SPA1, a WD-Repeat Protein Specific to Phytochrome A Signal Transduction

Ute Hoecker, James M. Tepperman, Peter H. Quail*

The five members of the phytochrome photoreceptor family of *Arabidopsis thaliana* control morphogenesis differentially in response to light. Genetic analysis has identified a signaling pathway that is specifically activated by phytochrome A. A component in this pathway, SPA1 (for "suppressor of phyA-105"), functions in repression of photomorphogenesis and is required for normal photosensory specificity of phytochrome A. Molecular cloning of the *SPA1* gene indicates that SPA1 is a WD (tryptophan–aspartic acid)-repeat protein that also shares sequence similarity with protein kinases. SPA1 can localize to the nucleus, suggesting a possible function in phytochrome A–specific regulation of gene expression.

Plants have evolved several photoreceptors that allow adaptation of growth and development to the ambient light environment (*1*). The members of the phytochrome photoreceptor family monitor the red (R) and the far-red (FR) regions of the electromagnetic spectrum, with evidence for differential photosensory and physiological activities among members. Mutational analyses have demonstrated that phytochrome A (phyA) is responsible for perception of continuous FR light (FRc), whereas responses to continuous R light (Rc) are primarily mediated by phytochrome B (phyB) (*2*).

Initial insight into components functioning early in phytochrome signal transduction has been provided recently by the isolation of a phytochrome-interacting factor, PIF3, which is a basic helix-loop-helix protein (*3*). PIF3 appears to be involved in both phyA and phyB signaling, consistent with genetic evidence for a signal transduction pathway shared by phyA and phyB (*4, 5*).

By contrast, genetic data also suggest that the distinct photosensory specificities of phyA and phyB involve separate, probably early acting, signal transduction components that are specific to either phyA or phyB (*4, 6, 7*). An *Arabidopsis* mutant, *spa1*, identified in a *phyA* suppressor screen is hypersensitive only to light signals perceived by phyA (*7*). Analyses suggest that *SPA1* encodes a negatively acting factor that functions at an early step in phyA-specific signal transduction. Because phyA mediates deetiolation responses to both Rc and FRc in the absence of functional SPA1, it appears that SPA1 normally plays a key role in confining the sensory specificity of phyA to FRc (*7*).

We cloned the *SPA1* gene by positional cloning (Fig. 1, A and B). Sequence analyses revealed that a candidate gene in the 0.003-centimorgan region to which we mapped *SPA1* carries single base-pair substitutions in all five *spa1* mutant alleles (Fig. 1B). Each of the five mutations forms a stop codon in the predicted open reading frame. We therefore conclude that this gene is the *SPA1* gene. We isolated a full-length cDNA clone of the gene which contains an uninterrupted open reading frame of 3090 base pairs (bp) (*8*). The genomic structure of *SPA1* is displayed in Fig. 1B.

SPA1 encodes a novel 114-kD protein with four WD repeats in the COOH-terminal portion (Fig. 2A). WD repeats represent a motif com-

Department of Plant and Microbial Biology, University of California Berkeley, Berkeley, CA 94720, USA, and U.S. Department of Agriculture/Agricultural Research Service Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710, USA.

*To whom correspondence should be addressed. E-mail: quail@nature.berkeley.edu

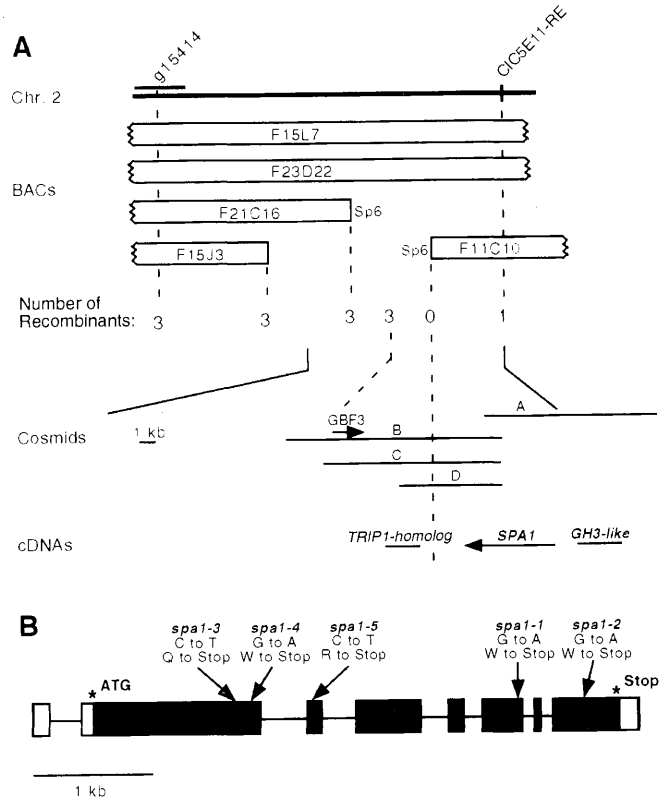

Fig. 1. Molecular identification of the *SPA1* gene by positional cloning. (**A**) Physical mapping of *SPA1* on chromosome 2. On the basis of analysis of 1200 recombinant chromosomes, *SPA1* was fine-mapped between the markers g15414 and CIC5E11-RE. Clones from the Institut für Genbiologische Forschung (Berlin) Bacterial Artificial Chromosome (BAC) library (*24*) were anchored to these markers, and a BAC contig was assembled based on information available at www.mpimp-golm.mpg.de/101/chr_cont/chr2_BAC_contigs.xls. BAC ends were isolated from the BAC clones F15J3, F21C16, and F11C10, converted into CAPS markers, and used to delimit the *SPA1* locus between the Sp6 end of F21C16 and CIC5E11-RE. The Sp6 end of F11C10 and CIC5E11-RE was used as a probe to isolate four distinct cosmid clones from a binary cosmid library (*25*). A fragment common to cosmids B and C contained the marker GBF3, which allowed further fine-mapping of the *SPA1* locus. Cosmid fragments were used to probe a seedling cDNA library (*22*), and clones representing three genes were isolated. The *SPA1* gene was identified by sequencing these candidate genes from *spa1* mutant and wild-type plants (*26*). (**B**) Structure of the *SPA1* gene and position of the five *spa1* mutations. The locations of the initiation (ATG) and stop codons are indicated. *SPA1* gene structure was derived from comparison of genomic and cDNA sequence. Filled boxes indicate the open reading frame, open boxes indicate untranslated regions, and lines between boxes indicate introns.

mon to many proteins with diverse functions and are thought to be involved in protein-protein interactions (*9*). A complete WD-repeat domain appears essential for SPA1 function, because COOH-terminal truncation in the fourth predicted WD repeat (*spa1-2*) causes a strong *spa1* mutant phenotype (*7*). In the WD-repeat region, SPA1 shares highest sequence similarity with COP1, a repressor of photomorphogenesis in dark-grown *Arabidopsis* (*10*), and the yeast transcriptional repressor TUP1 (*11*) (44% and 30% identity in amino acid sequence, respectively) (Fig. 2, A through C). Sequence similarity between SPA1 and COP1 or TUP1 is limited to the WD-repeat region. SPA1 contains a putative coiled-coil domain NH_2-terminal to the WD repeats (Fig. 2A). Such coiled-coil domains have been shown to promote protein-protein interactions, such as homo- or heterodimerization or oligomerization (*12*).

The NH_2-terminal sequence of SPA1 shows weak similarity to Ser/Thr and Tyr protein kinases, and is most closely related to human p68 protein kinase (Fig. 2B) (*13*). However, whereas SPA1 appears to contain several of the 11 domains forming the protein kinase motif, including a putative mononucleotide-binding motif (GXGXXGK) (Fig. 2, A and B) (*14*), it lacks several of the key amino acid residues that are

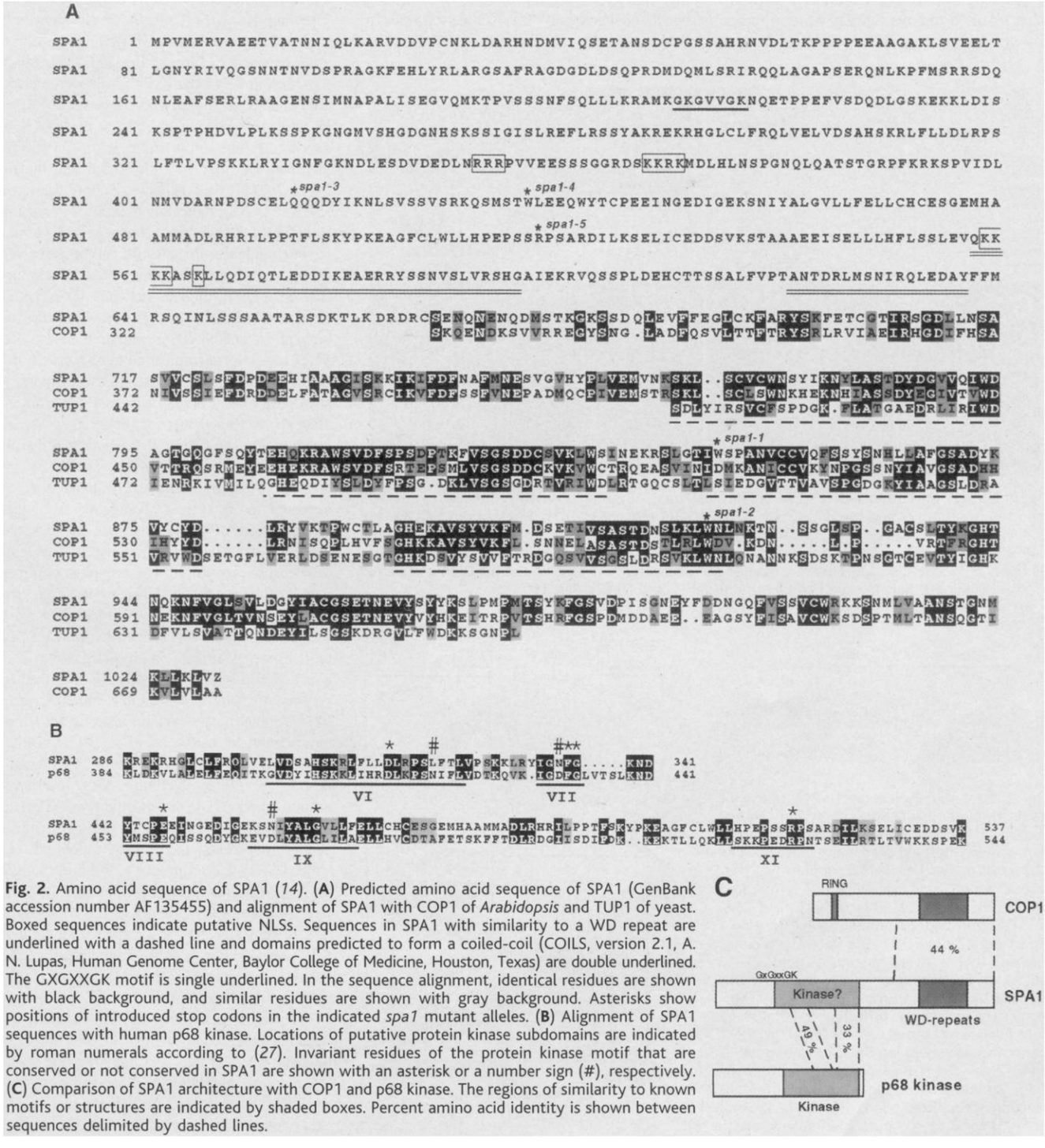

Fig. 2. Amino acid sequence of SPA1 (*14*). (**A**) Predicted amino acid sequence of SPA1 (GenBank accession number AF135455) and alignment of SPA1 with COP1 of *Arabidopsis* and TUP1 of yeast. Boxed sequences indicate putative NLSs. Sequences in SPA1 with similarity to a WD repeat are underlined with a dashed line and domains predicted to form a coiled-coil (COILS, version 2.1, A. N. Lupas, Human Genome Center, Baylor College of Medicine, Houston, Texas) are double underlined. The GXGXXGK motif is single underlined. In the sequence alignment, identical residues are shown with black background, and similar residues are shown with gray background. Asterisks show positions of introduced stop codons in the indicated *spa1* mutant alleles. (**B**) Alignment of SPA1 sequences with human p68 kinase. Locations of putative protein kinase subdomains are indicated by roman numerals according to (*27*). Invariant residues of the protein kinase motif that are conserved or not conserved in SPA1 are shown with an asterisk or a number sign (#), respectively. (**C**) Comparison of SPA1 architecture with COP1 and p68 kinase. The regions of similarity to known motifs or structures are indicated by shaded boxes. Percent amino acid identity is shown between sequences delimited by dashed lines.

invariant among members of the protein kinase superfamily (Fig. 2B). These unusual amino acid substitutions make it unlikely that SPA1 functions as a classical protein kinase. On the other hand, several proteins with kinase activity but little or no sequence similarity to the kinase motif have been described in animals, with some displaying highly substrate-specific phosphorylation activity (*15*). Also, phytochrome from higher plants, though exhibiting only weak sequence similarity with His kinases and no similarity to Ser/Thr kinases (*16*), has been reported to have Ser/Thr protein kinase activity (*17*). It is therefore possible that SPA1 defines a novel class of protein kinases. Alternatively, the kinase-like domain of SPA1 may have evolved a new function.

Analysis of the predicted amino acid sequence of SPA1 suggests the presence of two putative nuclear localization sequences (NLSs), one bipartite NLS, and one SV40-type NLS (Fig. 2A) (*17*). Indeed, GUS:SPA1 fusion proteins were found almost exclusively in the nucleus when expressed in transiently transfected onion epidermal cells (Fig. 3A). A deletion derivative of SPA1, GUS:SPA1ΔN591, which contains only the COOH-terminus of SPA1 and none of the putative NLSs, did not preferentially localize to the nucleus (Fig. 3B). The β-glucuronidase (GUS) protein alone also remained cytoplasmic (*19*). No difference in subcellular localization of GUS:SPA1 was found between dark- and light-treated cells (*19*). The results demonstrate that SPA1 is a nuclear protein and, therefore, may directly control gene expression.

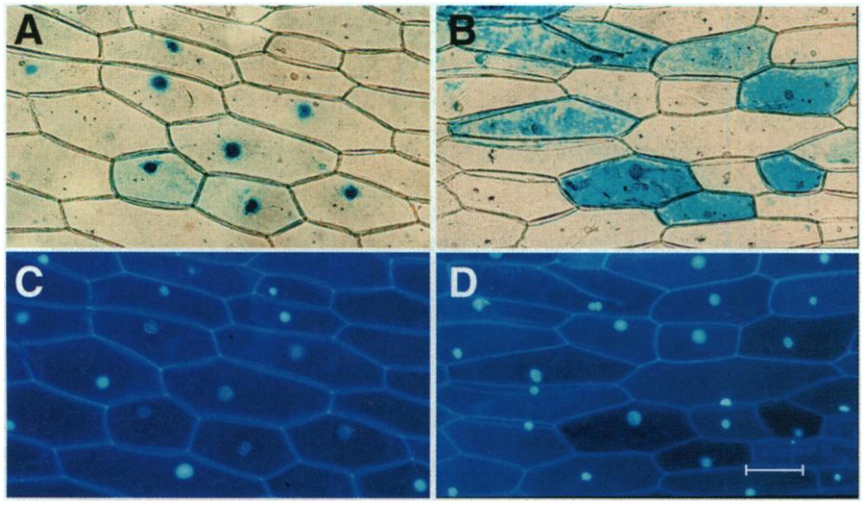

Fig. 3. Nuclear localization of SPA1 after transient transformation. Constructs encoding the GUS reporter fused to either the full-length *SPA1* cDNA sequence (GUS:SPA1) (**A** and **C**) or the COOH-terminal region of SPA1 (GUS:SPA1ΔN592) (**B** and **D**) (*28*), each under the control of the CaMV 35*S* promoter, were introduced into onion epidermal cells by particle bombardment. After 24 hours of incubation, the cells were stained for GUS activity (A and B) and for DNA using DAPI (4′,6′-diamidino-2-phenylindole) to identify nuclei (C and D) (*3*). Bar, 100 μm.

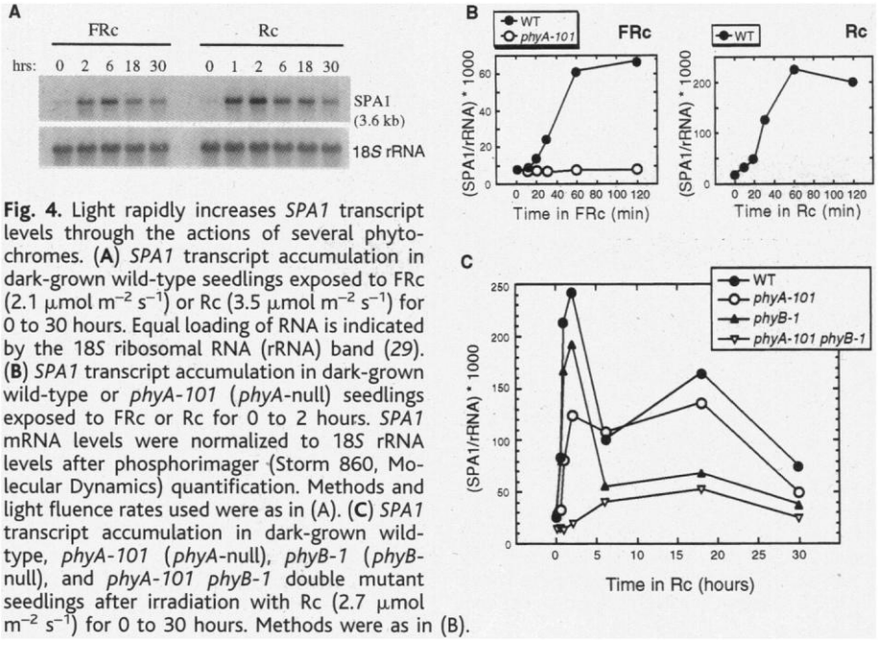

Fig. 4. Light rapidly increases *SPA1* transcript levels through the actions of several phytochromes. (**A**) *SPA1* transcript accumulation in dark-grown wild-type seedlings exposed to FRc (2.1 μmol m^{-2} s^{-1}) or Rc (3.5 μmol m^{-2} s^{-1}) for 0 to 30 hours. Equal loading of RNA is indicated by the 18*S* ribosomal RNA (rRNA) band (*29*). (**B**) *SPA1* transcript accumulation in dark-grown wild-type or *phyA-101* (*phyA*-null) seedlings exposed to FRc or Rc for 0 to 2 hours. *SPA1* mRNA levels were normalized to 18*S* rRNA levels after phosphorimager (Storm 860, Molecular Dynamics) quantification. Methods and light fluence rates used were as in (A). (**C**) *SPA1* transcript accumulation in dark-grown wild-type, *phyA-101* (*phyA*-null), *phyB-1* (*phyB*-null), and *phyA-101 phyB-1* double mutant seedlings after irradiation with Rc (2.7 μmol m^{-2} s^{-1}) for 0 to 30 hours. Methods were as in (B).

Because several, if not all, phytochromes mediate the light-induced increase in *SPA1* transcript abundance, the phyA-induced increase cannot be the sole mechanism by which *SPA1* participates specifically in phyA signaling. This conclusion is supported by the finding that, in contrast to *spa1 phyA* double mutants, *spa1 phyB* double mutants exhibit a strong *spa1* mutant phenotype in Rc and FRc (*19*), indicating that phyB is not essential for SPA1 function despite its role in regulating *SPA1* transcript accumulation. Thus, the specificity of SPA1 to phyA signaling must rely on a phyA-dependent change in addition to, or independent of, enhanced *SPA1* transcript levels. We propose that the key regulatory step may involve specific, phyA-induced modification of the SPA1 protein, thereby activating the repressor function of SPA1. Alternatively, SPA1 might regulate the activity of phyA or an intermediate of phyA-specific signal transduction.

The importance of the phytochrome-induced increase in *SPA1* mRNA levels is yet to be determined. However, it may provide negative feedback regulation of phyA signaling. We speculate that the rapid Rc-induced increase in *SPA1* transcript levels may play an important

role in switching off phyA signaling in Rc and, therefore, in conferring the FRc-specific sensitivity of phyA.

It is notable that the WD-repeat regions of SPA1 and COP1 show high sequence similarity, because these two proteins are also related by function. Both proteins are repressors of photomorphogenesis whose activities are controlled by light. Because functional analysis of COP1 suggests that the WD-repeat domain mediates the repression of photomorphogenesis (*20*), it is conceivable that the WD-repeat regions of SPA1 and COP1 may have related functions. However, the contrasting phenotypes of *spa1* and *cop1* mutants demonstrate differences between the light-regulation of the two proteins. Whereas *spa1* mutations affect only light-grown seedlings, *cop1* mutations have a greater effect in dark-grown seedlings. Also, *spa1* mutations specifically affect phyA signaling, whereas *cop1* mutations affect signaling from multiple phytochromes as well as other photoreceptors (*7, 21*). SPA1 and COP1 are therefore likely to function at different points in the light-signaling pathway, but potentially through related mechanisms.

Genetic evidence has for some time suggested the existence of signaling pathways specific to individual phytochrome family members. The cloning of *SPA1* now provides initial molecular identification of such a pathway specific to phyA, and the nuclear localization of the SPA1 protein suggests a possible function in regulating gene expression. This suggestion is consistent with evidence from the recently identified phyA- and phyB-interacting protein PIF3 (*3*) that early events in phytochrome signaling involve nuclear-localized components.

References and Notes

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29. Total RNA was extracted from 3-day-old seedlings that had been transferred to light for the indicated time using the Qiagen RNeasy Plant Miniprep kit. We separated 5 μg of total RNA on a Mops-RNA gel containing 6.7% formaldehyde and subsequently transferred the RNA to MSI Nylon membrane. The membranes were hybridized with a random prime-labeled PCR product containing the full-length *SPA1* cDNA and washed with a final wash with 0.2× saline sodium citrate and 0.1% SDS at 65°C. Membranes were subsequently hybridized with a pea 18*S* rRNA probe.

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Control of mRNA Decay by Heat Shock–Ubiquitin-Proteasome Pathway

Gaurav Laroia,[1] Rafael Cuesta,[1] Gary Brewer,[2] Robert J. Schneider[1]*

Cytokine and proto-oncogene messenger RNAs (mRNAs) are rapidly degraded through AU-rich elements in the 3′ untranslated region. Rapid decay involves AU-rich binding protein AUF1, which complexes with heat shock proteins hsc70-hsp70, translation initiation factor eIF4G, and poly(A) binding protein. AU-rich mRNA decay is associated with displacement of eIF4G from AUF1, ubiquitination of AUF1, and degradation of AUF1 by proteasomes. Induction of hsp70 by heat shock, down-regulation of the ubiquitin-proteasome network, or inactivation of ubiquitinating enzyme E1 all result in hsp70 sequestration of AUF1 in the perinucleus-nucleus, and all three processes block decay of AU-rich mRNAs and AUF1 protein. These results link the rapid degradation of cytokine mRNAs to the ubiquitin-proteasome pathway.

In eukaryotes, gene expression can be regulated by the selective decay of mRNAs. The mRNAs of proto-oncogenes and cytokines have half-lives of 5 to 30 min, whereas stable mRNAs have half-lives of several hours (*1*). The selective destabilization of these short-lived mRNAs is facilitated by an AU-rich element (ARE) in the mRNA 3′ untranslated region (3′UTR) (*1*, *2*). A family of ARE-binding proteins known as AUF1 or hnRNP-D proteins (*3*) appears to promote ARE-mRNA decay. AUF1 consists of four protein isoforms of 37, 40, 42, and 45 kD that differ in NH_2- and COOH-terminal domains and hence may have different RNA binding specificities (*4*). The role of AUF1 in decay

[1]Department of Microbiology and Biochemistry, New York University School of Medicine, New York, NY 10016, USA. [2]Department of Microbiology and Immunology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA.

*To whom correspondence should be addressed. E-mail: schner01@mcrcr6.med.nyu.edu