinct 40S and 60S ribosomal subunit peaks, as well as 80S monosome and polysome peaks, could be detected in the wild-type *FUN12* extract (Fig. 1A, upper panel). The isogenic *fun12*Δ strain showed a dramatic reduction in the larger polysomes and a corresponding increase in the 80S monosome peak (Fig.1A, lower panel). The polysome-to-monosome ratio in the *fun12*Δ strain was reduced by 70% compared with the ratio in the isogenic wild-type strain (Fig. 1A). The 80S monosomes that accumulated in the *fun12*Δ strain appear to be inactive 80S particles composed of 40S and 60S subunits but lacking mRNA because, when these extracts were sedimented in high-salt sucrose gradients, the 80S particles dissociated into 40S and 60S subunits (*9*). In contrast, the 80S monosomes in the wild-type *FUN12* extract were relatively stable in the high-salt gradients (*9*), as expected for translationally active 80S ribosomes (*13*). The reduction in large polysomes and the increase in 80S monosomes in the *fun12*Δ strain are indicative of a translation initiation defect. Consistent with yIF2 functioning in general translation initiation, indi-

Table 1. Stimulation of first peptide bond synthesis by yIF2 and eIF2 in a reconstituted system from rabbit reticulocytes. The AUG-directed synthesis of methionyl-puromycin was assayed as described in (*16*). Each reaction mixture contained ribosomes and, where indicated, translation factors (eIF1A, eIF5, and eIF5A) purified from rabbit reticulocytes. The GST-yIF2 fusion protein (5 μg) and the GST control protein (5 μg) were purified from yeast crude extracts (*15*); eIF2 (5 μg) was purified from rabbit reticulocytes. The GST-yIF2 fusion protein lacks the NH_2-terminal region of yIF2 with the GST protein fused directly to the GTP binding domain of yIF2. Results shown are typical of three independent assays.

Additions	Methionyl-puromycin synthesis (pmol)
eIF2 (alone)	0.05
eIF2 + factors	0.59
GST-yIF2 (alone)	0.08
GST-yIF2 + factors	0.54
GST + factors	0.02

S. K. Choi, J. H. Lee, T. E. Dever, Laboratory of Eukaryotic Gene Regulation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892–2716, USA.
W. L. Zoll and W. C. Merrick, Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106, USA.

*To whom correspondence should be addressed: E-mail: tdever@box-t.nih.gov

rect immunofluorescence analysis with immunopurified polyclonal antibodies revealed a diffuse cytoplasmic localization for yIF2 (9).

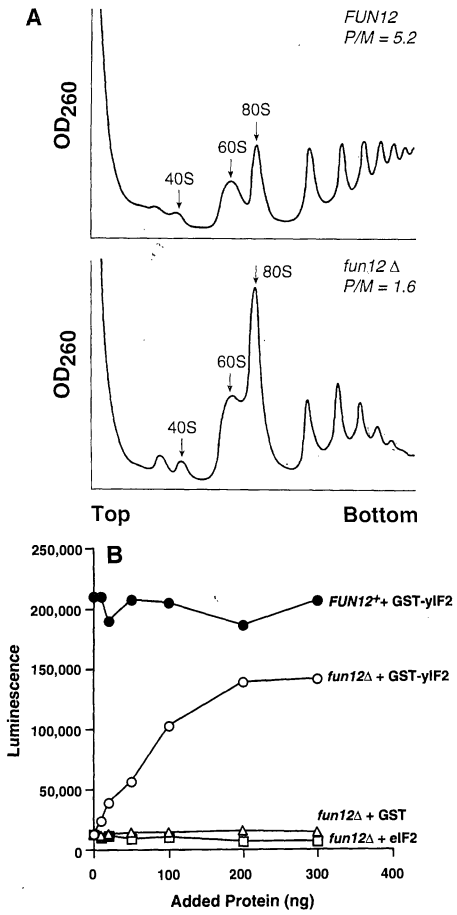


Fig. 1. Translation initiation defect in strains lacking *FUN12*. (**A**) Polysome profile analysis of wild-type *FUN12* and *fun12*Δ strains. Extracts were prepared from the *fun12*Δ strain J133 (*10*) carrying the low–copy number plasmid pC479 (*24*) containing wild-type *FUN12* (upper panel) or the vector pRS316 only (lower panel). Extracts were centrifuged on 7 to 47% sucrose gradients for 2.5 hours at 39,000 rpm in an SW41 rotor. Gradients were fractionated while they were being scanned at 254 nm; the absorbance profiles are shown with the top of the gradient at the left. Positions of 40*S*, 60*S*, and 80*S* ribosomal species are indicated. Ratios of polysomes (P) to monosomes (M) were calculated by measuring the area under the 80*S* peak and the polysomes (up to the point where the absorbance tracing terminated). (**B**) yIF2 is required for efficient protein synthesis in yeast in vitro translation assays. In vitro translation extracts from the wild-type and *fun12*Δ strains described in (A) were prepared and incubated with 200 ng of luciferase mRNA and the indicated amounts of recombinant GST or GST-yIF2 fusion protein or purified yeast eIF2 (*15*). Translational activity was determined by measuring luminescence after 15 min of incubation at 26°C. Results are representative of at least three independent experiments. Extract from the wild-type cells was about 1.25-fold more concentrated than extract from the *fun12*Δ strain.

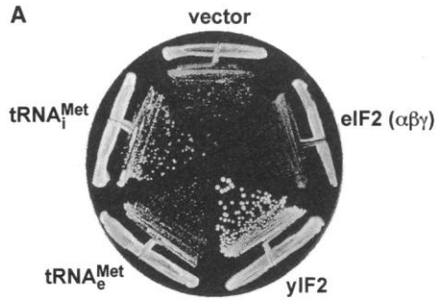
To determine whether yIF2 was directly involved in translation, we examined the translational activities of whole cell extracts from isogenic *FUN12*$^+$ and *fun12*Δ strains. Protein synthesis was measured in crude S30 extracts with capped and polyadenylated firefly luciferase (*LUC*) mRNA synthesized in vitro (*14*). In control experiments the amount of translation of *LUC* mRNA, measured by a luciferase assay, in both the wild-type and *fun12*Δ extracts was dose dependent for mRNA and increased linearly with time for up to 20 min; however, in both experiments the *fun12*Δ extract was less active than the wild-type extract (9). As shown in Fig. 1B, in the absence of added proteins, wild-type extract had about 15-fold greater translational activity than did the *fun12*Δ extract.

The translation initiation defect in the *fun12*Δ strain could occur because yIF2 functions directly in translation initiation or indirectly by altering the expression or function of the cellular translational machinery. When a recombinant glutathione *S*-transferase (GST)–yIF2 fusion protein (*15*) was added to the in vitro translation extract from the *fun12*Δ strain, we observed a dose-dependent recovery in translational activity (Fig. 1B). This recovery was specific for added yIF2, as addition of GST or purified yeast eIF2 failed to stimulate translation in extracts from the *fun12*Δ strain (Fig. 1B). In addition, the in vitro and the in vivo functions of yIF2 were both dependent on an intact GTP binding domain (9). Based on immunoblot analyses, the 200 ng of recombinant yIF2 required to restore translational activity in the *fun12*Δ extract was roughly equal to the amount of yIF2 present in the extract from the wild-type strain (9). The addition of excess yIF2 or eIF2 to the wild-type extract had no effect on translation (Fig. 1B) (9). Thus, yIF2 appears to be a general translation factor.

Both IF2 and eIF2 can promote Met-tRNA$_i^{Met}$ binding to the small ribosomal subunit (*1*, *16*). To determine whether yIF2 has a similar activity, we examined its ability to catalyze the synthesis of the dipeptide methionyl-puromycin by using a reconstituted mammalian translation system. In this assay the AUG-dependent conversion of [^{3}H]Met-tRNA$_i^{Met}$ to methionyl-puromycin as directed by rabbit reticulocyte ribosomes and ancillary factors was monitored. As shown in Table 1, addition of eIF2 resulted in >25-fold stimulation in methionyl-puromycin synthesis compared with assay mixtures containing the control protein GST. Recombinant GST-yIF2 fully substituted for eIF2 in the stimulation of methionyl-puromycin synthesis (Table 1); in titration experiments, eIF2 and yIF2 showed similar specific activities (*17*). In addition, yIF2 showed the same dependency as eIF2 for the translation factors eIF1A, eIF5, and eIF5A, indicating that yIF2 functions in the standard translation initiation pathway. Whereas these results suggest that yIF2 and eIF2 are functionally interchangeable in binding Met-tRNA$_i^{Met}$ to the ribosome, the polysome profile analyses and the in vitro translation experiments demonstrated that yIF2 is required for general translation initiation, even in the presence of eIF2.

Genetic analyses provide additional evidence that yIF2 functions in general translation initiation and facilitates Met-tRNA$_i^{Met}$ binding to ribosomes. The severe slow-growth phenotype of *fun12*Δ strains was partially suppressed by introduction of a high–copy number plasmid carrying the yeast *IMT4* gene that encodes tRNA$_i^{Met}$ (Fig. 2A). This suppression was specific for tRNA$_i^{Met}$ because overexpression of elongator tRNA^{Met} failed to suppress the slow-growth phenotype of the *fun12*Δ strain (Fig. 2A). Thus, the yIF2 role to deliver Met-tRNA$_i^{Met}$ to the ribosome can be partially compensated by increasing the abundance of Met-tRNA$_i^{Met}$ in the cell. Co-overexpression of all three subunits of eIF2 exacerbated the growth defect in the *fun12*Δ strain (Fig. 2A), demonstrating that eIF2 and yIF2 are not functional isozymes and that the two proteins perform distinct functions in the translation initiation pathway. We propose that the overexpressed eIF2 further inhibits translation in the *fun12*Δ strain by sequestering other initiation factors in nonproductive complexes. Overexpression of yIF2 did not cause any noticeable phenotypes in yeast nor was it able to suppress defects caused by mutations in the genes encoding eIF2 subunits (9).

Regulated translation reinitiation at four short upstream (u) ORFs in the *GCN4* mRNA leader governs the expression of *GCN4* in yeast [reviewed in (*18*)]. In wild-type cells *GCN4* expression was increased about ninefold under amino acid starvation (DR) conditions; however, this derepression was completely blocked in the *fun12*Δ strain (Fig. 2B). This defect was due to altered translational regulation because the *fun12*Δ had little or no effect on *GCN4* expression from a reporter that lacked the uORFs (Fig. 2B). Ribosomes are thought to translate uORF1 in the *GCN4* mRNA and then resume scanning; translation reinitiation at uORFs 2 to 4 depends on high levels of eIF2·GTP·Met-tRNA$_i^{Met}$ ternary complexes. Only ribosomes that bypass uORFs 2 to 4 without reinitiating are competent to translate *GCN4*. Therefore, the block in *GCN4* expression in the *fun12*Δ strain could be due to a failure to reinitiate any translation after uORF1 or an inability to



B

	GCN4-lacZ Activity (U)			
	FUN12+		fun12Δ	
	R	DR	R	DR
p180	14	129	3	7
p227	1115	514	838	480
p209	383	n.d.	310	n.d.

Fig. 2. Genetic evidence for translation initiation defects in *fun12Δ* strains. (**A**) Overexpression of initiator tRNA^{Met} alleviates the slow-growth phenotype of *fun12Δ* strains. The *fun12Δ* strain J129 (*25*) was transformed with the high–copy number plasmid YEp24 (vector); pC44, YEp24 carrying *IMT4* encoding yeast tRNA$_i^{Met}$; pCGS42-EMT, high–copy number plasmid carrying *EMT4* encoding yeast elongator tRNA^{Met} [tRNA$_e^{Met}$ (*26*)]; p1780, YEp24 carrying genes encoding α, β, and γ subunits of yeast eIF2 [eIF2(αβγ) (*27*)]; pC479, low–copy number plasmid carrying wild-type *FUN12* as indicated. Transformants were streaked on minimal SD medium supplemented only with the required nutrients and incubated 5 days at 30°C. (**B**) Defective *GCN4* translational control in a *fun12Δ* strain. Isogenic *FUN12*$^+$ and *fun12Δ* strains were constructed by transforming the *fun12Δ* strain J129 with either the low–copy number *FUN12* plasmid pC480 (*FUN12*$^+$) or the vector pRS315 (*fun12Δ*). The resulting strains were then transformed with plasmids containing *GCN4-lacZ* fusions with the wild-type leader (p180), a leader lacking all four uORFs (p227), or a leader lacking uORFs 2 to 4 (p209), as indicated. Boxes, wild-type ORFs; ×, point mutations that eliminate the AUG start codons of the ORFs. β-Galactosidase activities were measured as described in (*28*) in extracts of the indicated transformants grown under nonstarvation conditions (R) or under histidine starvation conditions (DR) imposed by the histidine analog 3-aminotriazole. β-Galactosidase activities are expressed as nanomoles of *o*-nitrophenyl β-D-galactopyranoside hydrolyzed per minute per milligram of total protein. Results shown are averages of assays conducted on three independent transformants, and individual measurements deviated from the average values shown here by 20% or less; n.d., not determined.

bypass uORFs 2 to 4. The *fun12Δ* had no effect on *GCN4* expression from a reporter containing only uORF1 (Fig. 2B), demonstrating that lack of yIF2 does not result in a general defect in translation reinitiation. We propose that the defect in *GCN4* expression in the *fun12Δ* strain results from increased translation of uORFs 2 to 4 either because of ribosomes scanning past the UORFI AVG start codon without initiating translation or because of an increased relative affinity of reinitiating ribosomes versus free 40S subunits for binding ternary complexes. This in vivo defect in *GCN4* expression in the *fun12Δ* strain is consistent with our biochemical analyses, indicating that yIF2 functions along with eIF2 in delivering Met-tRNA$_i^{Met}$ to the ribosome.

It has been thought that eukaryotes and prokaryotes use different mechanisms and structurally distinct protein factors to deliver the Met-tRNA$_i^{Met}$ to the ribosome during translation initiation (*1*, *19*). The identification of yIF2 suggested two possibilities: yIF2 may function independently of eIF2 to translate a subset of cellular mRNAs or it may act with eIF2 in virtually all initiation events. Although *FUN12* was not essential for cell viability, the *fun12Δ* strain had a severe slow-growth phenotype and polysome profiles showed defects in general translation initiation. This translation defect was also observed in extracts from the *fun12Δ* strain for an mRNA with no specialized features, and translational activity could be fully restored by the addition of recombinant yIF2. On the basis of these findings, we propose that yIF2 is a general translation factor that works in conjunction with eIF2 to promote binding of Met-tRNA$_i^{Met}$ to the small ribosomal subunit. yIF2 may bind to the 40S ribosomal subunit and direct binding of the eIF2·GTP·Met-tRNA$_i^{Met}$ ternary complex to the ribosomal P site, or it may bind after eIF2 and stabilize binding of ternary complexes or the Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit. The biochemical characteristics of yIF2 described here are similar to those described for translation factor eIF2A from rabbit reticulocytes (*16*, *20*). In addition, we have identified expressed sequence tags from mammalian cDNA libraries that show strong sequence similarity to IF2 (*9*). Although further experiments are necessary to determine whether the mammalian *FUN12* homolog and eIF2A are the same protein, the identification of IF2 homologs in yeast and archaea suggests that IF2 is universally conserved. Thus, this central step of the translation initiation pathway, delivery of Met-tRNA$_i^{Met}$ to the ribosome, and the IF2 factors that promote this step in various organisms may represent one of the most conserved elements in gene expression throughout evolution.

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10. A 3.55-kb Bst BI DNA fragment containing the *FUN12* gene was isolated from the λ clone PM-5080 (70512 from ATCC, Rockville, MD) and inserted at the Cla I site of the high-copy-number *URA3* plasmid pRS426 [T. W. Christianson, R. S. Sikorski, M. Dante, J. H. Shero, P. Hieter, *Gene* **110**, 119 (1992)] to create the plasmid pC473. A *fun12::LEU2* deletion disruption allele was generated by inserting a 2.4-kb Bgl II–*LEU2* fragment from YEp13 in place of the internal 1.7-kb *FUN12* Bgl II fragment in pC473 to create pC481. A 4.3-kb Aat II–Nco I fragment from pC481 was used to transform to Leu$^+$ the diploid strain H1056 (*MAT***a**/*MATα leu2-3/leu2-3 leu2-112/leu2-112 ura3-52/ura3-52*). The transformant diploid was sporulated and subjected to tetrad analysis. Tetrads contained four viable spores with the two Leu$^+$ *fun12::LEU*$_2$ spores showing a marked slow-growth phenotype. Disruption of *FUN12* in the slow-growing spores was confirmed by polymerase chain reaction (PCR) and could be complemented by transforming with a low–copy number plasmid carrying wild-type *FUN12*. Two *fun12::LEU2* ascospores from the same tetrad were isolated and designated J133 and J135.
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39°C as described [R. B. Wickner, *Methods Mol. Genet.* **3**, 141 (1994)]. Translation extracts (cytoplasmic S30 extracts) were prepared exactly as described (*21*) and stored in liquid N_2. Capped and polyadenylated luciferase mRNA was prepared with the T7 luciferase (T7LUC) vector pRG166 (a gift of S. Green and Charles Moehle, RiboGene, Hayward, CA) and the Ampliscribe T7 transcription kit (Epicentre Technologies). mRNA was purified with the RNeasy total RNA kit (Qiagen). Translation assays were performed as described (*21*), except that the micrococcal nuclease treatment of the translation extracts was omitted. Luminescence was measured by adding 10 μl of translation mix to 100 μl of LUC assay reagent (Promega) and measuring the emission for 10 s on a Turner TD-20e luminometer.

15. To express the GST-yIF2 fusion protein in yeast under control of the yeast *GAL1* promoter, we inserted a portion of the *FUN12* cDNA from the plasmid fun12-1 [P. Sutrave, B. K. Shafer, J. N. Strathern, S. H. Hughes, *Gene* **146**, 209 (1994); a gift from P. Sutrave and S. Hughes, National Cancer Institute, Frederick, MD] into the yeast GST fusion expression vector pEGKT [D. A. Mitchell, T. K. Marshall, R. J. Deschenes, *Yeast* **9**, 715 (1993)], creating the plasmid pC485. GST and GST-yIF2 were purified from the *fun12*Δ strain J130 (*22*) transformed with the plasmids pEGKT and pC485, respectively. The GST-yIF2 fusion protein containing yIF2 amino acid residues 396 to 1002, starting 16 residues before the GTP binding domain and extending to the COOH-terminus of the protein, was able to fully complement the slow-growth phenotype of the *fun12*Δ strain, indicating that the fusion protein is functional in vivo. Crude protein extracts from transformants grown in synthetic minimal medium containing 10% galactose and 2% raffinose were incubated with glutathione Sepharose 4B and washed; the GST and GST-yIF2 proteins were eluted in buffer containing 10 mM reduced glutathione. The purified proteins were dialyzed against sample buffer [20 mM tris-HCl (pH 7.5), 100 mM KCl, 1 mM DTT, 10% (v/v) glycerol] and stored in liquid nitrogen. Purified yeast eIF2 [G. D. Pavitt, K. V. A. Ramaiah, S. R. Kimball, A. G. Hinnebusch, *Genes Dev.* **12**, 514 (1998)] was kindly provided by Graham Pavitt.
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25. The *fun12::LEU2* allele in strain J135 (*10*) was disrupted by transforming to Ura^+ prototrophy with a Bgl II fragment containing the *leu2::hisG::URA3::hisG* cassette from the plasmid pNKY85 (Nancy Kleckner, Harvard University, Cambridge, MA). A Ura^+ Leu^- transformant was identified and then replica plated to 5-fluoroorotic acid (5-FOA) medium to select for a Ura^- segregant, generating the haploid strain J129 (*Matα leu2-3 leu2-112 ura3-52 fun12::leu2::hisG*).
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The Ubiquitin-Related Protein RUB1 and Auxin Response in *Arabidopsis*

J. C. del Pozo, C. Timpte,* S. Tan, J. Callis, M. Estelle†

The AXR1 (auxin-resistant) protein, which has features of the ubiquitin-activating enzyme E1, is required for normal response to the plant hormone auxin in *Arabidopsis thaliana*. ECR1 functions together with AXR1 to activate members of the RUB/NEDD8 family of ubiquitin-related proteins. Extracts from mutant seedlings lacking AXR1 did not promote formation of the RUB-ECR1 thiolester, indicating that AXR1 is the major activity in this tissue. AXR1 was localized primarily to the nucleus of dividing and elongating cells, suggesting that the targets of RUB modification are nuclear. These results indicate that auxin response depends on RUB modification of one or more nuclear proteins.

Plant growth and development depends on the coordinated action of several phytohormones. One of these hormones, indole-3-acetic acid (IAA or auxin), has been implicated in the control of diverse developmental processes (*1*). Auxin regulates these processes by promoting changes in cell division and cell elongation (*2*). Recessive mutations in the *AXR1* gene result in decreased auxin response, including reduced auxin-induced gene transcription (*3*). A screen for suppressors of the *axr1* phenotype resulted in identification of the *SAR1* gene. By genetic criteria, *SAR1* acts downstream of *AXR1* (*4*). Mutations in another gene, *TIR1*, also result in a defect in auxin response (*5*). Genetic studies indicate that *AXR1* and *TIR1* function in the same or overlapping pathways.

The molecular characterization of *AXR1* and *TIR1* implicates the ubiquitin pathway in auxin response. *AXR1* encodes a protein related to the NH_2-terminal half of the ubiquitin-activating enzyme, E1 (*6*), and proteins related to AXR1 can be found in yeast and mammals (*7*). Despite extensive similarity with E1, all lack the active-site cysteine required for thiolester bond formation with ubiquitin. *TIR1* encodes an F-box protein related to yeast Grr1p and human SKP2 (*5*). F-box proteins have been shown to function in a ubiquitin-ligase complex (E3) called the SCF (for Skp1 Cdc53 F-box). These results suggest that auxin response is mediated by ubiquitin or a ubiquitin-like protein modification.

Ubiquitin (UBQ) is one of the most conserved proteins among eukaryotes. Covalent attachment of UBQ to other proteins targets them for degradation by the 26S proteosome (*8*). UBQ is activated by E1 in an adenosine 5′-triphosphate (ATP)–dependent reaction in which a thiolester bond is formed between the COOH-terminus of UBQ and a cysteine within the E1 enzyme. The UBQ moiety is transferred to a cysteine residue on a UBQ-conjugating enzyme (E2). Finally, UBQ is covalently attached to a target protein by an isopeptide linkage directly from the E2 or by a UBQ-protein ligase such as the SCF (*9*).

Two conserved families of UBQ-related proteins (Smt3p/SUMO and RUB/NEDD8) have also been identified (*10*). In *Saccharomyces cerevisiae*, activation of Smt3p is performed by the E1-like heterodimer Aos1p/Uba2p rather than E1 (*11*). Aos1p is a member of the AXR1 family, while Uba2p is similar to the COOH-terminal half of E1 and contains the active-site cysteine. Smt3p is conjugated in vivo to other proteins, but these targets are yet to be identified (*11*). A protein related to Smt3p in

J. C. del Pozo, C. Timpte, M. Estelle, Department of Biology, Indiana University, Bloomington, IN 47405–6801, USA.
S. Tan and J. Callis, Section of Molecular and Cellular Biology, University of California–Davis, 1 Shields Avenue, Davis, CA 95616, USA.

*Present address: Department of Biological Sciences, University of New Orleans, New Orleans, LA 70148, USA.
†To whom correspondence should be addressed. E-mail: mestelle@bio.indiana.edu