

## Transcriptional Regulation of *PS-IAA4/5* and *PS-IAA6* Early Gene Expression by Indoleacetic Acid and Protein Synthesis Inhibitors in Pea (*Pisum sativum*)

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The transcription of two genes, *PS-IAA4/5* and *PS-IAA6*, in pea is induced by indoleacetic acid (IAA) and protein synthesis inhibitors such as cycloheximide (CHX) and anisomycin (ANI). Induction by IAA is rapid, taking 5 and 7.5 minutes for *PS-IAA4/5* and *PS-IAA6*, respectively, and is independent of IAA concentration and whether IAA has a free or esterified carboxyl group (ethyl-IAA). The rate of mRNA accumulation, however, is dependent on hormone concentration, and is greater with IAA than with ethyl-IAA. The turnover rates ( $t_{1/2}$ ) of the *PS-IAA4/5* and *PS-IAA6* mRNAs are 60 and 75 minutes, respectively, and are not affected by IAA.

CHX or ANI induce the transcription of *PS-IAA4/5* and *PS-IAA6* more slowly than IAA (5 to 10 minutes for *PS-IAA4/5* and 20 minutes for *PS-IAA6*). While protein synthesis inhibitors stabilize both mRNAs, the rapidity of induction by CHX and ANI cannot be accounted for solely by mRNA stabilization. The relationship between mRNA induction and protein synthesis inhibition does not obey Michaelis-Menten kinetics, but rather is best described by a hyperbolic curve, suggesting the release of transcriptional repression by the inhibition of protein synthesis. RNA expression experiments with transgenic tobacco seedlings or with transfected pea protoplasts using *PS-IAA4/5* promoter *GUS* or *CAT* fusions reveal that CHX transcriptionally activates *PS-IAA4/5* gene expression. Thus, protein synthesis inhibitors have a dual effect on *PS-IAA4/5* and *PS-IAA6*. (1) They stabilize both mRNAs (possibly by a translational arrest-linked process or by preventing the synthesis of a labile nuclease(s)). (2) They activate transcription (possibly by preventing the synthesis or function of a repressor).

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Abbreviations used: IAA, indole-3-acetic acid; ethyl-IAA, ethyl indole-3-acetic acid; CHX, cycloheximide; ANI, anisomycin; EME, emetine; UTR, untranslated region; *AuxRR*, *AuxRD*, *AuxRE*, auxin responsive region, domain, and element, respectively; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside.

### Introduction

The plant growth hormone auxin, typified by indole-3-acetic acid (IAA), regulates various aspects of plant growth and development such as cell division, vascular differentiation, apical dominance, morphogenesis, oncogenesis and tropisms (Went & Thimann, 1937) and is generally considered to be responsible for regulating plant cell growth (Taiz, 1994). Growth in response to auxin, mediated by cell elongation, is one of the fastest hormonal responses, occurring with a lag period of 15 to 25 minutes (Evans & Ray, 1969). At the molecular level,

auxin mediates its regulatory role by modulating membrane function (Goldsmith, 1993) and gene expression (Guilfoyle, 1986; Theologis, 1986). However, the primary mechanism of auxin action is unknown.

Auxin specifically induces gene expression earlier than the initiation of cell growth, suggesting that the activation of gene expression may be responsible for initiating the growth process. During the past ten years, several primary auxin responsive genes have been identified (Venis & Napier, 1995). Their auxin responsive promoter element(s) (*AuxRE*(s)) have the potential to be used as molecular probes to identify the components of the signal transduction pathway responsible for their activation by IAA using biochemical, molecular, and reverse genetics approaches (Ballas *et al.*, 1993). We are focusing our efforts to elucidate the mechanism of gene activation by auxin using two early IAA-regulated genes from pea, *PS-IAA4/5* and *PS-IAA6*. Both genes have been structurally characterized (Oeller *et al.*, 1993) and the auxin responsive domains (*AuxRDs*) of one of them, *PS-IAA4/5*, have been identified (Ballas *et al.*, 1993, 1995). Their encoded proteins, which are highly similar to the proteins encoded by other auxin-inducible genes from various plant species (Oeller *et al.*, 1993), are short-lived, posttranscriptionally regulated, nuclear localized, and contain a putative  $\beta\alpha\alpha$  motif reminiscent of the  $\beta$ -sheet DNA-binding domain of prokaryotic repressor polypeptides (Abel *et al.*, 1994; Oeller & Theologis, 1995). The data thus far obtained suggest a regulatory function of the *PS-IAA4/5* and *PS-IAA6* proteins.

The induction of both genes by IAA qualifies as a primary response to IAA: it is rapid, specific, and insensitive to protein synthesis inhibition (Ringold, 1979; Theologis *et al.*, 1985), suggesting that the IAA signal is transmitted to the nucleus *via* preexisting components. In fact, protein synthesis inhibitors such as cycloheximide (CHX), anisomycin (ANI), and emetine (EME) are the only other known inducers of these genes (Theologis *et al.*, 1985), a common characteristic of many mammalian oncogenes (Herschman, 1991). The rapid and specific induction of *PS-IAA4/5* and *PS-IAA6* mRNAs by IAA has been attributed to (1) activation of transcription, (2) posttranscriptional processing, or (3) selective stabilization (Theologis *et al.*, 1985). Their induction by CHX and ANI has been attributed to the loss of a short-lived repressor which specifically interacts with the auxin responsive region (*AuxRR*) of the genes and limits their transcription. Alternatively, CHX or ANI may alter the functionality of a repressor by preventing the synthesis of a labile repressor-modifying enzyme (Theologis *et al.*, 1985). According to these views, IAA induces both genes by altering the functionality of the putative repressor, whereas CHX or ANI alters its level or function (Ballas *et al.*, 1993, 1995; Theologis, 1986). Despite the extensive molecular characterization of *PS-IAA4/5* and *PS-IAA6* gene expression, no direct experimental evidence exists to indicate whether the IAA-, CHX-, or ANI-mediated

mRNA induction is due to transcriptional or post-transcriptional effects or both. Here, we present a detailed characterization of *PS-IAA4/5* and *PS-IAA6* gene expression by IAA and protein synthesis inhibitors.

## Results

### Determination of the obligatory latent (lag) period required for the induction of *PS-IAA4/5* and *PS-IAA6* mRNAs by IAA

Since the induction of *PS-IAA4/5* and *PS-IAA6* mRNAs by IAA appears to be the result of the primary mechanism of IAA action (Theologis, 1986; Theologis *et al.*, 1985), it is crucial to know precisely the minimum time required for their induction. This period is defined as the latent period (period of no increase in rate). We have previously determined that in non-abraded pea tissue segments, the latent periods for the induction of the *PS-IAA4/5* and *PS-IAA6* mRNAs are 10 and 15 minutes, respectively (Theologis *et al.*, 1985). Since the tissue cuticle is a potential barrier for hormone penetration into the tissue (Anker, 1971; Ray, 1985), we compared the latent period of mRNA induction in the absence (abraded) and presence (non-abraded) of the cuticle. The kinetics of induction during the first 30 minutes after IAA application were determined by RNA hybridization analysis and the results are shown in Figure 1. The latent period of *PS-IAA4/5* mRNA induction is 10 minutes in non-abraded and 5 minutes in abraded tissue (Figure 1, compare panel Ia with Ic). Similar results were obtained with the *PS-IAA6* mRNA. The latent period of its induction is 15 minutes in non-abraded and 7.5 minutes in abraded tissue (Figure 1, compare IIa with IIc).

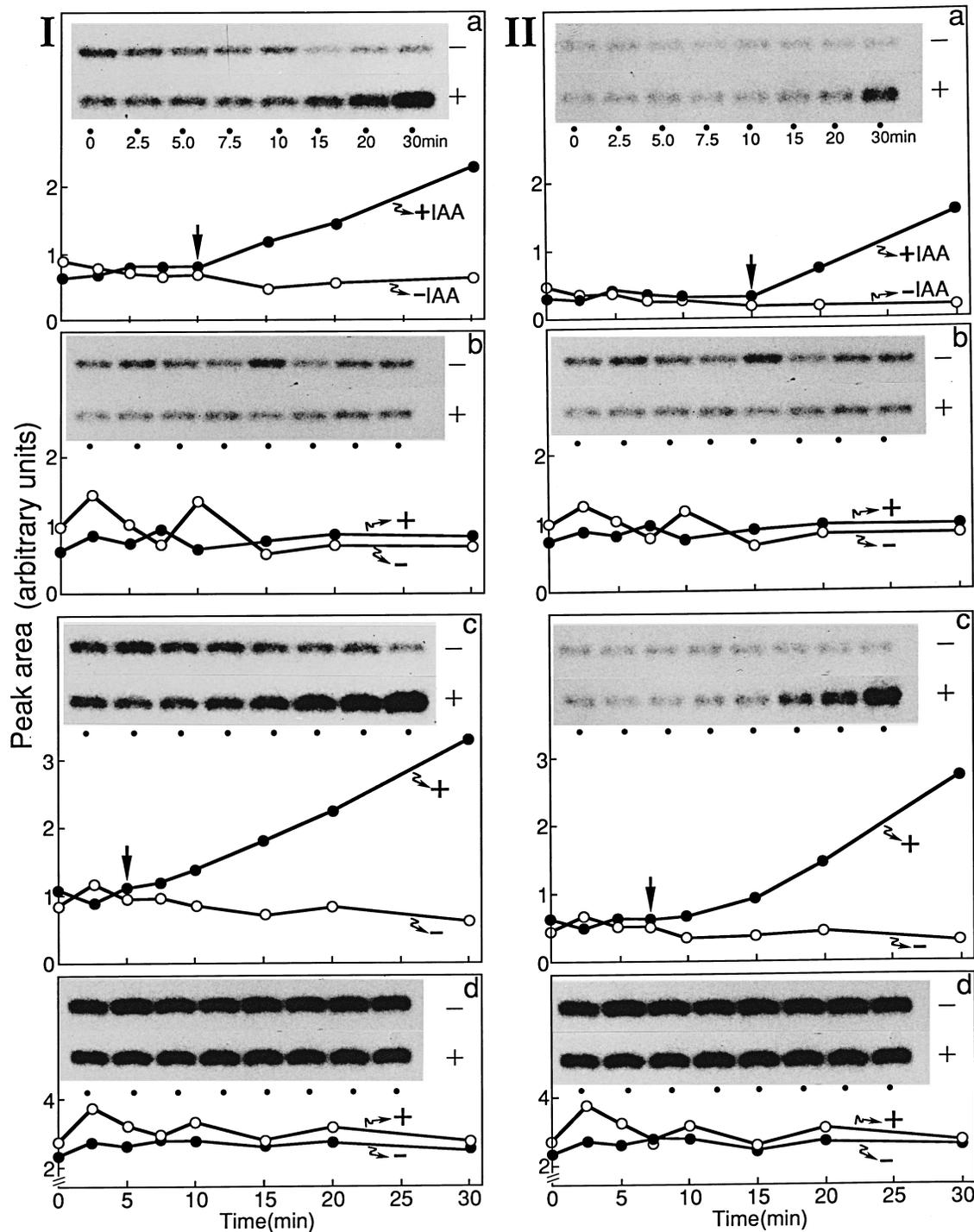
### Induction kinetics with IAA and ethyl-IAA

To determine whether the latent periods of *PS-IAA4/5* and *PS-IAA6* mRNAs induction are affected by the IAA concentration, detailed time course experiments were carried out using three different hormone concentrations (2, 20, and 500  $\mu$ M). The results are shown in Figure 2I. Clearly, their latent periods of induction are independent of IAA concentration (Figure 2, Ia (*PS-IAA4/5*) and Ib (*PS-IAA6*)). However, the rate of mRNA accumulation is concentration dependent (Figure 2, Ia and Ib; compare the slopes of the lines with various IAA concentrations).

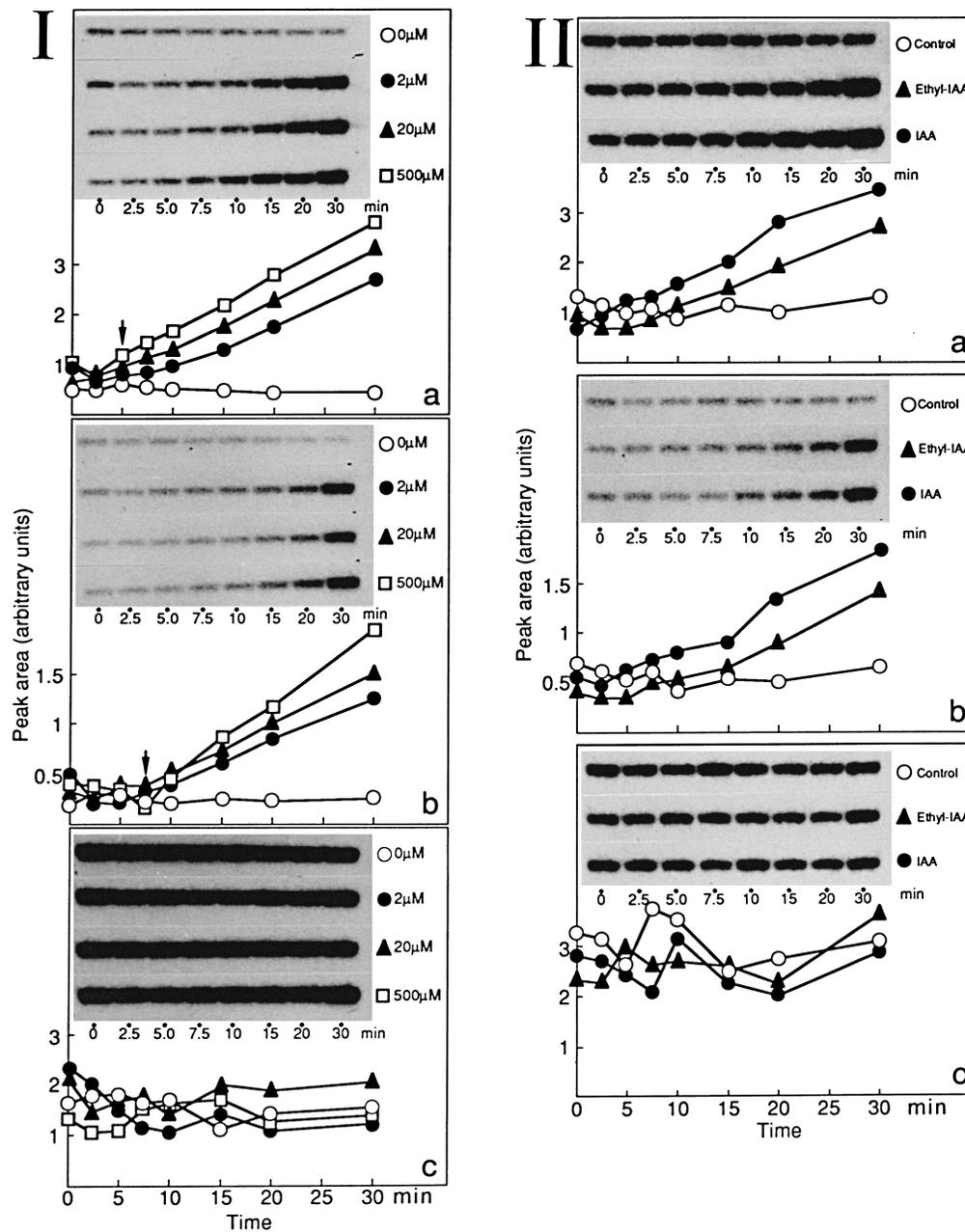
The effect of a non-ionizable (neutral) auxin, such as ethyl indole-3-acetic acid (ethyl-IAA) on the latent periods of induction of *PS-IAA4/5* and *PS-IAA6* mRNAs was determined and is shown in Figure 2II. Since ethyl-IAA is an uncharged molecule, it has been postulated, based on the chemiosmotic polar mechanism of auxin transport, that its penetration through the pea tissue is faster than that of IAA (Goldsmith *et al.*, 1981). Contrary to the predicted expectation, the latent periods of both mRNAs are the same with IAA or ethyl-IAA (Figure 2, IIa and

IIb). However, the rate of mRNA accumulation with ethyl-IAA is slower than that with IAA (Figure 2, IIa and IIb; compare the slopes of the lines with

ethyl-IAA and IAA). Dose response curves show that ethyl-IAA is a less effective IAA analog than IAA (data not shown).



**Figure 1.** Induction kinetics of *PS-IAA4/5* (panel I) and *PS-IAA6* (panel II) mRNAs in: a, non-abraded and c, abraded pea tissue segments treated without (-) or with (+) 20  $\mu$ M IAA. Total nucleic acids were isolated from tissue segments at various time intervals and electrophoretically separated and transferred to diazophenylthioether paper, as described in Materials and Methods. Each lane contains 25  $\mu$ g of total nucleic acids. The filters were successively hybridized with  $^{32}$ P-labeled *pIAA4/5*, *pIAA6* plasmid DNAs. The accuracy of loading was accessed by hybridization with the non-IAA responsive cDNA *pW11* and the results are shown in b and d in both panels. The vertical arrows in a and c in both panels indicate the latent (lag) period of mRNA induction in non-abraded and abraded tissue. Ib and IIb are the same; Id and IIc are also identical. They were duplicated in order to make the figure symmetrical. Probes and exposures: *pIAA4/5*,  $1.69 \times 10^6$  cpm/ml for seven hours; *pIAA6*,  $1.8 \times 10^6$  cpm/ml for 10 hours; *pW11*,  $2 \times 10^6$  cpm/ml for six hours.



**Figure 2.** I, Effect of IAA concentration on the latent period of induction of: a, *PS-IAA4/5*, b, *PS-IAA6*, and c, *pW11* mRNAs. Abraded pea tissue segments were treated without IAA, or with three different concentrations of IAA (2, 20 and 500  $\mu$ M). Probes and exposure: *pIAA4/5*,  $1.4 \times 10^6$  cpm/ml for 17 hours; *pIAA6*,  $1.8 \times 10^6$  cpm/ml for 19 hours; *pW11*,  $2 \times 10^6$  cpm/ml for four hours. II, Effect of ethyl-IAA on the latent period of induction of: a, *PS-IAA4/5*, b, *PS-IAA6*, and c, *pW11* mRNAs. Abraded pea tissue segments treated without or with 20  $\mu$ M IAA or ethyl-IAA. All lanes contains 25  $\mu$ g total nucleic acids. Experimental details are the same as described in the legend of Figure 1. Probes and exposures: *pIAA4/5*,  $2 \times 10^6$  cpm/ml for five hours; *pIAA6*,  $1.8 \times 10^6$  cpm/ml for 10 hours; *pW11*,  $2 \times 10^6$  cpm/ml for 10 hours.

#### Studies on the turnover rate of *PS-IAA4/5* and *PS-IAA6* mRNAs *in vivo* and in isolated pea nuclei

In order to test the possibility that IAA acts posttranscriptionally, for example, by interacting with an mRNA processing component required for maturation of the primary transcript or by

stabilizing the mRNA by inhibiting the action of a nuclease, we carried out experiments for determining the effect of IAA on the turnover rate ( $t_{1/2}$ ) of *PS-IAA4/5* and *PS-IAA6* mRNAs *in vivo*. In addition, we determined whether the genes are differentially transcribed in nuclei isolated from pea epicotyl tissue treated with or without IAA.

The effect of IAA on the  $t_{1/2}$  of the two mRNAs was determined using the following experimental design. The *PS-IAA4/5* and *PS-IAA6* mRNAs were induced by incubating pea tissue segments with 20  $\mu$ M IAA for one hour. Subsequently, 5  $\mu$ M  $\alpha$ -amanitin, an inhibitor of RNA polymerase II activity (Lindell *et al.*, 1970), was added to prevent the synthesis of new mRNA, and the segments were divided in two. One sample continued to receive IAA; the other was incubated without hormone after removing the IAA by extensive washing during the first 20 minutes. The decay of the mRNAs was followed over a period of three hours by RNA hybridization analysis, and the results are shown in Figure 3. Clearly, IAA does not affect the  $t_{1/2}$  of *PS-IAA4/5* (Figure 3A) or *PS-IAA6* (data not shown) mRNAs. Quantitation of the hybridization data yields a  $t_{1/2}$  of 60 minutes for the *PS-IAA4/5* mRNA (Figure 3, insert in panel A) and a  $t_{1/2}$  of 75 minutes for the *PS-IAA6* mRNA (data not shown).

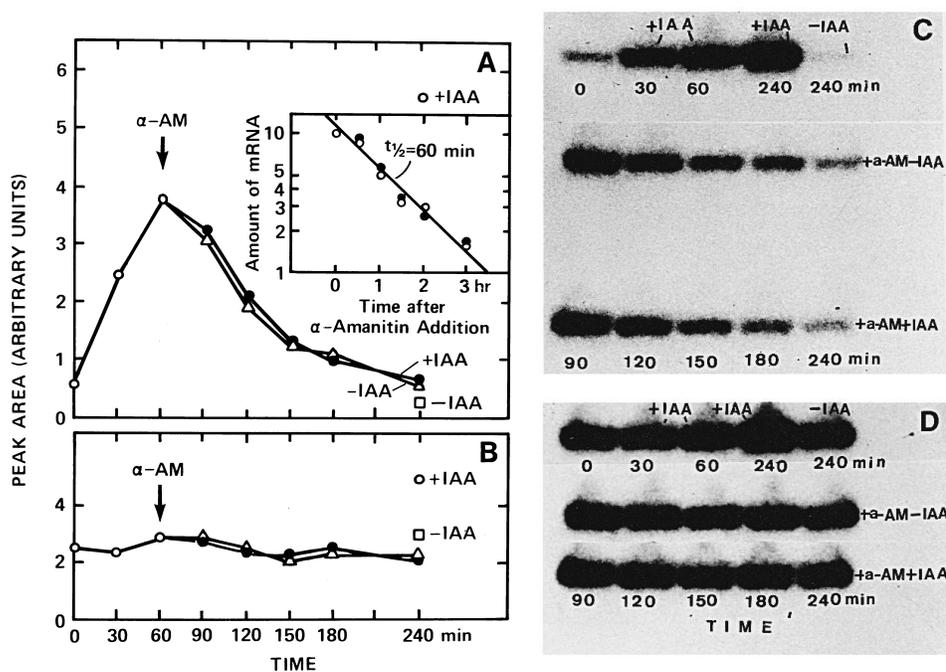
To supplement the *in vivo* experiments presented in Figure 3, we carried out *in vitro* transcription with pea nuclei isolated from 3rd internode pea epicotyl segments treated with or without 20  $\mu$ M IAA for 40 minutes. The results show that IAA enhances the amount of run-on *PS-IAA4/5* and *PS-IAA6* transcripts after hormone treatment (data not shown). The mRNA enhancement is completely abolished by 5  $\mu$ M  $\alpha$ -amanitin (data not shown).

### Studies on the regulation of expression of *PS-IAA4/5* and *PS-IAA6* mRNA by protein synthesis inhibitors

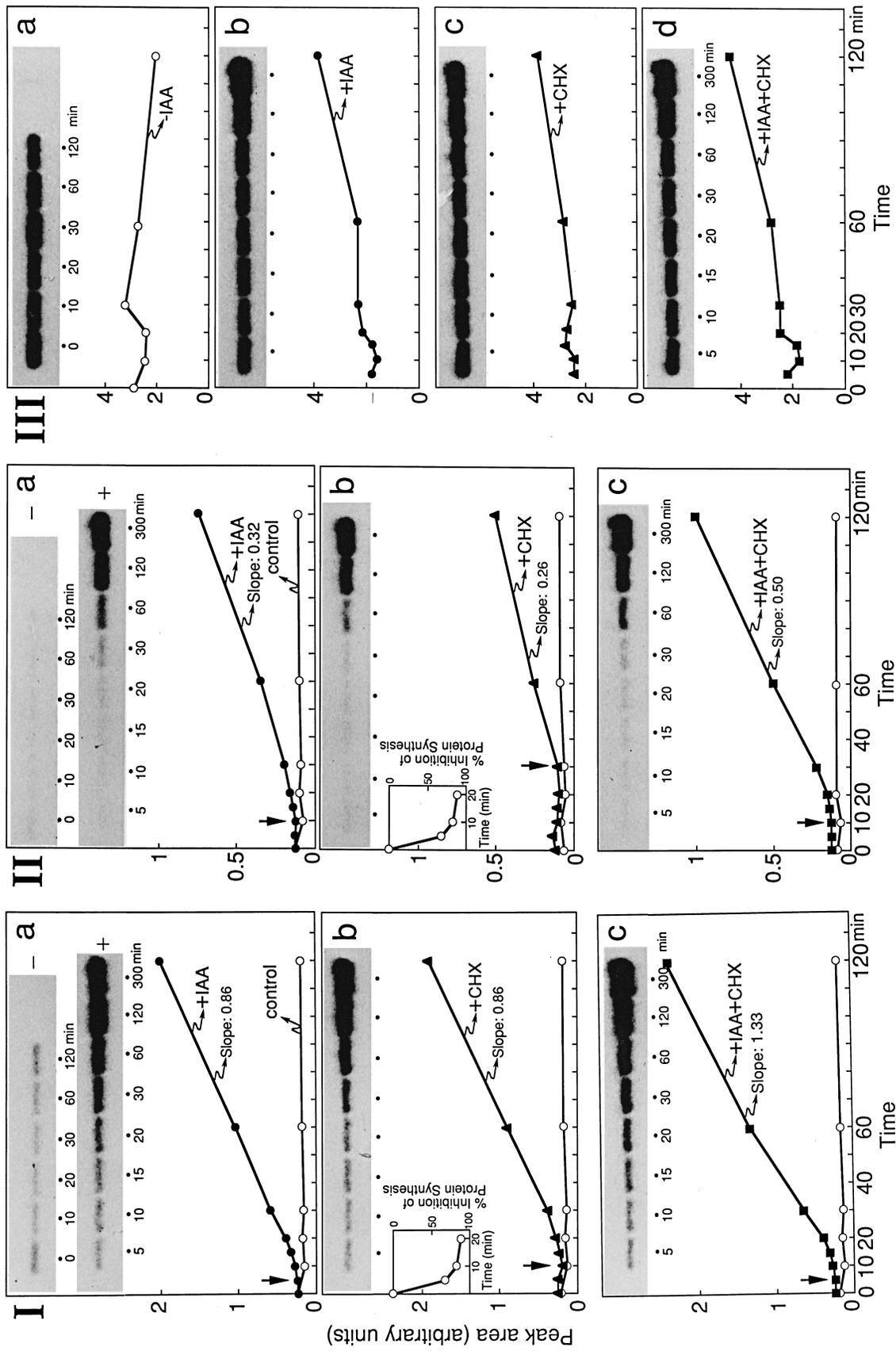
Protein synthesis inhibitors, such as CHX, ANI, and EME, induce the *PS-IAA4/5* and *PS-IAA6* mRNAs in the absence of IAA (Theologis *et al.*, 1985). We imagine that the expression of these genes is under the control of a labile negative regulator whose level or function is altered by protein synthesis inhibitors. In order to better understand the CHX or ANI inducibility, we further characterized this response by carrying out the following experiments.

#### *Induction kinetics of the PS-IAA4/5 and PS-IAA6 mRNAs by CHX and ANI*

If a short-lived repressor is inactivated by protein synthesis inhibition, then the latent period of induction will depend on the rapidity of inhibition of protein synthesis and on the turnover rate of the putative repressor. We determined the kinetics of induction of both mRNAs in the presence of IAA alone, CHX or ANI alone, and IAA + CHX or IAA + ANI. Figure 4I, shows that the latent period of *PS-IAA4/5* mRNA induction by CHX is five minutes longer than in the presence of IAA alone (Figure 4, compare panel Ib with Ia). The longer lag



**Figure 3.** A and C, Effect of IAA on the stability ( $t_{1/2}$ ) of the *PS-IAA4/5* mRNA. Total nucleic acids were isolated and RNA hybridization was successively carried out using  $^{32}$ P-labeled *pIAA4/5* and *pW11* cDNAs. B and D, The effect of the non-differential clone *pW11*. The inset in A shows the  $t_{1/2}$  of *PS-IAA4/5*. It was calculated by plotting the percentage of the mRNA remaining at various times after  $\alpha$ -amanitin addition (0 minutes) on a semilog graph ( $\circ$ , +IAA -  $\alpha$ -amanitin;  $\square$ , -IAA -  $\alpha$ -amanitin;  $\bullet$ , +IAA +  $\alpha$ -amanitin;  $\triangle$ , -IAA +  $\alpha$ -amanitin). The induction of the mRNAs in the absence of  $\alpha$ -amanitin was also monitored during the course of the experiment (C, top, *PS-IAA4/5*; D, top, *pW11*). Each lane contains 25  $\mu$ g total nucleic acids.



**Figure 4.** Induction kinetics of I, PS-IAA6 mRNAs in the presence of a, 100  $\mu$ M IAA, b, 100  $\mu$ M CHX and c, IAA + CHX in abraded pea tissue segments. III, Induction kinetics of the non-differential clone pW11. The RNA filter papers were successively hybridized with  $^{32}$ P-labelled pIAA4/5, pIAA6 and pW11 cDNAs, as described in Materials and Methods. Each lane contains 25  $\mu$ g total nucleic acids. The rapidity of protein synthesis inhibition by CHX is shown as an inset in Ib and IIb. Probes and duration of exposure: pIAA4/5, 1.45  $\times$  10<sup>6</sup> cpm/ml for three hours; pIAA6, 2  $\times$  10<sup>6</sup> cpm/ml for 3.5 hours; pW11, 2  $\times$  10<sup>6</sup> cpm/ml for 4.5 hours.

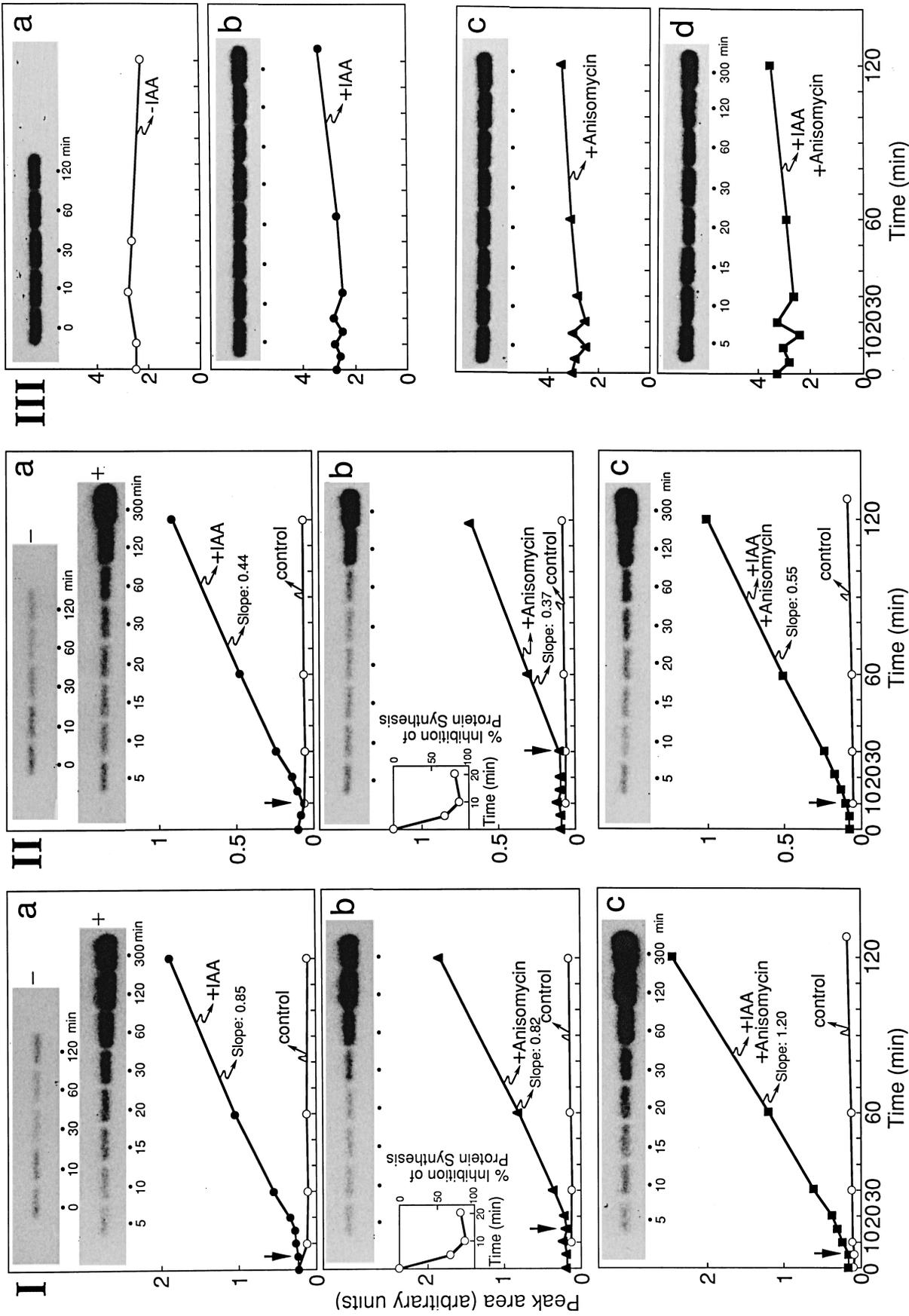
period of induction by CHX may be due to the inability of CHX to inhibit protein synthesis fast enough to exert its effects, because five minutes after CHX addition protein synthesis is inhibited only 75%; after 10 minutes the inhibition is 95% (Figure 4, see insert in Ib). The induction of PS-IAA4/5 is almost simultaneous with complete protein synthesis inhibition. The addition of CHX in the presence of IAA (Figure 4, Ic) enhances the rate of the PS-IAA4/5 mRNA accumulation by 65% (super-induction; Figure 4, compare the slope of the line in Ic with that in Ia). Furthermore, the latent period in the presence of IAA + CHX is identical to that observed in the presence of IAA alone (Figure 4, compare panel Ic with Ia). The results obtained with the PS-IAA6 mRNA are shown in Figure 4II. The latent period of PS-IAA6 mRNA induction is 30 minutes in the presence of CHX, 20 minutes longer than that observed with IAA alone (Figure 4, compare I Ib with I Ia). However, this longer lag period cannot be attributed to slow inhibition of protein synthesis by CHX, because protein synthesis inhibition is complete after 10 minutes. The longer lag of PS-IAA6 mRNA induction compared to that of PS-IAA4/5 mRNA may reflect differences in the turnover rate of the components responsible for the induction of these genes by CHX. The addition of CHX in the presence of IAA (Figure 4, IIc) enhances the rate of accumulation of PS-IAA6 mRNA observed with IAA alone (Figure 4, compare the slope of the line in IIc with that in IIa). The same experiment as the one shown in Figure 4 was carried out with ANI, and the results are shown in Figure 5. The results are similar to those observed with CHX (compare Figure 4 with 5). ANI enhances the IAA-mediated accumulation of PS-IAA4/5 (Figure 5, compare the slope of the line in Ic with that in Ia) and PS-IAA6 (Figure 5, compare the slope of the line in IIc with that in IIa) mRNAs. The latent periods of induction of both mRNAs are longer by 10 minutes for PS-IAA4/5 and 20 minutes for PS-IAA6 in the presence of ANI than those observed with IAA (Figure 5, compare Ia with Ib and IIa with I Ib). The longer lag periods, however, cannot be attributed to slow inhibition of protein synthesis by ANI, because protein synthesis inhibition is complete (>95%) 10 minutes after the addition of inhibitor.

#### *Effect of CHX and ANI on the turnover rate ( $t_{1/2}$ ) of the PS-IAA4/5 and PS-IAA6 mRNAs*

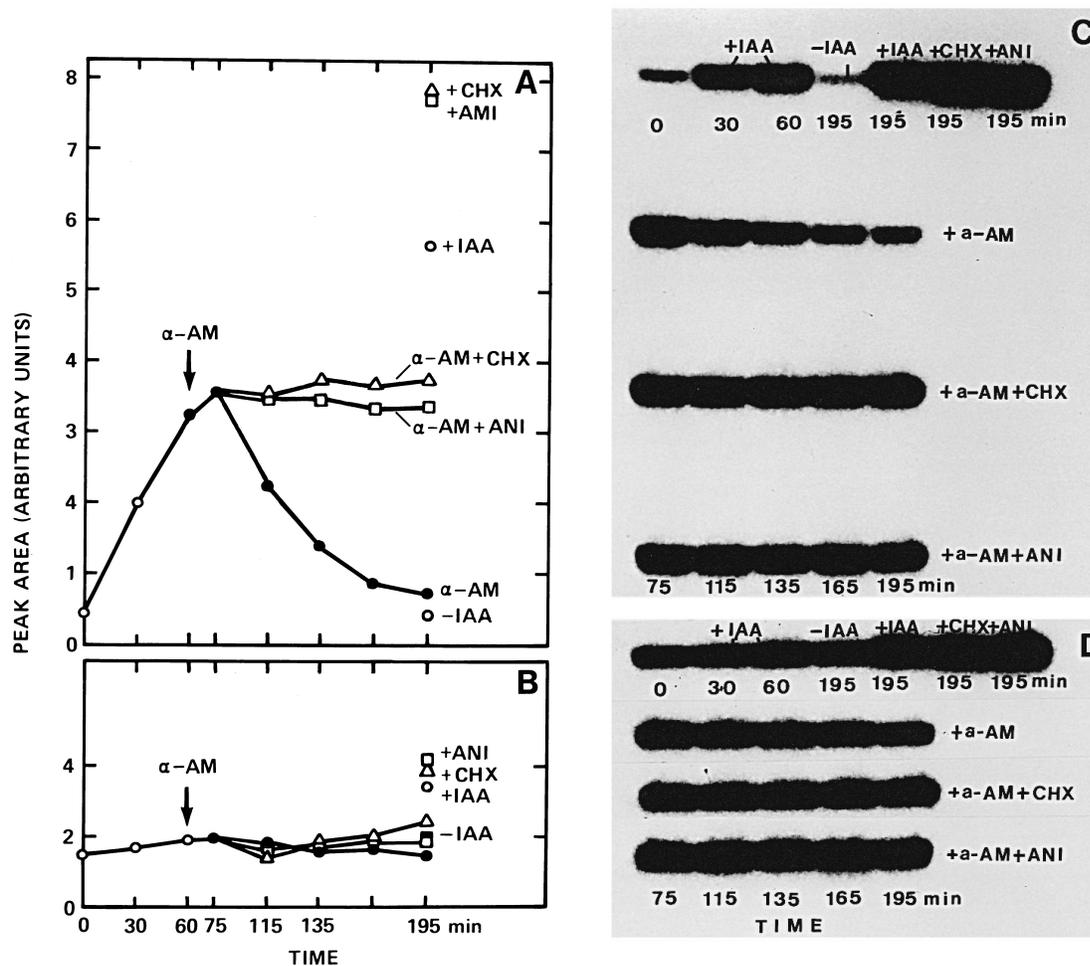
The rapid induction of both mRNAs by CHX or ANI alone may be attributed to transcriptional activation, mRNA stabilization, or both. In order to determine whether CHX and ANI alter the stability of the PS-IAA4/5 and PS-IAA6 mRNAs, experiments similar to those shown in Figure 3 were carried out and are shown in Figure 6. Tissue segments were incubated with 20  $\mu$ M IAA for one hour, followed by the addition of 5  $\mu$ M  $\alpha$ -amanitin to inhibit RNA synthesis. After 15 minutes of preincubation with  $\alpha$ -amanitin, one third of the tissue received CHX,

one third ANI, and the other third was incubated with  $\alpha$ -amanitin alone. The decay of the mRNAs was followed by RNA hybridization analysis. The results show that both inhibitors prevent the degradation of PS-IAA4/5 (Figure 6A) and PS-IAA6 (data not shown) mRNAs. The data in Figure 6 show that CHX and ANI increase the  $t_{1/2}$  values of both mRNAs at least 10-fold ( $t_{1/2} = 10$  to 12 hours). The upper limits of  $t_{1/2}$  were not clearly established by these experiments, because prolonged incubation of the tissue with CHX or ANI is deleterious.

The results shown in Figure 6 raise the question of whether the induction of the PS-IAA4/5 and PS-IAA6 mRNAs by protein synthesis inhibitors is solely mediated by mRNA stabilization (Figures 4 and 5). In order for such a proposition to be valid, the genes should be constitutively transcribed, but their transcripts should not accumulate because of their high turnover rate. The nuclear run-on experiments, however, do not support such a view. Furthermore, the observed initial rates of mRNA accumulation in the presence of protein synthesis inhibitors alone cannot be achieved solely by mRNA stabilization. This second conclusion is based on calculations using the turnover equation (Hargrove *et al.*, 1991),  $dR/dt = \kappa_1 - \kappa_2 R$ , which describes the kinetics of induction, from the end of the lag period until the plateau is finally reached, in terms of the two rate constants  $\kappa_1$  and  $\kappa_2$ .  $R$  is the relative rate of PS-IAA4/5 or PS-IAA6 mRNA synthesis at time  $t$ ,  $\kappa_1$  is a zero order rate constant for mRNA synthesis and  $\kappa_2$  is a first order rate constant for mRNA degradation. The value of  $\kappa_1$  can be estimated from the initial slope of the induction curves (Figures 4 and 5) and  $\kappa_2$  can be estimated from the relationship  $\kappa_2 = \ln 2 / t_{1/2} = 0.693 / t_{1/2}$ , where  $t_{1/2}$  is the half-life of the mRNA. As originally observed by Schimke *et al.* (1964), the first order turnover constant,  $\kappa_2$ , is the sole determinant of the amount of time required to progress from the basal level to the new steady state or plateau level. Furthermore, values for  $\kappa_1$  can be independently estimated using the relationship  $\kappa_1 = \kappa_2 R_i$ , where  $R_i$  is the fully induced plateau level of the corresponding mRNA. Since, at the plateau,  $dR/dt$  approaches zero, the value of  $R$  at the plateau ( $R_i$ ) becomes equal to  $\kappa_1 / \kappa_2$ . Consequently, the turnover equation becomes  $\ln R_i - R_t / R_i - R_0 = -\kappa_2 t = -0.693 / t_{1/2}$ , where  $R_0$  is the uninduced, basal level and  $R_t$  is the level at time  $t$  after the administration of CHX or ANI. Linearized induction curves with a slope of  $-\kappa_2$  can be regenerated by plotting the data of Figures 4 (Ib or IIb) and 5 (Ib and IIb) as  $\ln R_i - R_t / R_i - R_0$  versus time ( $t$ ) using this equation. Such curves yield  $t_{1/2}$  values for the mRNAs in the presence of CHX or ANI that are much smaller (PS-IAA4/5,  $t_{1/2} = 0.9$ ; PS-IAA6,  $t_{1/2} = 1.3$ ) than the  $t_{1/2}$  determined experimentally in Figure 6, indicating that the observed kinetics of induction can not be due solely to CHX- or ANI-induced mRNA stabilization. Consequently, the prospect arises that protein synthesis inhibitors have a dual effect: (1) they activate the transcription of PS-IAA4/5 and PS-IAA6 genes, and (2) they also stabilize their transcripts.



**Figure 5.** Induction kinetics of I, PS-IAA4/5 and II, PS-IAA6 mRNAs in the presence of a, 100  $\mu$ M IAA, b, 500  $\mu$ M ANI and c, IAA + ANI in abraded pea tissue segments. III, Induction kinetics of the non-differential clone pW11. All lanes contain 25  $\mu$ g total nucleic acids. Probes and duration of exposure: pIAA4/5,  $1.5 \times 10^6$  cpm/ml for five hours; pIAA6,  $2 \times 10^6$  cpm/ml for 10 hours; pW11,  $2 \times 10^6$  cpm/ml for 4.5 hours.



**Figure 6.** Effect of CHX and ANI on the stability ( $t_{1/2}$ ) of: A and C, *PS-IAA4/5*, and B and D, *pW11* mRNAs, in abraded pea epicotyl tissue. ○, +IAA; ●, -IAA +  $\alpha$ -amanitin; △, +CHX +  $\alpha$ -amanitin; □, -CHX +  $\alpha$ -amanitin. All lanes contain 25  $\mu$ g total nucleic acids. Probes and periods of exposure: *pIAA4/5*,  $1.5 \times 10^6$  cpm/ml for six hours; *pW11*,  $2 \times 10^6$  cpm/ml for 4.5 hours.

### Relationship between protein synthesis inhibition and mRNA induction

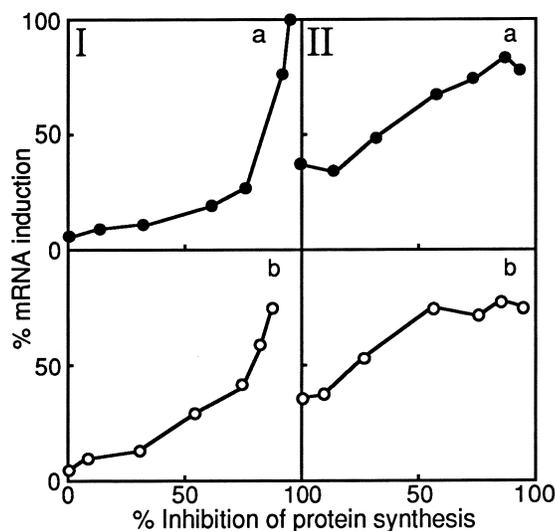
In order to establish whether protein synthesis inhibitors activate the transcription of *PS-IAA4/5* and *PS-IAA6* genes, the following experiments were carried out. First, we determined the extent of protein synthesis inhibition by monitoring the incorporation of [ $^{35}$ S]methionine into TCA-insoluble material under various inhibitor concentrations. Concomitantly, the extent of mRNA induction was assessed by RNA hybridization analysis. IAA induces the *PS-IAA4/5* and *PS-IAA6* mRNAs without having an inhibitory effect on protein synthesis (data not shown). CHX is the most effective inhibitor in inducing both mRNAs at relatively low concentrations. Protein synthesis is almost completely inhibited with 10  $\mu$ M CHX, and both mRNAs are induced at the same level as with optimal IAA concentration (10 to 100  $\mu$ M; data not shown). ANI is 10- to 50-fold less effective than CHX in inhibiting protein synthesis, but enhances both mRNAs at the same level as IAA or CHX when protein synthesis inhibition is complete (data not shown). EME is the

least effective and non-specific protein synthesis inhibitor.

When the percentage of *PS-IAA4/5* mRNA induction is plotted versus the percentage inhibition of protein synthesis by CHX or ANI, it becomes apparent that the relationship between these two parameters follows a hyperbolic curve (see Figure 7, Ia and Ib, respectively). However, the same relationship for the *pW11* mRNA follows a curve reminiscent of the Michaelis-Menten kinetics of enzyme action (Figure 7, IIa and IIb). Similar curves to those of *PS-IAA4/5* are obtained with the *PS-IAA6* mRNA (data not shown).

### Transcriptional activation of the *PS-IAA4/5* gene by CHX

To determine directly whether the induction of the *PS-IAA4/5* or *PS-IAA6* genes by CHX is also due to transcriptional activation, chimeric *PS-IAA4/5* or *PS-IAA6* promoter *GUS* or *PS-IAA4/5* *CAT* gene fusions lacking *PS-IAA4/5* or *PS-IAA6* coding sequences were constructed and tested for CHX inducibility in transgenic tobacco seedlings or in CHX-responsive pea protoplasts.



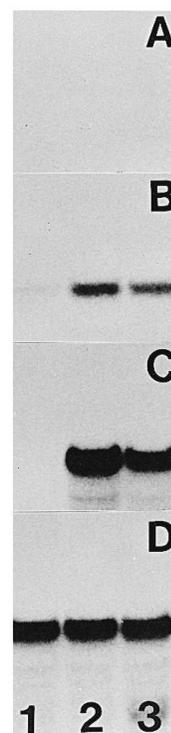
**Figure 7.** I, Relationship between *PS-IAA4/5* mRNA induction and inhibition of protein synthesis in the presence of two different protein synthesis inhibitors; a, cycloheximide; and b, anisomycin. II, Relationship between these two parameters for the non-IAA responsive gene *pWII* using the same inhibitors. The values of % inhibition of protein synthesis and % induction of *PS-IAA4/5* or *pWII* mRNAs were obtained from detailed dose response experiments. See Results section.

#### Transgenic seedlings

Transgenic tobacco plants harboring 2.4 or 5 kb of the *PS-IAA4/5* and *PS-IAA6* promoter region, respectively, fused to the *GUS*·*NOS* reporter gene were introduced into the tobacco genome by *Agrobacterium tumefaciens* mediated transformation. Seven-day-old etiolated transgenic tobacco seedlings were treated with IAA or CHX for three hours. RNA hybridization shows that the *GUS* gene driven by the *PS-IAA4/5* or *PS-IAA6* promoter is highly induced by IAA as well as by CHX (compare Figure 8B and C, lanes 2 and 3, respectively). IAA or CHX treatment does not affect the constitutive expression of the *GUS* mRNA from the *CaMV 35S* promoter (Figure 8D). The expression of the *GUS* gene is nil in the presence of IAA (lane 2) or CHX (lane 3) in seedlings harboring the pB101.1 vector alone (Figure 8A).

#### Transient expression in pea protoplasts

As an alternative experimental approach for determining the effects of CHX on *PS-IAA4/5* transcription, we used pea protoplasts to co-transfect the constitutive *35S*·*CAT*·*NOS* and the IAA inducible *PS-IAA4/5*·*CAT*·*NOS* genes (Ballas *et al.*, 1993). The effects of CHX, as well as IAA, on the induction of the *CAT* gene driven by the *PS-IAA4/5* promoter with or without 5' untranslated region (UTR) versus the *CAT* gene driven by the *35S* promoter are shown in Figure 9. Primer extension analyses with RNAs isolated from control, IAA, and CHX treated pea tissue segments defined three main transcription



**Figure 8.** Induction of *GUS* mRNA by IAA and CHX in etiolated transgenic tobacco seedlings. Seven-day-old etiolated seedlings harboring: A, pB101.1 vector alone, B, *PS-IAA4/5*·*GUS*·*NOS*, C, *PS-IAA6*·*GUS*·*NOS*, and D, *35S*·*GUS*·*NOS* were incubated for three hours in buffer alone (lane 1), 20 μM IAA (lane 2) and 20 μM CHX (lane 3). Each lane contains 30 μg total RNA. RNA hybridization analysis was carried out using <sup>32</sup>P-labeled *GUS*·*NOS* as a probe. All lanes were evenly loaded determined with a 17 S rice ribosomal probe (data not shown).

initiation sites in the *PS-IAA4/5* gene, 93, 95, and 99 bp upstream of the ATG codon (Figure 9, panel I). IAA or CHX treatment greatly enhances the *CAT* transcript expressed from the *PS-IAA4/5* promoter (–532/+85) (Figure 9, IIC, compare lanes 2 (IAA), and 3 (CHX) with lane 1 (control)). Furthermore, the same three transcription initiation sites utilized by the endogenous *PS-IAA4/5* gene are also utilized for transcription initiation of the chimeric *PS-IAA4/5*·*CAT* + *L* gene (Figure 9, compare IIC with IB). Similar primer extension analysis with RNA isolated from protoplasts transfected with the *35S*·*CAT* and *PS-IAA4/5*·*CAT* + *L* (*L* represents the 5' UTR) genes treated with IAA or CHX for two hours shows that the *CAT* transcript derived from the *35S* promoter is only slightly induced by IAA or CHX (Figure 9, IIC, compare lane 1 (control) with 2 (IAA) and 3 (CHX)). The transcription initiation site of the *35S*·*CAT* transcript is the same as previously determined for the authentic *CaMV 35S* gene (Ballas *et al.*, 1993).

The possibility exists that the presence of the 85 bp 5' UTR region of the *PS-IAA4/5* promoter is the site of action of the putative short-lived negative regulator. To test this possibility, a primer extension analysis (similar to that shown in Figure 9III) was



carried out using a leaderless *PS-IAA4/5-CAT-L* gene that contains only 3 bp of the 5' UTR. Removal of the 5' UTR leader does not affect the induction of the CAT transcript by CHX (Figure 9, compare lane 3 in IIC with that in IIIC) or IAA (Figure 9, compare lane 2 in IIC with that in IIIC). The only noticeable effect of removing the 5' UTR is the utilization of a few additional transcription initiation sites compared to those utilized by the endogenous *PS-IAA4/5* (Figure 9, compare IIIC with IB) or by the *PS-IAA4/5-CAT + L* chimeric gene (Figure 9, compare IIIC with IIC).

## Discussion

### Auxin inducibility

#### Transcriptional activation

IAA is known to rapidly enhance the level of several mRNAs in various plant tissues. The major mechanism accounting for this enhancement is transcriptional activation (Key, 1989). During the course of this study, we found that IAA enhances the relative rate of *PS-IAA4/5* and *PS-IAA6* mRNA synthesis. We conclude that a major component of *PS-IAA4/5* and *PS-IAA6* mRNA induction by IAA is due to transcriptional activation at the level of transcription initiation. Our experiments, however, do not exclude the possibility that the hormone may also act at a posttranscriptional processing step.

### Induction kinetics

In order to understand the details of an inductive process, it is crucial to know its obligatory lag period. We found that the lag periods of *PS-IAA4/5* and *PS-IAA6* mRNA induction by IAA are 5 and 7.5 minutes, respectively. The presence of the tissue cuticle extends these periods by 5 and 7.5 minutes, respectively, probably because the cuticle acts as a barrier to hormone penetration (Ray, 1985). Anker (1971) estimated that the resistance of the *Avena* coleoptile cuticle to the uptake of auxin from a bathing solution is 3000 times greater than that of a cut surface. Interestingly, the lag periods are not affected by the concentration of IAA or whether IAA has a free or esterified carboxyl group (ethyl-IAA). According to the chemiosmotic polar mechanism of auxin transport (Goldsmith *et al.*, 1981), the neutral ethyl-IAA molecule should penetrate faster than the charged IAA molecule and, consequently, the lag period should be shorter with ethyl-IAA than with IAA if the lag is due to diffusion. The results, however, show that the lag for mRNA induction is independent of the auxin analog used. The neutral ethyl-IAA may be as effective as IAA because it is hydrolyzed by an esterase, giving rise to IAA, but we believe this to be unlikely, because the dose response experiments with IAA and ethyl-IAA show that the two compounds have different activities (IAA > ethyl-IAA).

The relatively short lag periods raise the question of their potential components. One component is the time required for the penetration of the hormone

**Figure 9.** Transcriptional activation of the *PS-IAA4/5* promoter by IAA and CHX in transfected pea epicotyl protoplasts. I, Endogenous *PS-IAA4/5* gene. A, Dideoxynucleotide chain termination sequencing reaction with pH3 plasmid DNA containing the 5 kb fragment that carries the entire *PS-IAA4/5* gene using the P2 (30 mer) primer (the location of the primer is at +85 to 114 of the *PS-IAA4/5* gene; Oeller *et al.*, 1993). Lanes 1 to 4, reactions A, C, G, T, respectively. B, Primer extension analysis. 5'-[<sup>32</sup>P] P2 primer was hybridized with 30 µg total nucleic acids from abraded pea epicotyl tissue segments, untreated for two hours (lane 1), 40 µM IAA-treated for two hours (lane 2) or 40 µM CHX treated for two hours (lane 3), and extended with reverse transcriptase. The nucleotide sequence in the vicinity of the transcription start site is shown on the right of the primer extension reaction. The bold residue and large arrow represent the major start of transcription. C, Schematic diagram indicating the position of the P2 primer relative to the transcription and translation start sites, and a schematic representation of the primer extended product using the same primer. The stippled box represents the 96 bp 5' UTR of the endogenous *PS-IAA4/5* gene. II, *35S-CAT* and *PS-IAA4/5-CAT + L* hybrid genes. A, Dideoxynucleotide chain termination sequencing reaction with *35S-CAT* (-430) plasmid DNA using the cat (24 mer) primer (the location of the primer is at 253 to 276 of the *CAT* gene; Alton and Vaprek, 1979). B, Dideoxynucleotide chain termination sequencing reaction with 5'D-532 plasmid DNA containing the *PS-IAA4/5-CAT + L* gene that has 85 bp of the 5' UTR using also the same cat primer. Lanes 1 to 4 are A, C, G, T, respectively, in both A and B. C, Primer extension analysis. 5'-[<sup>32</sup>P]cat primer was hybridized with 30 µg total nucleic acids isolated from protoplasts and extended with reverse transcriptase, as described in Materials and Methods. Thirty samples of 6 × 10<sup>6</sup> protoplasts each were cotransfected with a mixture of *PS-IAA4/5-CAT + L* (53 µg) and *35S-CAT* (2 µg) plasmid DNAs as described in Materials and Methods. After transfection and PEG removal, the protoplast samples were combined and sampled into 30 new samples, and 10 of these samples were incubated for two hours in K<sub>3</sub>S medium alone (lane 1), 40 µM IAA (lane 2), or 40 µM CHX (lane 3). The nucleotide sequences in the vicinity of the transcription start sites of the *PS-IAA4/5-CAT* and *35S-CAT* hybrid genes are shown on the right side of the primer extension reactions. The bold residues and large arrows represent the major transcription start sites in these two genes, respectively. D, Schematic representation, as in IC. The stippled box represents 85 bp of 5' UTR of *PS-IAA4/5*; the hatched box in the *PS-IAA4/5-CAT* gene represents 31 bp of a polylinker, whereas the same shape box in the *35S-CAT* gene represents 20 bp of a polylinker. IIIA, The same as in IIA. B, The same as in IIB, except that the sequencing reaction was carried out with the plasmid *PS-IAA4/5-CAT-L* which does not have the 85 bp of the 5'-UTR of the *PS-IAA4/5* gene. C, The same as in IIC, except that the primer extension reaction was carried out with RNA from cotransfected protoplasts with the *35S-CAT* and *PS-IAA4/5-L* genes. D, The same as in IID, except that the hatched box in the *PS-IAA4/5-CAT-L* gene represents 9 bp, three of which are from the 5' UTR and the remainder constitute a *Bam*HI site. The filled dots in A and B define the extent of the DNA sequence shown on the right hand side of C.

into the cells. Another component is the special "biochemistry" that must take place before the mRNA can be detected. If the lag depends on the passive diffusion of IAA into the cells, its duration should be concentration dependent. Since the lag period is independent of the externally supplied concentration of IAA over a wide range, whereas the rate of IAA absorption is concentration dependent (Poole & Thimann, 1964), the penetration of IAA does not seem responsible for the observed lag. Interestingly, the lag period for IAA action on cell elongation is also independent of auxin concentration (Evans & Ray, 1969), and the induction of  $\beta$ -galactosidase in *Escherichia coli* by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; a classical inductive system) is also independent of inducer concentration (Pardee & Prestidge, 1961). We are thus left with the task of explaining the components of the lag periods and their different durations (*PS-IAA4/5*, 5 minutes; *PS-IAA6*, 7.5 minutes). One of the components is the time required for the synthesis of the primary transcripts by RNA polymerase II. The sizes of the mature *PS-IAA4/5* and *PS-IAA6* mRNAs are approximately the same, 917 nt versus 803 nt (Oeller *et al.*, 1993). The length of the unprocessed mRNAs, however, is quite different; the *PS-IAA4* transcript is 1513 nt, whereas the *PS-IAA6* is 2469 nt. This is due to the longer introns of the *PS-IAA6* gene (Oeller *et al.*, 1993). The longer lag period required for the appearance of the *PS-IAA6* mRNA may be due to the additional time needed for its transcription. Gene length can function as a delay timer in *Drosophila* (Thummel, 1992). Based on the estimation of transcription rate in *Drosophila*, we estimate the time required for the synthesis of the *PS-IAA4* and *PS-IAA6* RNA transcripts to be  $\sim 1.5$  and  $\sim 2.5$  minutes, respectively.

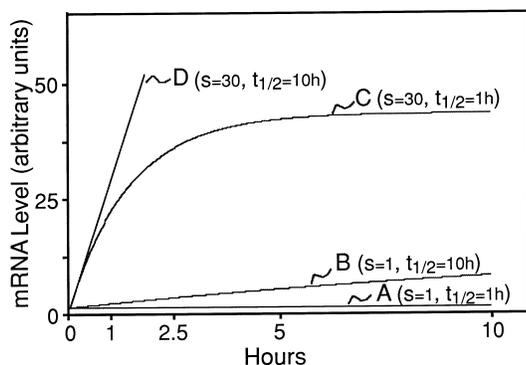
The remaining time of the lag periods (*PS-IAA4/5*, 3.5 minutes; *PS-IAA6*, 5 minutes) may represent the time required for the activation of the signal transduction pathway(s) responsible for their synthesis and the processing of adequate amounts of mRNA to be detected by RNA hybridization analysis. One may wonder what is occurring in the remaining time. (1) The possibility exists that IAA is sensed by the transcriptional machinery, e.g. via a kinase cascade which is activated by the interaction of the hormone with a receptor located in the plasma membrane (Hill & Treisman, 1995; Venis & Napier, 1995). Recent experimental evidence indicates that the signal via the three-component prokaryotic histidine kinase cascade (three proteins are involved) is transmitted to the DNA in 100 ms (M. Simon, personal communication), a very rapid process. Since eukaryotic cells contain a nuclear membrane (a transport barrier), the three to five minute lag period may be required for the transfer of a phosphorylated or dephosphorylated intermediate via the nuclear pore. Protein nuclear transport systems have a  $t_{1/2}$  of eight to ten minutes (Bader & Wietzerbin, 1994; Picard & Yamamoto, 1987), a relatively slow process. (2) IAA may generate second messages, such as  $\text{Ca}^{2+}$  or inositol triphosphates, that

diffuse into the nucleus and activate transcription by a nuclear kinase cascade, a very rapid process (Clapham, 1995). (3) The simplest view is that auxin, after entering the cells by an efficient and very rapid active transport mechanism, diffuses into the nucleus and is directly sensed by a protein that directly or indirectly interacts with the auxin responsive region (*AuxRR*) of the genes (Ballas *et al.*, 1995; Theologis, 1986). A small solute such as auxin, with a diffusion coefficient,  $D$ , of  $10^{-5}$  cm<sup>2</sup>/s, can diffuse across a 50  $\mu\text{m}$  typical plant cell in 0.6 second, a very rapid process (Nobel, 1970). Finally, it is tempting to speculate that the differential length of the lag periods of *PS-IAA4/5* and *PS-IAA6* gene induction may reflect differences in the biochemical properties of the components of the signaling apparatus responsible for their transcriptional activation and processing of their transcripts.

### Stability of the *PS-IAA4/5* and *PS-IAA6* mRNAs

Differential rates of mRNA turnover strongly influence the overall pattern of gene expression in the eukaryotic cell (Atwater *et al.*, 1990; Peltz *et al.*, 1991; Sachs, 1993). The importance of mRNA decay in differential gene expression is exemplified by the transformation phenotype associated with mRNA stabilization mutations in the *c-myc* and *c-fos* mRNAs (Schiavi *et al.*, 1992). There are numerous examples of hormonal and developmental regulation of gene expression exerted at the level of transcription (Wilson *et al.*, 1990). Examples in which hormones and developmental factors influence mRNA stability upon transcriptional activation are fewer in number (Guyette *et al.*, 1979; Brock & Shapiro, 1983; Paek & Axel, 1987). Examples of the effects of plant hormones on mRNA stability are missing (Sullivan & Green, 1993). In the present study, we have measured the half-lives of *PS-IAA4/5* and *PS-IAA6* mRNAs under conditions of limited RNA synthesis. The  $t_{1/2}$  values of both mRNAs are relatively short, 60 and 75 minutes, respectively, and IAA does not alter their stability.

The measurement of mRNA stability following transcription inhibition by actinomycin D or  $\alpha$ -amanitin has been reported to cause overestimation of mRNA half-lives in plants and mammalian systems (Peltz *et al.*, 1991; Sullivan & Green, 1993), presumably by inhibiting short-lived degradative enzymes. We believe the effect of transcriptional inhibition on  $t_{1/2}$  determination is minimal in our experiments for two reasons. (1) The decay rates of both mRNAs during deinduction (after the removal of IAA) in the absence of any transcriptional inhibitors are approximately the same as those determined in this study (see Figure 7 of Theologis *et al.*, 1985), indicating that auxin does not affect the stability of the mRNAs and confirming that the  $t_{1/2}$  values determined in the presence of  $\alpha$ -amanitin are accurate. Furthermore, studies of transcriptionally inducible genes have shown that the period required to attain new levels of individual mRNAs is related to their unique half-lives (Hargrove & Schmidt,



**Figure 10.** Predicted time course for induction of *PS-IAA4/5* mRNA using the STELLA<sup>®</sup> II microcomputer program for kinetic modeling of gene expression (Hargrove *et al.*, 1993). Four theoretical accumulation curves were computed based on its half life,  $t_{1/2}$  = one hour (Figure 3A).  $S$  is the transcriptional rate. A, Basal level of *PS-IAA4/5* gene expression;  $S = 1$  and  $t_{1/2} = 1$  hour. B, mRNA stabilization by CHX;  $S = 1$  and  $t_{1/2} = 10$  hours. C, Transcription activation by IAA;  $S = 30$  and  $t_{1/2} = 1$  hour. D, Dual effect of CHX;  $S = 30$  and  $t_{1/2} = 10$  hours.

1989; Schimke *et al.*, 1964). The shorter the half-life, the sooner the new steady state is reached (Hargrove & Schmidt, 1989). Based on this generally accepted model for the kinetics of the induction processes, half-maximal induction should be detected after the equivalent of one  $t_{1/2}$ , assuming that the degradation rate is not changed by the inducer. Indeed, the half-times of induction and deinduction of *PS-IAA4/5* and *PS-IAA6* mRNAs are almost the same as the  $t_{1/2}$  values determined in the presence of  $\alpha$ -amanitin. Furthermore, the experimentally determined half-lives satisfy the requirement that the time required for an inducible mRNA to achieve a new steady state is five times its half-life, because the new steady state levels of *PS-IAA4/5* and *PS-IAA6* mRNAs after IAA induction are approximately four to five hours (Figure 7 of Theologis *et al.*, 1985, and Figures 4 and 5 of this work). (2) A quantitative model has been developed that predicts the rate of mRNA accumulation as a function of mRNA  $t_{1/2}$ . Using the microcomputer program STELLA<sup>®</sup> (Hargrove *et al.*, 1993), we were able to simulate the kinetics of induction of *PS-IAA4/5* mRNA using the experimentally determined  $t_{1/2}$  in Figure 3. The simulated curve C in Figure 10 quantitatively fits the experimental data shown in Figures 4A and 5A and Figure 7 of Theologis *et al.* (1985).

Nucleolytic breakdown of mRNA is a highly specific process, and it varies widely for different mRNA molecules. Some mRNAs have  $t_{1/2}$  values that span several hours, or even days (Sachs, 1993; Peltz *et al.*, 1991), while others (Franco *et al.*, 1990; Greenberg *et al.*, 1986; Laird-Offringa, 1992) have half-lives in the range of 30 minutes. The  $t_{1/2}$  values of *PS-IAA4/5* and *PS-IAA6* make them fall into the unstable category, which includes the phytochrome and *SAURs* transcripts in plants (Sullivan & Green, 1993) and several protooncogene mRNAs in mam-

malian cells (Atwater *et al.*, 1990; Greenberg *et al.*, 1986; Sachs, 1993). mRNA instability is often associated with genes that must be rapidly and stringently controlled, like those encoding products involved in regulating cell growth and differentiation. Recently, two different elements, DST and AU-rich sequences, have been functionally identified in plant mRNAs responsible for mRNA instability (Newman *et al.*, 1993; Ohme-Takagi *et al.*, 1993). The *PS-IAA4/5* and *PS-IAA6* mRNAs do not contain any DST elements throughout their primary transcripts, but do contain one and two AUUUA elements, respectively, in their 3' UTRs (Oeller *et al.*, 1993). Whether these elements are responsible for the instability of *PS-IAA4/5* and *PS-IAA6* mRNAs remains to be determined.

### Inducibility by protein synthesis inhibition

A unique property of the *PS-IAA4/5* and *PS-IAA6* genes is their inducibility by various structurally and mechanistically dissimilar protein synthesis inhibitors such as CHX, ANI, and EME (Theologis *et al.*, 1985). This is also a property of other auxin regulated genes such as the *SAURs* of soybean and *Arabidopsis* (Franco *et al.*, 1990; Gil *et al.*, 1994). While this property is unique to auxin-regulated genes in plants, it is widespread in mammals where numerous early genes, including various oncogenes, are induced by protein synthesis inhibitors (Makino *et al.*, 1984; Mitchell *et al.*, 1985, 1986; Müller *et al.*, 1984). In many systems, including the *PS-IAA4/5* and *PS-IAA6* genes, the concomitant presence of the inhibitor and the inducer result in superinduction of the mRNAs (Herschman, 1991).

Protein synthesis inhibitors have traditionally been used to unmask biochemical capacity responsible for early gene activation. Induction of early genes does not depend on *de novo* synthesis, indicating a primary response to the inducer *via* preexisting components (Ringold, 1979; Herschman, 1991). Furthermore, inhibition of protein synthesis potentiates the strength and duration of many signal-induced transcriptional responses by eliminating attenuating factors that are often synthesized as part of the response (Hill & Treisman, 1995). The rationale for stabilizing certain mRNAs in response to stresses like translation inhibition seems straightforward; the cell might need the stabilized mRNA for the synthesis of essential proteins once the translation block is relieved (Brewer & Ross, 1989).

The molecular mechanisms responsible for the induction and superinduction of *PS-IAA4/5* and *PS-IAA6* mRNAs by protein synthesis inhibitors are unknown. Our studies indicate that protein synthesis inhibitors have a dual effect: they activate transcription and also stabilize the inducible mRNAs. Similar experimental evidence has been obtained by other investigators who have described transcriptional activation of genes and mRNA stabilization following protein synthesis inhibition (Ikeda *et al.*, 1990; Lazar *et al.*, 1990; Reed *et al.*, 1987; Wilson & Treisman, 1988). The relative contribution of the two processes during the early stages of mRNA

induction cannot be estimated from our experiments. Since the rate of accumulation in the presence of IAA plus CHX or ANI is greater (superinduction) compared to that in the presence of CHX or ANI alone during the early stages of induction, we speculate that stabilization by CHX or ANI acts early on the IAA-induced mRNAs.

#### *Transcriptional activation by protein synthesis inhibition*

Protein synthesis inhibitors, such as CHX, ANI, EME and pactamycin (Pestka, 1971), superinduce a variety of mRNAs by transcriptional activation (Greenberg *et al.*, 1986; Lusska *et al.*, 1992; Messina, 1990; Whittemore & Maniatis, 1990). A popular interpretation of this effect is that a short-lived transcriptional repressor is degraded following protein synthesis inhibition, resulting in transcriptional activation. According to this model, the CHX-mediated induction of *PS-IAA4/5* and *PS-IAA6* is due to the decrease in the amount of the repressor, whereas IAA alters its function (directly or indirectly; Ballas *et al.*, 1993, 1995). The experiment, the results of which are shown in Figure 7, is a titration experiment where a negative regulator is removed upon the addition of various concentrations of CHX and ANI. The shape of the curves in Figure 7 is reminiscent of that describing *lacZ* activity under various concentrations of the *lac* repressor (Zubay *et al.*, 1965). It is also quite possible that there is not a labile repressor, but rather that the inhibition of protein synthesis has biochemical consequences that lead to the activation of positive, or the deactivation of negative, transcription factors (Sen & Baltimore, 1986). In such a scenario, the labile repressor could be either a phosphatase or a kinase that modifies the activity of transcription factors, or a molecule that blocks the site of phosphorylation/dephosphorylation by direct interaction with the positive or negative transcription factor(s).

The lag periods of the two mRNAs are quite different in the presence of CHX and ANI (Figures 4 and 5). This finding suggests that distinct proteins with different degradation rates negatively regulate the levels of *PS-IAA4/5* and *PS-IAA6* mRNAs. Similar results have been obtained with the *bcl2* and *c-myc* mRNAs in lymphocytes (Reed *et al.*, 1987) and the *ACS2* and *ACS5* mRNAs in *Arabidopsis* (Liang and Theologis, unpublished results). Is it possible that such short-lived proteins exist? There are several examples: the  $\sigma^5$  subunit of RNA polymerase in *E. coli* has a half life of 1.4 minutes (Lange & Hengge-Aronis, 1994); the *cII* repressor of phage lambda has a half-life of <one minute (Gottesman & Maurizi, 1992); *Mat $\alpha$ 2* of yeast, has a half-life of five minutes (Gottesman & Maurizi, 1992); the *fushi tarazu* (*ftz*) protein, a central developmental regulator in *Drosophila*, has a half-life of five minutes (Gottesman & Maurizi, 1992); the  $\text{I}\kappa\text{B}$  repressor has a half-life of 1.5 minutes (when it is dissociated from NF- $\kappa\text{B}$ ; Henkel *et al.*, 1993); many cellular and viral oncogenic proteins, including Myc, Myb, and Fos,

have been shown to have extremely short half-lives *in vivo* (Gottesman & Maurizi, 1992). By comparison, the products of the *PS-IAA4/5* and *PS-IAA6* genes have half-lives of eight and six minutes, respectively (Abel *et al.*, 1994).

#### *Stabilization of PS-IAA4/5 and PS-IAA6 mRNAs by protein synthesis inhibition*

The second contributing factor to the rapid induction and eventual superinduction of the *PS-IAA4/5* and *PS-IAA6* mRNAs by CHX and ANI is mRNA stabilization. The rapid induction kinetics upon protein synthesis inhibition (Figures 4 and 5) cannot be explained solely by mRNA stabilization. Computer simulation of the *PS-IAA4/5* induction kinetics with the STELLA<sup>®</sup> program (Figure 10) clearly shows that a 10-fold increase in  $t_{1/2}$  by CHX or ANI without any change in transcriptional activity ( $S = 1$ ) is not sufficient for rapid mRNA accumulation and does not agree with the experimental evidence obtained. Even if mRNA stability is enhanced 100-fold ( $t_{1/2} = 100$  hours), the rate of accumulation (slope) will be slightly higher, but the final high level of mRNA accumulation will be achieved only after a few days (data not shown). This is predicted by the kinetic model for inducible mRNAs and has been experimentally verified (Hargrove & Schmidt, 1989). The time required for an inducible mRNA to achieve a new steady state is inversely proportional to its  $t_{1/2}$  (Hargrove *et al.*, 1991). It is noteworthy that a 30-fold enhancement in transcriptional rate ( $S = 30$ ) by CHX or ANI with a concomitant 10-fold increase in  $t_{1/2}$  ( $t_{1/2} = 10$ ) predicts a mRNA accumulation curve very similar to the experimental evidence obtained (Figure 14, curve D). It is of great interest that the initial slopes (rates) of curves C (representing the IAA-mediated *PS-IAA4/5* mRNA accumulation) and D (representing the CHX- or ANI-mediated mRNA accumulation) in Figure 10 are the same as those experimentally observed in Figures 4 and 5.

Two major mechanisms have been proposed to explain mRNA stabilization by various protein synthesis inhibitors. (1) Translational arrest, which prevents the nucleolytic breakdown of the mRNAs by a mechanism not clearly understood. (2) Translational inhibition, which prevents the synthesis of labile nucleases responsible for RNA degradation (Sachs, 1993). The precise mechanism of *PS-IAA4/5* and *PS-IAA6* mRNA stabilization by protein synthesis inhibition remains to be determined.

## Materials and Methods

### Plant material

Pea seeds (*Pisum sativum*, cultivar Alaska) were planted in vermiculite and grown for seven days at 25°C in the dark, as previously described (Theologis *et al.*, 1985). Abraded segments 8 mm long, beginning 3 mm below the top of the apical hook, were prepared and incubated in "depletion" and "incubation" media as described by Theologis *et al.* (1985). 15-segment samples (0.3 g fresh

weight) were treated with or without IAA, ethyl-IAA or protein synthesis inhibitors in incubation medium at concentrations indicated in the Figure legends. After incubation, the segments were frozen in liquid N<sub>2</sub> and stored at -80°C. Seven-day-old etiolated transgenic tobacco seedlings were obtained by germinating 0.1 g of transgenic seeds on agar plates containing MS medium (Murashige & Skoog, 1962) in the dark at 25°C. Seedlings were harvested and incubated for three hours in incubation medium with or without 20 µM IAA or 20 µM CHX. After treatment, the tissue was frozen and stored at -80°C.

### Plasmid construction

All plasmids used for transfection were constructed in the pKS<sup>+</sup> vector using standard recombinant DNA techniques (Sambrook *et al.*, 1989) and their authenticity was confirmed by DNA sequencing (Sanger *et al.*, 1977). All plasmids were purified by CsCl gradient centrifugation (Davis *et al.*, 1980). The construction of the chimeric *CAT-NOS* genes driven by the *CaMV 35S* promoter (*35S-CAT*; -430) and the *PS-IAA4/5* promoter (*PS-IAA4/5-CAT + L*; -532/+85) with 85 bp of the 5' untranslated region have been previously described (Ballas *et al.*, 1993). The *PS-IAA4/5* promoter (-532/+3) without the 5' untranslated region was synthesized by PCR as a *Hind*III (5')/*Bam*HI (3') fragment and subcloned into pKS<sup>+</sup>. Subsequently, the *CAT-NOS* gene was isolated as a *Bam*HI fragment from *PS-IAA4/5-CAT + L* (Ballas *et al.*, 1993) and subcloned downstream of the promoter, giving rise to *PS-IAA4/5-CAT-L*; (-532/+3).

### Construction of *PS-IAA4/5* and *PS-IAA6* promoter *GUS-NOS* gene fusions in pBI101.1 and generation of tobacco transgenic plants

The construction of the *PS-IAA4/5* promoter (-2309/+85) fused to *GUS-NOS* has been described previously (Ballas *et al.*, 1993). The promoter of *PS-IAA6* (-4500/+42) was obtained from the genomic clone  $\lambda$ -*PS-IAA6B* (Oeller *et al.*, 1993) and an *Nco*I site was placed at the initiating methionine. The 4.5 kb, *Eco*RI/*Nco*I *PS-IAA6* promoter fragment was linked to the *GUS* gene of JS8 (a gift from Dr Maliga, Rutgers University) through the *Nco*I site at the ATG of *GUS*, giving rise to pNS25. The *PS-IAA6-GUS* gene was subcloned as a *Hind*III/*Sna*BI fragment into the pBI101.1 vector, giving rise to the *pBI-PS-IAA6-GUS-NOS* plasmid. The construct was mobilized into *A. tumefaciens* strain LBA4404 by direct transformation, and the tobacco (*Nicotiana tabacum*, cultivar Samsun) was transformed using the leaf disk method described by Rogers *et al.* (1986). Transgenic tobacco seeds with the promoterless pBI101.1 and pBI-IAA4/5-*GUS-NOS* genes have been previously reported (Ballas *et al.*, 1993).

### mRNA half-life ( $t_{1/2}$ ) determination

mRNA half-life determinations were performed with abraded depleted tissue segments under conditions of limited mRNA synthesis. The *PS-IAA4/5* and *PS-IAA6* mRNAs were induced by treating the tissue in an incubation medium containing 20 µM IAA for one hour at 25°C in the dark. Subsequently, 5 µM  $\alpha$ -amanitin (Lindell *et al.*, 1970) was added to inhibit RNA synthesis, and the mRNA decay was followed in the presence or absence of IAA over a period of three hours by RNA hybridization analysis. The IAA was removed in the -IAA samples by washing the tissue four times, for five minutes each time, during the first 20 minutes after  $\alpha$ -amanitin addition. The

effect of protein synthesis inhibitors CHX and ANI on the stability of the two mRNAs was determined with the same experimental approach, except that the inhibitors were added after 15 minutes of  $\alpha$ -amanitin addition.

### Isolation of pea protoplasts, transfection and hormonal treatment

Pea protoplasts were isolated and transfected with various plasmids as described by Ballas *et al.* (1993).

### Isolation of nucleic acids and RNA hybridization analysis

Total nucleic acids were isolated using the phenol/chloroform extraction procedure described by Theologis *et al.* (1985). RNA hybridization analysis with various <sup>32</sup>P-labeled probes was carried out as described by Theologis *et al.* (1985). The autoradiograms were quantified using a LKB laser densitometer (Bromma, Sweden), and the results are graphically presented below or next to the autoradiograms in the various figures. The RNA filters were successively reprobbed with various <sup>32</sup>P-labeled probes by melting off each probe as described by Theologis *et al.* (1985). Autoradiograms from three different low exposures were quantified to ensure linearity in the film response. The results were consistent with those assessed by visual inspection.

### Primer extension analysis

The transcription initiation sites of the endogenous *PS-IAA4/5* and the transfected *PS-IAA4/5-CAT* or *35S-CAT* genes were determined by primer extension analysis with 30 µg heat denatured RNA and 5' end-labeled gene specific primers, as previously described (Oeller *et al.*, 1993; Ballas *et al.*, 1993).

### Determination of [<sup>35</sup>S]methionine incorporation into protein

*Dose response of inhibition of protein synthesis by CHX, ANI, and EME*

Duplicate samples of 15 abraded segments were depleted of endogenous IAA (as described above) and incubated in 1 ml incubation medium containing various concentrations of IAA, CHX, ANI, and EME in the presence of 20 µCi [<sup>35</sup>S]methionine (906 Ci/mmol). After two hours of incubation at 25°C, the segments were washed three times, for one minute each time, with 1.5 ml of incubation medium and frozen in liquid N<sub>2</sub>. The frozen segments were homogenized with Polytron for two minutes with 5 ml of 10 mM Hepes (pH 7.5) at 4°C and centrifuged at 13,000 rpm at 4°C for 10 minutes. Total radioactivity was determined by counting 10 µl of the supernatant. The incorporation of [<sup>35</sup>S]methionine into protein was determined according to Mans and Novelli (1971) using 10 µl samples.

### Kinetics of inhibition of protein synthesis

Duplicate samples were depleted of auxin; for the last 15 minutes of the 30 minutes pre-incubation period (Theologis *et al.*, 1985) the segments were incubated in the presence of [<sup>35</sup>S]methionine (40 µCi/ml). Subsequently, the protein synthesis inhibitor was added to the medium at the appropriate concentration indicated in the Figure legends. Tissue samples were removed every five minutes

for the first 20 minutes. The segments were briefly washed and frozen. The total uptake of [<sup>35</sup>S]-methionine and its incorporation into the trichloroacetic acid-insoluble protein fraction was determined as described above.

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