RNA polymerase I (pol I) transcribes the repeated genes that encode the precursor of 17–18, 5.8, and 25–28 S ribosomal RNA (rRNA). Pol I transcription is up-regulated in growing cells and down-regulated in quiescent cells, presumably reflecting the demand for ribosomes and protein synthesis. However, the signal transduction pathways responsible for pol I regulation are poorly understood. We tested the effects of exogenously applied plant hormones on promoter-dependent rRNA transcription in Arabidopsis thaliana. Gibberellic acid, abscisic acid, auxin, and ethylene had no detectable effect on rRNA transcription, but kinetin (a cytokinin) stimulated rRNA transcription within 1 h of treatment. Increased steady-state levels of accurately initiated rRNA transcripts, detected by S1 nuclease protection, were paralleled by increased levels of nascent rRNA transcripts in isolated nuclei. Therefore, the primary effect of cytokinin appears to be at the level of transcription initiation rather than rRNA stability. Pol I accounts for 60% following cytokinin treatment. The specific responsiveness of pol I transcription to kinetin suggests that cytokinins may act as general regulators of protein synthetic capacity and growth status in plant cells.

Eukaryotes have evolved three nuclear RNA polymerases with specialized functions. RNA polymerase I transcribes the tandemly repeated genes encoding the precursors of 17–18, 5.8, and 25–28 S ribosomal RNA (1–3) (rRNA size varies with species). Pol1 II transcribes protein-coding genes and most small nuclear RNAs (4–6), and pol III transcribes 5 S rRNA, tRNAs, and one or more small nuclear RNAs (6–8). All three polymerases are needed to produce the rRNAs and proteins of a functional ribosome (9, 10), although the rRNAs transcribed by pol I are thought to comprise the ribosome's catalytic core (11).

Ribosomal RNA transcription and cell growth rate tend to be positively correlated (12–16). In Escherichia coli and other prokaryotes, the cellular concentration of guanosine tetraphosphate (ppGpp), reflects the level of rRNA transcription, although cause and effect are unclear (17). In eukaryotes, there is no strong evidence for an analogous compound. However, as in prokaryotes, rRNA transcription is down-regulated upon nutritional challenge (amino acid, carbon, or nitrogen limitation; serum starvation), cycloheximide treatment, or upon reaching stationary phase (18–24; recently reviewed in Ref. 12). Glucocorticoid hormones have also been shown to stimulate or repress pol I transcription in different tissues (25–28). These physiological responses appear to be brought about by phosphorylation or other modifications of the polymerase or its auxiliary transcription factors (21, 22, 29–31), but the causative signal transduction pathways remain obscure.

Using the model plant species, Arabidopsis thaliana, we have begun to define the rRNA gene loci and cis-acting DNA sequences essential for accurate pol I transcription initiation (32–36). Recent progress toward dissecting hormone signaling pathways in A. thaliana prompted us to investigate reports that plant hormones such as auxin and cytokinin affect pol I activity in cell-free extracts, isolated chromatin, or nuclei (37–41). Consequently, we examined hormonal effects on transcription initiation at the rRNA gene promoters as well as elongation of nascent rRNA transcripts. We show that exogenous cytokinin induces pol I transcription within 1 h of treatment, and the stimulatory effect persists for at least 24 h. The effect of cytokinin on rRNA transcription is consistent with its role in growth regulation and cell division.

**EXPERIMENTAL PROCEDURES**

**Sterile Growth of Arabidopsis Seedlings—**A. thaliana Columbia seeds were sterilized in 95% ethanol (five washes, 5 min each), followed by 3% sodium hypochlorite wash (10 min) and three washes in sterile distilled water (5 min each). Seeds were recovered by filtration (Nalge sterile filter unit) and dried for 2 days in a desiccator containing sterile Dri-Rite. Approximately 100 seeds were sprinkled onto semisolid germination medium (42) in 75-mm diameter Petri dishes. Seeds were germinated and plantlets grown for 14–21 days in a growth chamber (16 h day length, 22 °C, 70% relative humidity) prior to hormone treatment.

**Hormone Treatment of Plants—**Plant hormones were purchased from Sigma and dissolved as 0.1 M - 0.2 M stocks. Kinetin was initially dissolved in 1 N NaOH then diluted with water to 0.1 M. Stocks of abscisic acid, gibberellic acid, and 2,4-dichlorophenoxyacetic acid were prepared in 100% ethanol. All hormone stocks were made just prior to use and filter-sterilized. Plants were sprayed to run-off with a fine mist, essentially according to Guilfoyle (37) and harvested by pulling them from the agar with forceps. Plantlets were frozen in liquid nitrogen for RNA isolation or homogenized immediately for nuclear isolation.

**RNA Isolation—**For S1 nuclease assays, RNA was isolated according to Chirgwin et al. (43) with minor modifications. RNA was resuspended in 400 μl of diethyl pyrocarbonate-treated water and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1; v/v) followed by extraction with 1 volume of chloroform:isoamyl alcohol (24:1, v/v). Sodium acetate (pH 5.2) was added to 0.25 M and nucleic acids precipitated by addition of 2.5 volumes of 100% ethanol and centrifugation at 14,000 × g for 20 min (4 °C). Pellets were resuspended in 300 μl of diethyl pyrocarbonate-treated water followed by addition of 600 μl of ice-cold 4 M lithium chloride to precipitate RNA. Tubes were vortexed and incubated overnight on ice. Following centrifugation as...
above, pellets were resuspended in 100 μl of diethyl pyrocarbonate-treated water and quantified by absorbance at 260 nm. All RNA samples were also checked by agarose gel electrophoresis and ethidium bromide staining to verify their quality and equivalent concentrations.

**S1 Nuclease Protection**—rRNA transcripts were detected using S1 nuclease protection (44) and 5′ end-labeled probes as described previously (32, 33). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit mRNAs were detected using an oligonucleotide perfectly complementary to the A. thaliana ATs35 clone, but capable of hybridizing to transcripts of all four (known) genes (45). The sequence of the oligonucleotide is 5′ CCACAGCCGCGAGGAGCATAGAG-GAACGATTATTCTTGTGCGGCGC 3′; the underlined nucleotides in the 3′ tail are not homologous to any Rubisco mRNAs and are removed by S1 nuclease, discriminating the longest protected fragments from undigested probe.

**Nuclei Isolation**—Nuclei were isolated according to Feinbaum and Ausbel (46, 47) with modifications. Arabidopsis plants (~800) were harvested from 8 to 10 agar plates, washed in ice-cold sterile water, and kept at 4°C. Plants were submerged in cold diethyl ether for 5 min, then washed twice (5 min each) in ice-cold sterile water. Plants were homogenized in 100 ml (approximately 3 volumes) of nuclei isolation buffer (1 M sucrose, 50 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, 5 mM KCl, 10 mM 2-mercaptoethanol, 0.2 mM PMSF) with a motorized homogenizer (Fisher Scientific Powersher 700). The homogenate was filtered through eight layers of cheesecloth, one layer of Miracloth (Calbiochem), and one layer of 50-μm nylon mesh. The filtrate was centrifuged at 14,000 × g for 15 min at 4°C. The pellet was resuspended in 10 ml of nuclei isolation buffer using a 15-ml Dounce homogenizer (A pestle), and the final volume was measured. One volume of “100%” Percoll solution (34.23 g of sucrose, 5 ml of 1 M Tris-HCl (pH 7.2), 0.5 M of 1 M MgCl₂, 0.5 ml 1 x KCl, 34 μl of 2-mercaptoethanol, 100 μl of 2 mM PMSF, and Percoll to 100 ml) was added to 19 volumes of nuclei buffer. The cells were then layered onto a four-step discontinuous Percoll gradient with 5 ml of layers of 15, 30, 45, and 60% Percoll solutions (diluted from the 100% Percoll solution using nuclei isolation buffer). Gradients were centrifuged at 2000 rpm for 10 min, then 8000 rpm for 10 min, and washed twice (15 min each) in ice-cold sterile water. Plants were kept at 4°C. Plants were submerged in cold diethyl ether for 5 min. 15 ml of total nuclei were used. All nuclear run-on reactions were for 10 min (unless otherwise stated) at 30°C in a final volume of 50 μl (40 μl of run-on reaction buffer, 10 μl of nuclei). Nuclear run-on reaction buffer was: 50 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, 5 mM KCl, 5x SSC, 0.1% SDS, 50% glycerol. A aliquots were transferred to prechilled 1.5-ml microcentrifuge tubes, frozen in liquid nitrogen, and stored at −80°C.

**Nuclear Run-on**—Frozen nuclei were thawed on ice, stained with 4′,6-diamidino-2-phenylindole, and counted in a hemacytometer using fluorescence microscopy. Nuclear run-ons for filter hybridizations involved 1 × 10⁶ nuclei; for total incorporation experiments, 1 × 10⁷ nuclei were used. All nuclear run-on reactions were for 10 min (unless otherwise stated) at 30°C in a final volume of 50 μl (40 μl of run-on reaction buffer, 10 μl of nuclei). Nuclear run-on reaction buffer was: 50 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, 5 mM KCl, 75 mM (NH₄)₂SO₄, 1 mM MnCl₂, 300 μM each ATP, GTP, and UTP, 4.5 mM CTP, 0.5 mM [α-³²P]CTP, 50 mM dithiothreitol, 0.2 mM PMSF, 2 units of RNase inhibitor (Promega), 10% glycerol, and 150 μg/ml o-aminotri (inclusion in the amanitin level to 500 μg/ml was found to have no additional effect). 5 units of RNase-free DNase (Promega RQ1 DNase) was added and the incubation continued 10 min. 250 μl of 2 mg/ml Pronase, 1% SDS was added, and reactions were incubated for 30 min at 55°C. Reactions were extracted twice with phenol/chloroform and once with chloroform. Ammonium acetate was added to a final concentration of 2 M, and RNA was precipitated with 2.5 volumes of ice-cold 100% ethanol. Following centrifugation, RNA was pelleted and washed twice with 70% ethanol. Dried pellets were resuspended in filter hybridization solution, formamide RNA loading buffer, or TE (10 mM Tris-HCl, 0.1 mM EDTA) (pH 7.2) for hybridization, polyacrylamide gel electrophoresis, or scintillation counting, respectively.

**Contribution of Pol I to Total Nucleus**—Nuclear run-ons using 1 × 10⁶ nuclei were performed in the presence of 0 or 150 μg/ml o-amanitin (Sigma). Purified RNA was resuspended in 10 μl of TE (pH 7.2) and spotted onto DES1 paper (Whatman; ~1 cm²). Filters were washed five times in 300 ml of 5% NaHPO₄, and twice with 250 ml of sterile Milli-Q water. Washes were at room temperature, 5 min each. Filters were rinsed briefly in 100% ethanol to aid drying. After air-drying, filters were subjected to liquid scintillation counting.

**Filter Hybridizations for Nuclear Run-on Experiments**—435 μg of plasmid DNA (with or without cloned rDNA) was denatured for 10 min in 40 ml of boiling 0.4 M NaOH and loaded onto a 44-mm diameter circle of Zeta-Probe (Bio-Rad) nylon membrane by vacuum filtration in a Millipore filter apparatus. A wash with 40 ml of 0.4 M NaOH was followed by two washes with 200 ml of sterile water. DNA was covalently cross-linked to the membrane using UV light (Bio-Rad Gene Linker GS; program C1). Filters were neutralized with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and rinsed in water. Circular filters of ~7-mm diameter were cut from the large filter using a sterilized hole punch. Each filter contained ~15 μg of DNA. Filters were coded for later identification and were stored under vacuum.

**Filters** were pretreated with sterile water and submerged in 0.4–0.6 ml of hybridization solution (50% denatured formaldeh, 5 × Denhardt’s solution, 5 × SSC, 10 mM EDTA, 0.25% SDS, 50 μg/ml yeast tRNA) in 15-ml plastic snap-cap tubes for 1 h at 43°C. Each 15-ml tube contained a plain filter (no DNA), two filters with pHosphate (Stratagene) plasmid DNA, and two filters with cloned rRNA gene sequences (essentially a complete gene cloned in pHosphate). Radioactive RNA from a nuclear run-on reaction was resuspended in 200 μl of hybridization solution, denatured at 65°C, 10 min and added to a tube with a set of filters. Hybridization reactions were incubated overnight at 43°C. Filters were rinsed in 2 × SSC, incubated in 20 μg/ml RNase A (in 2 × SSC) at 30°C for 30 min to remove unhybridized RNA, then washed at high stringency (0.2 × SSC, 0.1% SDS, 65°C, 1 h). Filters were subjected to autoradiography and quantitation by phosphorimaging (Molecular Dynamics).

**RESULTS**

The A. thaliana rRNA gene promoter is located within the intergenic spacer approximately 2 kilobases upstream of the 18S coding sequences (Fig. 1). Noncoding sequences are removed in several processing steps, the first involving cleavage within the external transcribed spacer (ETS) (9, 48, 49). ETS processing occurs rapidly and is thought to be a co-transcriptional event (50–55). The promoter-proximal ETS RNA is then rapidly degraded, unlike coding sequences which can have half-lives longer than a cell cycle. Because ETS RNA does not accumulate, steady-state levels of transcripts just downstream of the promoter are expected to closely reflect rRNA gene transcription activity. Consequently, we measured transcripts accurately initiated at +1 to initially survey plant hormones for effects on rRNA gene transcription.

Aqueous solutions of gibberellic acid, 2,4-dichlorophenoxyacetic acid, abscisic acid, and kinetin were sprayed onto 3-week-old A. thaliana plantlets until run-off, according to the experimental protocol of Guilfoyle (37). Concentrations tested ranged from 10⁻⁷ to 10⁻³ M. Ethylene was also tested by gassing plants at 10–60 ppm in a specially designed chamber.
Cytokinin Induction of rRNA Gene Transcription

FIG. 2. Steady-state levels of accurately initiated rRNA transcripts increase in a time- and dosage-dependent manner following a single kinetin treatment. A, sterile-grown Arabidopsis plants were sprayed to run-off with the indicated concentrations of kinetin. Mock-treated plants were sprayed with a solution lacking hormone. 6, 12, or 24 h later, RNA was isolated, and nascent rRNA transcripts were detected by S1 nuclease protection using a 5' end-labeled probe spanning the promoter region (~520 to +92, labeled at +92; see Fig. 1 for the position of the probe). The same amount of total RNA (15 μg) was probed for each treatment. The panels show are from the same autoradiographic exposure of the same gel. S1 signals were also quantified by phosphorimaging; the -fold increase over the mock-treated plants is shown below each lane. The signal in the 24-h, 10 M kinetin treatment (marked with an asterisk below the lane) is low due to a pinched well in the acrylamide gel and loss of part of the sample during loading; no quantitation is provided for this one lane. B, ribosomal RNA and Rubisco transcript levels respond differently to kinetin treatment. Total RNA isolated from plants 12 h after spraying with various kinetin concentrations was hybridized to the rRNA gene promoter probe or to an oligonucleotide probe designed to hybridize to transcripts of the Rubisco gene family. The multiple S1 nuclease-protected bands observed with the Rubisco probe are as expected based on the positions where mRNA sequences of the different family members diverge from the probe sequence in their 5'-untranslated region.

(with the kind assistance of Dr. Harry Klee and the Monsanto Company, St. Louis, MO). RNA was isolated from control and treated plants at various times after treatment, and rRNA transcripts were detected using S1 nuclease protection as described previously (32, 33). Only cytokinin exerted a response in this initial survey (data not shown).

Cytokinin treatment induced nascent rRNA transcripts in a dose- and time-dependent manner (Fig. 2). Six hours after treatment, transcript levels were increased by kinetin at concentrations of 10^{-5} M or higher (Fig. 2A, lanes 3–6), reaching their maximum with 10^{-3} M exogenous hormone (lane 5). However, if RNA was isolated 12 or 24 h following treatment, an effect was discerned at concentrations as low as 10^{-4} M (lane 2). Note that these are the concentrations sprayed onto the exposed upper surfaces of the plants; internal concentrations are expected to be at least 100-fold lower based on surface area to volume estimations. Maximal induction of accurately initiated rRNA transcripts was approximately 6-fold and was observed 24 h after treatment at the highest kinetin concentrations (Fig. 2A, lanes 5 and 6).

The experiment of Fig. 2A showed that pre-rRNA transcripts increase within a fixed amount of total RNA in response to cytokinin. However, because ~80% of total RNA is ribosomal RNA coding sequences, our estimates of cytokinin responsiveness would be underestimated if the total ribosome pool were increased by cytokinin. At early time points, the ribosome pool is so large that such effects might not be significant. Nonetheless, we compared transcription initiation from the rRNA gene promoters to initiation from promoters of the Rubisco small subunit gene family, among the most highly expressed genes in green leaves. rRNA and ribulose bisphosphate carboxylase gene promoters showed different responsiveness to kinetin treatment (Fig. 2B). Whereas nascent rRNA transcript levels were increased severalfold per unit of total RNA in response to increased exogenous cytokinin concentrations (lanes 2–6), transcripts from the Rubisco gene family were unaffected or reduced at high concentrations. The decrease in Rubisco transcripts could be due to a kinetin-dependent inhibition of transcription, as reported for phytochrome mRNA (56), or a decrease in Rubisco RNA relative to the total RNA pool. Nonetheless, the data suggest that increased steady-state levels of rRNA transcripts is not part of a general positive response to cytokinin.

Although steady-state levels of nascent rRNA transcripts are thought to reflect transcription rates, due to the rapid turnover of ETS sequences, this has not been demonstrated in plants. Therefore, we also performed transcription run-on assays with nuclei of control and hormone treated plants (Fig. 3). Plants were again mock-treated (solution lacking hormone) or sprayed with various concentrations of cytokinin. Twelve hours later, nuclei were isolated and treated in four different ways. [32P]GTP was added to all reactions, but the remaining three nucleotides were withheld from half of the tubes (reactions 1 and 2, Fig. 3A) to control for possible nontranscriptional incorporation of the label. The remaining reactions (reactions 3 and 4) were provided with unlabeled ATP, CTP, and UTP to allow transcript elongation. In half of the reactions, α-amanitin was added to 150 μg/ml to inhibit pol II and pol III transcription, but not pol I (reactions 2 and 4). After a 10-min period to allow nascent transcripts to be elongated by template-engaged polymerases, RNA was purified and hybridized to an excess of DNA affixed to filters. Duplicate filters (lanes a and b) were used for each reaction. The DNA bound to the filters was either a denatured ribosomal gene clone or denatured pBluescript plasmid DNA, the latter serving as a control for the specificity of the hybridization conditions. After washing at high stringency, filters were exposed to x-ray film and were also quantified by phosphorimaging. As can be seen in Fig. 3, A and B, reactions 1 and 2, no signal was obtained if only the labeled nucleotide was provided to the nuclei. In contrast, adding all four nucleotides allowed the synthesis of radioactive transcripts that hybridized to rRNA gene filters (reactions 3 and 4), but not to pBluescript DNA filters (B). Note that run-on transcription signals were unaffected by α-amanitin (compare reactions 3 and 4), as expected for pol I transcription of rRNA. Importantly, the amount of run-on transcription was increased by kinetin treatment prior to nuclei isolation. Nuclear run-on signals were maximal following 10^{-3} M kinetin treatment and were 3.8-fold higher than in mock-treated plants (Fig. 3A). An increase in the kinetin concentration to 10^{-2} M decreased the response, suggesting inhibition at excessive concentrations.

The agreement between the nuclear run-on results and the S1 protection results of Fig. 2A is noteworthy (the 12-h time point of Fig. 2A is the relevant comparison). At 10^{-6} M kinetin, nuclear run-on assays showed a 1.4-fold increase over control nuclei (based on the average signals from the duplicate filters quantified by phosphorimaging), whereas S1 protection detected a 1.5-fold change. Likewise, relative signals at 10^{-5} M kinetin were 2.1- versus 1.9-fold; at 10^{-4} M were 2.7- versus 3.5-fold; and at 10^{-3} M were 3.8- versus 4.9-fold. For unknown reasons, the only large discrepancy was at the highest concentration of kinetin tested, 10^{-2} M, yielding a signal 1.7-fold higher than the control in the nuclear run-on assay, but 3.8-fold higher in the S1 protection assay. Nonetheless, we conclude that in Arabidopsis, as in other species, steady-state levels of rRNA transcripts initiated at +1 (detected by S1
nuclei of mock-treated plants; the -fold increases caused by kinetin are shown.

autoradiography, radioactivity bound to the filters was quantified by phosphorimaging. Average signals from duplicate filters were compared with

remaining nucleotides.

sprayed with 10

kineticsofthecytokininresponse.Plantsweremock-treatedor

protection) closely reflects levels of transcription.

Using the sensitive nuclear run-on assay, we examined the kinetics of the cytokinin response. Plants were mock-treated or sprayed with 10^{-4} \text{M} kinetin, and nuclei were isolated 1 or 6 h later (Fig. 3B). One hour after hormone treatment, rRNA transcription was stimulated 1.8-fold relative to control nuclei. At 6 h, the response was 2.4-fold in agreement with other experiments (Figs. 2A and 2A). For technical reasons, time periods shorter than 1 h were impractical. Nonetheless, these data suggest that induction of rRNA transcription by cytokinin occurs rapidly.

Increased rRNA synthesis in nuclear run-on assays could be due to transcription of more rRNA genes, a higher polymerase density on the same number of genes or more rapid transcript elongation. The latter possibility seems unlikely given that hormone-induced increases in transcripts initiated at +1 (detected by S1 protection) were paralleled by increased transcription throughout the body of the gene (detected by nuclear run-on). These data are most easily explained by kinetin affecting the frequency of polymerase I initiation rather than the rate of elongation. Time courses of nuclear run-on reactions also support the hypothesis that more RNA polymerase I molecules are engaged in transcription in hormone-treated plants.

In the experiment of Fig. 4, nuclear run-on reactions were performed using our standard conditions, but early time points in the reaction were examined. Even at the earliest time point (about 12 s), there is an approximately 2-fold increase in label incorporated in nuclei of treated plants. We interpret this to mean that an increased number of polymerase molecules are stalled on the rDNA of kinetin-treated nuclei. Upon addition of nucleotides, these resume transcription, the higher starting level in hormone-treated nuclei presumably reflecting the increased number of engaged polymerases. In contrast, if the same number of polymerase molecules were template-engaged but altered in their transcription rates, total isotope incorporation would be similar initially but would diverge with time (i.e. the lines connecting the data points would converge if extrapolated to time 0). Note that the zero time point provides no data in this assay, because endogenous transcripts are not labeled until transcripts begin to elongate. For this reason, there are no time 0 data points in Fig. 4.

**Fig. 3.** Nuclear run-on experiments confirm that kinetin induces increased rRNA transcription within 1 h of spraying whole plants. Equal numbers of nuclei isolated from plants 12 h after treatment at various kinetin concentrations (A) or harvested at 1 or 6 h following treatment at 10^{-4} \text{M} kinetin (B) were tested in 10-min nuclear-run-on reactions. Reactions 1 and 2 were incubated with only [32P]GTP, but not the remaining nucleotides. Reactions 3 and 4 received all four nucleotides, allowing transcription to occur. Reactions 2 and 4 included α-amanitin at 150 \mu g/ml to inhibit pol II and pol III transcription. RNA transcripts synthesized during the 10-min nuclear-run-on reaction were hybridized to duplicate (lanes a and b) nylon filters with covalently bound denatured rRNA gene DNA or denatured pBluescript plasmid DNA. Following autoradiography, radioactivity bound to the filters was quantified by phosphorimaging. Average signals from duplicate filters were compared with nuclei of mock-treated plants; the -fold increases caused by kinetin are shown.

**Fig. 4.** The kinetics of nuclear run-on transcription suggests that kinetin increases the number of template-engaged RNA polymerase I enzymes. The time course of α-amanitin-resistant incorporation of [32P]GTP into RNA is compared for nuclei of kinetin-treated (10^{-4} \text{M}, nuclei isolated 12 h after treatment), and mock-treated plants. For this experiment, radioactive RNA was separated from unincorporated GTP by denaturing gel electrophoresis, and radioactive signals were quantified by phosphorimaging. Background signals were subtracted for all time points. The data were fitted to straight lines using CricketGraph software. As discussed in the text, the higher initial incorporation of GTP in nuclei of hormone-treated plants suggests that kinetin primarily affects the number of template-engaged polymerase I molecules rather than the rate of elongation.

An experiment done in parallel with the one represented by Fig. 4 was performed to estimate the number of nucleotides incorporated per second in the nuclear run-on assays; these data are worth mentioning, though the data are not shown. Nuclei were treated with DNase-free ribonuclease to digest nascent transcripts protruding from the bound polymerase molecules. Nuclei were then washed and incubated in run-on conditions. Aliquots were taken at various intervals (10–300 s), and labeled transcripts were separated on a sequencing gel and visualized by phosphorimaging. Despite substantial size heterogeneity, computer imaging allowed us to identify the average transcript size at each time point by identifying the size range where the radioactive signal was highest. Such analyses led to an estimated average elongation rate of ~2 nucleotides/s. This
is in the same range as elongation rates of ~5 nucleotides/s within isolated animal nuclei (57). Importantly, we observed no obvious difference in average transcript size over time in nuclei of hormone-treated and control plants. However, transcription signals were approximately twice as strong in kinetin-treated nuclei. These data are consistent with those of Figs. 2–4, suggesting that kinetin increases the number of polymerase I enzymes engaged in RNA transcription.

Because pol I accounts for a large proportion of all nuclear transcription, we estimated the effect of kinetin on overall transcription by measuring total and α-amanitin-resistant [32P]GTP incorporation in nuclei of control and treated plants, which is calculated. The counts/min shown have been adjusted for background, determined by spotting filters with RNA from reactions containing only the labeled nucleotide, but not the remaining three nucleotides needed for transcript elongation.

![Table](image)

**Table**: Transcription of rRNA genes as a percent of total transcription

| Trial # | α-Amanitin (μg/ml) | Mock (μg/ml) | Kinetin-α-Amanitin (μg/ml) | Kinetin effect (fold increase) |
|---------|-------------------|-------------|---------------------------|-------------------------------|
| 1       | -                 | 156,359     | 156,800                   | 1.0                          |
|         | +                 | 50,099      | 88,329                    | 1.8                          |
| % Pol I | -                 | 32%         | 56%                       |                               |
|         | +                 | 49,469      | 85,321                    | 1.7                          |
| % Pol I | -                 | 30%         | 63%                       |                               |
|         | +                 | 30%         | 63%                       |                               |

* = corrected for background

**FIG. 5.** Polymerase I transcription as a percent of total transcription nearly doubles in response to kinetin. Total incorporation of [32P]GTP into RNA (counts/min) in 10-min nuclear run-on reactions are given for two independent trials in the absence or presence of 150 μg/ml α-amanitin. The effect of kinetin and the relative proportion of α-amanitin-resistant transcription (polymerase I) in nuclei of control and treated plants is calculated. The counts/min shown have been adjusted for background, determined by spotting filters with RNA from reactions containing only the labeled nucleotide, but not the remaining three nucleotides needed for transcript elongation.

Cytokininresponsive to kinetin is reasonable in light of the role of cytokinins in cell division (cytokinesis). Dividing cells partition their ribosomes among daughter cells, thus RNA synthesis might need to be up-regulated to replenish the ribosome pool. However, up-regulation within 1 h of kinetin treatment is unlikely to be a consequence of cell division. Furthermore, DNA content in total nucleic acid extracts, detected by Hoechst dye binding and fluorometry, was not appreciably increased at 1, 6, or 12 h following cytokinin treatment and was only slightly increased at 24 h (data not shown).

We were surprised that other hormones did not exert an effect on RNA gene transcription in our initial screen. In particular, we thought auxin (specifically 2,4-dichlorophenoxyacetic acid) might elicit a positive response. Guifoyle (37) clearly showed that RNA polymerase I activity on heterologous template DNA (e.g. sheared calf thymus DNA) was about 10-fold higher in cell-free extracts of soybean hypocotyl treated with 2.5 × 10⁻³ M 2,4-dichlorophenoxyacetic acid. Lack of an auxin effect in our experiments might suggest that pol I levels alone are not limiting for promoter-dependent rRNA gene transcription within the plant cell. Perhaps increased levels or modifications of other transcription factors are also needed to facilitate promoter-dependent rRNA transcription, and these events are affected by cytokinin but not auxin.

Cytokinins are the least understood class of plant hormones in terms of their mechanisms of action and signal transduction pathways (59, 60). Few mutants affecting cytokinin metabolism are available. The A. thaliana mutant, amp1 (61), has endogenous cytokinin levels six times higher than normal and displays a rapid growth phenotype. Using the S1 protection assay, we found nascent rRNA transcripts in amp1 plants to be slightly higher than in wild-type plants (data not shown). However, kinetin responsiveness was similar in amp1 and wild-type plants. We also tested several mutants we have isolated that are resistant to cytokinin levels that inhibit seed germination and plantlet growth. Thus far, rRNA transcript levels are also increased in these lines in response to exogenous cytokinin treatment, although slight differences relative to control plants are suggested in some lines.2 Mutants disrupted in cytokinin signaling would clearly be valuable for future studies.

The specific responsiveness of rRNA gene transcription to kinetin suggests that in plants, cytokinins may be key signaling molecules involved in communicating cellular growth status to the transcription and protein synthetic machinery. It is intriguing that kinetin and ppGpp (in prokaryotes) are both modified purines. However, at present it is not clear if this is coincidence or a hint of a functional similarity.

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