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Polyribosome Formation in Relation to Cytokinin-induced Cell Division in Suspension Cultures of *Glycine max* [L.] Merr.[1]

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ABSTRACT

We have investigated the relationship between cell proliferation and protein synthetic capacity in a cytokinin-requiring strain of cultured soybean cells (*Glycine max* [L.] Merr. cv. Sodifuri, of cotyledonary origin) in suspension culture. When transferred to a defined medium lacking cytokinin, very little cell division or cell enlargement took place over the course of a 6-day culture period. Cells transferred to medium of the same composition, but containing 0.5 μM zeatin, exhibited rapid initial growth, with maximum mitotic activity occurring after 24 hours in culture, and a doubling of the cell population within the first 36 hours of the culture period. The polyribosomal RNA content of the cells decreased over the course of the first 24 hours of the growth cycle while the polyribosome to monoribosome (P/M) ratio increased. The increase in the P/M ratio was greater in the cytokinin-treated cells. This apparent relationship between cytokinin-induced cell proliferation and polyribosome formation was examined further. Polyribosome formation was stimulated when zeatin was added directly to cell populations which had been cultured for 24 hours in medium lacking a cytokinin. Transfer to fresh medium alone also stimulated polyribosome formation, whether this medium contained a cytokinin or not. The magnitude of transfer-induced polyribosome formation depended upon the initial cell density (number of cells/ml of medium). Regardless of the initial cell density and independent of the P/M ratios attained, the cytokinin-treated cell populations divided while the cytokinin-deprived cell populations did not. *In vivo* labeling with [^{35}S]methionine and slab gel electrophoretic separation of sodium dodecyl sulfate derivatives of the labeled polypeptides demonstrated qualitative changes in the spectrum of proteins synthesized by the cytokinin-treated cells. These qualitative changes were independent of the cell density (and hence, independent of the P/M ratio) but they preceded cytokinin-induced cell division.

Protein synthesis has been reported to be necessary for cell division (21, 41, 51). Presumably at least part of this relationship stems from the fact that specific cell division proteins must be synthesized according to a genetically determined program for a cell to progress through its division cycle (14, 15). This relationship does not necessarily mean that the regulatory mechanisms for the cell division cycle are related to protein accumulation (growth).

Cultured soybean cells of cotyledonary origin require cytokinin for growth and cell division (11, 12). Recently Short *et al.* (43) observed that the polyribosome content of these cultured cells was correlated with their mitotic activity. Furthermore, they found that cytokinin treatment of zeatin-deprived cells rapidly stimulated polyribosome formation, and that this polyribosome formation preceded cytokinin-induced cell division. Since there is evidence that cellular polyribosome content is a direct measure of protein synthetic activity in plants (1, 35, 46), these findings suggested that a cell's progress through its division cycle might be in some way regulated by the rate of protein synthesis. Cytokinin-induced growth and cell division thus would be a manifestation of the same hormonal effect, namely a stimulation of the over-all rate of protein synthesis.

The present study tests this hypothesis. The previous correlation between mitotic activity and polyribosome formation was obtained with agar plate-grown cells (43). An objection to these studies is that diffusion across the cell mass may cause nutrients to become growth-limiting as the cell mass increases in plate-grown cells. We examined growth, polyribosome content, and mitotic activity in soybean suspension cultures as a function of the presence or absence of cytokinin in the culture medium. These results confirm our previous findings that cytokinin is required for cell division in cultured soybean cells and that it stimulates polyribosome formation. The polyribosome levels of these cells also are stimulated by transfer to fresh medium, whether that medium contains cytokinin or not, and the P/M[2] ratio attained by a given soybean cell population is influenced by the culture density. It is clear that the attainment of a high P/M ratio is not a sufficient condition to permit cell division. We also found that cytokinin treatment brought about qualitative changes in the spectrum of proteins synthesized by cultured soybean cells which preceded hormone-induced cell division. We propose that cytokinin-treated cells divide because the hormone induces the synthesis of specific cell division proteins.

MATERIALS AND METHODS

Cell Cultures. Cells originally derived from cotyledons of *Glycine max* (L.) Merr, cv. Sodifuri were maintained on an agar-solidified, chemically defined medium designated SCF (12) with biweekly subculturing. To prepare suspension cultures, cell clumps were removed from the plates with a spatula placed in liquid SCF and shaken for approximately 1 hr. The cell suspension cultures were grown in the dark on a reciprocating shaker (45 cycles/min, 9.2-cm stroke) at 23 ± 2 C. The cells were then passed through a wire mesh screen, collected on Miracloth, washed with medium, and resuspended in fresh, liquid SCF. The cells were transferred every 6 days by collecting them on Miracloth, washing them with fresh medium, and dispensing aliquots of the wet cells to preweighted, sterile flasks. After determining the fresh wt of the cells in the flask, sufficient fresh SCF was added to give the desired cell density.

The actual cell density was estimated from the measured cell fresh wt or filtered cell volume. Cell number/g fresh wt and cell number/ml filtered cell volume were determined from representative cell populations. For routine transfers, cultures with

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[2] Abbreviations: P/M ratio: polyribosome to monoribosome ratio; EGTA: ethylene glycol-bis(2-aminoethyl ether)-tetraacetic acid.

an initial cell density of 0.1 to 0.25 × 10^6 cells/ml were established.

Staining Soybean Nuclei. The nuclei and chromosomes of the cultured soybean cells were stained by a modification of the Feulgen procedure (22). Cells were collected by filtration and resuspended in SCF medium containing 2% glutaraldehyde. After a 24-hr exposure to the fixative at 4 C, the cells were washed with water, treated with 0.5% $NaBH_4$ in 0.1% $NaH_2PO_4 \cdot H_2O$ for 1 hr, washed twice with water, and hydrolyzed by exposure to 6 N HCl for 12.5 min. After washing the cells twice with water they were stained in De Tomasi-Schiff reagent (37) for 2 hr. The stained cells were washed with water, suspended in 45% acetic acid, squashed onto gel-coated glass slides, and mounted.

The mitotic index was determined from the Feulgen-stained cells. One thousand cells were scored for each sample. Only cells in late prophase (nuclear envelope broken down, chromosomes condensed), metaphase, anaphase, and early telophase (before the reformation of the nuclear envelope) were scored as dividing.

Polyribosome Isolation. Cells were homogenized in a ribosome extraction buffer (50 mM tris-HCl, pH 8.5, 400 mM KCl, 20 mM Mg acetate) which was similar in composition to the high pH, high salt buffer systems devised by other investigators to minimize nuclease degradation of polyribosomes during extraction (2, 7, 39). The homogenate was centrifuged at 11,000 rpm for 15 min in a Sorvall SS-34 rotor at 0 to 4 C. The supernatant was layered over 1.8 M sucrose prepared in resuspension buffer (50 mM tris-HCl, pH 7.8, 200 mM KCl, 10 mM Mg acetate) and centrifuged for 3 hr at 50,000 rpm in the Beckman SW 50.1 rotor or for 4.7 hr in the Beckman SW 36 rotor (5.4 × 10^7 g-min of centrifugal force in either case). After pelleting the ribosomes, the supernatant was decanted, the tube walls were dried with a cotton swab, and the ribosomes were resuspended in 500 μl of resuspension buffer lacking sucrose. This solution was clarified by centrifuging out any unresuspended material, and the clear supernatant was removed and saved. In some cases the resuspended ribosomes were frozen for later analyses. We have found that the polyribosome patterns and the P/M ratios are virtually unaltered after storage for several weeks at −70 C, but that polyribosomes become extensively degraded within 8 hr if the resuspended ribosomes are frozen at −20 C.

Polyribosome Analysis. An aliquot of the resuspended ribosomes, containing between 30 and 300 μg of RNA, was layered onto a 10 to 40% sucrose gradient prepared in resuspension buffer and centrifuged for 35 min in a Beckman SW 50.1 rotor at 40,000 rpm or for 60 min at 36,000 in a Beckman SW 36 rotor. After centrifugation, 70% sucrose was pumped into the bottom of the centrifuge tube, forcing the gradient through a flow cell where the A at 260 nm was monitored continuously with a Beckman Acta III recording spectrophotometer. The P/M ratio was calculated by Xeroxing the A_{260} profile, drawing the base line (obtained by fractionating a blank gradient) under the profile, and then cutting out and weighing the paper representing the areas under the monoribosome and polyribosome regions of the profiles.

Extraction of Whole Cell RNA. Whole cell nucleic acids were extracted by a modified phenol-detergent procedure (17, 38). All glassware and reagents, except the SDS and phenol-chloroform mixtures, were autoclaved. The cells were collected on Miracloth filters, placed on aluminum foil squares, and frozen between blocks of dry ice. The frozen cells were partially thawed in RNA extraction buffer (50 mM tris-HCl, pH 8.5, 0.14 M NaCl, 10 mM EDTA, and 1% SDS). Then 2 volumes of a phenol-chloroform (2:1) mixture containing 4% isoamyl alcohol were added and the cells were homogenized with a Teflon-glass homogenizer at room temperature. The homogenate was centrifuged for 5 min at 10,000 rpm in the Sorvall SS-34, the aqueous phase saved, the organic phase was discarded, and the interphase was resuspended in fresh RNA extraction buffer (0.5 ml/g of starting material) with the aid of a Dounce homogenizer. Two volumes of fresh phenol-chloroform mixture were added and the preparation was shaken again. After centrifugation (10,000 rpm, 5 min), the aqueous phase was removed and transferred to the tube containing the first aqueous phase. The combined aqueous phases were extracted one additional time with the phenol-chloroform mixture and then twice with chloroform alone. The aqueous phase was removed by pipette and the nucleic acids were precipitated with 2.5 volumes of ethanol at −20 C overnight. The precipitated nucleic acids were collected by centrifugation (10,000 rpm, 10 min), dried with a stream of N_2 gas, and dissolved in 0.15 M sodium acetate (adjusted to pH 6 with acetic acid). Material insoluble in 0.15 M sodium acetate was removed by centrifugation, and the nucleic acids were again precipitated with 2.5 volumes of cold ethanol. Finally, the nucleic acids were collected by centrifugation, dried, and dissolved in Loening's (31) electrophoresis buffer containing 0.2% SDS or Sarkosyl.

To estimate the loss of extracted RNA during the course of its purification, 50 μl of a solution containing a total of 35 μg of soybean ^{3}H-labeled rRNA (1,760 cpm/μg of RNA) was added to each sample before homogenization. After purification by the above described procedure, the nucleic acids, dissolved in electrophoresis buffer, were precipitated from a 50-μl aliquot by the addition of ice-cold trichloroacetic acid (5%). The precipitated nucleic acids were collected on a glass fiber filter disc and washed three times with cold 5% trichloroacetic acid. After air-drying, the radioactivity in the precipitate was determined by liquid scintillation in a Triton X-100-toluene cocktail. The efficiency of RNA extraction was estimated from the percentage of the ^{3}H counts recovered.

The UV absorption spectrum of the purified nucleic acid preparations in electrophoresis buffer was determined between 235 and 290 nm. The A_{260}/A_{280} ratios of the samples ranged from 2.07 to 2.16. The nucleic acid concentration of these solutions was determined from the A_{260} reading, which was converted to weight of nucleic acid using the assumption that 20 A_{260} units = 1 mg nucleic acid/ml of solution. The DNA content of the solutions was determined by the diphenylamine reaction (3) using salmon sperm DNA (Calbiochem) as a standard. The RNA content was determined by subtracting the DNA content and the amount of added [^{3}H]rRNA recovered from the total nucleic acid content of the samples.

RNA Extraction from Isolated Ribosomes. Isolated ribosomes were resuspended in RNA extraction buffer containing 1% Sarkosyl. Two volumes of phenol-chloroform (2:1) containing 4% isoamyl alcohol were added and the mixture was shaken vigorously at room temperature. After centrifugation at 10,000 rpm for 1 min, the organic phase was removed and discarded. Fresh phenol-chloroform was added and the mixture was shaken, centrifuged, and the organic phase discarded as above. The aqueous phase plus interphase then was extracted two to three times with chloroform until the interphase was transparent. Finally, the aqueous phase was removed and the RNA was precipitated with 2.5 volumes of ethanol at −20 C. The precipitated RNA was washed once with 70% ethanol containing 0.1 M sodium acetate and 0.5% Sarkosyl, dried with a stream of N_2 gas, and dissolved in electrophoresis running buffer for further analysis.

Gel Electrophoresis. Whole cell and polyribosomal RNAs were separated by polyacrylamide gel electrophoresis (31). Samples were dissolved in electrophoresis buffer (36 mM tris, 30 mM NaH_2PO_4, 1 mM Na_2 EDTA, pH 7.8) containing 0.2% SDS and 10% glycerol. Aliquots were loaded onto 2.2% acrylamide gels in glass tubes (0.5 × 10 cm) which had been

pre-electrophoresed for 2 hr at 6 mamp/gel in electrophoresis buffer containing SDS. After electrophoresis for 3 hr at 6 mamp/gel in a water-cooled Hoefer electrophoresis apparatus, the gels were removed from the tubes, dialyzed for 1 hr against several changes of distilled H_2O, and scanned at 260 nm with a Beckman Acta III spectrophotometer equipped with a Beckman gel-scanning attachment.

DNase Digestion. The precipitated, dried RNA samples were dissolved in 10 mM tris-HCl, pH 7.4, containing 0.3 M NaCl and 5 mM $MgCl_2$. An aliquot containing two A_{260} units of nucleic acids was removed and 25 μl of a DNase (Calbiochem) solution in the same buffer (2.5 mg/ml) was added. This solution was incubated at 37 C for 45 min. The reaction was terminated by shaking the solution for 5 min with a 0.5 ml of chloroform. The aqueous phase was removed, the nucleic acids were precipitated with 2.5 volumes of cold ethanol, the precipitate was dried with N_2 gas, and the nucleic acids were redissolved in electrophoresis running buffer for analysis.

SDS Gel Electrophoresis. [^{35}S]Methionine (30 Ci/mmol) was prepared from [^{35}S]sulfate by the method of Graham and Stanley (13). Sufficient isotope was added to the soybean cell suspensions to give 2 μCi [^{35}S]methionine/ml. Approximately 70% of the isotope was taken up by the cells within 10 min of its addition to the cultures. After exposure to the isotope, the cells were washed with cold tris-HCl buffer (62.5 mM tris, pH 6.8) and homogenized in the same buffer containing 5% SDS, 1% mercaptoethanol, and 5% glycerol at a ratio of 1 ml of buffer/g of cells. The homogenate was heated in a boiling water bath for 5 min, cooled to room temperature, and centrifuged at 2,000*g* for 30 min. The 2-mercaptoethanol concentration of the supernatant was brought to 5% prior to electrophoresis. A 20-μl aliquot of the supernatant, which contained between 1.2 and 1.8×10^5 acid-precipitable cpm, was loaded into each slot of the slab gels for electrophoresis.

Gradient slab gels were prepared 8 to 24 hr prior to use by a modification of published procedures (23, 26). A 1.5-cm stacking gel (4% acrylamide) and a 13-cm resolving gel (8–13% acrylamide) were cast from a stock solution which contained 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bis-acrylamide. The gel solution also contained N,N,N',N'-tetramethylenediamine (TEMED) at a final concentration of 0.025% (v/v). The polymerization was catalyzed by the addition of sufficient 10% (w/v) ammonium persulfate to give a final concentration of 0.015% just prior to casting. Electrophoresis was conducted at a constant current of 10 mamp until the tracking dye was 1 cm from the bottom of the gel (about 12 hr). The gels were stained overnight in a 0.025% (w/v) Coomassie brilliant blue R in acetic acid-methanol-water (1:5:5) which was freshly prepared from a filtered 10× stock solution. Following destaining in 7.5% (v/v) acetic acid, the gels were photographed with Ektapan film, using a yellow filter. Autoradiographs of the dried gels were prepared by exposing them to Kodak X-O mat RXR5 medical x-ray film for 2 to 3 days. The mol wt of the soybean polypeptides was determined by comparing their mobilities to those of SDS derivatives of proteins of known mol wt (BSA, ovalbumin, and chymotrypsin) run on the same gel.

RESULTS

The response of soybean cells to cytokinin in suspension culture was examined during a 6-day growth cycle. Cells were harvested from suspension culture by filtration and used to inoculate flasks of fresh medium which either lacked a cytokinin or contained the cytokinin zeatin at a concentration of 0.5 μM. Samples were taken at daily intervals for determinations of polyribosome content, P/M ratio, mitotic index, cell number, and average cell size.

Growth. The cell population growth kinetics was linear over the course of the first 4 days after the cells were transferred to fresh medium containing 0.5 μM zeatin. The cell population doubled within 36 hr after transfer to cytokinin-containing medium and it doubled once again during the 6-day culture period. Only a slight increase in cell number was observed when cells were transferred to medium lacking a cytokinin (Fig. 1).

The highest mitotic index was observed in the cytokinin-treated cells 24 hr after their transfer to fresh medium. Mitotic activity declined in the zeatin-treated cells thereafter, with the mitotic index reaching approximately 1% by the 3rd day after transfer. The cytokinin-deprived cells exhibited a low mitotic index during the early part of the culture period, but no further mitotic activity after 72 hr of culture in the cytokinin-free medium (Fig. 2).

The possibility that some degree of mitotic synchrony occurred in the cultured soybean cells, with peaks at times other than the above mentioned sampling intervals, was examined. Suspension cultures were established as previously described. These were sampled at 4-hr intervals during the first 48 hr after transfer to fresh medium and the mitotic index of these samples was determined. The results of these experiments (data not shown) demonstrated that the mitotic index, began to rise 16 hr after the cells were transferred to medium containing 0.5 μM zeatin. A single mitotic index peak was observed at 24 hr, after which the mitotic index declined. The behavior of the cells in medium lacking cytokinin depended upon the age of the inoculum. When nondividing, stationary phase cells were transferred to medium lacking cytokinin, no subsequent cell division was observed. Low levels of mitotic activity were found during the first 48 hr when cells were transferred to fresh medium lacking cytokinin after 2 to 6 days in complete medium, with mitotic index values similar to those shown in Figure 2.

Cytokinin-related Changes in Average Cell Size. As another way of looking at the relationship between cellular growth and cytokinin, we determined the average cell diameter during the culture period following transfer. The average cell diameter decreased in the cells cultured in zeatin-containing medium. In contrast, there was no change in the average cell diameter of

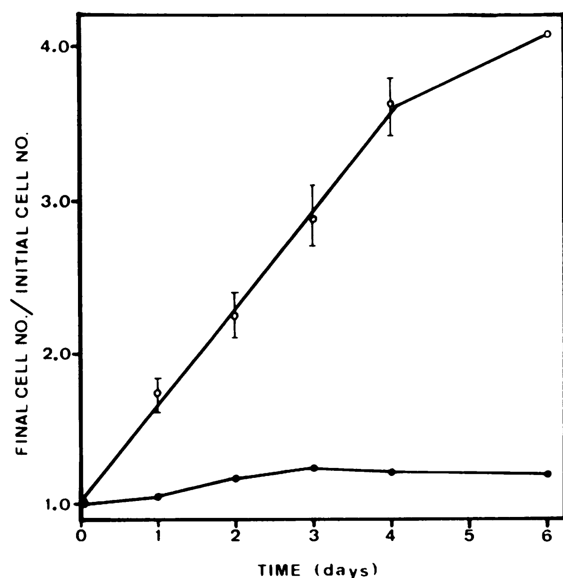

FIG. 1. Effect of cytokinin upon cell proliferation in soybean cell suspension cultures. Cells from 2-day-old suspension cultures were collected on Miracloth, washed with fresh medium, and transferred to fresh medium which either lacked a cytokinin (●——●) or contained 0.5 μM zeatin (○——○) at a density of 0.25×10^6 cells/ml.

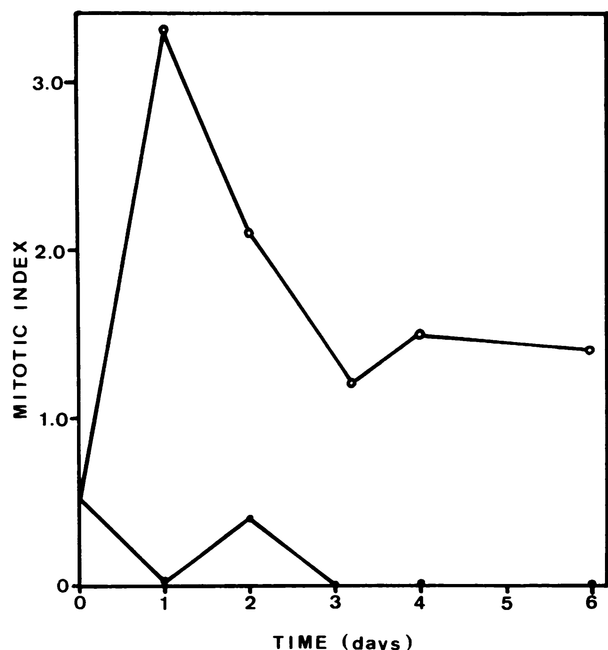

FIG. 2. Change in mitotic index as a function of cytokinin and time in culture. Cells from stationary phase suspension cultures were harvested on Miracloth, washed with fresh medium, and used to inoculate flasks of fresh medium which either lacked a cytokinin (●——●) or contained 0.5 μM zeatin (○——○). Initial cell density was 0.25×10^6 cells/ml.

Table I. Changes in average cell size in soybean cell populations cultured in liquid medium containing or lacking cytokinin.

At the time of inoculation and at intervals thereafter, cell populations cultured in media containing 0.5 µM zeatin, or lacking a cytokinin, were sampled. Cell diameter was determined by microscopy with the aid of a calibrated ocular micrometer. Approximately 35% of the cells in all of the populations examined were spherical. For the remainder of the cells, which were elliptical, minimum and maximum diameters were measured and an average cell diameter was determined. The data below represent the mean cell diameter from 100 determinations for each sample ± the standard deviation.

Days in Culture	Cultural Conditions	Mean Cell Diameter	Giant Cell Diameter	Frequency of Giant Cells
		µm		%
0 (inoculum)	-	73.1±10.4	111±4.2	2.1
1	no cytokinin	73.0±10.5	108±7.4	5.8
4	no cytokinin	72.6±9.6	110±6.7	11.1
1	0.5 µM zeatin	66.9±9.6	-	0
4	0.5 µM zeatin	68.9±10.5	-	0

the majority of the cells which were cultured in medium lacking cytokinin. The average cell of the inoculum has a diameter of 73 μm, as did the zeatin-deprived cells during at least the first 4 days of the culture period. Some cells were observed in cytokinin-deprived populations which were very much larger than the average cell. These "giant" cells tended to form a subpopulation in which the average cell diameter was nearly 3 standard deviations removed from the mean cell diameter of the bulk of the cell population. The frequency of these "giant" cells increased with increasing duration of culture in medium lacking cytokinin. They represented about 2% of the cells in the inoculum and about 11% of the cells in the population deprived of cytokinin for 4 days. No "giant" cells were observed in the cytokinin-treated cell population (Table I).

Polyribosome Content. Two measurements are necessary to determine the polyribosome content of cells. First, one must determine the P/M ratio, which is a measure of the proportion of the total ribosomes which are present in polyribosomal aggregates. Second, it is necessary to determine the total cytoplasmic RNA content of the cells. We made these determinations in cytokinin-treated and cytokinin-deprived soybean cells at intervals after transfer to fresh medium.

The P/M ratio increased in cells transferred to fresh medium, regardless of its composition. However, a larger increase was observed in the cytokinin-treated cells. The P/M ratio remained relatively constant throughout the culture period at slightly above 1.0 in cells cultured in medium lacking cytokinin. In contrast, the P/M ratio increased from 2.2 on the 1st day to 3.0 on the 4th day of the culture period in the cytokinin-treated cells (Fig. 3).

The RNA content of the extracted ribosomal material decreased over the first 24 hr of the culture period (Fig. 4). This

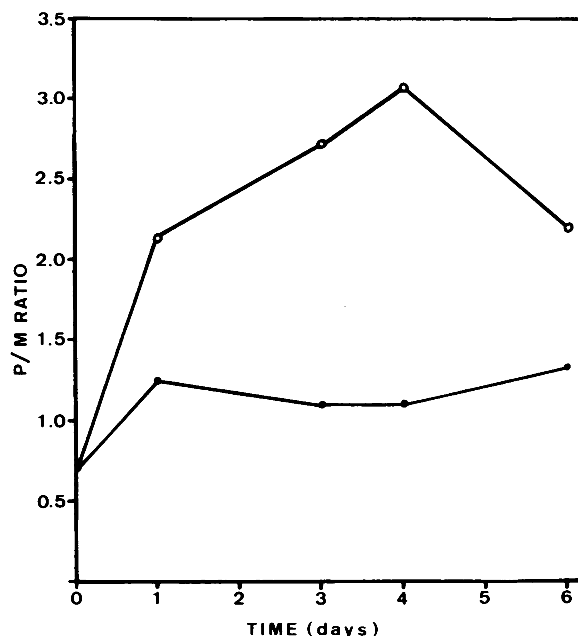

FIG. 3. Polyribosome content of soybean suspension cultures as a function of time in culture and composition of culture medium. Stationary phase soybean cells were inoculated into flasks of fresh medium which either lacked a cytokinin (●——●) or contained 0.5 μM zeatin (○——○) at a density of 0.25×10^6 cells/ml. Ribosomes were extracted from samples harvested at intervals thereafter.

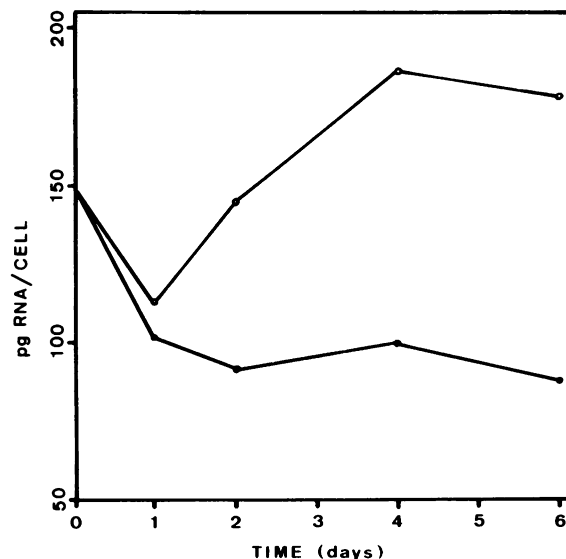

FIG. 4. Change in cellular RNA content as a function of time in culture and composition of culture medium. Stationary phase soybean cells were inoculated into flasks of fresh medium which either lacked a cytokinin (●——●) or contained 0.5 μM zeatin (○——○) at a density of 0.25×10^6 cells/ml.

decrease was most apparent in the cytokinin-deprived cells where it represented a loss of nearly 50% of the polyribosomal RNA found in the inoculum. After this initial decrease, the polyribosomal RNA content of the cytokinin-deprived cells remained constant throughout the remainder of the culture period. In the cytokin-treated cells, the initial slight decrease in ribosomal RNA content after 24 hr in culture was followed by an increase, which continued for 3 subsequent days.

Polyribosomal RNA as a Measure of the Whole Cell RNA Content. In the extraction of ribosomes from cultured soybean cells, the cells were broken with a Teflon-glass homogenizer. Microscopic examination of these homogenates indicated that not all of the cells were broken open by this procedure. The exact extent of cell breakage was difficult to determine. Certainly many cells survived intact after as many as 10 strokes of the motor-driven homogenizer. This fact raised the possibility that the decreased RNA content of the ribosomal pellets obtained from cells after 24 hr in culture could result from a change in the ease with which cells are disrupted, rather than representing a true change in the polyribosome content of the cells.

To investigate this possibility, cells were transferred to fresh liquid medium which either contained or lacked cytokinin, and at 0, 24, and 48 hr after transfer, the cultures were sampled. Whole cell RNA was extracted from half of each sample, while the ribosomes were pelleted from homogenates of the remaining cells. As was found in the previous experiment, the amount of polyribosomal RNA recovered from the cells declined during the first 24 hr after transfer to fresh medium. This decrease was paralleled by a similar decrease in the amount of extractable, whole cell RNA (Table II).

Although the amount of polyribosomal RNA we recovered from the cells by our extraction procedure tended to be a fairly constant percentage of the total cellular RNA, it represented somewhat less than 25% of the total. Recently Jackson and Larkins (18) reported that Ca^{2+} ions precipitated and reduced the yield of polyribosomes from tobacco leaves. They found that the incorporation of the chelator ethylene glycol-bis(2-aminoethyl ether) tetraacetic acid (EGTA) into the extraction buffer both improved the yield and decreased degradation in the isolation of polyribosomes from expanded leaf tissue. Therefore, we compared two EGTA-containing buffer systems to the one we routinely employ to see if we could improve the efficiency of polyribosome extraction from cultured soybean cells. Cells which had been in culture for 10 days in medium lacking cytokinin were collected on Miracloth, washed with one of three extraction buffers, and then homogenized in the same buffer, After pelleting the ribosomes through 1.8 M sucrose, the yield of polyribosomal RNA was determined from the A_{260} readings of the resuspended ribosomes.

The maximum yield of polyribosomal RNA was obtained with the buffer system we have devised for extracting polyribosomes from cultured soybean cells. The two EGTA-containing extraction buffers reduced the yield of polyribosomal RNA by 80 and 50%, respectively (Table III). Not only was the polyribosome yield reduced when soybean cells were homogenized in the EGTA-containing buffers, but the profiles obtained upon sucrose density gradient fractionation of the polyribosomes demonstrated that they were extensively degraded as well (Fig. 5).

Table II. A comparison of the yield of extractable, polyribosomal RNA to the whole cell RNA content of cultured soybean cells.

Soybean cells in suspension culture in medium containing or lacking cytokinin were harvested at the indicated times. The whole cell RNA content was determined from half of the cells while the polyribosomes were extracted and analyzed from the remaining cells as described in Materials and Methods.

Treatment	Whole Cell RNA		Polyribosomal RNA	
	pg RNA/cell	% Recovery	pg RNA/cell	% of whole Cell RNA
Inoculum	810.9	82.2	146.2	18.0
1 day in culture, no cytokinin	725.2	80.1	122.8	16.9
2 days in culture, no cytokinin	602.3	83.2	118.9	19.7
1 day in culture, 0.5 µM zeatin	639.6	79.4	124.8	19.5
2 days in culture, 0.5 µM zeatin	728.5	84.2	140.8	19.3

Electrophoretic Analysis of Polyribosomal and Whole Cell Nucleic Acid Preparations. Nucleic acid extracts prepared from polyribosomes and from whole cells were fractionated by polyacrylamide gel electrophoresis. The A_{260} profiles of the whole cell nucleic acid gels revealed nine distinct peaks or shoulders (Fig. 6A). Loening (30) has demonstrated that the mobility of RNA molecules in this gel system varies inversely with the log of their mol wt. By comparing the mobilities of these nine molecular species to those of RNAs of known mol wt (soybean ribosomal RNAs, yeast tRNA) in the same gel system, we were able to identify most of the components of the whole cell nucleic acid extracts (Table IV). Peak 1 probably represented DNA since it was no longer present when the extract was treated with DNase prior to electrophoresis. Peaks 2 and 3 were not identified although peak 3 is similar in mol wt to a rapidly labeled nuclear RNA which has been shown to represent a ribosomal RNA precursor in plants (29, 40). The mol wt of peak 2 is nearly the same as that of a large, rapidly labeled, methylated, nuclear RNA found in cultured *Acer* cells whose function has not been unambiguously determined (6).

Table III. The effect of the composition of the extraction buffer on ribosome yield

Weighed aliquots of 10-day-old soybean cells were homogenized in one of three ribosome extraction buffers as listed below. After pelleting the ribosomes by centrifugation as described in Materials and Methods, the RNA content of the resuspended ribosomal pellet was determined from its A_{260} reading. This figure was converted to µg of RNA using the conversion factor 20 A_{260} units = 1 mg RNA/ml.

Homogenization Buffer	µg RNA/gm Cells
Tepfer and Fosket buffer (unpublished - see Materials and Methods for composition)	19.2
Jackson and Larkins(18) buffer: 200 mM Tris-HCl pH 9, 400 mM KCL, 3.5 mM $MgCl_2$, 25 mM EGTA	3.5
Manning's buffer (personal communication): 30 mM Tris-HCl pH 7.5, 25 mM NaCl, 50 mM $MgCl_2$, 25 mM EGTA, 4 mM dithiothreitol, 0.58 M sucrose	11.4

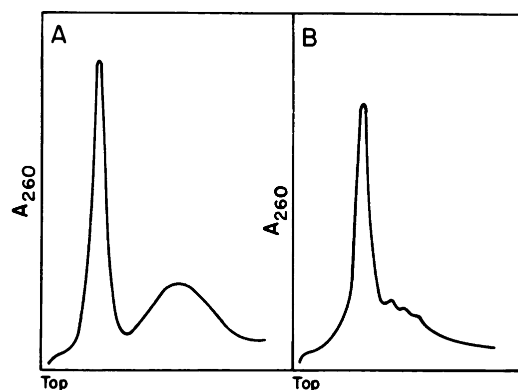

FIG. 5. Polyribosome profiles of ribosomal material extracted from soybean cells. Soybean cells were harvested after 10 days in culture in medium lacking cytokinin. They were weighted and then homogenized in either the ribosome extraction buffer described under "Materials and Methods" (A) or in the EGTA-containing buffer of Jackson and Larkins (18) (B). Resuspended ribosomal pellets were loaded onto 10 to 40% sucrose gradients which were centrifuged and fractionated as described under "Materials and Methods."

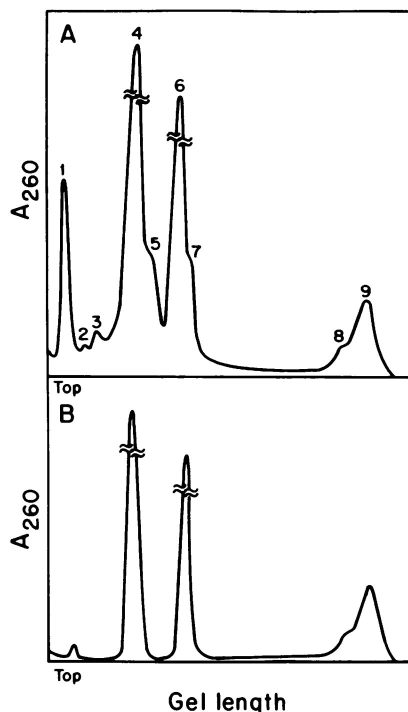

FIG. 6. Polyacrylamide gel electrophoresis of soybean whole cell and polyribosomal RNA. Fifty-μl aliquots containing either 65 μg of whole soybean cell nucleic acids (A) or 50 μg of soybean polyribosomal RNA (B), both dissolved in electrophoresis running buffer plus 0.2% SDS and 10% glycerol, were loaded onto 2.2% acrylamide gels and subjected to electrophoresis at 6 mamp/gel for 3 hr. After the run, the gels were soaked in distilled H_2O for 1 hr before scanning them at 260 nm with a Beckman Acta III spectrophotometer.

Table IV. Whole cell nucleic acids extracted from cultured soybean cells.

Nucleic acids were extracted from whole soybean cells as described in Materials and Methods. After subjecting aliquots of these extracts to polyacrylamide gel electrophoresis and scanning the gels at 260 nm, the mobility of the individual nucleic acids was determined. The molecular weight of these components was estimated from a semi-log plot of electrophoretic mobility against log (molecular weight), which was calibrated with RNAs of known molecular weight after electrophoresis under the same conditions. For peak number, see Fig. 3a.

Peak	Mol. Wt.	Identity of the Nucleic Acid
No.	Daltons	
1	5.0×10^6	DNA
2	3.5×10^6	unknown
3	2.85×10^6	unknown
4	1.4×10^6	25S rRNA
5	1.0×10^6	organelle RNA
6	0.7×10^6	18S rRNA
7	0.55×10^6	organelle RNA
8	4.0×10^4	5S rRNA
9	2.5×10^4	4S rRNA

The electrophoretic patterns of RNAs extracted from ribosomal pellets were considerably simpler. Two symmetrical peaks were observed which had the mobilities of 25S and 18S ribosomal RNAs, respectively (Fig. 6B), as well as a more diffuse peak with a shoulder representing the 4S and 5S RNAs. No evidence for contamination of the polyribosomal pellets by organellar RNAs or by nuclear RNAs was found in the gel scans, although on occasion a small DNase-sensitive peak was observed near the top of the gel. This peak, when it was present, corresponded to peak 1 in the whole cell electrophoretic patterns (compare Fig. 6, A and B).

Cytokinin-induced Polyribosome Formation and Cell Division. The data presented in Table V, as well as our previous work (43) demonstrated that the transfer of soybean cells to fresh medium is sufficient to induce polyribosome formation, whether or not the medium contains cytokinin. When stationary phase cells with a low P/M ratio were transferred to fresh medium lacking a cytokinin at a density of 2.5×10^6 cells/ml, the P/M ratio increased nearly 4-fold over the course of the subsequent 27 hr. When the same cells were transferred a second time to fresh medium, 24 hr after the first transfer, a further increase of 1.5-fold in the P/M ratio occurred over the course of the next 3 hr, while transfer to medium containing cytokinin brought about a 2-fold increase in the P/M ratio.

The magnitude of the transfer effect on polyribosome formation is influenced by a variety of factors, including the P/M ratio of the inoculum, the initial culture density, and the presence or absence of cytokinin in the culture medium. The significance of the latter two variables is illustrated by the data in Table VI. When cells were transferred to fresh medium lacking a cytokinin at either a high (2.5×10^6 cells/ml) or a low (0.25×10^6 cells/ml) density, only a small change in the P/M ratio was noted. At an intermediate density (1.25×10^6 cells/ml), the P/M ratio increased nearly 4-fold upon transfer to the same medium. Higher P/M ratios were observed when the cells were transferred to medium containing a cytokinin, but again the magnitude of the increase was dependent upon the initial cell density. The highest P/M ratios again were observed when the initial cell density was 1.25×10^6 cells/ml, with either lower or higher cell densities resulting in lower P/M ratios.

Cell division was not correlated with the P/M ratios attained. No cell division was observed in any of the cell populations cultured in medium lacking a cytokinin, despite the fact that a high P/M ratio was achieved in cultures with a density of 1.25×10^6 cells/ml. All of the cytokinin-treated cell populations divided, regardless of the initial cell density. There was no correlation between the mitotic index of the cytokinin-treated cell populations and their P/M ratios (Table VI.) At an initial density of 2.5×10^6 cells/ml, cell division ceased after 48 hr in culture, even though a very high P/M ratio was attained. The

Table V. The effect of transfer to fresh medium upon the polyribosome levels of cultured soybean cells.

Stationary phase cells (23 days old) were harvested from Petri plates, washed with medium and cultured in fresh medium lacking cytokinin at a density of 2.5×10^6 cells/ml. Twenty-four hr later some of the cultures were transferred to fresh medium which either contained 0.5 µM zeatin or lacked a cytokinin, while the cells of one culture remained in the original medium. Three hr after the second transfer, the cells were harvested from all three cultures, ribosomes were extracted, and the polyribosome/monoribosome ratios were determined as described in Materials and Methods.

Treatment	P/M Ratio
Inoculum (23-day-old plate-grown cells).	0.69
Cells cultured for 27 hr in medium lacking cytokinin.	2.67
Cells cultured for 24 hr in medium lacking cytokinin, followed by transfer to fresh medium lacking cytokinin and culture for an additional 3 hr.	4.09
Cells cultured for 24 hr in medium lacking cytokinin, followed by transfer to fresh medium containing 0.5 µM zeatin and culture for an additional 3 hr.	5.35

Table VI. The effect of density on polyribosome formation and cell division in suspension cultures of soybean cells

Cells from stationary phase suspension cultures were harvested on Miracloth, washed with fresh medium lacking cytokinin, and used to inoculate flasks of fresh medium, which either contained 0.5 µM zeatin or lacked a cytokinin, at the indicated density. Twenty-four hr later the cultures were harvested and their mitotic index determined as described in Materials and Methods. The P/M ratios represent data from a different experiment in which cells were cultured for 24 hr at the indicated cell densities and then harvested for determination of the P/M ratio.

Medium	Cell Density	Mitotic Index at 24 hr	P/M Ratio
	cells/ml	%	
Inoculum	--	--	1.08
SCF, no cytokinin	2.5×10^6	0	2.31
SCF, no cytokinin	1.25×10^6	0	4.06
SCF, no cytokinin	0.25×10^6	0	1.24
SCF, 0.5 µM zeatin	2.5×10^6	3.42	4.25
SCF, 0.5 µM zeatin	1.25×10^6	3.11	5.25
SCF, 0.5 µM zeatin	0.25×10^6	2.81	2.19

cytokinin-treated cells would continue to divide after the first 48 hr only when the initial cell density was between 0.25×10^6 and 0.1×10^6 cells/ml. At these densities, the increase in the P/M ratio upon transfer to fresh cytokinin-containing medium was comparatively small.

The data presented in Table VII demonstrate that cytokinin stimulates polyribosome formation even in the absence of a transfer to fresh medium. For this experiment, stationary phase cells were cultured in medium lacking a cytokinin at a density of 0.25×10^6 cells/ml. Twenty-four hr later, sufficient zeatin in sterile distilled H_2O was added to half of the cultures to give a final concentration of 0.5 μM zeatin. The same volume of sterile distilled H_2O was added to the remaining cultures. Three or 18 hr later the cultures were harvested to determine the yield of polyribosomal and nonpolyribosomal RNA and the P/M ratios. The P/M ratios observed in the cytokinin-treated cell population were 26% higher after 3 hr of treatment and between 88 and 104% higher after 18 hr, depending upon the amount of centrifugal force used to spin down the ribosomal material.

In the experiment described above we also investigated the effect of centrifugal force upon the observed P/M ratios since it has been shown that the P/M ratio obtained with plant tissues can be markedly altered by varying the centrifugal force used to obtain the initial ribosomal pellet (27, 28). The postmitochondrial homogenates from the 18-hr samples each were divided into two aliquots. These aliquots were subjected to two different centrifugal forces to obtain the ribosomal pellets which subsequently were analyzed to determine their P/M ratios. Decreasing the centrifugal force from that we routinely employed for this study decreased the yield of ribosomal material recovered in the ribosomal pellet (compare Tables II and VII). However, it also increased the observed P/M ratios. Regardless of the centrifugal force employed to obtain the ribosomal pellet, the P/M ratios obtained from cytokinin-treated cells were higher than those obtained from the cytokinin-deprived cells. Thus, cytokinin stimulated polyribosome formation when the hormone was added directly to the culture, without concomitant transfer to fresh medium, apparently by mobilizing existing cytoplasmic ribosomes into polyribosomal aggregates (Table VII).

Cytokinin-induced Qualitative Changes in Protein Synthesis. In order to determine whether or not cytokinin brings about qualitative as well as quantitative changes in soybean cell protein synthesis, we examined the pattern of newly synthesized proteins directly by isotope-labeling experiments. Stationary phase cells were transferred to fresh medium containing or lacking a cytokinin. The cells were exposed to [^{35}S]methionine. The proteins subsequently were solubilized in the presence of SDS and they were analyzed by electrophoresis on slab gradient polyacrylamide gels.

Autoradiographs of the gels showed at least four polypeptides whose synthesis was initiated by cytokinin treatment. These polypeptides, designated 1, 2, 3, and 4 in Figure 7, had mol wt of 82,000, 76,000, 67,000, and 44,500 daltons, respectively. In addition, there was at least one polypeptide (polypeptide 5, mol wt, 41,000) whose synthesis was repressed by cytokinin. These qualitative changes in the spectrum of proteins synthesized by the soybean cells appeared over the course of the first 24 hr after transfer to cytokinin-containing medium. The changes could be detected as early as 9 hr after transfer, although some qualitative differences were evident after only 3 hr of cytokinin treatment.

To see if these qualitative changes were correlated with elevated polyribosome levels or with cell division, cultures were prepared at three dilutions in medium lacking a cytokinin or containing 0.5 μM zeatin. Twenty-four hr later the cultures were exposed to [^{35}S]methionine and the proteins were subjected to SDS gel electrophoresis. An autoradiogram of the gel (Fig. 8) demonstrated that the same spectrum of proteins was

Table VII. Stimulation of polyribosome formation upon the addition of zeatin directly to cytokinin deprived soybean cell suspensions

Stationary phase soybean cells were transferred to fresh medium lacking a cytokinin at a density of 0.25×10^6 cells/ml. Twenty-four hr later an aliquot of a sterile zeatin solution was added aseptically to some of the cultures to give a final zeatin concentration of 0.5 μM. An equal volume of sterile distilled H_2O was added to control cultures. At 3 hr and at 18 hr after hormone addition, cultures were harvested for analysis. The cells were collected on Miracloth, washed with cold (0-4 C) medium, and then with cold extraction buffer before they were frozen on dry ice. The frozen cells were reduced to a powder with a prechilled mortar and pestle, the powder was resuspended and partially thawed in ribosome extraction buffer (2 ml/gm cells) and then homogenized with 10 strokes of a Dounce Tissue grinder (B pestle). The homogenate was centrifuged at 11,000 rpm for 15 min to obtain a post-mitochondrial supernatant. The supernatants from the 3-hr samples were centrifuged for 2.1×10^7 g/min with the Beckman SW 50.1 rotor, the post-ribosomal supernatant decanted and saved, while the ribosomal pellet was resuspended in ribosome extraction buffer. The post-mitochondrial supernatants from the 18-hr samples each were split into two aliquots. One aliquot was subjected to 2.1×10^7 g/min of centrifugation while the other was centrifuged for 5.4×10^7 g/min. In either case, the post-ribosomal supernatants were saved while the ribosomal pellets were resuspended in ribosome resuspension buffer. RNA was extracted from the post-ribosomal supernatants as described in Materials and Methods. The P/M ratio of the ribosomal pellets was determined from the polyribosome profiles as previously described while the RNA content of the ribosomal pellets was calculated from the A_{260} of the resuspended ribosomes.

	2.4×10^7 g/min centrifugation			5.4×10^7 g/min Centrifugation
Treatment	Polysomal RNA	Non-polysomal RNA	P/M	P/M
	pg/cell	pg/cell		
No cytokinin, 3 hr	100.8	58.1	2.93	-
0.5 μM zeatin, 3 hr	98.7	49.8	3.71	-
No cytokinin, 18 hr	85.0	55.8	3.01	1.36
0.5 μM zeatin, 18 hr	95.7	39.6	5.66	2.78

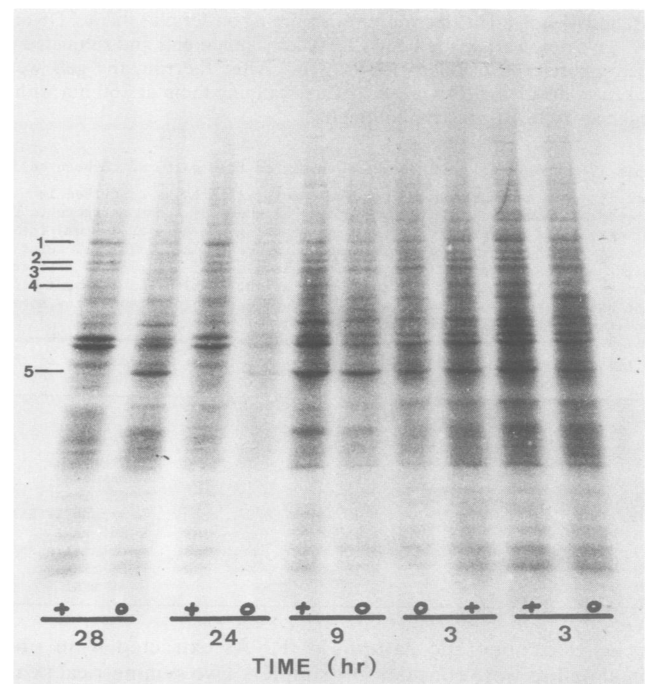

FIG. 7. Autoradiograph of a gradient polyacrylamide slab gel after SDS electrophoretic separation of proteins from soybean cells cultured for different periods of time in medium containing or lacking a cytokinin. Stationary phase cells were transferred to medium lacking a cytokinin. Cells were collected 24 hr later, washed with medium, and inoculated into fresh medium containing 0.5 μM zeatin or lacking a cytokinin at a density of 1.25×10^6 cells/ml. At 3 hr, 9 hr, and 24 hr after transfer, the cells were given a 10-min exposure to [^{35}S]methionine (20 μCi/10 ml culture). SDS derivatives of the proteins were prepared and separated on 2-mm-thick linear gradient polyacrylamide slab gel which was subsequently subjected to autoradiography. +: Extracts from cells cultured in medium containing 0.5 μM zeatin; 0: extracts from cells cultured in medium lacking a cytokinin.

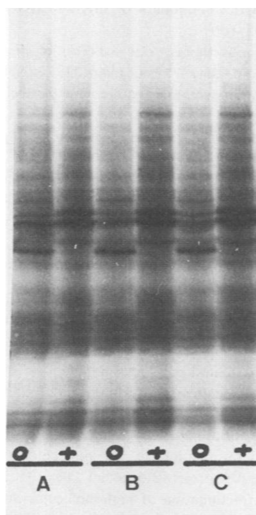

FIG. 8. Autoradiograph of a gradient polyacrylamide slab gel after SDS electrophoretic separation of ^{35}S-labeled proteins from soybean cells cultured under growing and nongrowing conditions. Stationary phase cells from plates were transferred to fresh medium lacking a cytokinin. Twenty-four hr later the cells were collected on Miracloth, washed with fresh medium, and transferred to fresh medium which either contained 0.5 μM zeatin or lacked a cytokinin at one of three culture densities. After an additional 24 hr in culture, 20 μCi of [^{35}S]methionine (30 Ci/mmol), prepared by the method of Graham and Stanley (13), was added to each culture. The cells were harvested 4 hr later and SDS derivatives were made of their protein which was separated on a 1-mm-thick gradient polyacrylamide gel in which the lower third was exponential while the upper two-thirds were linear. +: Extracts from cells cultured in medium containing 0.5 μM zeatin; 0: extracts from cells cultured in medium containing 0.5 μM zeatin; A: culture density of 2.5×10^6 cells/ml; B: culture density of 1.25×10^6 cells/ml; C: culture density of 0.25×10^6 cells/ml.

synthesized by the cytokinin-treated cells, regardless of the cell dilution. The cytokinin-deprived cultures all exhibited the same pattern of labeled proteins, different from that of the cytokinin-treated cells, despite the fact that the dilution effects on polyribosome formation might be expected to result in as much as a 4-fold difference in the P/M ratios of these cultures.

DISCUSSION

Soybean cells in suspension culture exhibited linear growth kinetics during the first 4 days of culture in medium containing 0.5 μM zeatin. Linear growth kinetic behavior is characteristic of a stem cell population (such as the vascular cambium) in which, after each division, one daughter cell continues into a subsequent division cycle while the other daughter cell leaves the proliferative cycle (5). Although linear growth kinetic behavior is unusual in cultured cells, Verma and Marcus (48) observed a similar growth pattern in peanut cell suspension cultures (also of cotyledonary origin). The latter cells exhibited a lag period of several days upon transfer to fresh medium. When linear growth began at approximately 3.5 days after transfer, the culture had 2.5×10^5 cell/ml. Cell number doubled by day 5 (36 hr later), with a second doubling occurring near the end of the linear growth phase on day 8. The chief difference between these peanut cell cultures and our soybean cell cultures is that the soybean cells do not exhibit any significant lag period between the time of transfer and the onset of growth.

It is possible that the 16-hr period between the time of transfer and the onset of cytokinin-stimulated mitotic activity represents, at least in part, a short lag period. It is also possible that this interval represents the expected duration of the phases of the cell cycle preceding mitosis. The fact that the cell population doubled within the first 36 hr after transfer and that the total duration of the cell cycle in other species of cultured plant cells varies between 24 and 48 hr (10, 24) suggests that the latter possibility may be the case. The simplest hypothesis which would explain our data is that cytokinin overcomes a block in the cell cycle so that most, if not all, of the cells enter the proliferative cycle upon transfer to fresh medium containing the hormone.

Cytokinins are characterized by their ability to stimulate cell division in plant tissue cultures (44). Numerous studies have shown that the cytokinins also stimulate protein and/or RNA synthesis in various plant tissues (8, 25, 32, 34, 45, 47, 50). Short *et al.* (43) demonstrated that polyribosome formation is rapidly stimulated when stationary phase soybean cells are treated with cytokinin. They also observed a correlation between the polyribosome content of agar plate-grown soybean cells and cell proliferation. Furthermore, Jouanneau (21) has shown that protein synthesis is necessary for cytokinin-induced cell division in tobacco suspension cultures.

Does cytokinin trigger cell division through its effect on protein synthesis? There are at least two ways this could occur. (*a*) It is possible that the relationship between accelerated rates of protein synthesis and the initiation of proliferation, which has been observed repeatedly (4, 19, 42, 48, 49), is the result of some common feature of eucaryotic cell cycle regulation. That is, cells usually grow during their cell cycle, or growing cells usually divide, because the rate of protein synthesis is important in determining whether or not critical steps in the cell cycle take place. (*b*) Cytokinins have been shown to induce the *de novo* synthesis of specific proteins (9, 16, 20) and could initiate qualitative changes in protein synthesis which would result in the synthesis of the specific cell division proteins which are necessary for a cell to progress through its division cycle (14, 15).

If the first hypothesis were true, one would expect to observe a close correspondence between the polyribosome content of a cell population and its mitotic activity. The results of the present investigation show that polyribosome content and mitotic activity are not closely correlated in soybean cell suspensions. The maximum rate of cell division was observed 24 hr after transfer to fresh medium containing zeatin. While the P/M ratio had approximately doubled by this time, the maximum value for this parameter was not observed until the 4th day after transfer to fresh medium. By this time the mitotic index had declined from the peak observed on the 1st day of the culture period. More significantly, although we demonstrate that cytokinin treatment by itself can stimulate polyribosome formation, transfer to medium lacking cytokinin also brought about an increase in the P/M ratio. At the proper cell density the magnitude of this increase was nearly as great in cultures diluted with medium lacking cytokinins as it was in cultures diluted with medium containing cytokinin. Yet the cytokinin-deprived cells did not divide. Thus, we can conclude that, although cytokinin can stimulate polyribosome formation, this is not the mechanism by which the hormone initiates cell division.

The validity of this conclusion depends upon a convincing demonstration that the isolated polyribosomes were not degraded or contaminated with nuclear material, and that they were representative of the polyribosomes in the intact cell. The A_{260} profile of the soybean polyribosomes which were isolated and analyzed by the methods we have described shows no evidence of degradation (33). Payne and Loening (36) have shown that the electrophoretic pattern of polyribosomal RNA is a sensitive indicator of polyribosome degradation. As we have shown in Figure 3, neither the 25S nor 18S rRNA molecules show any signs of degradation upon electrophoresis. The electrophoretic patterns of soybean polyribosomal RNA

indicated that the isolated ribosomal pellets contain very little, if any organelle RNA and that there is very little contamination with nuclear material.

We have shown that the analytical procedures we employed for the bulk of this study give an accurate representation of the polysome content of the extracted ribosomes. It is not, however, a complete representation of the cellular polysome content since we have only dealt with the free polysomes. The bulk of the membrane-bound ribosomes sediment with the mitochondria and cell debris during the initial centrifugation (27). Tepfer (unpublished data) has shown that the increased polyribosome formation upon transfer of soybean cells to fresh medium or treatment with cytokinin is confined to the free ribosome fraction.

The data we have presented demonstrate that cell division is not triggered simply by an acceleration of the rate of protein synthesis. The results of our *in vivo* labeling studies show that cytokinin brought about qualitative changes in the spectrum of proteins synthesized by the cultured soybean cells. Since these changes precede cytokinin-induced cell division, we propose that it is these changes that permit the cells to divide.

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