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Cytokinins and Auxins Control the Expression of a Gene in *Nicotiana plumbaginifolia* Cells by Feedback Regulation

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Both cytokinin (N^6-benzyladenine [BA]) and auxin (2,4-dichlorophenoxyacetic acid [2,4-D]) stimulate the accumulation of an mRNA, represented by the cDNA pLS216, in *Nicotiana plumbaginifolia* suspension culture cells. The kinetics of RNA accumulation were different for the two hormones; however, the response to both was transient, and the magnitude of the response was dose dependent. Runoff transcription experiments demonstrated that the transient appearance of the RNA could be accounted for by feedback regulation of transcription and not by the induction of an RNA degradation system. The feedback mechanism appeared to desensitize the cells to further exposure of the hormone. In particular, cells became refractory to the subsequent addition of 2,4-D after the initial RNA accumulation response subsided. A very different response was observed when the second hormone was added to cells that had been desensitized to the first hormone. Under such conditions, BA produced a heightened response in cells desensitized to 2,4-D and vice versa. These findings support a model in which cytokinin further enhances the auxin response or prevents its feedback inhibition. The hormone-induced RNA accumulation was blocked by the protein kinase inhibitor staurosporin. On the other hand, the protein phosphatase inhibitor okadaic acid stimulated expression, and, in particular, okadaic acid was able to stimulate RNA accumulation in cells desensitized to auxin. This suggests that hormone activation involves phosphorylation of critical proteins on the hormone signaling pathway, whereas feedback inhibition may involve dephosphorylation of these proteins. The sequence of pLS216 is similar to genes in other plants that are stimulated by multiple agonists such as auxins, elicitors, and heavy metals, and to the gene encoding the stringent starvation protein in *Escherichia coli*. It is proposed that this gene family in various plants be called multiple stimulus response (*msr*) genes.

INTRODUCTION

Cytokinins and auxins act in concert to promote many growth and developmental processes in higher plants. The experiments of Skoog and Miller (1957) underscored the relationship between these two plant hormones and demonstrated that the course of plant regeneration in culture could be determined by the ratio of cytokinin and auxin. More progress has been made in understanding the molecular basis for the action of auxin than cytokinin because auxin stimulates rapid and easily measured physiological events. Auxin responses are thought to involve receptors that may be represented by auxin binding proteins such as ABP1 (Hesse et al., 1989; Inohara et al., 1989). Antibodies specific to ABP1 reduce auxin-induced membrane hyperpolarization, and addition of ABP1 to tobacco protoplasts further sensitizes the membrane response (Barbier-Brygoo et al., 1989, 1991).

Auxin rapidly activates the expression of auxin-induced genes that have been described in various systems (reviewed by Guilfoyle, 1986; Theologis, 1986). cDNAs representing auxin-induced mRNAs have been cloned from soybean (Walker and Key, 1982; Hagen et al., 1984; Ainley et al., 1988; McClure et al., 1989), pea (Theologis et al., 1985), Arabidopsis (Alliotte et al., 1989), and tobacco (van der Zaal et al., 1987; Takahashi et al., 1989). Activation of some auxin-induced genes is very rapid and occurs before the onset of other physiological responses such as cell elongation. For several auxin-induced genes that have been studied, activation is transcriptional (Hagen and Guilfoyle, 1985; Theologis et al., 1985; Hagen et al., 1988) and is tissue specific (Gee et al., 1991; van der Zaal et al., 1991).

There has been less progress to date in understanding the cytokinin response and in finding cytokinin receptors. Several groups have described cytokinin binding proteins (Brinegar et al., 1988; Romanov et al., 1988; Jayabaskaran, 1990); however, in the absence of a functional test, it has been difficult to attach physiological roles to any of the cytokinin binding proteins. The primary action of cytokinins has been associated

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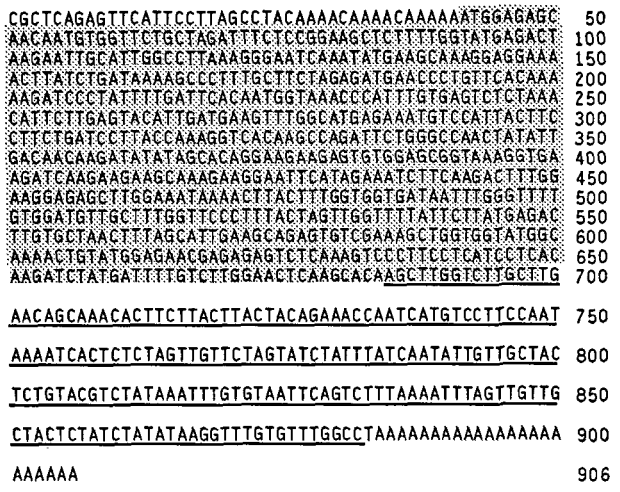

Figure 1. Sequence of the cDNA Insert in pLS216.

Shadowed region shows the 657-bp open reading frame. Underlined region marks the limits of the gene-specific probe present in pLS216GS.

with enhanced calcium ion influx and the action of calmodulin (Hepler and Wayne, 1985; Poovaiah and Reddy, 1987).

The addition of cytokinins to cultured plant material results in the appearance of a number of new mRNAs and proteins (Fosket and Tepfer, 1978; Chen and Leisner, 1985; Chatfield and Armstrong, 1986; Bauw et al., 1987; Chen et al., 1987; Crowell et al., 1990). Certain plant cultures (tobacco and Lemna) respond to cytokinin by greening in the light and accumulating the light-harvesting chlorophyll *a/b* binding proteins and their mRNAs (Axelos et al., 1984; Teyssendier de la Serve et al., 1985; Flores and Tobin, 1986, 1988). Flores and Tobin (1986, 1988) have argued that in Lemna, cytokinin stimulation of chlorophyll *a/b* binding protein gene expression is regulated primarily at the post-transcriptional level. Cytokinins also induce cytokinin oxidase in bean cell cultures (Chatfield and Armstrong, 1986). The enhancement of cytokinin oxidase was blocked by RNA synthesis inhibitors, suggesting that cytokinin regulation of cytokinin oxidase expression was transcriptional.

We have found that both cytokinin and auxin stimulate the expression of a gene in *Nicotiana plumbaginifolia* cell suspension cultures related to a family of genes regulated by a number of stimuli including auxin, pathogens (elicitors), heat shock, and heavy metal treatment (Czarnecka et al., 1984, 1988; Hagen et al., 1984; Hagen and Guilfoyle, 1985; Taylor et al., 1990; van der Zaal et al., 1991). A feedback inhibition mechanism desensitizing the cells to further exposure to the hormone appeared to limit the hormone response. It is proposed that cytokinin and auxin interact by further enhancing or by preventing the feedback inhibition of the other hormone's response system.

RESULTS

An *N. plumbaginifolia* cDNA library was screened for clones that were differentially expressed in response to cytokinin. Among the isolated clones were four related cDNAs (pJD201, pJD216, pJD238, and pJD269), none of which was full length based on the length of the RNA (1 kb) to which the cDNA hybridized. pJD216 was then used as a probe to screen another cDNA library to select for a full-length cDNA clone, pLS216. The cDNA insert in pLS216 was 906 bp, including a 23-bp polyadenylated tail. A large open reading frame of 657 bp was found encoding a protein of 219 amino acid residues, as illustrated in Figure 1. The derived amino acid sequence was used to search the Protein Identification Resource data base (Pearson and Lipman, 1988). As shown in Figure 2, surprising similarity was found between pLS216 and proteins encoded by genes that have been reported to be regulated by other stimuli. The soybean gene, *Gmhsp26A*, was originally obtained as cDNA (pCE54) by screening for heat shock genes (Czarnecka et al., 1984). Despite its reported heat shock response, the gene product bore little resemblance to heat shock proteins from other

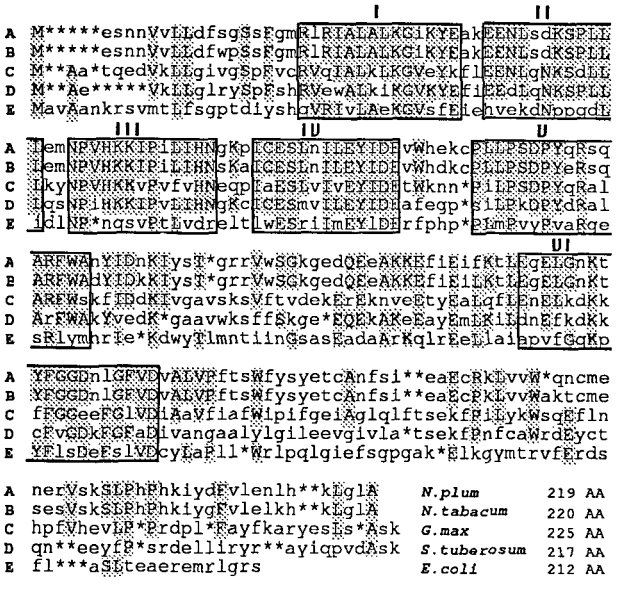

Figure 2. Derived Amino Acid Sequence of the Protein Encoded by pLS216.

The protein encoded by pLS216 from (A) *N. plumbaginifolia* is compared to similar proteins derived from (B) *N. tabacum* (Takahashi et al., 1989), (C) soybean (*Glycine max*) (Czarnecka et al., 1988), (D) potato (*Solanum tuberosum*) (Taylor et al., 1990), and to the SSP from (E) *Escherichia coli* (Serizawa and Fukuda, 1987). Amino acid residues identical in three of four proteins are capitalized and shadowed. Blocks of sequences conserved among the plant proteins are designated I to VI.

organisms (Czarnecka et al., 1988). The same gene from soybean was independently cloned as cDNAs (GH2 and GH4) by screening a library for auxin-regulated genes (Hagen et al., 1984; Hagen and Guilfoyle, 1985). Recently, cDNAs representing similar genes from *N. tabacum* and potato have been isolated on the basis of their auxin- or elicitor-stimulated expression (Takahashi et al., 1989; Taylor et al., 1990; van der Zaal et al., 1991).

The proteins encoded by these genes have modest sequence similarity to the stringent starvation protein (SSP) of *Escherichia coli*. The *N. plumbaginifolia* protein (219 amino acids) and the *E. coli* SSP (212 amino acids) are similar in size and 22% identical in protein sequence. The plant proteins for which sequence information is available show blocks of sequence homology that are largely clustered in the N-terminal half of the protein and that we have arbitrarily designated as regions I to VI. The sequence match between the *N. plumbaginifolia* and *E. coli* proteins is much higher in several of these regions (I, 40%; IV, 54%; V, 37.5%). The *N. plumbaginifolia* and *E. coli* proteins also have other properties in common. For example, the two proteins have comparable hydropathy profiles (data not shown).

To determine the number of gene copies that hybridize to pLS216 in the *N. plumbaginifolia* genome, pLS216 was hybridized under standard stringency conditions to total *N. plumbaginifolia* DNA digested with several restriction enzymes that do not cut within the probe cDNA. In most cases, pLS216 hybridized to two DNA bands instead of one, indicating that there are probably two or more copies of the gene in the *N. plumbaginifolia* genome (data not shown). In an effort to obtain a gene-specific probe, a DNA segment derived almost exclusively from the 3′ untranslated region of the cDNA was used to probe *N. plumbaginifolia* DNA. As shown in Figure 3, this probe (pLS216GS), represented by the underlined sequence in Figure 1, hybridized to only a single DNA band in each restriction enzyme digest.

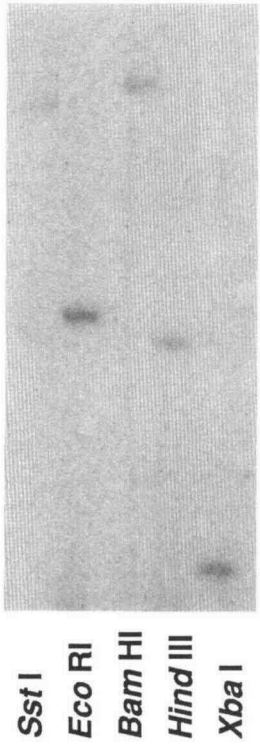

Figure 3. DNA Gel Blot of *N. plumbaginifolia* Leaf DNA.

The *N. plumbaginifolia* leaf DNA was digested with various restriction enzymes and hybridized with the ^{32}P-labeled, gene-specific probe (pLS216GS) described in Figure 1. Lengths of DNA fragments were determined relative to the migration of markers in a 1-kb DNA ladder (Bethesda Research Laboratories): SstI, 6.3 kb; EcoRI, 3.2 kb; BamHI, 7.1 kb; HindIII, 3.0 kb; XbaI, 1.4 kb.

Regulation of Expression

pLS216 was selected because it represents an mRNA that accumulates in *N. plumbaginifolia* cells in response to cytokinin. However, the product encoded by pLS216 resembles the product of an auxin-induced gene. Therefore, we determined whether the gene to which pLS216GS hybridizes was stimulated by both plant hormones. The hormone response was examined under conditions ordinarily used to stimulate organ regeneration (shoot differentiation). Suspension cells were maintained in a proliferative state by growth in 2 μM 2,4-dichlorophenoxyacetic acid (2,4-D). To induce shoot differentiation, cells were deprived of 2,4-D by shifting the culture for 3 days to a preconditioning medium containing 0.5 μM naphthaleneacetic acid, and then cytokinin (N^6-benzyladenine [BA]) or 2,4-D was added at time 0. Under these conditions, we found, indeed, that adding either BA (5 μM) or 2,4-D (5 μM) stimulated pLS216 mRNA accumulation.

The kinetics of RNA accumulation in response to the two hormones was transient and differed for the two hormones. As shown in Figure 4, the 2,4-D response peaked at ~3 to 6 hr, whereas the BA response peaked at ~24 to 48 hr. Similar patterns of RNA accumulation have been observed in five repeats of this experiment. In shorter time course experiments, it was observed that the onset of the gene expression response was quite fast. Although it takes hours for the RNA accumulation response to peak, the onset of response to either hormone could be detected within 15 min of adding the hormone (data not shown). We were curious whether the timing of the response was characteristic of the hormone or was dose dependent. With both hormones, the magnitude of the response varied with hormone concentration (over a 10- to 40-fold range); however, the timing of the peak response was about the same at the different hormone doses (Figure 4). Therefore, even at BA concentrations (20 μM) that exceeded the optimum used in growth or shoot induction experiments by

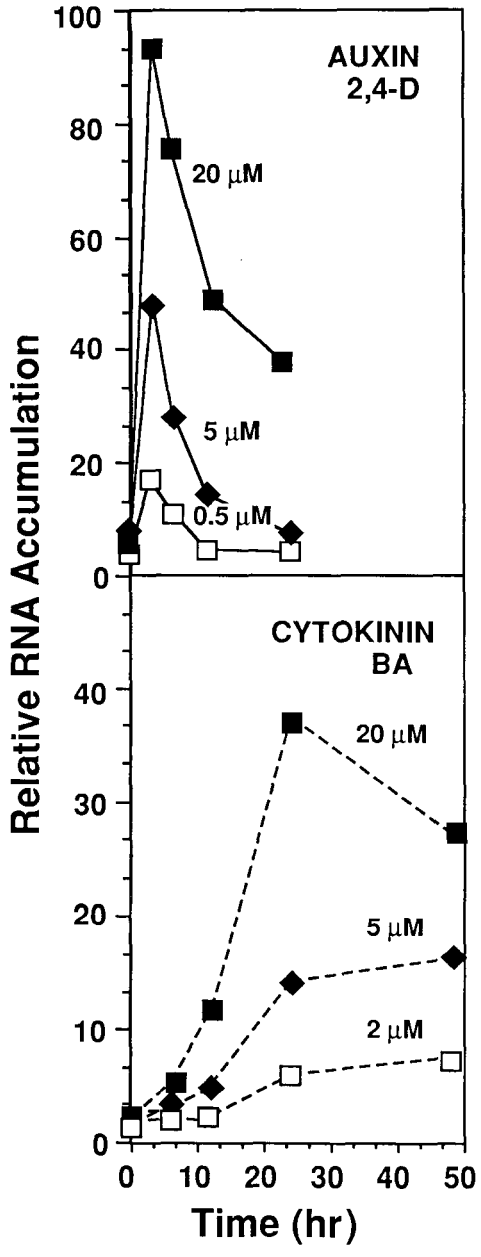

Figure 4. Time Course for the Accumulation of pLS216-Specific RNA in Response to 2,4-D and BA.

BA or 2,4-D was added at time 0, and 10-mL samples were taken at indicated times thereafter. pLS216-specific RNA was determined from RNA or slot blot analysis of the extracted RNA hybridized to the pLS216GS probe. Five micrograms of total RNA was used per sample, and the amount of RNA hybridizing to the gene-specific probe was quantified using a PhosphorImager. Data from the PhosphorImager are relative values indicating the amount of radioactivity in each band or sample and representing the amount of RNA accumulated. These values are expressed with relationship to the peak value obtained in response to 20 μM 2,4-D, which was set graphically at somewhat less than 100.

fourfold, the time to reach a peak BA response never matched that of 2,4-D. Hence, the timing of the two responses appeared to be different: characteristic of the hormone systems and not obviously dose dependent.

The transient accumulation of pLS216-specific RNA could be due to changes in transcription or turnover of RNA. Therefore, we determined by runoff transcription assays whether there were changes in the rate of transcription following hormone addition and, if so, whether those changes correlated with the pattern of RNA accumulation. As shown in Figure 5, we found that there was an expected correlation between changes in the rates of transcription and the pattern of RNA

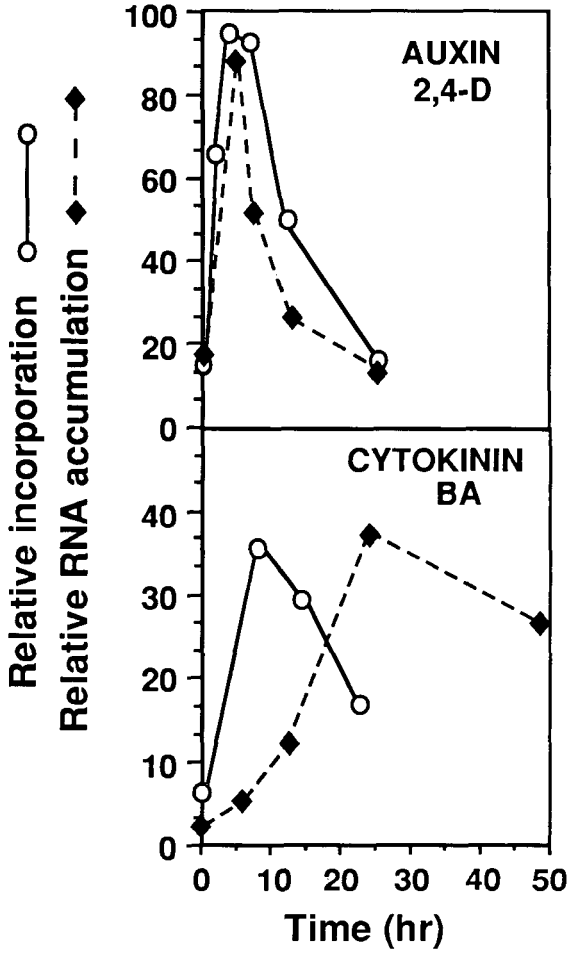

Figure 5. Comparison of the Rates of Transcription to the Pattern of pLS216-Specific RNA Accumulation after Adding 5 μM 2,4-D and 20 μM BA.

Relative transcription rates (^{32}P-UTP incorporation) were determined in nuclear runoff assays carried out on nuclei isolated from cells at the times indicated. RNA extracted from nuclei was hybridized to 5 μg of filter-immobilized pLS216 plasmid DNA. The amount of ^{32}P-UTP incorporated into RNA hybridizing to pLS216 was quantified with a PhosphorImager. The accumulation of pLS216-specific RNA was determined as described in Figure 4.

accumulation; that is, for the gene corresponding to pLS216, transcription rates were highest when the rate of RNA accumulation was the greatest. Peak rates were observed ~2 to 3 hr after adding auxin (5 μM 2,4-D) and ~6 to 10 hr after adding cytokinin (20 μM BA). In short time courses, it was clear that peak rates of RNA synthesis were reached faster in response to auxin than in response to cytokinin (data not shown). On first approximation, it seemed reasonable to conclude that changes in transcription rate largely account for the patterns of RNA accumulation, assuming a constant rate of RNA turnover. However, because RNA accumulation peaked much later than transcription following BA addition, the rate of RNA turnover following BA may be slower. This matter needs further study. In any case, a mechanism involving the induction of a specific system that degrades pLS216-specific RNA is not needed to explain the transient nature of the response.

Two other explanations were put forward to explain the transient nature of the hormone response. One was that the plant hormones were inactivated, inaccessible, or destroyed after adding to the culture. If the synthesis of pLS216-specific RNA depended on the continued presence of the hormone, then the loss or inactivation of the hormone would shut off RNA synthesis and RNA levels would drop through normal RNA turnover after reaching some peak level. The second explanation was that the response was desensitized after adding the plant hormones.

To test these possibilities, we added the two hormones in various sequences and times, as shown in Figure 6. In the first case, 2,4-D was added twice, once to elicit the initial RNA accumulation response and again to determine whether a second response could be elicited after the first response had subsided. As a control, 2,4-D was added at 24 hr (time 0) and cells were harvested 6 hr later (at 30 hr). The amount of pLS216-specific RNA accumulated was determined by RNA gel blot analysis and set at 100% (Figure 6, line 1). As a further control, 5 μM 2,4-D was added to the cell culture at 0 hr and cells were harvested at 30 hr. pLS216-specific RNA declined as expected by 30 hr to 20% peak levels (Figure 6, line 2). When 2,4-D was added twice, no stimulation of RNA accumulation was observed upon second addition of the hormone (Figure 6, line 3). In this experiment, 5 μM 2,4-D was added to the cell culture at 0 hr and again at 24 hr after the mRNA accumulation response had subsided. Cells were harvested 6 hr later, at 30 hr. Hence, the experiment demonstrated that after the initial mRNA accumulation response subsided, the response system was refractory to further addition of 2,4-D. (Note: the second addition of 2,4-D raised the final auxin concentration in the culture from 5 to 10 μM, but that increase in concentration should not block the response, but make it stronger. We have shown that the magnitude of the response increases up to 20 μM 2,4-D; see Figure 4.) Therefore, the results did not support the first hypothesis that the response was transient because 2,4-D was rapidly inactivated, made inaccessible, or destroyed. Instead, it appeared that cells were indifferent to the presence or absence of the hormone and became incapable of responding to 2,4-D soon after the initial 2,4-D response

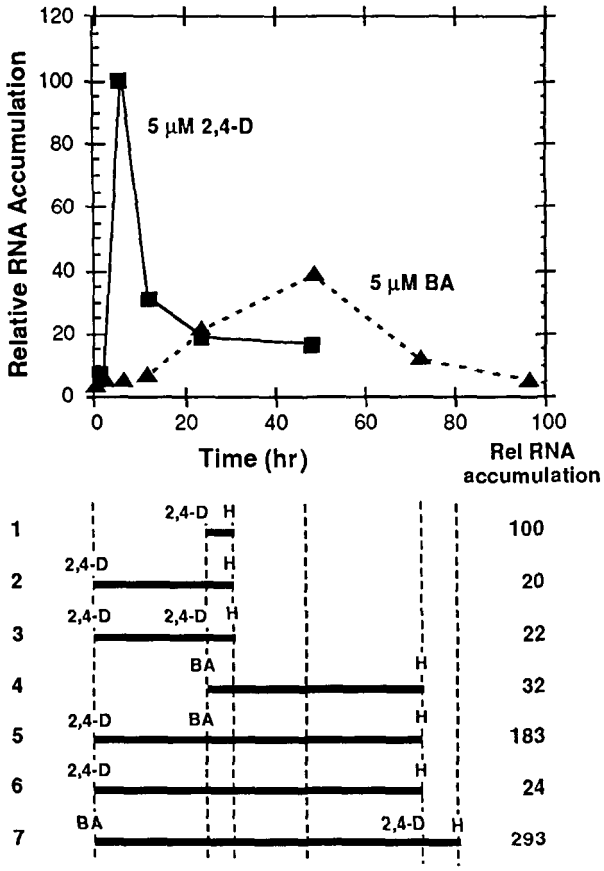

Figure 6. Scheme for the Addition of Hormones (BA or 2,4-D) at Various Times.

(Top) Time course for the accumulation of pLS216-specific RNA when 2,4-D or BA was added at time 0. RNA levels were determined as described in Figure 4.

(Bottom) The two hormones (5 μM BA or 2,4-D) were added at the times indicated, and the cells were harvested for RNA extraction (H) as shown. Relative RNA accumulation levels were determined as described in Figure 4 and expressed on an arbitrary scale set at 100% for the peak 2,4-D response.

subsided. For technical reasons, it was difficult to add BA twice because a long period of time is required for RNA levels to peak and decline. Using the same regimen as in the 2,4-D experiments, the time involved in adding BA twice exceeded the time in which we would ordinarily passage cells.

We then asked what would happen if we added the second hormone (BA) after the peak RNA accumulation response to 2,4-D had subsided. In the control, 5 μM BA was added at 24 hr and cells were harvested 48 hr later (at 72 hr). Under these conditions, pLS216-specific RNA accumulated to a level typical for a BA response (Figure 6, line 4). In the experiment, 5 μM 2,4-D was added at time 0, and then 5 μM BA was added at 24 hr, during the auxin refractory period. pLS216-specific mRNA levels, measured at 72 hr (48 hr after the addition of

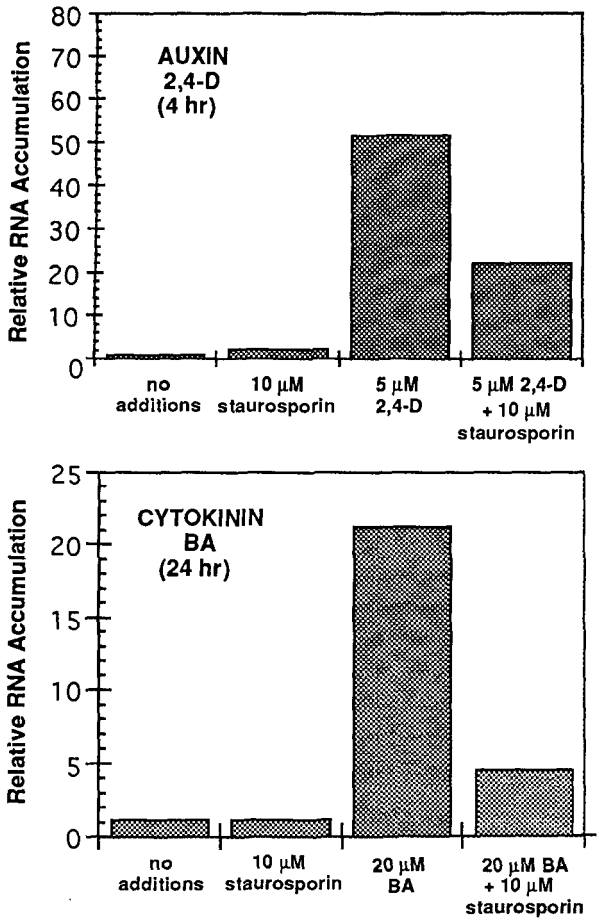

Figure 7. Inhibition of pLS216-Specific RNA Accumulation by 10 μM Staurosporin.

Hormone and staurosporin were added at time 0, and cells were harvested at 4 hr for cultures to which auxin (5 μM 2,4-D) was added and at 24 hr for cultures to which cytokinin (20 μM BA) was added. pLS216-specific RNA was determined as described in Figure 4.

BA), were elevated beyond expectation (Figure 6, line 5), that is, higher than the levels reached if BA had been added on its own without prior addition of 2,4-D (Figure 6, line 4) or if 2,4-D had been added 72 hr earlier without subsequent addition of BA (Figure 6, line 6). Thus, after the 2,4-D response had subsided, cells were still able to respond to cytokinin and in doing so accumulated RNA at much higher levels than expected.

To determine what effect prior addition of BA had on the subsequent 2,4-D response, the order of hormone addition was reversed. In this case, 5 μM BA was added at time 0, and then 5 μM 2,4-D was added at 72 hr when the BA response was in decline; cells were harvested 6 hr later. Interestingly, the 2,4-D response was very strong (Figure 6, line 7). This experiment demonstrated that prior addition of BA did not block the 2,4-D–stimulated accumulation of pLS216-specific mRNA; to the contrary, prior BA addition markedly stimulated the 2,4-D response.

Effect of Agents That Influence Protein Phosphorylation

To understand the hormone signaling pathway leading to the expression of the gene corresponding to pLS216, it was of interest to identify agents that might alter the auxin or cytokinin response. One group of agents that was effective was the broad spectrum protein kinase inhibitor staurosporin that affects protein phosphorylation (Tamaoki, 1991). In plant cell suspension cultures, staurosporin has been shown to block elicitor-induced protein phosphorylation (Felix et al., 1991). At 10 μM, staurosporin had little effect on basal expression, but it was able to reduce by 57% auxin-induced pLS216-specific RNA accumulation measured at 4 hr following 2,4-D addition, as shown in Figure 7. At the same concentration, staurosporin reduced by 79% cytokinin-induced pLS216-specific RNA accumulation measured at 24 hr after the addition of BA. The inhibition by staurosporin suggests that protein phosphorylation is critical for hormone-induced pLS216-specific mRNA accumulation.

We tested whether a protein phosphatase inhibitor might have the opposite effect, that is, to stimulate pLS216-specific mRNA accumulation. Okadaic acid is an inhibitor of type 1 and 2a protein phosphatases (Haystead et al., 1989). We found

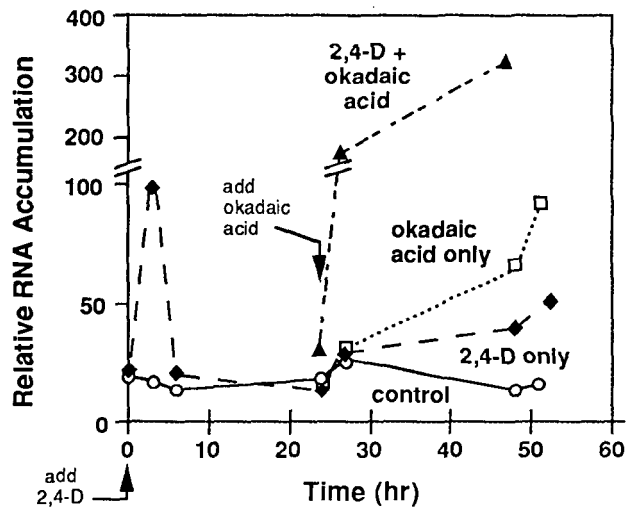

Figure 8. Stimulation of pLS216-Specific RNA Accumulation by Okadaic Acid.

In the control sample, no hormone or okadaic acid was added. In the 2,4-D only sample, 5 μM 2,4-D was added at 0 hr. In the okadaic acid only sample, 0.2 μM okadaic acid was added at 24 hr. In the 2,4-D + okadaic acid sample, 5 μM 2,4-D was added at 0 hr and 0.2 μM okadaic acid was added at 24 hr. The accumulation of pLS216-specific RNA was determined as described in Figure 4.

that adding okadaic acid to control cultures in which no hormone was added slightly stimulated pLS216-specific mRNA accumulation, as seen in Figure 8. On the other hand, adding okadaic acid to cultures in which the auxin response had been densensitized greatly stimulated pLS216-specific mRNA accumulation. Thus, okadaic acid was only able to modestly stimulate the RNA accumulation response on its own, but it was very effective in overcoming the feedback-inhibited or feedback-desensitized state of the auxin response. These findings suggest a critical role for protein phosphorylation in pLS216-specific gene expression and argue that protein dephosphorylation might be involved in feedback inhibition of the hormone response system.

DISCUSSION

The actions of cytokinin, auxin, and other agonists are closely intermeshed in the regulation of expression of the gene in *N. plumbaginifolia* represented by pLS216. This cDNA was selected because it was derived from a gene that was stimulated by adding cytokinin to cell cultures. The sequence of pLS216 closely matches the sequence of genes in a gene family induced by other stimuli in other plants. A cDNA (pCE54) representing one of the gene family members was first cloned from soybean by Czarnecka et al. (1984, 1988). The cDNA represented an mRNA that accumulated in response to heat shock in soybean hypocotyls. Using the cDNA as probe, it was found that the mRNA accumulated in response to many different stimuli, including abscisic acid, 2,4-D, polyethylene glycol, polyamines, sodium arsenite, and heavy metals. The soybean gene encoding this mRNA, *Gmhsp26-A*, is an intron-containing gene, and intron processing is inhibited by several heavy metals (Czarnecka et al., 1988). The gene product does not appear to be a typical heat shock protein and bears only faint resemblance to heat shock proteins from other organisms (Czarnecka et al., 1988). cDNAs (pGH2 and pGH4) representing the same gene were selected by Hagen and Guilfoyle (1985) on the basis of the gene's response to auxin in soybean plumules. They found that the gene was transcriptionally regulated and responded to heavy metals, but not to heat shock (Hagen et al., 1988).

Recently, similar genes have been identified on the basis of their auxin inducibility, such as the protoplast auxin regulated (*par*) gene (Takahashi et al., 1989, 1990) and the 103 gene (van der Zaal et al., 1991) in *N. tabacum*, or by their elicitor inducibility, such as the *prp1* gene encoding proline-rich protein 1 (Taylor et al., 1990) in potato. There are several members of the gene family in various plant species. It is not known whether each gene member responds to the full range of environmental stimuli or whether one member responds more fully to one stimulus than to another. In any case, it is proposed that these genes be designated multiple stimulus response (*msr*) genes and that the gene corresponding to pLS216 in *N. plumbaginifolia* should be called the *msr1* gene.

In our experiments, the accumulation of pLS216-specific mRNA in *N. plumbaginifolia* cells was transient in response to either 2,4-D or BA. Three different explanations were put forward to account for the transient nature of the response: (1) an RNA degradation system was induced that counteracted the accumulation of pLS216-specific RNA; (2) the plant hormones were rapidly destroyed, inactivated, or compartmentalized after adding them to the culture; or (3) the response was desensitized after adding the plant hormones. The first alternative, that a specific RNA degradation system was activated following the rise in pLS216-specific RNA transcription, seemed unlikely for two reasons. First, following hormone addition, the rates of transcription measured in runoff experiments rose and fell in a manner consistent with the RNA accumulation patterns, assuming continuous RNA turnover. Second, if an RNA degradation system had been activated by the first hormone, then one might not expect to see a response from the second hormone. That was clearly not the case. A very robust response was observed when BA was added following the 2,4-D response and vice versa.

The second explanation that the hormones were rapidly destroyed or inactivated seemed unlikely because these hormones are routinely used to sustain growth or promote development in long-term cultures. Also, we found that when 2,4-D was added a second time, after the peak RNA accumulation response subsided, there was no further stimulation of RNA accumulation. Thus, after the initial response subsided, the cells were refractile, incapable of responding to the hormone agonist.

By elimination, it appeared that the third alternative was the likely explanation, that the response was desensitized after adding the hormone. Desensitization of cells to hormonal stimuli is an adaptive response common in animal systems (Benovic et al., 1988). Two major patterns of desensitization describe the action of multiple agonists in a hormone response: desensitization can be "homologous" and agonist specific or "heterologous" and agonist nonspecific (Sibley and Lefkowitz, 1985). In homologous desensitization, the agonist attenuates the response only to the desensitizing hormone, whereas the response to other hormone activators is unimpaired. The response described in this study is clearly a case of homologous desensitization. The response elicited by auxin desensitizes the cells to auxin and not to cytokinin and vice versa. This feedback regulation is illustrated in Figure 9 in a model in which the hormone response is regulated by a negative feedback mechanism. In this model, it is envisioned that the feedback loop for auxin is fast, rapidly shutting down RNA accumulation soon after RNA synthesis begins. The cytokinin response might be similarly regulated, although it appears that the feedback response is slower. This model shows single, unbranching pathways for illustration only. It does not mean that there might not be multiple receptors and alternative pathways.

The response to the hormone dose demonstrated how tightly the system was feedback regulated. The magnitude of the response increased with dose, but the timing of the response generally did not (Figure 4). Because the timing of the peak

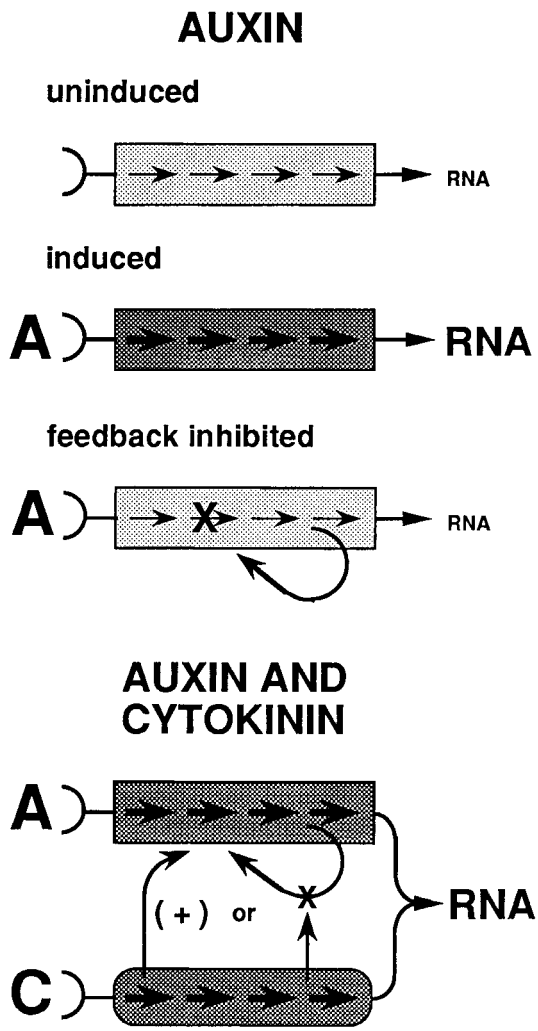

Figure 9. Scheme Showing Feedback Mechanisms Proposed To Regulate *msr1* Gene Expression (pLS216-Specific RNA Accumulation Response).

A and C represent auxin and cytokinin, respectively, and the shaded boxes represent auxin and cytokinin signal transduction pathways leading to *msr1* gene expression and RNA accumulation. Scheme shows little *msr1* gene expression and RNA accumulation in the uninduced state (absence of hormone). Auxin stimulates *msr1* gene expression and RNA accumulation, but the response is quickly feedback inhibited and further RNA accumulation is prevented. Cytokinin stimulates *msr1* gene expression and RNA accumulation on its own and by acting positively (+) to sensitize the auxin response or by blocking the feedback inhibition of the auxin response.

response remained approximately the same regardless of dose, the system must be able to desensitize in proportion to the intensity of the signal. This feedback mechanism makes the system somewhat insensitive to the absolute concentrations of the growth regulators. However, because the two hormone response systems "cross talk" to each other, the system might be relatively insensitive to the absolute concentration of the two growth hormones but quite sensitive to ratios of their concentrations. This, too, is another classical property of the way in which these growth hormones act.

Given the desensitization properties of the individual hormone systems, the interaction between the two hormone responses is a curious property. A simple explanation for the action of the second hormone is that either it sensitizes or contributes to the response of the first hormone or it interferes with the feedback mechanism limiting the first hormone response (Figure 9). Whatever the case, the response reveals an interesting synergism between auxin and cytokinin and may reflect the manner in which these hormones interact with each other in long-term developmental events.

In animal systems, desensitization often involves turnover of receptors or modification of receptors or signaling chain components by phosphorylation. We found that staurosporin, a protein kinase inhibitor, significantly inhibited hormone-induced, pLS216-specific RNA accumulation. On the other hand, okadaic acid, a protein phosphatase inhibitor, stimulated pLS216 gene expression. In particular, okadaic acid was able to stimulate expression when pLS216 gene expression was in refraction from prior 2,4-D stimulation. These findings suggest that phosphorylation of a critical protein(s) may be required for the induction of pLS216 gene expression and dephosphorylation of that protein (or another protein) may be involved in feedback inhibition.

Efforts are underway in several laboratories, including our own, to study the operation of *msr* genes through functional promoter analyses. The issue is at what level does the integration of information from multiple stimuli occur? Do *msr* genes have multiple, independent promoter elements each of which facilitates responses to a different stimulus? Or is information from multiple stimuli integrated at a different level and transmitted to the gene as a general signal? The promoters from *msr* genes in *N. tabacum*, the *par* gene, and the 103 gene (Takahashi et al., 1990; van der Zaal et al., 1991) are being studied by deletions and sequence comparison in an effort to identify functional domains.

It is possible that the pLS216 gene product may play some important role in hormonal and growth regulatory responses. The gene product is modestly similar in sequence to the stringent starvation protein in *E. coli*. However, the similarity becomes more significant when blocks of conserved sequences among the plant proteins are compared to the bacterial protein sequence. Furthermore, the proteins are similar with respect to other attributes including size and hydropathy profiles.

The stringent response in *E. coli* is a global regulatory response mechanism that adjusts the growth of the bacterium to nutrient conditions (Cashel and Rudd, 1987). The response manifests itself most dramatically in the down regulation of rRNA synthesis under conditions of amino acid starvation. Under starvation conditions, a major product of protein synthesis is the SSP. The function of the protein is not known, and its involvement in the response itself is still under study (Fukada et al., 1988). Nonetheless, the protein has been reported to bind to *E. coli* RNA polymerase holoenzyme and to alter the

transcriptional activity of the polymerase (Serizawa and Fukuda, 1987). The response in *N. plumbaginifolia* to plant hormones and in *E. coli* to nutritional conditions are both growth regulatory responses. It will be interesting to know whether the pLS216 gene product has a similar role in the two organisms.

Finally, the hormone shifts we have used to reveal the adaptational responses in this study are neither normal nor physiological. However, to understand the forces that govern the normal levels of gene expression in plant cells, one needs to perturb a steady state system. By doing so, we have uncovered an adaptational response that undoubtedly operates at steady state in plants but eludes detection by the near-static state of most systems.

METHODS

Plant Materials and Cell Culture

Suspension cultures of *Nicotiana plumbaginifolia* cells were obtained by regenerating leaf mesophyll protoplasts according to Maliga (1984). The cell line used in these experiments, DNP 7, was continually selected for rapid growth and screened for its ability to differentiate (undergo greening) in response to cytokinin when cultured in continuous light. Cells were maintained on a Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2 μM 2,4-dichlorophenoxyacetic acid (2,4-D).

The response of cells was studied under conditions that promote shoot regeneration in plated cells. Cells grown in the presence of the strong auxin 2,4-D were shifted for 3 days to preconditioning medium containing a weak auxin, 0.5 μM naphthaleneacetic acid. Cytokinin or auxin was then added to the culture at a time designated as time 0. Benzyladenine (BA) in DMSO was usually added to a final concentration of 5 μM. Other hormones or the same quantity of DMSO was added to control cultures.

RNA and DNA Extractions

To isolate RNA for cDNA production and for some routine analysis, a modification of the guanidinium/CsCl cushion procedure was used (Ausubel et al., 1988). Poly(A) RNA was obtained by binding total RNA to Hybond membranes (Amersham Corp.) and eluting according to manufacturer's instructions. Double-stranded cDNA was synthesized using a kit from Amersham Corp. according to manufacturer's instructions.

For routine samples, RNA was obtained in a scaled-down phenol/chloroform extraction procedure modified from Dudley and Northcote (1978). A sufficient volume of cell culture was harvested by filtration through a 30-μm nylon filter to give ~0.3 to 0.4 g of cells. The cell mat was folded into the filter, blotted with paper towels to remove excess medium, and frozen with liquid N_2. Frozen cells were ground to a fine powder with prechilled mortars in pestles, and sufficient powder was transferred to prechilled 1.5-mL microcentrifuge tubes to fill the tubes half-full. Added to the tube were 0.45 mL of HB solution (100 mM Tris-HCl, pH 8, 4% SDS, 200 μg/mL heparin, 50 μM aurintricarboxylic acid [ATA]) and 0.25 mL of water-saturated phenol, and the tube was vortexed immediately. All solutions were treated with 0.05% diethylpyrocarbonate or prepared with water that had been similarly treated.

The mixture was centrifuged in a microcentrifuge for 5 min at room temperature, and the upper phase was transferred to a new tube. The upper phase was reextracted three more times with 0.4 mL of phenol/chloroform/isoamyl alcohol (25:24:1). The upper phase was made 0.3 M with sodium acetate, pH 5, and RNA was precipitated with 2 volumes of cold ethanol at −70°C for 30 min.

Precipitated RNA was collected by centrifugation in a microcentrifuge at room temperature for 10 min. The RNA-containing pellet was dispersed in 1 mL of cold 3 M sodium acetate, pH 5, 5 mM EDTA using a pipette tip to break up the pellet. The dispersed pellet was collected again by centrifugation in a microcentrifuge for 5 min at room temperature. The pellet was once again dispersed in 1 mL of cold 2 M LiCl, 5 mM EDTA, 50 μM ATA, and the dispersed material was allowed to sit on ice for 30 min. The RNA-containing pellet was collected by centrifugation for 5 min at 4°C and washed with 1 mL of 70% ethanol. The pellet was collected again by centrifugation for 5 min at 4°C. The pellet was dried by vacuum centrifugation and dissolved in 200 μL of 50 μM ATA solution with vortex mixing. The concentration of RNA was calculated from the A_{260} reading but corrected for the contribution from ATA using the following formula:

$$\text{corrected } A_{260} = A_{260} - (0.464 \times A_{310}).$$

For RNA gel blot analysis, 5-μg RNA samples were subjected to electrophoresis on agarose formaldehyde gels and transferred to nylon membrane filters. For slot blots, 5-μg RNA samples were denatured at 60°C for 15 min in a solution containing 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 12.5% formaldehyde. RNAs on filters were hybridized to ^{32}P-labeled, cloned cDNAs using standard conditions (Ausubel et al., 1988). RNA hybridizing to the gene-specific probe was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

DNA was extracted from the leaves of vegetatively propagated *N. plumbaginifolia* plants (Ausubel et al., 1988); this was the same plant line used for the suspension cultures. DNA digested with various restriction enzymes was separated on 0.6% agarose gels, and after exposure to short UV light (254-nm peak) for 40 sec on an illumination box (UVP, Inc., San Gabriel, CA), the DNA was transferred to Nytran nylon filters (Schleicher & Schuell) using standard conditions. Cloned cDNA probes were hybridized to the filters at 42°C in hybridization buffer containing 10% dextran sulfate (w/v) (Klessig and Berry, 1983).

cDNA Cloning and Screening

Double-stranded cDNAs, used in the construction of cDNA libraries, were synthesized from cells 24 hr after the addition of 1 μg/mL BA, selected for fragment lengths of >300 bp on agarose gels, and cloned in λ phage Zap vectors (Stratagene). Phage plaques were screened using ^{32}P-labeled cDNA probes prepared from RNA obtained from cells 24 hr after the addition of 1 μg/mL BA and from control cells to which no BA was added. Hybridization and screening techniques were performed essentially as described by Theologis et al. (1985).

DNA excised as a Bluescript SK− plasmid was sequenced by dideoxy chain termination techniques using Sequenase polymerase (U.S. Biochemicals Corp.) according to manufacturer's instructions.

Nuclear Isolation and Runoff Transcription Reactions

Nuclei were isolated by techniques adapted from Paul et al. (1987) and Lissemore and Quail (1988). Cells (3 g) collected by filtration were ground for 1 min in an ice cold mortar with pestle with 5 mL of grinding buffer

(2.5% Ficoll 400, 5% Dextran T40, 250 mM sucrose, 25 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM mercaptoethanol + 0.1% Triton X-100). The ground cells were filtered through Miracloth and washed once with another 5 mL of grinding buffer. Nuclei were collected by centrifugation at 1400*g* for 3 min at 4°C in a rotor (model No. HB-4; Sorvall Instruments Div., Newton, CT). The nuclei were gently resuspended in 10 mL of grinding buffer and sedimented again at 650*g* for 3 min at 4°C in the rotor. The nuclei were resuspended in residual supernatant liquid in a volume of ~300 μL, adjusted to give 5×10^7 nuclei per milliliter, frozen in liquid nitrogen, and stored at −80°C.

Runoff transcription reactions (100 μL) containing 2.5×10^6 nuclei in 4 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 0.3 μM phosphocreatine, 2.5 μg creatine phosphokinase, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 80 units RNasin, 150 μCi ^{32}P-UTP were incubated at room temperature for 30 min. Ten microliters of RQ1 DNase (1 unit per microliter; Promega) was added and incubated at room temperature for an additional 15 min. Two volumes of 1% SDS, 10 mM EDTA, 50 μM ATA were added, and RNA was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). RNA was precipitated twice on dry ice with 2.5 M NH_4 acetate and 2.5 volumes of cold ethanol. The RNA-containing pellet was resuspended in 300 μL of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, plus 50 μM ATA. The pellet was washed twice with 70% ethanol, dried briefly, and resuspended in 50 μL of 10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5. Aliquots of the samples were precipitated with 20% trichloroacetic acid, collected on glass fiber filters, and washed first with ice cold 5% trichloroacetic acid, 1.5 mM sodium pyrophosphate, and then with 70% ethanol. Incorporation of ^{32}P-UTP was determined by scintillation counting.

^{32}P-labeled RNA (0.5 to 1×10^6 cpm) was hybridized to various plasmid DNAs immobilized on Magnagraph nylon filter strips (Micron Separations, Inc., Westboro, MA) under the conditions described by Lissemore and Quail (1988). The hybridization reactions (200 μL) in 1.5-mL microcentrifuge tubes were overlaid with 200 μL of mineral oil and incubated at 42°C for 40 to 48 hr.

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