Phase-Specific Polypeptides and Poly(A)$^+$ RNAs during the Cell Cycle in Synchronous Cultures of Catharanthus roseus Cells$^1$

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ABSTRACT

This study shows an overall analysis of gene expression during the cell cycle in synchronous suspension cultures of Catharanthus roseus cells. First, the cellular cytoplasmic proteins were fractionated by two-dimensional gel electrophoresis and visualized by staining with silver. Seventeen polypeptides showed qualitative or quantitative changes during the cell cycle. Second, the rates of synthesis of cytoplasmic proteins were also investigated by autoradiography by labeling cells with $[^35]S$-methionine at each phase of the cell cycle. The rates of synthesis of 13 polypeptides were found to vary during the cell cycle. The silver-stained electrophoretic pattern of proteins in the G$\text{2}$ phase in particular showed characteristic changes in levels of polypeptides, while the rates of synthesis of polypeptides synthesized during the G$\text{2}$ phase did not show such phase-specific changes. This result suggests that posttranslational processing of polypeptides occurs during or prior to the G$\text{2}$ phase. In the G$\text{1}$ and S phases and during cytokinesis, several other polypeptides were specifically synthesized. Finally, the variation of mRNAs was analyzed from the autoradiograms of in vitro translation products of poly(A)$^+$ RNA isolated at each phase. Three poly(A)$^+$ RNAs increased in amount from the G$\text{1}$ to the S phase and one poly (A)$^+$ RNA increased preferentially from the G$\text{2}$ phase to cytokinesis.

There have been also a number of reports on gene expression considered in terms of variations in mRNAs during the cell cycle in mammalian cells and yeast. Several genes related to DNA synthesis, such as those that encoded dihydrofolate reductase (20), thymidine kinase (38), and histones (6), and histones (22, 34), which are associated with transient increases in levels of their respective mRNAs during the cell cycle. It has been suggested that proto-oncogenes are involved in the intracellular events that govern cellular proliferation in mammalian cells (19). Altered expression of other specific genes has been detected via the appearance of new, translatable mRNAs after stimulation of growth (21). Genes activated by mitogenic stimuli have also been investigated by differential screening of cDNA libraries prepared from RNA of resting cells exposed to serum or growth factors, and a number of mRNAs have been shown to appear at specific intervals between stimulation and DNA synthesis (12, 26).

On the other hand, only a few reports on studies of gene expression during the cell cycle of higher plant cells have appeared. Newly synthesized proteins in tobacco mesophyll protoplasts, cultivated in vitro, have been investigated by two-dimensional gel electrophoresis, but none of the proteins seen on the gels was specifically synthesized during the S, G$\text{2}$, or M phases (27). In cytokinin-requiring strains of soybean cells in suspension cultures, it was shown by Fosket et al. (17) that an increase in mitotic activity occurred after treatment of the cells with cytokinin. Deprivation of cytokinin caused mitotic arrest and the addition of cytokinin was followed by a change in the spectrum of proteins synthesized by the cells. Jouanneau (23) also reported similar changes in proteins in tobacco suspension cultures. By contrast, Wang et al. (39) reported that in soybean suspension cultures deprivation of cytokinin did not cause arrest at any particular point in the cell cycle, and that no qualitative changes occurred in patterns of proteins examined by IEF.

The cells of Catharanthus roseus (periwinkle) contain several indole alkaloids, which have been applied in the treatment of circulatory diseases and leukemia. Many studies on the

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$^3$ Abbreviation: IEF, isoelectric focusing; PCNA, proliferating cell nuclear antigen.
accumulation of indole alkaloids in *C. roseus* cells have been reported (14). Several investigations in terms of cell proliferation have also been performed in *C. roseus* cells grown in suspension (13, 16).

Amino et al. (3) have established a system of synchronous cell division in suspension cultures of *C. roseus* cells. Using this system, this paper presents a comprehensive study of the changes in gene expression during the cell cycle of a higher plant by providing a catalog of constituent polypeptides, of their relative rates of synthesis and of the population of translatable mRNA. Possible roles in the cell cycle of the polypeptides and mRNAs that have been revealed by this study, are discussed.

**Materials and Methods**

**Cells and Synchronized Growth**

Cultures of cells of *Catharanthus roseus* L. (G) Don. were maintained at 27°C, in the dark, in Murashige-Skoog medium which contained 3% (w/v) sucrose and 2.2 × 10^-5 M 2,4-D. Cells were subcultured every 7 d, and were synchronized in early G1 by the double phosphate starvation method (3). Cells were cultured in phosphate-free medium at an initial cell density of about 3 × 10^7 cells/mL for 3 d. Pi was then added to the medium at a final concentration of 0.14 mM. Eighteen h later the cells were washed with phosphate-free medium and transferred to phosphate-free medium at a cell density of about 2 × 10^7 cells/mL. Pi was added again after 3 d in culture, and the synchronized division of cells was observed. In this system, the total duration of the cell cycle was about 30 h, and 70 to 80% of the cells usually divided within a 4-h interval. This period of cell division was designated 'cytokinesis.' The duration of each phase of the cell cycle was determined as previously described (3). The average duration of the G1 phase was 12 h, that of the S phase was 13 h, and that of the G2 plus M phases was 7 h. The degree of synchrony in this system was checked by analyzing the cell number and mitotic index of synchronized populations, and the DNA content of synchronized cells was also measured by flow cytometrical analysis as previously reported (4).

**Harvesting and Labeling of Cells**

Cells were collected on Miracloth at the following times after the addition of Pi: 15 h (S phase); 27h (G2 phase); 29 h (cytokinesis); and 35 h (G1 phase). Cells in the first G1 phase (0–12 h) were not used, because the protein pattern in those cells is affected by the addition of phosphate after starvation. Two hundred mg of cells were harvested for the preparation of soluble proteins and 11 to 13 g of cells harvested for the isolation of poly(A)^+ RNA. Both groups of cells were frozen in liquid nitrogen and stored at −80°C.

In order to detect the polypeptides synthesized at each phase, 1 mL of a suspension of synchronized cells (about 1 × 10^6 cells/mL) was incubated with 50 μCi [35S]methionine (specific activity 44.6 Tbq/mmol, Amersham) at 27°C for 60 min. This labeling was started at the midpoint of each phase, namely, 15 h (S phase), 25 h (G2 phase), 27.5 h (cytokinesis), and 36 h (G1 phase) after Pi was added. The labeled cells were collected by centrifugation at 300g for 2 min, frozen directly in liquid nitrogen, and stored at −80°C.

**Preparation of Cytoplasmic Proteins**

Aliquots of 200 mg of frozen unlabeled cells or 60 mg of frozen labeled cells were suspended in 600 μL of extraction buffer (10 mM potassium phosphate [pH 7.5], 5 mM MgCl₂, 5 mM 2-mercaptoethanol) that contained 20 mg polyvinyl-polypyridolone, and homogenized with a glass homogenizer. Large fragments of cell debris were eliminated from the homogenate by centrifugation at 6,000g for 2 min, and the supernatant was centrifuged again at 20,000g for 30 min. The supernatant was dialyzed in extraction buffer overnight at 4°C. The cleared supernatant was adjusted to a final concentration of 8 M urea, 2% (v/v) amphyolate (pH 3.5–10, Ampholine, LKB) and 2% (v/v) Nonidet P-40. The diluted samples were stored at −80°C. Protein concentrations were measured by Bradford's method (7). To measure rates of protein synthesis, the incorporation of [35S]methionine into hot-TCA-insoluble material was measured (35).

**Extraction of Poly(A)^+ RNA**

The high-mol wt RNA was extracted as described by Schmidt et al. (36). Eleven to 13 g of frozen cells were homogenized with 40 mL of 50 mM Tris-HCl (pH 9.0), 1% (w/v) SDS, and an equal volume of 90% (v/v) phenol in a Waring blander. The aqueous phase obtained upon centrifugation was extracted with an equal volume of phenol:chloroform (1:1, v/v) and extracted with an equal volume of chloroform. The final aqueous extract was then adjusted to pH 5.0 by the addition of acetic acid and made 0.2 M in NaCl. The nucleic acids were precipitated with 0.6 vol of isopropanol at −20°C overnight. The precipitated nucleic acids were dissolved in sterile H₂O, and the high-mol wt RNA was precipitated overnight at 4°C in 2 M LiCl. The precipitated high-mol wt RNA was washed twice with 70% (w/v) ethanol, and then dissolved in sterile water. Poly(A)^+ RNA was prepared from this RNA fraction by chromatography on oligodeoxythymidylic acid-cellulose (Pharmacia) (5).

**Protein Synthesis In Vitro in the Wheat Germ System**

Wheat germ extract was prepared according to the method of Marcu and Dudock (25), and used to translate poly(A)^+ RNA in vitro (40). One μg of poly(A)^+ RNA and 10 μCi of [35S]methionine (specific activity 34.6 TBq/mmol, New England Nuclear) were added per 10 μL of wheat germ translation mixture and incubated at 30°C for 60 min. The translation reaction was stopped by the addition of 10 μg/mL RNase A (Sigma) followed by incubation at 30°C for 15 min. The reaction mixture was diluted with 60 μL of 9.5 M urea, 2% (v/v) Nonidet P-40 and 2% (v/v) amphyolate (pH 3.5–10, Ampholine, LKB) and stored at −80°C. The incorporation of [35S]methionine into hot-TCA-insoluble material was also measured.

**Two-Dimensional Gel Electrophoresis**

IEF in the first dimension (ampholytes, pH 4–7) and SDS/PAGE in the second dimension (12.5%, w/v, acrylamide) was
performed by the method of O’Farrell (31). Volumes of samples applied to the first dimension were adjusted as follows: samples containing 20 μg protein were used for analysis by silver-staining, samples containing 4 × 10^3 cpm of radioactive protein were used for the detection of polypeptides synthesized at each phase and sample consisted of 30 μL of diluted reaction mixture that contained translated polypeptides from about 0.5 μg poly(A)^+ RNA. Pol(A)^+ RNAs isolated from the cells at each phase of the cell cycle were translated with same efficiency. The aliquots of each reaction mixture, therefore, contained equal amounts of radioactivity as protein. After electrophoresis, the gels were fixed and stained with silver nitrate according to a modified version of the procedure of Oakley et al. (30). In the case of the detection of polypeptides labeled with [35S]methionine, gels were fluorographed (24), dried, and exposed to Kodak XAR 5 film at -80°C for 2 to 7 d. Mol wt were estimated from the migration on gels relative to mol wt markers from Biorad: lysosome (14,400 D), soybean trypsin inhibitor (21,500 D), carbonic anhydrase (31,500 D), ovalbumin (41,500 D), bovine serum albumin (66,200 D), and phosphorylase B (92,500 D).

RESULTS
Changes in Electrophoretic Patterns of Polypeptides Visualized by Silver-Staining

The measurements undertaken demonstrated several distinct changes in proteins at various stages of the cell cycle. The extraction procedure yielded primarily cytoplasmic proteins. Proteins bound to membranes and associated with chromatin were not extracted because of the low ionic strength of the buffer and the absence of a surface-active agent. Figure 1 shows the patterns of polypeptides at each phase of the cell cycle. Small differences can be seen between these four polypeptide patterns which have been visualized by staining with silver nitrate. Figure 2 shows the polypeptide map prepared from the two-dimensional electrophoretic pattern of cytoplasmic proteins in the G2 phase. The relative intensity of a polypeptide spot indicates the relative size of the pool of that polypeptide. Seventeen kinds of polypeptide, indicated by arrows, display qualitative and quantitative changes during the cell cycle. Figure 3, for example, shows that polypeptides 7, 9, 10, and 11 were clearly detectable in the S phase but hardly detectable during cytokinesis. Polypeptides 8 and 12 were detected only in the S and G2 phases, respectively. In Table I, the variations in polypeptides during the cell cycle are summarized with their mol wt. Polypeptides 4, 8, 12, and 15 each appeared at a specific phase in the cell cycle. Polypeptides 1, 2, 13, and 16 disappeared or their levels decreased at specific phases, but in other phases these polypeptides were detected at constant levels. Levels of polypeptides 5, 6, 14, and 17 increased at particular phases, and those of polypeptides 3, 7, 9, 10, and 11 increased gradually and reached maximum levels at particular phases. All other polypeptides...
Table 1. Relative Amounts of Polypeptides at Each Phase of the Cell Cycle

Results were obtained from the silver-stained electrophoretic patterns by visual estimation of the relative amounts of each polypeptide.

<table>
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<tr>
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<th>Mol wt</th>
<th>G\textsubscript{1}</th>
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<th>G\textsubscript{2}</th>
<th>C\textsubscript{a}</th>
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<td>17</td>
<td>42</td>
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a Polypeptide numbers correspond to those shown in Figure 1. b C, cytokinesis. c +++*, Clearly detected; ++, detected; +, trace; ±, obscure; --, not detected.

Changes in Rates of Synthesis of Cytoplasmic Polypeptides

Cells in the G\textsubscript{1}, S, and G\textsubscript{2} phases and cells during cytokinesis were labeled with \textsuperscript{13}S)methionine for 60 min. Figure 4 shows the two-dimensional gel electrophoretic patterns of polypeptides synthesized during each phase. Autoradiograms of labeled polypeptides from cells in the G\textsubscript{1}, S, G\textsubscript{2}, and during cytokinesis revealed that the rates of synthesis of 13 different polypeptides (A–M) varied during the cell cycle (Figs. 4 and 5). In Figure 6, the changes in the rates of synthesis of polypeptides (G–K) are shown. Polypeptide J was synthesized at a constant rate in the G\textsubscript{1}, S, and G\textsubscript{2} phases, but during cytokinesis the intensity of the corresponding spot on the gel was significantly reduced. Polypeptide G was detected clearly only in the G\textsubscript{1} phase. Changes in the rates of synthesis and the mol wt of these 13 polypeptides are listed in Table II. Polypeptides A, G, and H seemed to be synthesized exclusively during one specific phase of the cell cycle. The rate of
Table II. Relative Rates of Synthesis of Polypeptides at Each Phase of the Cell Cycle

Results were obtained from the autoradiograms by visual estimation of the relative intensities of the spots corresponding to the polypeptides.

<table>
<thead>
<tr>
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<th>G₂</th>
<th>C*</th>
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<tr>
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* Polypeptide letters correspond to those shown in Figure 5.  
  b C, cytokinesis.  
  c See footnote in Table I.

Figure 7. Comparison between a silver-stained gel and an autoradiogram. Silver-stained pattern of polypeptides in the G₂ phase (A). Autoradiogram of polypeptides synthesized during the G₂ phase (B).

Synthesis of polypeptide I showed a remarkable increase during cytokinesis, while in the G₁, S, and G₂ phases polypeptide I was present at all times and synthesized at a relatively low rate. Synthesis of polypeptides B, J, and M ceased or their rates of synthesis decreased considerably during specific phases of the cell cycle. Polypeptides C, D, E, and F were periodically detected in the autoradiograms of cells in the G₁ and G₂ phases. Polypeptide K was synthesized at a maximum rate during cytokinesis, while polypeptide L was synthesized at a maximum rate during the S phase. Other unnamed polypeptides, representing about 95% of the total polypeptides seen on autoradiograms, were unchanged, in terms of the intensities of their respective spots, throughout the cell cycle.

Almost all spots detected on autoradiograms were also detected on silver-stained gels. However, the changes observed in the silver-stained electrophoretic patterns do not reflect changes in the rates of synthesis of polypeptides. Only one polypeptide, designated as 17 on the silver-stained gels (Fig. 2) and as M in the autoradiograms (Fig. 5) showed a parallel change in both its level and its rate of synthesis (Tables I and

Figure 8. Autoradiograms of polypeptides translated from poly(A)^+ RNA isolated at each phase of the cell cycle from C. roseus cells. Poly(A)^+ RNA was purified from total RNA as described in "Materials and Methods" and translated in a wheat germ system.

Figure 9. Two-dimensional map of polypeptides translated from poly(A)^+ RNA. The map was prepared by tracing the autoradiogram of polypeptides translated from poly(A)^+ RNA isolated from cells in the S phase. Letters (a–d) have been assigned to polypeptides for which qualitative or quantitative changes were observed during the cell cycle. Polypeptide d was not detected in the autoradiogram for the S phase, so in this figure there is no spot for this polypeptide, but its position is indicated by an arrow.
II. From a comparison of these two kinds of electrophoretic
pattern, the rate of turnover of each polypeptide can be
inferred. It appears that individual cytoplasmic polypeptides
turn over at different rates (Fig. 7). As shown in Figure 7A,
the open triangles indicate the polypeptides that were detected
in considerable amounts on the silver-stained gels but not on
the autoradiograms, and this difference suggests inactive turn-
over of these polypeptides. In Figure 7B, the open squares
indicate the polypeptides that were detected in considerable
amounts on autoradiograms but not on silver-stained gels,
and this difference suggests that active turnover of these
polypeptides. Polypeptides 1, 3, 4, 10, and 12, the levels of
which varied on the silver-stained gels, were also detected in
the same positions on autoradiograms, but the rates of syn-
thesis of these polypeptides were unchanged throughout the
cell cycle. It is probable that the turnover rates of these
polypeptides was different in each phase of the cell cycle (data
not shown).

Variation in the in Vitro Translation Products of Poly(A)+
RNA

Changes in the rate of synthesis of polypeptides (A–M)
could be due to either to variations in their amounts of mRNA
or to the regulation at the level of translation or posttransla-
tional mechanisms. Thus, in vitro translation products from
poly(A)+ RNA isolated at various phases in the cell cycle were
resolved on two-dimensional gels (Fig. 8). Figure 9 shows a

<table>
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*Polypeptide letters correspond to those shown in Figure 9. *$C$, cytokinesis. * See footnote in Table I.
two-dimensional map of in vitro translation products of mRNAs isolated in the S phase. Letters (a–d) indicated polypeptides for which qualitative and quantitative changes were observed during the cell cycle. If the intensities of labeling of the in vitro translation products reflects the levels of their corresponding mRNAs, the map indicates that the levels of translatable mRNAs encoding polypeptides a, b, and c increased preferentially before the S phase (Figs. 10 and 11), and decreased significantly prior to cytokinesis. We also observed that the amount of translatable mRNA for a unique polypeptide, d, increased considerably and transiently during the G2 phase and decreased rapidly after cytokinesis (Fig. 11). The changes in the levels of mRNA that encode the polypeptides a, b, c, and d are summarized in Table III. More than 99% of polypeptides produced by in vitro translation were associated with constant levels of their corresponding mRNAs during the cell cycle.

Several polypeptides translated in vitro were also detected at the same position on autoradiograms of polypeptides synthesized in vivo (Fig. 12). In Figure 12, the open circles indicate the same common polypeptides synthesized in vivo and translated in vitro. Among 13 polypeptides (A–M) shown in Figure 5, five polypeptides, A, B, C, J, and L, were observed on autoradiograms of polypeptides translated in vitro, but the amounts of their corresponding mRNAs were unchanged throughout the cell cycle.

**DISCUSSION**

The progression of the cell cycle is primarily controlled by sequential expression of various genes. It is important for the understanding of the mechanisms of cell proliferation of higher plants to identify and characterize genes which encode proteins that have the specific functions for the cell cycle. Using synchronous cultures of cells from a higher plant, Catharanthus roseus, the expression of genes during the cell cycle was revealed by the analyses of the levels of polypeptides, of the rates of synthesis of polypeptides and of translated products of mRNA isolated from each phase of the cell cycle. We have found that the levels of 17 kinds of polypeptide vary, and the rates of synthesis of 13 kinds of polypeptide change in a cell-cycle-dependent manner. In the case of mammalian cells and yeast, there have been, until recently, no reports of proteins whose synthesis is limited to a specific phase of the cell cycle. However, the expression of cellular proteins throughout the cell cycle has recently been studied in more detail, and a few proteins whose synthesis is cell-cycle-dependent, such as ‘divisin’ (10), HSP70 (28), and a pair of 21 kD proteins (41), have been reported in mammalian cells. Furthermore, proliferating cell nuclear antigen (PCNA, also called cyclin), whose synthesis increases in the S phase (8), has been shown to be identical to a DNA polymerase-δ auxiliary protein (9, 32). Thus, the polypeptides whose levels or rates of synthesis varied throughout the cell cycle, as revealed in this study, are probably also involved in DNA replication or mitosis.

The analysis of silver-stained, two-dimensional gels revealed that several cytoplasmic polypeptides displayed characteristic changes in the G2 phase: polypeptides 4, 12, and 15 appeared, while polypeptides 2, 13, and 16 disappeared, and polypeptides 3, 6, and 17 reached a maximum level (Fig. 1; Table I). However, the rates of synthesis of cytoplasmic polypeptides showed no characteristic changes in the G2 phase (Table II). These results suggest that posttranslational processing, including modifications, degradations, reconstructions, and changes in intracellular localization of proteins (28) occurred during the G2 phase.

The increased levels of mRNA for translated polypeptides, a, b, and c in the cells at S phase reflect the possibility that they were involved in DNA replication. Several studies have demonstrated that genes involved in DNA synthesis undergo transient expression during the cell cycle in mammalian cells and yeast (6, 20, 22, 34, 38). The levels of mRNA coding for calmodulin (11), and the transcription product of the ts11 gene which complements the ts11 mutation (18), a temperature-sensitive mutation in mammalian cells, were increased in the period from the G1 to the early S phase. Expression of the mRNA that encodes p53 (33) and the mRNA that encodes PCNA (2), increased at the G1/S boundary. HSP70 mRNA (28) was also expressed preferentially in the S phase. These genes expressed from the G1 to the S phase are thought to play important roles in the progression of the cell cycle.

We also observed that the amount of translatable mRNA for a unique polypeptide, d, increased considerably and transiently during the G2 phase and decreased rapidly after cytokinesis (Fig. 11). The mutation promoting factor (MPF), which is thought to induce mitosis and meiosis, disappears as cells from higher organisms exit from mitosis (37). Two other proteins have been described which are synthesized specifically in the G2 phase in hamster cells and then disappear after cell division (41). However, it is not known whether the mRNAs coding for these proteins are also expressed preferentially in the G2 phase or not.

We described 13 polypeptides (A–M) whose rates of synthesis varied during the cell cycle. In the autoradiograms of the polypeptides translated in vitro, the levels of translated products corresponding to the five polypeptides (A, B, C, J, and L) were approximately constant during the cell cycle (Fig. 12). Furthermore, almost all the major translatable species of mRNA did not change in level; the levels of only a small population of mRNAs, such as the mRNAs for the polypeptides a, b, c, and d, vary during the cell cycle. Thus, for the polypeptides (A–M) whose rates of synthesis changed during the cell cycle, most of the changes in rates of synthesis must be the result of changes in translational or posttranslational regulation, but not in transcriptional one.

This paper is the first report on overall analysis of the regulation of gene expression in the cell cycle of a higher plant. Nothing is known at present about the relationship between these mRNAs or their translation products and the cell-cycle-specific proteins and mRNAs identified in mammalian cells and yeast. However, the polypeptides and mRNAs described in this paper may be candidates for markers of each phase of the cell cycle. The results obtained in this paper show the possibility to isolate the genes whose expression are cell-cycle-dependent. The isolation of cell-cycle-dependent cDNAs is under investigation. These will lead to elucidation of molecular mechanisms of progression of the cell cycle in higher plants.
Changes in Polypeptides and mRNAs During the Cell Cycle

LITERATURE CITED

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