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.

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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 18S rRNA probe.

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

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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

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is suggested by in vivo correlations of ARE-mRNA decay rates with amounts of AUF1 protein and ARE binding (*5*). Moreover, ARE-mRNA decay is promoted by addition of p37 AUF1 (the 37-kD isoform of AUF1) to an in vitro decay system, where it binds to the ARE (*5*). ARE-mRNAs can be transiently stabilized by release of stored Ca^{2+}, stimulation of the c-Jun NH_2 kinase (JNK) signaling pathway (*6*), or overexpression of another ARE binding factor, HuR (*7*).

Control of ARE-mRNA decay and the mechanism of degradation are not understood. Several lines of evidence implicate translation in the activation of decay in animal cells (*2*). Inhibition of ARE-mRNA translation by removal of start codons (*8*), or by insertion of secondary structure, stabilizes mRNAs containing the granulocyte-macrophage colony-stimulating factor (GM-CSF) ARE (*9*). However, translation of an ARE-mRNA can be uncoupled from its decay, and certain ARE-mRNAs may not be rapidly degraded in a translation-dependent manner (*9*, *10*).

The hsp70 mRNA contains a 3′UTR ARE, yet the RNA is not rapidly degraded during heat shock despite its active translation (*11*). This suggests that the ARE-mRNA decay machinery might be inactivated during heat shock, or that hsp70 mRNA may be resistant to accelerated decay. To discriminate between these possibilities, we constructed matched sets of β-galactosidase (β-gal) reporter mRNAs containing the destabilizing GM-CSF ARE or a nonfunctional mutant ARE (GC control) in the 3′UTR. The effect of heat shock on GM-CSF ARE-mRNA stability is not known; however, mRNAs contain a 5′UTR that is either translated during heat shock (cap-independent) or is blocked by heat shock (cap-dependent) (*12*).

HeLa cells were transiently transfected and the effects of heat shock on reporter ARE-mRNA decay were determined by mRNA steady-state and half-life analysis. At normal temperature (37°C), amounts of reporter ARE-mRNA were reduced to 5 to 10% of those in stable controls (Fig. 1A), because of a shortened cytoplasmic half-life (Fig. 1B; $t_{1/2}$ = 30 min) relative to stable GC-control mRNAs without an ARE ($t_{1/2}$ > 9 hours). Cap-independent ARE-mRNAs were largely stabilized ($t_{1/2}$ ~ 5 hours) and well translated during heat shock (Fig. 1, B and C), excluding stabilization by heat shock–mediated nuclear retention of the mRNA. Thus, heat shock is associated with a block in the degradation of ARE-mRNAs.

Heat shock of HeLa cells blocks cell protein synthesis after 30 to 60 min, with maximal appearance of heat shock proteins by 2 hours (*13*). Hsp70 protein facilitates the nuclear accumulation of cytoplasmic proteins during heat shock by mediating conformational changes that promote membrane translocation (*14*). AUF1 distribution was therefore examined with antisera that recognize all four isoforms. At normal temperatures, AUF1 is a nuclear and cytoplasmic protein (*3*). After 2 hours of heat shock, most hsp70 and AUF1 was nuclear and perinuclear (Fig. 1D); this process was complete by 3 hours (*13*). Imaging of cytoplasmic α-tubulin as a control showed a slight change in cell morphology after 3 hours at 44°C. These results implicate hsp70 in the nuclear-perinuclear accumulation of AUF1 proteins.

To detect AUF1 binding proteins, we labeled HeLa cells with [^{35}S]methionine at 37°C or at the start of heat shock. Immunoprecipitation of AUF1 from cells revealed interacting polypeptides of 220 kD, 105 to 110 kD, and 46 to 70 kD that were synthesized at 37°C, and a 70-kD protein synthesized at 44°C (Fig. 2A) (*15*). AUF1 protein was labeled at 44°C because inhibition of translation does not occur until after 1 hour of heat shock. AUF1-interacting proteins were identified by immunoprecipitation of unlabeled AUF1 from control cells or from cells heat-shocked for 2 hours, followed by immunoblotting for suspected proteins. Immunoprecipitated AUF1 bound the constitutively synthesized hsc70 heat shock protein at normal and heat shock temperatures. At 44°C there was increased binding of AUF1 to 220-kD translation initiation factor eIF4G and to the 70-kD heat-inducible hsp70 protein (Fig. 2B). eIF4G was not identified by [^{35}S]methionine labeling at 44°C because it is not efficiently labeled in the short period before heat shock inhibition of translation. Ribonuclease A (RNase A) treatment did not disrupt protein complexes, excluding coprecipitation by mutual mRNA binding. Inhibition of translation by disruption of polysomes with puromycin also increased AUF1-eIF4G association (*13*). Immunoprecipitates of AUF1 were examined for poly(A) binding protein (PABP) because PABP binds eIF4G (*16*). PABP interacted with the AUF1-eIF4G precipitate, which was slightly decreased by heat shock. Thus, PABP is a member of the AUF1 complex of proteins, and PABP may directly interact with AUF1 because heat shock, which strongly enhances AUF1-eIF4G interaction, only modestly increased the presence of PABP. Blotting with antiserum to hsp105 (which is not in the

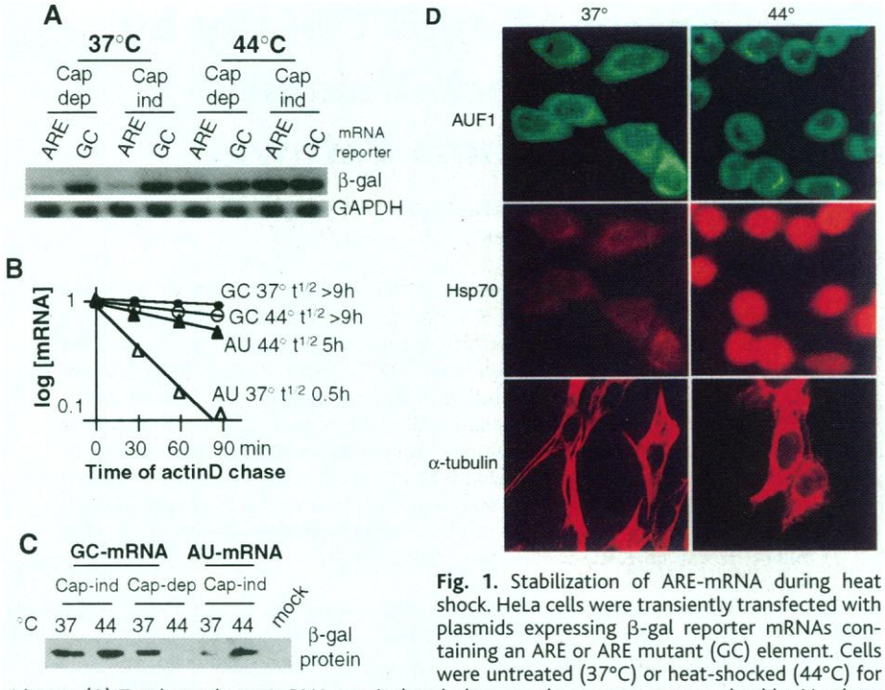

Fig. 1. Stabilization of ARE-mRNA during heat shock. HeLa cells were transiently transfected with plasmids expressing β-gal reporter mRNAs containing an ARE or ARE mutant (GC) element. Cells were untreated (37°C) or heat-shocked (44°C) for 4 hours. (**A**) Total steady-state RNA was isolated, then equal amounts were resolved by Northern analysis and were hybridized to labeled probes (Cap dep, cap-dependent; Cap ind, cap-independent; GAPDH, glyceraldehyde phosphate dehydrogenase). (**B**) Cells were heat-shocked for 3 hours, then actinomycin D (actinD) was added for the times indicated to block transcription. Equal amounts of total RNA were resolved by Northern analysis and quantified by densitometry. The plot averages three independent experiments for half-life derivation. (**C**) Cells were labeled with [^{35}S]methionine, then equal protein amounts were used for β-gal immunoprecipitation, resolved by gel electrophoresis, and fluorographed. Mock samples were not transfected. (**D**) HeLa cells grown on cover slips were untreated (37°C) or heat-shocked for 2 hours, fixed-permeabilized, and reacted with antibodies to AUF1 and hsp70 as described (*12*).

complex) and immunoprecipitation with preimmune serum demonstrated the specificity of complex formation. Moreover, AUF1 could also be cross-linked in vitro to hsc70-hsp70, eIF4G, and PABP (*13*). Cytoplasmic AUF1 protein was predominantly in the polysome fraction, which was eliminated by RNase A treatment (Fig. 2C). Thus, a protein complex containing AUF1, eIF4G, PABP, and hsc70-hsp70 forms on polysomal mRNA. The identities of the remaining AUF1-interacting proteins are not yet known.

Studies tested whether hsp70 sequestration of AUF1 is primarily responsible for the block in ARE-mRNA decay. HeLa cells were transfected with reporter constructs expressing cap-independent GC-control or ARE-mRNAs, then treated with the proteasome inhibitor MG132, which strongly stimulates synthesis of hsp70 protein at normal temperature (*17, 18*). MG132 strongly induced hsp70 protein after 3 hours and slightly impaired cell protein synthesis by a factor of 2 to 4 (*13*), as previously reported (*17*). Steady-state reporter ARE-mRNA amounts were identical to those of stable control mRNAs after 4 hours of MG132 treatment (Fig. 3). Because 4 hours of MG132 treatment does not affect transcription rates of the cellular pool of mRNAs by [^{3}H]uridine (*17*), inhibition of proteasome activity stabilized the ARE-mRNA. As observed during heat shock, MG132 mediated nuclear-perinuclear localization of hsp70 and AUF1 (*13*). Two independent lines of evidence therefore correlate hsp70-AUF1 binding with nuclear sequestration and inhibition of ARE-mRNA decay.

Proteasome inhibition with MG132 or heat shock of cells increased p37 AUF1 by a factor of 10 to 20 and led to slightly elevated amounts of p40/42 AUF1 (Fig. 4A). Thus, p37 AUF1 is rapidly degraded in proteasomes at 37°C. Treatment of cells with lysomotrophic agents such as 10 mM NH_4Cl had no effect on AUF1 amounts, excluding lysosomal involvement in AUF1 protein turnover (*13*).

The heat shock response is tightly linked to the ubiquitin-proteasome degradative network, in that the binding of hsc70 to proteins promotes ubiquitin-dependent decay, and heat shock appears to decrease proteasome activity (*17, 18*). We therefore investigated whether AUF1 promotes ARE-mRNA decay in a ubiquitin-proteasome–dependent manner. AUF1 was immunoprecipitated from HeLa cells, resolved by SDS–polyacrylamide gel electrophoresis (PAGE), and immunoblotted with antisera to polyubiquitin (Fig. 4B). In vivo polyubiquitination of AUF1 proteins was evident. In vitro studies showed the p37 and p40 isoforms of AUF1 to be predominant targets for ubiquitination (*13*). Thus, ubiquitination of AUF1 establishes a link between AUF1 protein turnover and that of the ARE-mRNA to which AUF1 binds.

The role of the ubiquitin-proteasome network in ARE-mRNA decay was confirmed in the mouse *ts*85 cell line, which bears a temperature-sensitive mutation in the ubiquitin-activating enzyme E1 (*19*). Cells were transfected with plasmids encoding GC-control or ARE-mRNAs and maintained at permissive (30°C) or restrictive temperature for E1 activity (39.5°C). Northern (RNA) analysis showed that at the restrictive temperature, amounts of β-gal reporter ARE-mRNA increased by a factor of 10 to 20, whereas amounts of stable GC-control mRNA only doubled (Fig. 4C). Inhibition of E1 enzyme function was shown by increased abundance of p53 protein (Fig. 4D), which is degraded in proteasomes. Inactivation of E1 led to much larger amounts of p37 AUF1 but only a slight increase in p40/42 AUF1. In wild-type parental cells at 39.5°C, hsp70 is not induced (*13, 17, 19, 20*) and ARE-mRNAs are short-lived (*13*). Because proteasome inhibition by MG132 had identical effects, ARE-mRNA turnover is related to ubiquitination and proteolysis of AUF1 protein rather than reduction in mRNA transport or secondary effects of MG132.

Our results demonstrate that the rapid turnover of an mRNA containing the GM-CSF ARE is associated with the ubiquitination and degradation of members of the AUF1 family of ARE-binding proteins. Codegradation of proteins associated with ubiquitin-dependent target proteins is well described, as observed for degradation of c-Fos with targeted turnover of c-Jun (*21*). Whether ubiquitinated AUF1 mediates rapid codegradation of associated PABP, resulting in ARE-mRNA turnover, is not known. Disruption of eIF4G binding to AUF1 by translation also suggests a complex interplay between proteasomes, PABP, heat shock proteins, and translational control in ARE-mRNA decay.

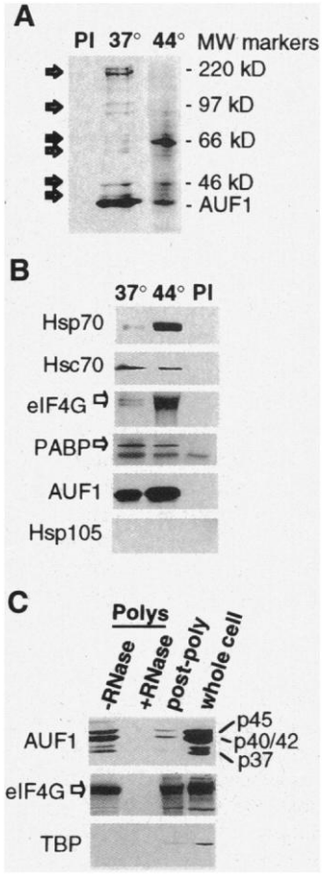

Fig. 2. AUF1 binding partners. **(A)** HeLa cells were untreated or heat-shocked at 44°C for 4 hours while labeling with [^{35}S]methionine. AUF1 was immunoprecipitated from equal protein amounts and resolved by gel electrophoresis, and labeled proteins synthesized during normal or heat-shock conditions that bind AUF1 were fluorographed. PI, preimmune serum control. **(B)** HeLa cells were untreated or heat-shocked at 44°C for 2.5 hours. AUF1 was immunoprecipitated from equal amounts of unlabeled HeLa cell lysates with RNase A treatment, resolved by gel electrophoresis, immunoblotted with antibodies specific for hsp70, hsc70, eIF4G, PABP, hsp105, or AUF1, and then developed using enhanced chemiluminescence (*15*). The lower band in the PABP samples is nonspecific. **(C)** Polysomal and postpolysomal fractions of HeLa cell cytoplasm were prepared (with or without RNase) (*15*), then 25% of each fraction was resolved by gel electrophoresis and immunoblotted with antisera to AUF1, eIF4G, and TATA-binding protein (TBP) as a control (TBP antibody, Santa Cruz).

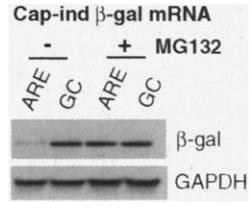

Fig. 3. Effect of proteasome inhibition on AUF1 and ARE-mRNAs. HeLa cells maintained at 37°C were transiently transfected with cap-independent β-gal reporter constructs containing a GC or ARE 3′UTR. Cells were treated with MG132 at 20 μM for 4 hours. Northern analysis of total RNA was carried out as described in Fig. 1 from untreated or MG132-treated cells.

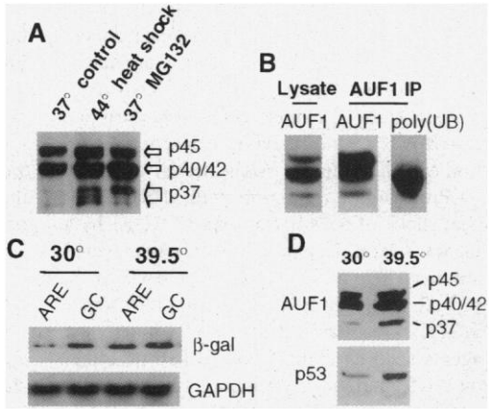

Fig. 4. (**A**) Stabilization of AUF1 by proteasome inhibition. HeLa cells at 37°C were untreated or treated for 4 hours with 20 μM MG132 or heat-shocked for 3 hours at 44°C. Equal amounts of lysates were resolved by gel electrophoresis and immunoblotted for AUF1. (**B**) AUF1 was immunoprecipitated from HeLa cells and immunoblotted with antibodies to ubiquitin (Zymed). (**C**) Mouse *ts*85 cells were transfected with plasmids encoding reporter β-gal GC-control or ARE-mRNAs for 36 hours, then maintained at nonrestrictive (30°C) or restrictive temperature (39.5°C) for 8 hours to inactivate the ubiquitin-activating enzyme E1. Total RNA was isolated and equal amounts used for Northern mRNA analysis. (**D**) Immunoblot analysis was performed using equal amounts of whole-cell lysates and antisera to AUF1 or p53 protein (p53 antibody; Santa Cruz sc99).

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Regulation of Mammalian Circadian Behavior by Non-rod, Non-cone, Ocular Photoreceptors

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Circadian rhythms of mammals are entrained by light to follow the daily solar cycle (photoentrainment). To determine whether retinal rods and cones are required for this response, the effects of light on the regulation of circadian wheel-running behavior were examined in mice lacking these photoreceptors. Mice without cones (*cl*) or without both rods and cones (*rdta/cl*) showed unattenuated phase-shifting responses to light. Removal of the eyes abolishes this behavior. Thus, neither rods nor cones are required for photoentrainment, and the murine eye contains additional photoreceptors that regulate the circadian clock.

Mammals use light both to generate a visual image of their environment and to provide time-of-day information. Internal circadian time is synchronized (entrained) with the solar day by light-induced resetting (photoentrainment) mechanisms, which correct for deviations in the period and phase of the endogenous clock (*1*). Eye loss in both human and nonhuman mammals abolishes photoentrainment, demonstrating that the eyes provide the primary source of light information to the clock (*2*). However, the retinal projections that convey light information to the visual and circadian centers