Possible role for mRNA stability in the ammonium-controlled regulation of nitrate reductase expression

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Ammonium, or a metabolite of ammonium, represses the expression of nitrate reductase (NR) in *Chlorella vulgaris*. The removal of ammonium and addition of nitrate (induction) resulted in a rapid (20 min) peaked synthesis of NR mRNA. Nitrate reductase protein and activity increased at a much lower rate, reaching their maxima by 8 h. Ammonium added to nitrate-grown cells resulted in a dramatic decrease in NR mRNA from a steady-state level to undetectable levels within 15 min of ammonium addition. Nitrate reductase activity and protein levels decreased to 20% and 40% of initial levels respectively over 8 h. The half-life for NR mRNA under these conditions was estimated to be less than 5 min, compared with 120 min for NR protein. Such rapid decreases in NR mRNA suggested a degradation and/or cessation in NR mRNA transcription. No apparent difference in NR mRNA-specific RNAase activity of crude cell extracts (NR-induced or repressed) was observed. However, a significant difference in the susceptibility to degradation of NR mRNA from long-term nitrate-grown cells compared with the NR mRNA isolated from short-term induced cells (20 min in nitrate) was observed. NR mRNA isolated from long-term-nitrate-grown cells was completely degraded by RNAases in cell extracts under conditions in which the NR mRNA isolated from short-term induced cells was resistant to degradation. These results suggest that mRNA stability may be an important factor in the metabolic regulation of assimilatory nitrate reductase in *Chlorella*.

INTRODUCTION

The nitrate assimilatory pathway is the major route by which inorganic nitrogen is converted into a biologically useful organic form in plants and micro-organisms (Solomonson and Barber 1990). This pathway generates approx. 2×10^4 megatons of organic nitrogen per year (Guerrero et al., 1981), two orders of magnitude greater than that generated by nitrogen fixation (Gallon and Chaplin, 1987). Two enzymes, nitrate reductase (EC.1.6.6.1) and nitrite reductase (EC.1.7.7.1), are required to reduce nitrate to the end product, ammonium, which is then incorporated into amino acids (eight reducing equivalents are consumed to reduce nitrate to ammonium). Nitrate reductase (NR) is considered to be the key enzyme in this assimilatory pathway, and the reduction of nitrate to nitrite is considered to be the regulated and rate-limiting step (Beevers and Hageman, 1969). Efficient regulation of this step is necessary to assure efficient utilization of available nitrate from the environment and to avoid toxic accumulation of the intermediate, nitrite, or the end product, ammonia. Therefore, considerable interest has focused on understanding how NR is regulated at the molecular level.

Both nitrate and ammonium (or a product of ammonium) play a role in regulating NR synthesis, with nitrate acting as an inducer and ammonium the repressor (Solomonson and Barber, 1990). In algae and nitrate-assimilating yeasts, NR synthesis occurs with the removal of ammonium (Cannons et al., 1986; Cannons and Hipkin, 1987; Zeiler and Solomonson, 1989) without a need for nitrate inducer. Indeed, in *Chlorella*, the presence of ammonium prevents NR expression even if the inducer, nitrate, is also present (Zeiler and Solomonson, 1989), suggesting that ammonium (or a product of ammonium produced in the cell) has a repressive effect on NR expression, rather than nitrate having an inducing effect. In higher plants, nitrate is required for NR expression (Calza et al., 1987; Cheng et al., 1986; Crawford et al., 1988; Gowri and Campbell, 1989; Friemann et al., 1991; Melzer et al. 1989), while a maximal level of NR expression is achieved with nitrate and light (Deng et al., 1989). Other environmental factors that are involved in regulating NR expression include diurnal rhythms (Deng et al., 1989), hormones (Schmerder and Borriss, 1986; Lu et al., 1990), phytochrome in etiolated seedlings (Melzer et al., 1989) and sucrose (Cheng et al., 1992). These factors appear to act primarily at the transcriptional level. Other modulators, including cyanide and superoxide radicals (Solomonson and Spehar, 1979; Vargus et al., 1987), act post-transcriptionally, and there is reversible light modulation involving adenine nucleotides (Kaiser et al., 1992).

To better understand how NR is regulated in *Chlorella*, we have investigated the role of ammonium nitrogen in controlling the levels of NR mRNA, protein and activity. Our results suggest that NR mRNA levels change very rapidly in response to ammonium addition or removal, and that NR mRNA stability may play a significant role in the overall regulation of nitrate assimilation.

EXPERIMENTAL

Cells and media

Chlorella vulgaris cells were grown at 25 °C with continuous white light as previously described by Zeiler and Solomonson (1989). Cells maintained on mineral-salts medium containing 20 mM NH_4Cl were referred to as repressed for nitrate reductase and were induced by harvesting the cells at 4 °C, washing with nitrogen-free medium and resuspension in medium containing 20 mM potassium nitrate. Repression experiments were done in a similar manner except that cells were initially maintained on medium containing 20 mM potassium nitrate and then transferred to medium containing 20 mM NH_4Cl.

Abbreviations used: NR, nitrate reductase; poly(A)$^+$, polyadenylated.

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Analysis of NR protein and activity in cell extracts

Aliquots (50 ml) containing 5×10^9 cells were removed at designated time points, harvested by centrifugation, washed with 10 mM potassium phosphate buffer, pH 7.5, and resuspended in 3 ml of the same buffer. Cells were disrupted in a French pressure cell (Aminco) at 69 MPa and the extract clarified by centrifugation at 13 000 *g* at 4 °C. The supernatant was either used immediately or beaded in liquid nitrogen. The presence of immunoreactive NR in the extracts was determined by Western blotting. Equal amounts of protein (75 μg) of the crude extracts were mixed with 2X Laemmli sample buffer (Laemmli, 1970) and electrophoresed through an SDS/10 %-(w/v)-polyacrylamide gel. The proteins were transferred to nitrocellulose and immunoreactive NR detected as described previously (Cannons et al., 1991). Quantification of NR subunit was determined by densitometric scanning of the processed blot. NADH:NR activity was determined in the cell-free extracts by measuring the rate of NADH oxidation in the presence of nitrate (Solomonson et al., 1986).

Ammonium uptake

The rate of ammonium uptake by cells was determined by measuring the disappearance of ammonium from the medium. Samples of 10 ml (density = 10^8/ml) were removed at designated times, filtered rapidly and the ammonium level determined by measuring the oxidation of NADH as a result of the reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase (Dewan, 1938), (Sigma ammonia diagnostic kit).

NR mRNA expression

Total RNA was extracted from 100 ml cells (10^8 cells/ml) using the method described previously (Cannons et al., 1991). NR mRNA levels were determined in these samples by means of the RNAase protection assay (Ausubel et al., 1991). An antisense RNA probe to NR was transcribed from the *Nco*I-linearized pCVNR1 clone (Cannons et al., 1991) using T7 RNA polymerase and [α-^{32}P]UTP. Following DNAase I digestion the probe was purified by phenol/chloroform extraction and ethanol precipitation. Equal amounts (10 μg) of RNA from each time point of induction/repression were hybridized with 5×10^5 c.p.m. of riboprobe for 16 h at 42 °C in hybridization buffer [80 % formamide/40 mM Pipes (pH 6.4)/0.4 mM NaCl/1 mM EDTA]. RNA hybrids were treated with RNAase A at 40 μg/ml followed by proteinase K digestion. RNAase-resistant hybrids were then purified by phenol/chloroform extraction and ethanol precipitation and analysed by electrophoresis on a 6 %-polyacrylamide gel containing 7 M urea. Densitometric scans of a 280 bp protected fragment were performed to quantify levels of NR mRNA. As a control, levels of the ADP/ATP translocator protein mRNA (Hilgarth et al., 1991) were measured by Northern analysis. Samples of total RNA (10 μg), as prepared above, were separated on denaturing agarose/formaldehyde gels (Derman et al., 1981), transferred to Genescreen Plus (du Pont) by capillary blotting (Sambrook et al., 1989) and probed with a ADP/ATP translocator cDNA labelled with ^{32}P by random prime labelling.

In vitro assay for NR RNAase activity

Extracts were prepared from *Chlorella* cells grown on nitrate medium (induced) or nitrate medium and transferred to ammonium medium for 10 min (repressed). The cells were harvested, washed with 10 mM Tris, pH 7.0, and resuspended in a minimal volume of the same buffer. The cells were broken in a French pressure cell at 83 MPa, followed by centrifugation at 13000 *g*. The supernatant was taken as a source of RNAase. Samples of supernatant containing 10 μg of protein were incubated with total RNA samples for 0 or 30 min, followed by phenol/chloroform extraction, Residual RNA in the sample was then precipitated with ethanol and the NR mRNA level assessed by RNAase protection assay described above.

RESULTS

Expression of NR during induction

Changes in levels of NR mRNA, protein and activity due to the removal of ammonium with nitrate addition (induction) are shown in Figure 1. *Chlorella* cells were grown on ammonium as the sole nitrogen source and then transferred to nitrate-containing medium immediately following the removal of ammonium. It should be noted that nitrate is added to prevent the cells being nitrogen-starved during later time points. However, it has been shown that the removal of ammonium is sufficient for NR expression (Zeiler and Solomonson, 1989). Samples were taken over the next 8 h and assayed for NR mRNA by RNAase protection assay, NR protein by Western blotting and NADH-NR activity (Figure 1). Cells grown on ammonium (repressed) did not contain detectable NR mRNA, protein or activity. The removal of ammonium and addition of nitrate as the sole source of nitrogen resulted in the expression of NR. Within 20 min of the removal of ammonium, NR mRNA can be measured and the expression appears to have attained a peak by this time during the time course studied (Figure 1). Later time points of the induction show detectable stable levels of NR mRNA which are significantly lower than the initial burst at 20 min. NR protein was undetectable at 20 min, but could be detected by Western blotting after 60 min of induction (Figure 1). Levels of NR protein continued to increase over the time course, reaching a

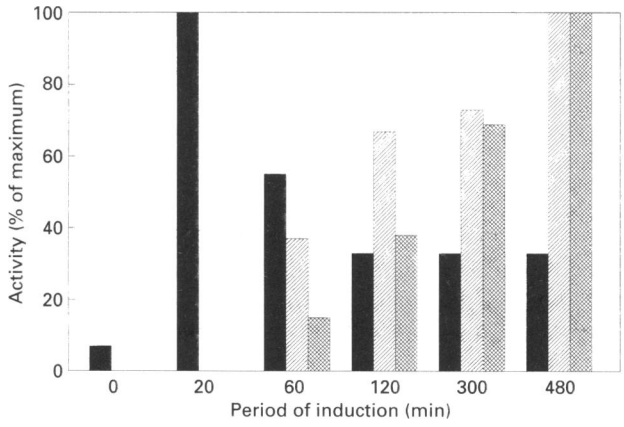

Figure 1 Induction of nitrate reductase

Chlorella cells, grown on ammonium medium (repressed conditions), were transferred to nitrate medium and samples taken for the next 8 h for the detection of NR mRNA, protein and activity. NR mRNA was measured by the RNAase protection assay. A clone to *Chlorella* NR was used to synthesize a 300 bp complementary ^{32}P-labelled RNA that was then hybridized to RNA isolated from the time course. Following RNAase digestion and separation by denaturing PAGE, the protected species was detected by autoradiography. NR protein was measured by Western blotting. Cell-free extracts prepared at the time points indicated were separated by SDS/PAGE, followed by blotting and probing the blot with anti-(*Chlorella* NR) to detect the 100 kDa subunit. Values for NR mRNA and protein were determined by densitometric scanning. NADH:NR activity was measured in the same cell-free extracts used for the Western blotting and were assayed in triplicate. 100% maximal activity in this case was 0.12 μmol/min per of protein. The results shown are representative of three separate experiments. Key to bars: ■, NR mRNA; ▨, NR protein; ⊠, NADH:NR activity.

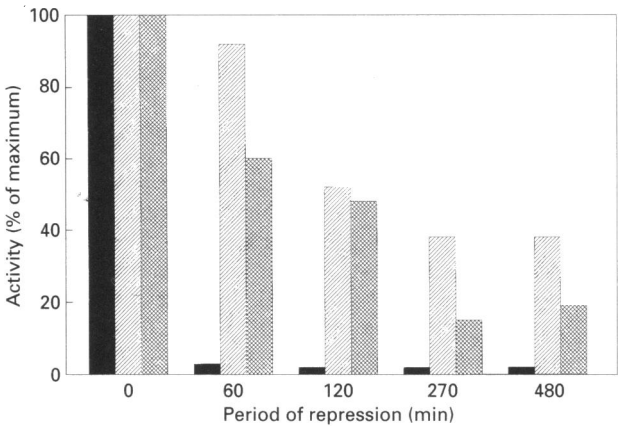

Figure 2 Repression of nitrate reductase

Chlorella cells grown continuously on nitrate medium were transferred to ammonium medium, and NR mRNA, protein and activity measured over the next 8 h. NR mRNA, protein and activity levels were measured as described in Figure 1. Maximal (100%) NR activity was 0.11 μmol/min per mg of protein. The results shown are representative of three separate experiments. Key to bars: ■, NR mRNA; ▨, NR protein; ⊠, NADH:NR activity.

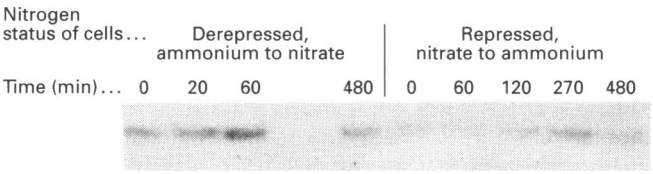

Figure 3 Detection of mitochondrial ADT/ATP-translocator-protein mRNA during induction and repression

RNA samples used in Figures 1 and 2 were analysed by Northern blotting and probing with ^{32}P-labelled translocator cDNA.

peak by 8 h (Figure 1). NR activity can also be detected after 60 min of ammonium removal, Figure 1, and continues to rise reaching a peak at 8 h. Levels of NR protein increase faster than NR activity initially suggesting a post-translational modification event is involved for full expression of NR (Zeiler and Solomonson, 1989).

Expression of NR during repression

Chlorella cells that had been grown under continual inducing conditions (nitrate medium) and then transferred to repressing conditions (ammonium medium) were assayed for changes in NR mRNA, protein and activity as described above. Under induced conditions, NR mRNA, protein and activity are high (Figure 2). The removal of nitrate and addition of ammonium, however, resulted in a dramatic loss of NR mRNA within 1 h of ammonium addition to levels that were barely detectable using the RNAase protection assay (Figure 2). In contrast, levels of NR protein and activity did not decrease as rapidly. The level of NR protein decreased slowly over the time course to approx. 40% of initial levels by 8 h (Figure 2). Immunoreactive fragments could be detected that were smaller than the NR subunit. However, these fragments did not increase, or decrease, in concentration during the time course, suggesting that NR was not being broken into fragments by specific proteolytic digestion (results not shown). NR activity declines to about 20% of

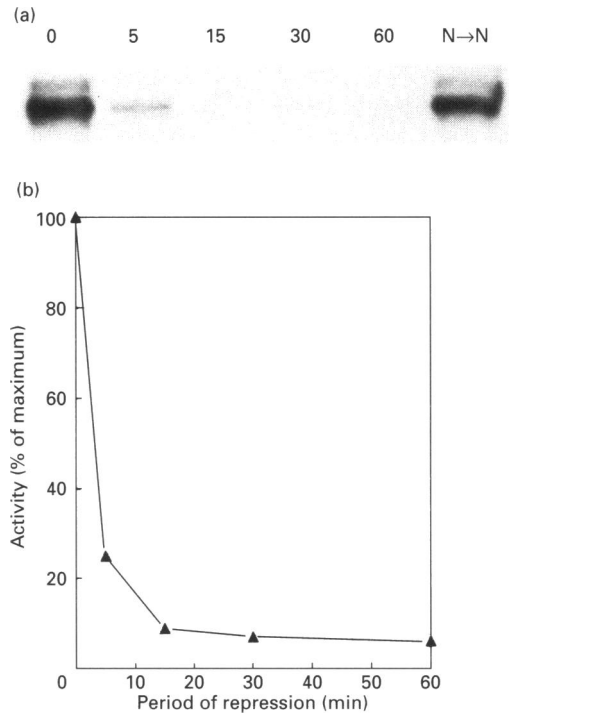

Figure 4 NR mRNA levels during the first 60 min after repression

Total RNA extracted from cells following repression was probed for NR mRNA by the RNAase protection assay as in Figure 1. (**a**) Gel of NR mRNA levels. Numbers above lanes refer to times (in min) after ammonium addition. N → N refers to cells transferred from nitrate medium to fresh nitrate medium. (**b**) Kinetics of NR mRNA stability during repression determined by scanning a representative gel from (**a**).

induced levels by 120 min (Figure 2), a higher rate than that of the protein, suggesting inactivation of the enzyme could be occurring (Zeiler and Solomonson, 1989).

To show that the changes in NR mRNA reflect specific responses to nitrogen source, the RNA samples isolated for these induction and repression time courses were probed by Northern analysis for a putative non-nitrogen controlled mRNA (Figure 3). The ADP/ATP translocator protein mRNA was found to be present at all stages of nitrogen status and, though the levels fluctuated slightly, there was no significant change as a result of ammonium addition or removal.

Stability of NR mRNA under repressing conditions

To study the time course of NR mRNA stability in more detail, NR mRNA turnover was measured during the first hour of repression. *Chlorella* cells grown on nitrate medium were transferred to medium containing ammonium and the NR mRNA levels measured in cells repressed for 0, 5, 15, 30 and 60 min. As shown in Figure 4(a), NR mRNA decreased to 25% of initial levels within 5 min of addition of repressor, and by 15 min essentially all of the transcript had disappeared. A plot of densitometric scans of this gel is shown in Figure 4(b) and illustrates this dramatic decrease in NR mRNA, suggesting that the half-life of NR mRNA under repressing conditions is less than 5 min. This compares with half-lives of the protein and activity of approx. 120 min (Figure 2c). Under these conditions, *Chlorella* cells could take up ammonium at a rate of 10 nmol/min per 10^8 cells within the first 5 min of transferring from nitrate to ammonium medium (results not shown).

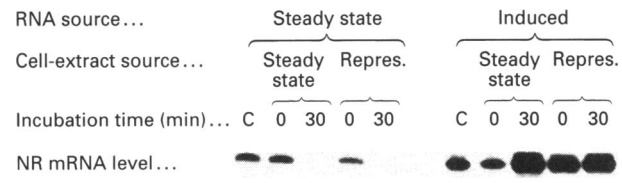

Figure 5 Stability of NR mRNA *in vitro*

Chlorella RNA isolated from cells exposed to nitrate for a 20 min period ('Induced') or from cells grown continuously on nitrate ('Steady state') was incubated with cell-free extracts prepared from nitrate-adapted cells ('Steady State') or from cells exposed to ammonium for a 20 min period ('Repres'). Following phenol/chloroform extraction, the presence of NR mRNA was detected by RNAase protection assay and gel analysis. 'C' refers to control, where no cell-free extract was added to the RNA source. Numbers above the lanes refer to the period of incubation (min) with the extract.

Detection of NR mRNA RNAase *in vitro*

To determine possible reasons for the striking loss in NR mRNA during repression, an *in vitro* assay for identifying specific NR mRNA RNAases was employed. Here RNA was isolated from *Chlorella* cells grown under fully induced conditions (nitrate medium) or short-term derepressed/induced conditions (cells transferred from ammonium medium to nitrate medium for 20 min). The former is referred to as 'steady-state' NR mRNA, while the latter is referred to as 'induced' NR mRNA. This RNA was then incubated with cell-free *Chlorella* extracts made from cells grown on nitrate medium, potentially a low source of NR mRNA-specific RNAase(s), or cells transferred from nitrate medium to ammonium medium for 20 min, potentially a rich source of NR mRNA-specific RNAase(s). Following incubation the extracts were assayed for NR mRNA by RNAase protection assay. The results shown in Figure 5 demonstrate that steady-state NR mRNA was completely degraded by both of the cell-free extracts, indicating that RNAase(s) capable of degrading NR mRNA was present in both nitrate-grown cells and short-term repressed cells. While NR mRNA isolated from nitrate-grown cells (steady-state) was susceptible to RNAase degradation, NR mRNA isolated from short-term induced cells (20 min in nitrate) appeared to be resistant to RNA digestion by both of the cell extracts when incubated under identical conditions (Figure 5). In fact the level of nitrate-induced mRNA present after treatment with the cell-free extracts was consistently elevated compared with the control (no extract added) and zero-time samples. This experiment has been repeated four times with the same results. The reason for this is not known, but it may be due to an extract component 'protecting' the already stable mRNA during the following RNAase protection assay. In each case, identical amounts of RNA were added to the assay.

DISCUSSION

The results shown here suggest that, in *Chlorella vulgaris*, NR expression was significantly regulated by nitrogen source. The level of control appears to be primarily transcriptional, although post-transcriptional controls also operate to determine high or low levels of the enzyme. Under repressed conditions, when ammonium was the sole nitrogen source, there was no detectable NR mRNA, protein or activity. This is similar to the situation in the mould *Neurospora* (Okamoto et al., 1991), *Candida nitratophila* (Cannons and Hipkin, 1987) and higher plants (Calza et al., 1987; Cheng et al., 1986; Crawford et al., 1988; Solomonson and Barber, 1990). Previously it has been found that NR mRNA (using *in vitro* translation) and NR activity can be detected in *Chlorella* grown continuously in ammonium (Sherman and Funkhouser, 1989). However, NR mRNA was undetectable using the RNAase protection assay employed for this study. Following ammonium removal and nitrate addition, NR mRNA increases substantially within 20 min. This was a peaked increase, since the level of NR mRNA decreases after 60 min to what appears to be a steady-state level which is approx. 30% of the peak level. This rapid peaked increase in NR mRNA has also been seen in *C. nitratophila* (Cannons and Hipkin, 1987) and in *Neurospora* (Okamoto et al., 1991), in which maximal NR mRNA concentration was observed 15 min after depression/induction. The rapid increase in *Chlorella* NR mRNA during induction was not reflected in other mRNAs. Probing for the ADP/ATP translocator mRNA indicated that nitrate addition and ammonium removal had no substantial effect on the level of this mRNA species. A similar expression of NR mRNA has been identified in higher plants. In rice (*Oryza sativa*) seedlings NR mRNA reached a peak after 6 h of induction, after which the level decreased (Hamat et al., 1989). In barley (*Hordeum vulgare*) the induction profile of NR mRNA is organ-specific. Barley roots show a peak of expression, with the highest level after 2 h of nitrate addition. In leaf tissue NR mRNA reaches a maximum after 4–12 h (Melzer et al., 1989). Light is also an important factor to consider in NR expression, since it augments the induction of NR in higher plants (Solomonson and Barber, 1990). NR mRNA expression is also under the control of a circadian rhythm (Deng et al., 1989). How light influences expression of NR mRNA in *Chlorella* is not known at this stage, since the cells were grown under continuous illumination.

The subsequent increases in NR protein and activity were similar to those observed before in *Chlorella* (Zeiler and Solomonson, 1989), *Neurospora* (Okamoto et al., 1991) and *C. nitratophila* (Cannons et al., 1986). The increases in NR protein and activity were not detectable until 60 min after the removal of ammonium. Sherman and Funkhouser (1989) have reported that detectable NR protein can be observed within 40 min of induction. The increase in protein occurs before the increase in activity. This has been previously reported for *Chlorella* (Zeiler and Solomonson, 1989), *C. nitratophila* (Cannons et al., 1986) and corn (*Zea mays*) (Oaks et al., 1988) and seems to suggest a post-translational modification is required for full NADH:NR activity. The incorporation of a molybdenum cofactor is a likely candidate for this limiting step in expression (Zeiler, 1989).

Changes in levels of NR mRNA, protein and activity as a result of repression (i.e., addition of ammonium to nitrate-grown cultures) are easier to follow in single-celled organisms like *Chlorella* than in higher plants. *Chlorella* does not store nitrate in vacuoles, as may occur in higher plants (Solomonson and Barber, 1990) and so the adaptation of the cell to a change in nitrogen source can be determined. *Chlorella* grown continuously on nitrate medium exhibited a high NR mRNA content, presumably representing a steady-state level. Following the addition of ammonium, NR mRNA decreased within 60 min to a barely detectable level. This decrease was subsequently found to be within 15 min, suggesting a half-life of NR mRNA to be less than 5 min, a value that is comparable with that observed for *Neurospora* (Okamoto et al., 1991). Changes in NR protein and activity were not as immediate as the mRNA. Previously it has been shown that NR activity decreases more rapidly than NR protein level (Zeiler and Solomonson, 1989). This is apparently due to a reversible inactivation process, possibly involving formation of an inactive NR–cyanide complex (Lorimer et al., 1974), since the enzyme can be re-activated by ferricyanide (Zeiler and Solomonson, 1989). Estimates suggest a half-life for

NR protein, under these conditions, of approx. 120 min. How NR is degraded in the cell is not known. Certainly NR can be inactivated by limited proteolysis (Hamano et al., 1985; Solomonson et al., 1986), but how this type of regulation occurs *in vivo* is not known. Our results using Western blotting suggest that NR can be degraded into discrete products. However, these products did not appear to increase or decrease during induction/repression (results not shown). This has been shown before for derepressed *Chlorella* cells (Sherman and Funkhouser, 1989; Zeiler and Solomonson, 1989).

There could be a number of factors that contribute to the rapid loss of NR mRNA during repression. Cells respond rapidly to changes in the environment by altering steady-state levels of mRNAs coding for proteins affected by these changes (Atwater et al., 1990). Essentially the rate of transcription and/or the stability of the mRNA could be affected in the cell. Determinants that could affect mRNA stability include the polyadenylated [poly(A)$^+$] tail, involvement of a poly(A)$^+$-binding protein and nuclease digestion (Atwater et al., 1990). Here we looked at the stability of NR mRNA to identify determinants involved in maintaining levels of NR mRNA (Figure 5). NR mRNA could be degraded by a cell-free extract, presumably containing a mix of nucleases. However, the sensitivity of NR mRNA to the extracts did not appear to be governed by the nitrogen status of the extracts containing the RNAase(s), but rather by the source of the mRNA. This suggests that the NR mRNA itself confers stability/instability. It is known that the structure of the mRNA itself can be responsible for its stability. Polynucleotide phosphorylase RNA in *Escherichia coli* is more resistant to endonucleolytic attack when unprocessed at the 5′ end (Takata et al. 1992) and stem–loop formation confers stability in certain RNAs (Adams and Stern, 1990; McLaren et al., 1991). Alternatively, specific sites can impart RNA instability, e.g. the RNA-instability-determinant sequence of c-myc RNA (Cole and Mango, 1990) and the translated N-terminal nucleotides of β-tubulin mRNAs (Theodorakis and Cleveland, 1992). There is also evidence that the binding of certain factors can affect mRNA stability (Bohjanen et al., 1992; Monod et al., 1992). Whether any of these determinants contribute to the stability/instability of NR mRNA in *Chlorella* under differing nitrogen sources is unknown. Northern-blot analysis did not indicate any difference in size of NR mRNA isolated from *Chlorella* under different conditions (steady state as against newly induced), and we have not identified any sequence differences as yet (results not shown). Also, Southern analysis has identified a single gene coding for NR in *Chlorella* (results not shown). There is no evidence to suggest that NR mRNA is regulated by degradation by specific RNAase(s) that are nitrogen-regulated. The identification of specific RNAases that are nitrogen-controlled, using SDS/PAGE (Yen and Green, 1992), was unsuccessful and inconclusive. However, there is evidence to suggest that, in corn, levels of NR transcript are higher in induced tissue when cycloheximide is present (Gowri et al., 1992). This could be the result of inhibition of synthesis of NR RNAases, thus resulting in higher levels of NR mRNA. Alternatively, nitrogen-regulated RNAase inhibitors may be involved. Whether such mechanisms operate in *Chlorella* is not known. The results described here suggest that mRNA stability may be an important factor in the nitrogen-controlled regulation of assimilatory nitrate reductase. The physiological significance of this regulatory mechanism is not understood at the present time, but could be important for rapid responses to changes in nitrogen source.

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