Role of Silicon in Diatom Metabolism. Messenger RNA and Polypeptide Accumulation Patterns in Synchronized Cultures of Cylindrotheca fusiformis

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Patterns of in vitro translation products from isolated mRNA and in vivo polypeptide accumulation in synchronized cultures of Cylindrotheca fusiformis were analysed by two-dimensional gel electrophoresis. The way in which the availability of silicon, the specific cell cycle stage, or the illumination conditions affected the pattern of gene expression was distinguished by comparing the timing of polypeptide and mRNA accumulation in cultures synchronized by two different methods. A rapid and dramatic shift in the relative abundance of in vitro translation products from mRNA followed either the removal or the readdition of silicate to the media as well as the transition from dark to light. Eleven mRNAs appeared to be expressed specifically between mid-S phase and cell separation, as their increase was observed at this stage in both synchronies. In addition, three mildly acidic polypeptides from the soluble protein fraction of C. fusiformis, each representing about 0.05% of the total protein, increased several-fold between mid-S and cell separation. Thus, silicon appears to affect gene expression both directly and, due to its effect on cell cycle progression, indirectly. Both effects are primarily at a level before translation.

INTRODUCTION
The diatom Cylindrotheca fusiformis does not initiate DNA synthesis in the absence of silicon (Darley & Volcani, 1969; Sullivan & Volcani, 1973), and this may be due, in part, to a requirement for silicon in the synthesis of several proteins including two nuclear DNA polymerases (Okita & Volcani, 1978, 1980). An increase in the level of cAMP may also be involved, as this increase also requires silicate in the media (Borowitzka & Volcani, 1977; Aline et al., 1984). Two-dimensional gel electrophoretic analysis of polypeptide labelling during synchronized growth (Okita & Volcani, 1980) has shown that the synthesis of specific polypeptides depends on the illumination conditions, the cell cycle stage, and the presence or absence of silicon. Furthermore, the absence of silicon affects the pattern of polypeptide synthesis well before the onset of DNA replication. However, it was not possible to compare accurately the timing of changes in polypeptide synthesis between light–dark and silicon-starvation synchronized cultures, since in order to label polypeptides sufficiently with $^{35}$SO$_4^-$, labelling times were of necessity long in relation to the length of the cell cycle stages (Okita & Volcani, 1980). At what stage (i.e. transcriptional, translational, etc.) protein synthesis was affected by silicon was also not ascertained.

As compared with analysis of polypeptide labelling patterns, direct analysis of the mRNA population has the advantages of more clearly defining the level at which silicon controls protein synthesis and of allowing the timing of changes to be more accurately compared between synchronies. This is important since the two methods available for synchronizing C. fusiformis

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cultures, light–dark cycles or light–dark cycles followed by silicon starvation and recovery (Darley & Volcani, 1969), could be subject to artifacts induced by the imposed regime. An accurate comparison of the timing of changes during the two alternative synchronies should theoretically allow events induced by one or the other synchronization regime to be identified.

In this study, the relative abundance of mRNAs during synchronized growth was analysed by two-dimensional electrophoretic resolution of *in vitro* translation products. The accumulation of polypeptides was also analysed on silver-stained two-dimensional gels. The results show that silicon affects protein synthesis at a pre-translational level, the specific effects depending on both the illumination conditions and cell cycle stage.

**METHODS**

*Culture conditions.* Maintenance, growth and synchronization of *C. fusiformis* were as previously described (Darley & Volcani, 1969; Paul & Volcani, 1976).

*Polysomal RNA isolation.* Polysomes and polysome profiles were obtained by an adaptation of the method of Jackson & Larkins (1976). All procedures were carried out at 4 °C. Cells were washed and suspended in polysome isolation buffer (Jackson & Larkins, 1976) to a density of $5 \times 10^6$ cells ml$^{-1}$ and passed through an ice-cold pressure cell (Yeda Research Development Ltd, Rehovot, Israel) at 27-6 MPa (4000 lbf in$^{-2}$) of $N_2$. After centrifugation at 12000g for 10 min the supernatant was brought to 1% (v/v) with respect to Triton X-100, layered over 60% (w/v) sucrose and centrifuged for 90 min at 200000g. Polysomal RNA was obtained by extracting polysomes suspended in 10 mM-Tris/HCl, pH 8, 200 mM-KCl, with an equal volume of chloroform/phenol (1:1, v/v) and precipitating RNA at $-20 \, ^\circ \text{C}$ with 2.5 vols ethanol.

*In vitro translation.* RNA was prepared for translation by reprecipitating with ethanol in the presence of 200 mm-potassium acetate, washing with 80% (v/v) ethanol, drying in vacuo, and dissolving in sterile water to 1 g l$^{-1}$. A rabbit reticulocyte translation kit (Bethesda Research Labs) was used according to the manufacturer’s instructions with $[^{35}\text{S}]{\text{methionine}}$ (4.8 $\times 10^{13}$ Bq (1300 Ci) mol$^{-1}$) (New England Nuclear) as the labelled amino acid. Optimal incorporation was obtained with 1.2 mm-Mg$^{2+}$ and 135 mm-K$^+$ and polypeptides as large as 20000 daltons were synthesized efficiently. Routinely, 10 µg of polysomal RNA was added to a 30 µl reaction volume, this amount being just saturating. Up to 20 µg of RNA was not inhibitory with respect to amino acid incorporation.

*Extraction of polypeptides.* Cells were harvested, washed and diluted to $5 \times 10^6$ cells ml$^{-1}$ in a buffer containing 0.6 mM-ammonium sulphate, 50 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 1 mM-mercaptoethanol and 1 mM-phenylmethylsulphonyl fluoride. This suspension was passed through the pressure cell at 27.6 MPa (4000 lbf in$^{-2}$) and centrifuged at 12000g for 10 min. To obtain soluble protein, the supernatant was centrifuged at 100000g for 2 h, saturated ammonium sulphate solution (2 vols) was added to the supernatant, and after 1 h on ice the precipitate was sedimented at 20000g for 10 min. The 20000g pellet was washed twice with methanol and then resuspended in, and dialysed overnight against, 1 mM-2-mercaptoethanol. The dialysed protein, including the precipitate, was lyophilized. To obtain particulate protein, the 12000g pellet obtained above was gently resuspended in the 0.6 M-ammonium sulphate buffer with a glass Teflon homogenizer and resedimented at 12000g for 10 min. This pellet was suspended in 10 mM-Tris/HCl, pH 7.5, 2% (v/v) Triton X-100. After 30 min at room temperature debris was removed at 12000g for 10 min, the supernatant was precipitated with 10 vols acetone and centrifuged for 5 min at 5000 g. The pellet was treated exactly as was the 20000g soluble protein pellet described above.

*Two-dimensional gel electrophoresis.* Mixtures of polypeptides produced by *in vitro* translation reactions were prepared for electrophoresis by precipitation with 10 vols ice-cold 80% (v/v) acetone followed by air drying. These samples, as well as the lyophilized cell protein fractions, were dissolved in lysis buffer and electrophoresed as described by O’Farrell (1975), except that samples were loaded on the acidic end (reverse polarity) and the final ampholyte (LKB) concentrations were 0.45% (pH range 2.5-4), 1.65% (pH 4-6) and 0.9% (pH 6-8). Two-dimensional gels were 12% (w/v) polyacrylamide. For *in vitro* translation products, gels were fixed and impregnated with EN$^3$HANCE (New England Nuclear) according to the manufacturer’s instructions, dried onto Whatman 3 MM paper under reduced pressure and exposed at $-70 \, ^\circ \text{C}$ to Kodak XRP-1 X-ray film. Exposure time was 1 d for every 3 $\times 10^6$ c.p.m. of hot TCA precipitable material applied to a gel. For unlabelled cellular proteins, gels were silver stained (Morrisey, 1981). In our hands, the threshold of detectability for bovine serum albumin, β-lactoglobulin, or carbonic anhydrase (all from Sigma) was 5-10 ng per spot, and between 10 and 500 ng a two-fold increase could be detected visually.

**RESULTS**

For both light–dark and silicon starvation–recovery synchrony, the timing of increase in cell number and DNA content was essentially as previously reported (Paul & Volcani, 1976; Borowitzka & Volcani, 1977). Zero time denotes the transition from the last dark period into
Fig. 1. Cell cycle stage-specific polypeptides. Enlargements of the region between pI values 5.5 to 6.8 and molecular masses 30 to 50 kDal from silver-stained two-dimensional gels. Soluble polypeptides were isolated at 8 h (a) and 12 h (b) following light-dark synchronization and at 2 h (c) and 4 h (d) after addition of silicon to starved cells. Numbered spots are discussed in the text. In this figure and in Figs 2 and 3, the pI increases from right to left and migration in the second dimension was from top to bottom.

continuous light for the light–dark synchrony or the point at which silicate is added to 24 h silicon-starved cells for the silicon recovery synchrony. The period of increase in DNA content (S phase) occurs between about 8 h to 13 h in the light–dark synchrony or 0.5 h to 3.5 h during silicon recovery. Cell separation occurs between 13 h and 17 h or 4 h to 7 h in the respective synchronies. (The above changes are shown in Fig. 5c, d.) Cells between 0 h and S phase are in G1 by definition, and from the point just after S phase to before the onset of cell separation the culture is assumed to consist of cells in G2 and/or mitosis. Finer distinction of cell cycle stages is not presently possible.

Patterns of polypeptide accumulation

To determine the extent to which changes in polypeptide labelling patterns (Okita & Volcani, 1980) were reflected in changes in accumulation patterns, polypeptides at various stages of synchronized growth were resolved on two-dimensional gels and visualized by silver-staining.
Of the total protein, 33% was particulate and the remainder soluble. The two fractions contained distinct sets of polypeptides, except for some abundant polypeptides that were seen in both fractions. Approximately 600 soluble and 400 particulate polypeptides were detected.

Despite the fact that accumulation is a less sensitive parameter than rate of synthesis for detecting periodicity of individual proteins, significant changes in the relative abundance of polypeptides were observed in both synchronies. In particular, silicon starvation caused both increases and decreases, some apparent after 6 h; after 24 h the pattern differed considerably from that seen during the light–dark synchrony. The appearance of some polypeptides (e.g. a group at approximately 20 kDa and an approximate pI of 5) was clearly specific to silicon starvation, since they accumulated dramatically during the starvation, but were not observed at all during the light–dark synchrony or exponential growth.

Following addition of silicate to silicon-starved cells, the pattern remained unchanged for 2 h and was nearly unchanged after 4 h. This lack of change was presumably due to the low sensitivity of accumulation as a measure of periodic synthesis. However, three novel polypeptides accumulated between 2 and 4 h after silicate addition (Fig. 1c, d) and these same three increased between 8 and 12 h of the light–dark synchrony (Fig. 1a, b). As the increases occurred between mid-S phase and cell separation in both synchronies, they are apparently cell cycle stage-specific. Before their increase, these polypeptides were below the level of detection with silver stain and thus represented less than 0.01% of the soluble protein. Comparison of the maximum spot densities with those produced by known amounts of standard proteins indicated that the minimum increases for the three polypeptides were about 10-fold for two of them (spots 1 and 3, Fig. 1) and 3- to 5-fold for the other. Each polypeptide at maximum concentration represented about 0.05% of the cell protein.

**Patterns of mRNA accumulation**

Because of limitations with in vivo polypeptide labelling in *C. fusiformis* (Okita & Volcani, 1980), mRNAs were analysed. This not only permitted the extent of the control of gene expression by silicon to be better defined, but also allowed the timing of individual changes to be more precisely determined. Thus, RNA extracted from polysomes isolated during synchrony was translated in vitro and the resulting products analysed by two-dimensional gel electrophoresis. Polysome profiles obtained from *C. fusiformis* (not shown) were essentially identical to those seen for other lower eukaryotes such as *Chlamydomonas reinhardtii* (Baumgartel & Howell, 1976). Addition of cycloheximide (20 μg ml⁻¹) 5 min before isolation improved the recovery of translational activity and was used routinely. This isolation procedure yielded only cytoplasmic polysomes. As there is no evidence that organelles are involved in the silicon requirement, organellar polysomes were excluded. A variable degree of background streaking on the two-dimensional gels made analysis of certain regions difficult. However, about 300 in vitro translation products were analysed qualitatively. As we were primarily interested in comparing the timing of changes between the two synchronies, quantification was not attempted. Between 5 to 20 μg, the amount of RNA added to the translation reaction did not significantly affect the pattern of translation products observed.

In order to distinguish changes due specifically to the effects of silicon from changes due to other causes, variations in the amounts of mRNAs during silicon starvation or recovery were compared to variations that occurred during light–dark synchrony. The most dramatic change in the mRNA population during the light–dark synchrony occurred between 0 and 3 h, when only about 10% of the translation products were common to both time points. There was little further change between 3 h and 6 h, i.e. a new steady-state was rapidly attained upon illumination. This indicated that most of the initial changes were due to the transition into the light and not to cell cycle progression per se. When silicon-starved and unstarved cultures were compared (Fig. 2), the initial dramatic change in the mRNA population showed important differences. As examples in Fig. 2 indicate, specific mRNAs increased or decreased in the presence (Fig. 2a) or absence (Fig. 2b) of silicon. Again, all the changes had occurred by 3 h and there was no further change by 6 h. Therefore, a subset of the genes whose expression was altered by the dark to light transition were also affected by silicon, with additional genes
Fig. 2. Effect of silicon starvation on the mRNA population. The patterns shown are of in vitro translation products produced by polysomal RNA isolated after 3 h in the light in the presence (a) and absence (b) of silicon. Numbers at the right refer to molecular mass markers. Arrows labelled X are examples of spots which were present at $t = 0$ and decreased in either the presence or absence of silicon. Arrows labelled with an asterisk are spots which increased in the presence of silicon and decreased in its absence. Other arrows indicate spots which were absent at $t = 0$ and increased under only one of the two conditions.
Fig. 3. Examples of changes in the mRNA population during recovery from silicon starvation. Panels (a)-(c) enlarge the two-dimensional gel region between pI 5.7 to 6.9 and molecular masses 25 kDa to 45 kDa; panels (d)-(f) enlarge the region between pI 4.5 to 5.3 and molecular masses 20 kDa to 45 kDa. Translation products programmed by polysomal RNA from just before silicon addition (a, d), and 1 h after addition (b, e) and 4 h after addition (c, f) are shown. Spots labelled with arrows are discussed in the text.

possibly being specific to silicon starvation. The pattern of the mRNA population continued to change during silicon starvation, so that by 24 h it was considerably different from the pattern at 6 h.

The amounts of several mRNAs also showed stage-specific variation during the recovery from silicon starvation, and these changes were compared to changes seen between 6 and 12 h of the light–dark synchrony. The time intervals in both synchronies spanned the period from immediately before initiation of DNA synthesis to just before cell separation. Examples of changes seen on the two-dimensional gels during silicon recovery (Fig. 3) and light–dark synchrony (Fig. 4) are shown. In addition to differences in timing which are discussed below, these figures show that even within a group of spots showing the same timing, there were significant differences in the extent of increase or decrease. Though undoubtedly important, these differences were ignored for the purpose of comparing synchronies.
The *in vitro* translation products that displayed significant temporal variation during light–dark and silicon recovery synchrony are summarized diagrammatically in Fig. 5(a, b) with patterns of qualitative temporal change shown in relation to the increases in DNA content and cell number shown in Fig. 5(c, d). Most of the changes were seen in one or the other synchrony, but not both. For example, spots M and N increased between 9 and 12 h of the light–dark synchrony, but did not change during silicon recovery. Several spots (e.g. P and Q) which increased rapidly upon addition of silicate to silicon-starved cells were not detected during light–dark synchrony. Five spots (e.g. S and T) increased between 6 and 9 h of light–dark synchrony and subsequently decreased, but these were absent during both silicon starvation and recovery. Two spots (V and W) varied in both synchronies, but the timing of the changes relative to DNA content and cell number did not coincide in the two synchronies. Eleven spots (A–K), however, did show corresponding increases in both synchronies, the increases occurring between mid-S phase and cell separation.
Fig. 5. Diagrammatic representation of changes in individual mRNAs in synchronized *C. fusiformis* cultures. Panels (a) and (b) show the two-dimensional gel location of individual translation products which varied significantly and reproducibly during the light–dark (a) and silicon recovery (b) synchronies. Panels (c) and (d) show qualitative patterns of temporal change relative to the increase in DNA (○) and cell number (○) for light–dark (c) and silicon (d) synchronies. Symbols correspond to categories of temporal change as follows: I or V (○), II or VI (□), III or VII (△), IV or VIII (○). Letters in panels (a) and (b) identify translation products discussed in the text. DNA and cell number were measured as previously described (Darley & Volcani, 1969). The multiplication factor varied less than 5% between replicate synchronies for initial cell densities between 6–8 × 10^5 cells ml⁻¹.

Four of the spots that appeared rapidly (within 1 h) after addition of silicate to silicon-starved cells were readily detected during the light–dark synchrony at the time of initiation of DNA synthesis (arrows in Fig. 5b). These spots apparently represent mRNAs that require silicon for their accumulation, and because of the timing of their accumulation, they might code for functions required for DNA synthesis (Okita & Volcani, 1978).

**Discussion**

The silicon requirement for DNA synthesis in *C. fusiformis* (Darley & Volcani, 1969) appears to be at least partly due to a silicon requirement for the synthesis of proteins, including DNA polymerase (Okita & Volcani, 1978, 1980). In this investigation we have shown that silicon affects the pattern of protein synthesis at a level before translation. By analysing the timing of mRNA and polypeptide accumulation we have been able to identify changes in individual mRNAs and polypeptides that are specific to silicon starvation and recovery or to light–dark synchrony, or that are common to both synchronies and thus possibly cell cycle specific.
Silicon and gene expression in C. fusiformis

Discontinuous synthesis and accumulation of mRNAs and polypeptides have often been observed in light–dark synchronized algal cultures (Howell et al., 1977; Okita & Volcani, 1980; Ares & Howell, 1982). However, the pronounced effect that induction synchronization can have upon cellular metabolism has only recently been fully appreciated (Elliott & McLaughlin, 1983; Rollins et al., 1983). Our results indicate that the majority of changes in mRNA and protein accumulation that occur during synchronized growth of C. fusiformis are due to the synchronization procedure and not to a cell cycle ‘programme’. However, eleven mRNAs and three polypeptides accumulated between mid-S phase and cell separation in cultures synchronized in two different ways, suggesting that these increases may be cell cycle stage-specific. Thus, our results support the notion that changes during induction synchrony should be interpreted with caution, but also suggest that comparing the timing of events between synchronies allows events which result directly from the perturbation to be distinguished from those which may be related to cell cycle stage-specific events.

The effect of silicon on the expression of many genes appears to be direct, and independent of the cell cycle as indicated by the many mRNAs and polypeptides that varied in relative abundance following silicon starvation and recovery and that did not display a corresponding variation during light–dark synchrony. Most of these changes also occurred rapidly upon the removal or readdition of silicate. Silicon starvation has been shown to increase protein turnover in C. fusiformis (Okita & Volcani, 1980), a phenomenon observed in other organisms when starved of essential nutrients. Thus, it will be important to determine whether any of the genes affected only during silicon starvation and recovery are truly ‘silicon-specific’ or are also affected when C. fusiformis is starved of other essential nutrients.

Silicon also affects the expression of some genes as a result of the interruption and reinception of the cell cycle during silicon starvation and readdition, respectively. This was indicated by the fact that the cell cycle stage-specific increase in the eleven mRNAs and three polypeptides was delayed during silicon starvation, increasing at the appropriate stage only after readdition of silicate. Why cell cycle stage-specific increases were observed only between mid-S phase and cell separation is unknown. This period within the cell cycle includes the stage of cell wall synthesis. In addition to being the stage of the cycle which consumes the largest amount of silicon to form the siliceous component of the wall, this stage also includes the synthesis of the organic component of the wall (Volcani, 1981), which contains a large amount of protein. It is also possible that mitosis and cytokinesis in particular demand the rapid accumulation of specific proteins. Tubulin mRNA accumulates at this stage in Chlamydomonas (Ares & Howell, 1982) and a polypeptide of unknown function accumulates dramatically just before mitosis in Physarum polycephalum (Pahlic & Tyson, 1983).

In summary, the effect of silicon on gene expression in C. fusiformis is at a level before translation, and depends on both the cell cycle position and the illumination conditions of the culture. These results have led to the construction of cDNA libraries representing the mRNA populations of cells during both silicon starvation and recovery (J. R. Ludwig & B. E. Volcani, unpublished). Representative cDNA probes will be used to define more precisely the timing of expression of specific genes in both synchronized and asynchronous cultures following a shift in growth conditions.

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REFERENCES


