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Transcriptional Control of the Inducible Nitrate Reductase Isoform in Soybean, Glycine Max

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TRANSCRIPTIONAL CONTROL OF THE INDUCIBLE NITRATE REDUCTASE ISOFORM IN SOYBEAN, *GLYCINE MAX*

by

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VITA

The author, John J. Callaci, is the son of Lee and Carmella Callaci. He was born July 9, 1962 in Oak Park, IL. His elementary education was completed in the Westchester public school system in Westchester IL. His secondary education was completed at Proviso West High School in Hillside IL, in June of 1980.

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ABSTRACT

Nitrate reductase (NR) is the first enzyme of the nitrate assimilatory pathway of higher plants. It catalyses the reduction of nitrate to nitrite, which is generally regarded as the rate limiting step in nitrate assimilation. We have measured steady-state mRNA levels of the inducible nitrate reductase isoform in soybean seedlings. Ten (10) day old seedlings were irrigated with nutrient media differing in nitrate content. These nutrient media contained: 1) zero nitrate 2) 10 mM glutamine 3) 10mM glutamine and 50 mM KNO_3 4) 50mM KNO_3. Poly A+ mRNA was isolated from primary leaves forty eight (48) hours after nutrient treatment, and slotted onto a nylon membrane. Hybridization was performed with a 1.2 kb cDNA clone for squash NR. It was found that seedlings treated with zero nitrate or 10mM glutamine possessed no measurable amounts of inducible NR transcript. Those treated with both 10mM glutamine and 50mM KNO_3 showed intermediate transcript levels, and seedlings treated with 50 mM KNO_3 showed high levels of inducible NR transcript. We also examined inducible NR transcript levels at several time points after treatment, including a point during the dark portion of the photoperiod. We observed NR specific transcript appearing two (2) hours after treatment, peak levels were seen twenty-four (24)

and forty-eight (48) hours after treatment. Much reduced levels of inducible NR transcript were observed in mRNA samples isolated from dark harvested leaves. This may indicate some form of light specific control regulating inducible NR transcript levels, in addition to the regulation of transcript levels observed in the nutrient treatment groups. Nuclear run-on transcription assays performed with nuclei isolated from seedlings treated as above, provide evidence indicating that fluctuations seen in steady state NR transcript levels due to nitrate treatment and light are controlled at the level of transcription.

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CHAPTER I

INTRODUCTION

Normal plant growth requires a variety of nutrients, one of the most important being nitrogen. Most higher plants assimilate the majority of their nitrogen in the form of nitrate obtained from the soil. Once in the plant, nitrate is reduced to ammonia by a two step pathway. The first reaction requires the enzyme nitrate reductase (NR); the second reaction is catalyzed by nitrite reductase (NiR).

$$NO_3^- \xrightarrow[\text{NADH or NADPH}]{\text{NR}} NO_2^- \xrightarrow[\text{Ferredoxin}]{\text{NiR}} NH_4^+$$

These nitrate reduction reactions occur predominately in the leaves of most plants (1,2). The first step in this pathway, catalyzed by nitrate reductase, is considered to be rate limiting (1,15), and thus is highly regulated (1,15). Nitrite reductase (NiR) has also been shown to be coordinately regulated with nitrate reduction (1,15). Factors such as light and availability of nitrate have been shown to be very important in modulating NR activity (3,4). Soybean leaves have been shown to contain three isoforms of NR (5). Two

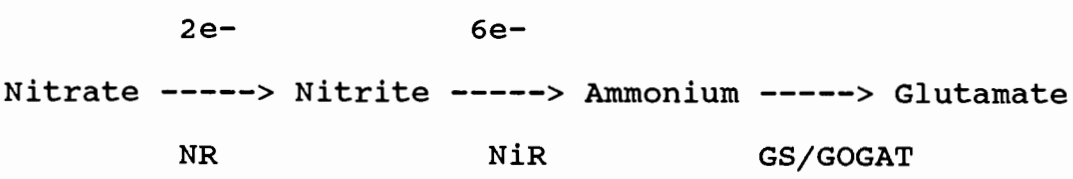
of the isoforms have been termed constitutive as their activity does not require the presence of nitrate (6,17). However their activities do increase in nitrate treated plants (6). The third isoform is termed inducible; the presence of nitrate is prerequisite for its expression and activity (6).

This study focuses on the molecular events which regulate expression levels of the inducible nitrate reductase isoform in soybeans. The overall objective was to determine if expression of the inducible NR isoform was controlled at the level of transcription. To satisfy this objective, both steady state and nuclear RNA levels specific for the inducible NR isoform were quantitated. The steady state experiments included both slot blot and Northern blot analyses. Nuclear RNA levels were analyzed by performing <u>in vitro</u> transcription assays with nuclei isolated from soybean primary leaves. A 1.2 kb cDNA clone for squash nitrate reductase (7), available in our laboratory was used as a probe for inducible NR mRNA (8). This clone was used to quantitate RNA levels for both the steady state mRNA experiments and nuclear run-off transcription assays. Further information regarding the molecular control mechanisms involved in NR synthesis, may ultimately lead to the development of crop plants capable of using soil nitrate more efficiently.

CHAPTER II

REVIEW OF RELATED LITERATURE

The majority of inorganic nitrogen converted into organic nitrogen by higher plants is derived from the assimilation of nitrate (2). In most plants, nitrate assimilation has been found to occur in the leaves (1,2). The nitrate assimilatory pathway in plants can be diagrammed as follows:



The first step in this pathway, the reduction of nitrate to nitrite, is generally regarded as the rate limiting step in nitrate assimilation (1,15). The enzyme which catalyzes this reaction, nitrate reductase, is highly regulated (1,15).

Nitrate reductases are soluble electron transferring proteins (1). In most higher plants, the enzyme is a homodimer of 110-115 KDa (18). Each subunit is associated with three prosthetic groups, flavin (FAD), cytochrome b_{557} heme, and molybdenum; each group comprising a redox center. These groups transfer electrons between the

pyridine nucleotide oxidation site and the nitrate reduction site (1). A sulfhydryl group is responsible for transferring the reducing power from the flavin site, where the oxidation of NADH of NADPH occurs, to the catalyst (19). There is evidence that the three redox centers in the enzyme are divided into distinct domains (18). Nitrate reductase is located in the cytoplasm of plants, whereas the other enzymes of the nitrate assimilation pathway are chloroplastic (20).

There is also evidence of apparent nonphysiological activities of nitrate reductase. One is a dehydrogenase activity where nitrate reductase catalyzes the pyridine nucleotide linked reduction of various electron acceptors such as ferricyanide, cytochrome c and dichlorophenolindophenol (14). Another activity reduces nitrate with electrons provide by such donors as viologen dyes, reduced flavins, or methylene blue (14). Another demonstrated activity of nitrate reductase is its ability to catalyze the reduction of a ferriphytosiderophore from barley (27,28). The significance of this iron reductase activity of NADH:nitrate reductase has not yet been demonstrated.

Nitrite reductase (NiR) is the second enzyme of the nitrate assimilatory pathway, and is located in the chloroplast stroma. Nitrite reductase catalyzes the 6-electron reduction of nitrite to ammonia. The best

characterized nitrite reductase has been purified to homogeneity from spinach leaves (29,30). Spinach nitrite reductase contains a tetranuclease iron-sulfur cluster (29), and a siroheme prosthetic group (31). Siroheme is an iron tetrahydroporphyrin of the isobactercteriochlorin type which functions as the site where nitrite is bound and reduced (29). This nitrite reducing unit has been found in association with a 61,000 molecular weight protein, where ferredoxin serves as the electron donor.

Soybean plants have been shown to contain three isoforms of nitrate reductase (5). One activity is NADH-linked and has a pH optimum of 6.5. A second NR activity can utilize NADH or NADPH as electron donors, and also has a pH optimum of 6.5. These activities were first described by Aslam (16) and have been termed constitutive as their activity does not require the presence of nitrate (6,17), although their activities do rise in the presense of nitrate (6). The activity of the third isoform is NADH-linked, and has a pH optimum of 7.5. This activity is dependent on nitrate induction and is termed inducible (18). The nitrate assimilation pathway is one of the few systems documented in plants that has a substrate inducible system (18).

Streit et al. (17) separated and purified the three soybean NR isoforms using Blue Sepharose and ion exchange chromatography. Several properties of these isoforms were

investigated. All three isoforms had one predominant band on SDS-PAGE. This band was 107 kD for the constitutive isoforms, 109 kD for the inducible isoform. The significance of this size difference is not known. Specific activities of the three isoforms varied considerably. Inducible NADH:NR (pH 7.5) had the highest specific activity, while NAD(P)H:NR had the lowest. Cytochrome b type heme was shown to be a component of the three NR isoforms by their UV/visible absorption spectra. The oxidized forms of the enzymes had peaks at 273 and 413 nm. Addition of NADH reduced the enzymes, revealing peaks at 424, 527 and 556nm, which are characteristic of b-type Cytochrome.

Early studies of the effects of metabolites on NR activity were performed using the ascomycete fungus Neurospora crassa. These studies indicated that nitrate induces nitrate reductase activity, while glutamine and other reduced nitrogen sources repress nitrate reductase activity even in the presence of nitrate (14). Many studies have also been performed studying the effects of metabolites on higher plant nitrate reductase activity. Nitrate application generally enhances NR activity in higher plants (6,21). However, since plants store large amounts of nitrate in their vacuoles, the flux of nitrate through plant cells has also been implicated in enhancing nitrate reductase activity (40). Many laboratories have

demonstrated the repression of nitrate reductase activity in higher plants by reduced nitrogen compounds such as glutamine (6, 21, 32-35).

The effects of metabolites on soybean nitrate reductase expression and activity have been studied by many groups. Soybean cell culture grown with 20mM glutamine or 20mM ammonium citrate failed to develop significant NR activity when transfered to media containing nitrate and glutamine or ammonium citrate (36).

Another study noted that soybean cotyledon suspension cultures grown with glutamine as the sole source of nitrogen displayed minimal in vivo NR activity, but when the cells were transfered to culture media containing 25mM nitrate, NR activity increased significantly (37). Curtis and Smarrelli (6) working with soybean seedlings, found that seedlings not supplied with nitrate display virtually no NADH-linked NR activity, at pH 7.5. When nitrate was supplied to the seedlings, a 30 fold enhancement was seen in pH 7.5 NADH-linked NR activity. This peak in enzyme activity was seen 48 hours after nitrate treatment. Addition of glutamine to the nitrate treatment medium, resulted in a 38% decrease in inducible NR activity at 48 hours. These results suggest that nitrogenous metabolites play important roles in the regulation of nitrate reductase activity in soybeans.

Much recent work in the field has focused on the molecular events controlling NR synthesis. cDNA's for squash (7), Arabidopsis (18), barley (26), and tobacco (22) nitrate reductase have been isolated and characterized. A Northern blotting experiment showed an increase in steady state levels of a 3.5 kb mRNA for nitrate reductase from barley, in response to nitrate induction (26). Using squash, Crawford et al. (7) performed a Northern blotting experiment which identified a 3.2 kb mRNA transcript for NR which appeared only after nitrate treatment. Dot blots of poly A+ mRNA obtained from squash cotyledons, probed with the 1.2 kb cDNA clone of the NR gene, show NR specific mRNA levels to be detectable only in plants supplied with exogenous nitrate (38). The same study also shows a correlation between changes in NR activity and changes in steady state levels of NR specific mRNA. Earlier work in our laboratory studying the inducible NR isoform in soybeans, also showed a correlation between its activity and steady state mRNA levels (8 and unpublished results).

The role of light in control of nitrate reductase synthesis has also been studied. Light has been shown to enhance the expression of a number of plant genes (45). The role of light in modulating NR synthesis is somewhat complex. In the absence of nitrate, light causes only small increases in nitrate reductase protein (23) and NR

specific mRNA (24) in etiolated plants. However, when etiolated plants were given nitrate and light together, nitrate reductase protein, activity, and NR specific mRNA all increased to a high level, and in proportion to one another(23,24). These studies suggest that although light may stimulate synthesis of NR, it is not able to exert this influence unless nitrate has previously activated the NR gene (25).

One mechanism studied for the influence of light on NR is the phytochrome system. In a study by Rajasebhar et al. (25), it was shown that etiolated squash seedlings given pulses of red light showed increases in NR protein and activity over dark grown plants. This influence of red light could be reversed by giving a pulse of far red light, establishing that the classical phytochrome system was in operation. Other etiolated plants were given continuous far red light, which is another way to activate the phytochrome system. Continuous far red light increased NR protein, activity, and mRNA levels relative to etiolated plants kept in darkness, but this effect was only seen in plants also given nitrate. Thus, it appears that the phytochrome system is involved in a mechanism which directly influences levels of NR mRNA, but does not trigger the expression of the gene (39).

The same study also showed that continuous white light produced higher levels of NR mRNA than continuous

far red light, indicating that additional effects of light probably exist in addition to those seen in the phytochrome system. The effects of light on NR expression can be summarized by saying that nitrate triggers the expression of the NR gene, while light influences the level of expression of the gene.

Recently much work has been performed in the area of control of gene expression in higher plants. The most important level of control in gene expression is transcriptional (48). Most studies measure steady state mRNA transcript levels of the message of interest. The problem with this approach is that steady-state mRNA levels represent synthesis as well as degradation of mRNA. Hence are not a true reflection of the rate of transcription of a specific gene. Transcriptional control must be assessed by the rate of synthesis of specific nuclear RNA. Nuclear RNA is not subject to the same level of degradation suffered by mature transcripts in the cytoplasm. Thus its synthesis is an accurate indication of transcription of a gene of interest. An experimental method often used to access transcriptional control is nuclear run-off transcription assays. These <u>in vitro</u> assays use intact nuclei isolated from fresh tissue. The main advantage of using isolated nuclei for transcriptional studies is that it is a system which approximates what is occurring in the intact cell. Within

isolated nuclei, chromatin is maintained in its native state, and newly synthesized RNA remains associated with the nucleus, as it does in an intact cell. Therefore the activity measured in these assays reflects the activity of nuclei in intact cells; the same genes are expressed in the same relative amounts in both.

When nuclei are isolated there are many RNA polmerases actively transcribing RNA, the process of isolation is thought to "freeze" RNA polymerase in place on the gene it is transcribing (41,47). When given the proper conditions in an in vitro system, the polymerase continues transcription where it left off when the cell was disrupted (41,47). Thus RNA transcribed in vitro in these assays reflects completion of RNA chains initiated in vivo (41,47). Since the RNA chains are initiated in vivo, this system seems well-suited to studying how endogenous factors may modulate expression of nuclear genes, by comparing their effect to suitable controls.

Many recent studies have utilized nuclear run off transcription assays to analyze control of gene expressions. Hagen and Guilfoile (13) used this technique to demonstrate rapid induction of selective transcription by auxins. This study found increased transcription rates of four auxin regulated genes within 5 minutes of auxin regulation. In another study, the effect of the phytochrome system on gene expression was studied using

runoff transcription assays. Silverthorne and Tobin (42) found phytochrome stimulated transcription of the chlorophyll a/b binding protein,(CAB), rRNA, and the small subunit of ribulose - 1,5, bisphosphate carboxylase (RUBISCO). Nuclei harvested from the duckweed Lemna gibba given short bursts of red light showed increased transcription of these genes as compared with dark grown controls. This phenomenon was demonstrated to be phytochrome regulated, by its reversibility by treatment with far red light after red light exposure.

Lawton and Lamb (43) demonstrated transcriptional regulation of defense genes in Phaseolus vulgaris cell suspension cultures and hypocotyl sections, by also using runoff transciption in isolated nuclei. These researchers saw rapid induction of transcription in nuclei isolated from bean cells or hypocotyl sections after treatment with a fungal elicitor preparation. The increased transcription in nuclei from treated tissue as compared to control tissue was by genes known to function in plant disease resistance, such as phenylalanine ammonia-lyase (PAL), and chalcone synthase (CHS). These experiments again demonstrate the usefulness of nuclear run off transcription assays in assessing transcriptional regulation of specific nuclear genes.

CHAPTER III

MATERIALS and METHODS

Plant Material and Growth Conditions

Soybean seeds (Glycine max. var. Williams) were grown for 10 days in an environmental growth chamber at 25°C with a 16 hour photoperiod. Plants were watered with tap water throughout the 10 day period. On day 10 of growth, 4 hours after the start of the photoperiod, the plants were irrigated with a modified Hoaglands nutrient medium (9) containing various nitrogen sources: (no NO_3^- 10mM glutamine, 10mM glutamine & 50mM KNO_3, and 50mM KNO_3). Primary leaves were harvested at varying time points after nutrient treatment: (0, 2, 4, 10, 11, 24, 42, and 48 hours). Leaves were frozen immediately in liquid nitrogen and stored at -80°C. For Northern Blot analysis, leaves were given nutrient treatment on day 3 of growth, and harvested 48 hours later. For nuclei isolations, leaves were harvested from 4 to 6 days after planting. This tissue was used immediately, as freezing of tissue prior to nuclei isolation adversely affects the transcriptional activity of isolated nuclei (13).

RNA Isolation

Plants grown as described above were used for mRNA isolations. This mRNA was used to quantitate steady state levels of the inducible NR isoform. The RNA isolations were performed according to the procedure of Dodd *et al.* (10). All glassware was baked at 200°C for a minimum of 4 hours before use. All buffers were made with diethyl pyrocarbonate (DEPC) treated water, filter sterilized and autoclaved. Primary leaves (10g) were homogenized to a powder with a mortar and pestle in liquid nitrogen. The powder was then stirred for 30 minutes at 4°C in a mixture containing STE (0.1 M NaCl, 0.05 M Tris, 0.001 M ETDA pH 7.0), water saturated phenol, chloroform: isoamyl alcohol (24:1), 0.5% SDS, and 10mM 2-mercaptoethanol. This mixture was then centrifuged for 10 minutes at 10,000 rpm, and the aqueous phase collected and adjusted to 15% ethanol. This phase was then subjected to Cellex N-1 cellulose column chromatography which binds most of the double stranded RNA, and allows single stranded RNA to pass through the column. This total RNA fraction was then precipitated by adding 2.5 volumes of 100% ethanol and incubating at -20°C for 12 hours. Total RNA was then fractionated into poly (A+) and poly (A-) RNA, by oligo (dT)- cellulose column chromatography (11). The resulting RNA samples were stored at -80°C.

RNA samples were analyzed by electrophoresis through 1% agarose, stained with ethidium bromide, visualized and photographed under ultraviolet light.

Slot Blot Analysis

Poly (A+) mRNA was screened for quantities of inducible NR transcripts by slot blot analysis. Five micrograms of poly (A+) RNA from each treatment group were slotted onto a Gene Screen Plus membrane (DuPont), using a Schleicher & Schuell slot blot apparatus. The probe was the 1.2 kb cDNA for squash nitrate reductase isolated by Crawford et al. (7). The probe was radioactively labelled by nick translation (Amersham) using [a 32p] dCTP. Hybridization conditions were 50% formamide, 1% SDS, 50mM Tris, 1M NaCl, 0.5mg/ml denatured sheared salmon sperm DNA. Blots were incubated in the above solution for one hour, at which time the radioactively labelled probe was added. Hybridizations were carried out for 48 hours at room temperature. Washing of blots to remove unbound radioactive label was performed as follows:

1) Two times at room temperature for 15 minutes in 100 ml 2X SSC
2) Two times at 55^{o}C for 30 minutes in 100ml 2X SSC, 1% SDS

3) Two times at room temperature for 15 minutes in 100 ml 0.5X SSC

Hybridization between the labelled probe and mRNA was visualized by autoradiography and quantitated by densitometry. This procedure was repeated to encompass all treatments and time points, and duplicated to ensure the signals seen were reproducible.

Northern Blot Analysis

To determine the size(s) and number of the transcript(s) detected in slot blot analysis, a Northern transfer was performed. For this procedure RNA was denatured and electrophoresed as follows. Sixteen ug of total RNA from the zero nitrate and 50mM nitrate treatment groups was dissolved in 2ul DEPC treated H_2O. These RNA samples were denatured for 10 minutes at 60^{o}C in a solution containing 50% formamide, 16.5% formaldehyde, 8.25% glycerol, 40mM Triethanolamine and 2mM disodium ETDA (pH 7.5). Samples were then chilled on ice, and loaded immediately on a denaturing 1.5% agarose gel containing 3.1% formaldehyde. Suitable RNA markers were included on the gel (RNA ladder, BRL INC. Gaithersburg MD). Samples were electrophoresed at 70V in a buffer also containing 3.1% formaldehyde. When electrophoresis was complete the gel was removed from the

electrophoresis chamber, the RNA markers were excised and soaked overnight in 1ug/ml ethidium bromide in H_2O. The remainder of the gel was soaked twice for 30 minutes in 500ml of 10mM Na-PO_4 (pH 7.0). The transfer apparatus was assembled as described by Thomas (44). When the transfer was complete approximately 24 hours later the blot was removed and baked for 2 hours at 75°C under vacuum.

The blot was prehybridized at 42°C for 12 hours in 20 ml of a solution of 50% formamide, 5X SSPE (1X SSPE is 10mM NaH_2PO_4 and 1mM Na_2 ETDA), 5 X Denhardt's Solution (100X Denhardt's is 2% Bovine serum albumin, 2% Polyvinyl pyrrolidone and 2% Ficoll), 0.1% SDS , and 250 ug/ml denatured DNA. After prehybridization , the filter was hybridized at 42°C for 48 hours in 20 ml of a solution of 50% formamide, 5 X SSPE, 1 X Denhardt's Solution, 0.1% SDS, with the addition of the 1.2 kb cDNA probe labelled by random priming (Multiprime DNA labeling system Amersham Corp. Arlington Heights IL), at a concentration of 1 X 10^6 cpm/ml. After hybridization, the blot was washed twice at room temperature in 500 ml of 2X SSPE, 0.1% SDS. Next, the filter was washed 2 times at 50° C for 60 minutes in 0.1X SSPE, 0.1% SDS. After the washes were complete the blot was exposed to X-ray film at -70°C for 1 week.

Plasmid Purification

To procure sufficient quantities of the 1.2 kb cDNA to be used as a probe, a large scale plasmid purification was performed. A bacterial strain (JM105) was transformed with pCmc-1 a plasmid containing the 1.2 kb insert. A strain of E. coli (JM105), was grown and plasmid DNA was isolated according to the procedure of Maniatis et al. (12). The 1.2 kb insert was then isolated from the plasmid DNA using a standard procedure (47).

Five hundred micrograms of pCmc-1 was cut with the restriction enzyme EcoR I to liberate the 1.2 kb insert. This plasmid was electrophoresed through 1% agarose to separate the plasmid from the 1.2 kb cDNA insert. A slot was then cut in the gel in front of the leading band in the gel , which represents the 1.2 kb insert DNA. The DNA in the band was then electrophoresed into a piece of DEAE paper placed in the slot. DEAE paper binds the insert DNA so it can be removed from the gel , without any plasmid DNA contamination. Insert DNA was removed from the paper by incubating in a high salt buffer (1 M NaCl , 20mM Tris HCl pH 8. 1 mM EDTA) at 65^{o}C for 30 minutes. Ethidium bromide was removed from the DNA by extraction with water saturated butanol. DNA was precipitated by adding three volumes of 95% ethanol and incubating at 20^{o}C overnight. DNA was pelleted by centrifugation and then

reprecipitated. Insert DNA was resuspended in TE buffer (10mM Tris HCl, 1mM EDTA, pH 8) at a concentration of 1ug/ul.

Nuclei Isolations

In order to study nuclear RNA levels of inducible NR transcript, nuclear runoff transcription experiments were performed (13). The first step in this process was the isolation of transcriptionally active nuclei from soybean primary leaves. Plants were grown in exactly the same manner as previously described, except they were harvested from 4 to 6 days after planting and were utilized immediately. The procedure used for the isolation of nuclei was a modification of the method described by Hagen and Guilfoyle (13). Five grams of primary leaves were harvested and soaked in ice cold diethyl ether for 1 minute. The ether was removed and residual ether was removed with dry nitrogen gas. All of the following steps were performed at 2^{o} to 4^{o} C. Nucleus isolation buffer (10mM Tris HCl pH 7.2, 5mM $MgCl_2$, 10mM 2- mercaptoethanol, 1 M sucrose) was added to the tissue at 10 volumes per gram fresh tissue weight. The tissue was then homogenized for 45 seconds with a Brinkman Polytron at a medium setting. The homogenate was filtered through 4 layers of cheesecloth and then through 300,

100, and 50 micron mesh. Twenty five percent Triton was then added to the filtrate to achieve a final concentration of 1%, and the suspension was then filtered through a 20 micron filter. The filtrate was then subjected to centrifugation at 5000 rpm for 10 minutes in a HS-4 swinging bucket rotor, and the resulting pellet was resuspended in 5ml of nucleus isolation buffer, with a Dounce homogenizer. The suspension was then layered over a discontinuous Percoll gradient containing 5ml 50% Percoll and 5ml 25% Percoll in nucleus isolation buffer. The gradient was centrifuged at 5000 rpm for 30 minutes in a HS-4 rotor. Nuclei banded at the interface between the 25% and the 50% Percoll layers, and were removed with a Pasteur pipet. The nuclei were pelleted twice in nucleus isolation buffer to remove residual Percoll. Purity of isolated nuclei was monitored by light microscopy, The nuclei were resuspended at a concentration of 1 X 10^8 per ml in nuclear storage buffer (20mM HEPES pH 7.2, 5 mM $MgCl_2$, 2mM diothiothreitol (DTT), 50% Glycerol). Nuclei were stored at -80^{o}C.

Total Incorporation Assay

Transcriptional activity of isolated nuclei was measured by the total incorporation of [^{3}H] UTP in an *in vitro* transcription assay (13). Eighty units of RNasin (Promega Biotech Madison, WI) was added to 10^6 soybean nuclei and incubated at 27^{o}C. The nuclei were then added to a solution at 27^{o}C containing 20mM HEPES (pH 7.9) 12.5 mM $MgCl_2$, 100mM $(NH_4)_2$ SO_4, 1.0 mM DTT, 25% (v/v) glycerol, 0.5 mM each ATP, CTP, and GTP, and 100uCi of [^3_H] UTP (New England Nuclear Corp., Boston Mass.) Aliquots of the reaction mixture were removed at time zero and every five minutes thereafter throughout a 25 minute period. The aliquots were spotted onto Whatman glass filters (GFC) pre-wetted with ice cold 10% trichloroacetic acid (TCA). The filters were washed three times each with ice cold 10% TCA and dried with 95% ethanol. Labelled RNA bound to the filters was detected using liquid scintillation counting. Duplicate filters were counted for each time point and average CPM were recorded.

Nuclear Runoff Transcription Assays

Nuclei shown to be transcriptionally active by incorporation assays were then used for nuclear runoff

transcription assays which measured levels of inducible NR mRNA transcribed in vitro. These assays were performed in a manner similar to that described by Hagen and Guilfoyle (13).

Five x 10^6 nuclei were incubated in 200 ul of a solution containing 20 mM HEPES (pH 7.9), 12.5 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 1.0 mM DTT, 25% (vol/vol) glycerol, 0.5 mM each ATP, CTP, GTP, 100 uCi of [alpha ^{32}P] UTP, (600 Ci/ m mole) and 160 units RNasin. Assays were conducted at 30° C for 10 min. After 10 min., nuclei were treated with 12 ug of DNase I (Sigma Chemical Co., St. Louis, MO) for 5 min. at 25° C. Next, a 400ul volume of 7.5 M urea, 5% SDS, 20 mM EDTA, 100 mM LiCl and 10 mM Aurintricarboxylic acid (ATA) (pH 7.0) was added along with 600 ul of phenol:chloroform:isoamyl alcohol (25:24:1), and the solution was vortexed for 1 min. The mixture was then centrifuged for 15 min. at 4° C at maximum speed in a microfuge. The aqueous phase was collected and to it was added 200 ul of 4 M ammonium acetate, 20 ug wheat germ tRNA (Sigma Chemical Co.), and 2.5 volumes of 100% ethanol. The mixture was then incubated at -20° C for 12 hours. RNA was pelleted at 4°C in a microfuge. The pellet was washed with 70% ethanol, dried and resuspended in 10 ul sterile H_2O.

DNA Slot Blot Hybridization

To quantify levels of inducible NR transcript produced in the nuclear runoff assays, hybridizations were carried out in DNA excess, as described by Hagen and Guilfoyle (13). Five ug amounts of Eco R1 digested pCmc-1 were used for these hybridizations. After restriction endonuclease digestion, plasmid DNA was denatured by adding 2.0 M NaOH and incubating at 65^{o}C for 1 hour. Plasmid DNA was then placed on ice and diluted to a 200 ul volume by adding 20 X SSC to a final concentration of 6X. (1 X SSC is 0.15M NaCl, 0.015 M Na citrate; pH 7.0). Plasmid DNA was then blotted onto Gene Screen Plus membrane (DuPont) in 5ug amounts using a slot blot aparatus (Schleicher and Schuell, Inc., Keene, N.H.). After blotting, the membrane was allowed to air dry, and then was cut into individual slots. Prehybridization of filters was at room temperature for 1 hour in a prehybridization solution (50% formamide, 1% SDS,1.0 M NaCl, 0.1 M Tris HCl pH 7.5, and 0.5 mg heat denatured herring sperm DNA (Sigma), in a volume of one ml in sealed 1.5ml polypropylene microfuge tubes. The hybridization solution was identical to the prehybridization solution with the addition of the ^{32}p-labelled transcripts from the runoff assays. Hybridizations were carried out for 48 hours at room

temperature. After hybridization filters were washed twice for 15 minutes, in 200ml of 2 X SSC at room temperature, twice for 30 minutes, in 200 ml of 2 X SSC, 1% SDS at 55^{o}C and twice for 15 minutes in 200ml of 0.5 X SSC at room temperature. Filters were then exposed to x-ray film at -80^{o}C. Autoradiograms were exposed for 48 hours without the use of intensifying screens. Hybridization to slot blots was then quantified by densitometry.

CHAPTER IV

RESULTS

Isolation of mRNA

Total cellular RNA, and poly (A+) RNA were isolated from primary leaves of 10 day old soybean seedlings as described in Materials and Methods. The concentration and purity of the isolated RNA samples was determined spectrophotometrically. Samples were scanned from 300 nm to 240 nm. The 260/280 nm ratio was noted as it is used as an indication of purity in nucleic acid samples (12). Table I lists concentrations and 260/280nm ratios for poly (A+) RNA samples isolated from each treatment group. Note that all samples isolated had 260/280 ratios above 1.8, which is the purity standard used for nucleic acid samples (12).

To analyze for size and extent of degradation of the purified RNA samples, gel electrophoresis was performed. Figure 1 shows an agarose gel using 5 ug of poly (A+) RNA from each treatment group. The banding pattern seen in each lane is due to residual rRNA present in each RNA sample. The presence of intact rRNA in the samples as well as the presesnce of high molecular weight

RNA in the gel, suggests that the poly (A+) RNA comprising most of the sample is also intact. The two major bands seen in these RNA samples are 28s and 18s ribosomal RNA. The approximate sizes of these RNA's were calculated to be 3.4 and 1.8 kb respectively.

No visual differences were detected in poly (A+) RNA samples analyzed by gel electrophoresis. To detect the changes in the mRNA profile between NO_3^- treated, and control samples, more sensitive methods were utilized.

Dot Blot Analysis

A quantitative analysis of mRNA from each treatment group was performed to verify the presence or absence of NR specific transcripts in each sample. Equal quantities of poly (A+) RNA isolated from leaves harvested 48 hours after nutrient treatment were probed for the presence of inducible NR transcripts by hybridizing to the ^{32}p labelled 1.2 kb squash NR cDNA. Figure 2 shows the autoradiogram obtained from this hybridization. There was no hybridization between the cDNA probe and poly (A+) RNA isolated from leaves treated with zero nitrate nor 10mM glutamine as seen by the absence of signal for these treatments. Hybridization is evident between the probe and poly (A+) RNA isolated from leaves treated with 50mM KNO_3 or 50mM KNO_3 and 10mM glutamine. The 50mM KNO_3

treatment shows the strongest signal, indicating that the highest steady state levels of inducible NR mRNA were present in this sample. The 50 mM KNO_3/ 10mM glutamine treatment causes an intermediate level of hybridization between the probe and poly (A+) RNA samples, indicating that inducible NRmRNA is present in the sample, but at a lower level than in mRNA from leaves treated with nitrate alone.

Northern Blot Analysis

Soybean plants have been shown to possess three isoforms of nitrate reductase (5). For this reason Northern blot analysis was employed, to verify that the probe used was specific for only one RNA species, and to determine the size of the message seen in the previous experiment. RNA was isolated from five day seedlings 48 hours after nutrient treatment. Two treatment groups were utilized; 1) no nitrate in the nutrient medium, and 2) 50 mM KNO_3 added to the nutrient medium.

An autoradiogram of this experiment is seen in Figure 3. A single band of approximately 3.3 kb was observed in the lane containing RNA from nitrate treated plants. No band was observed in the adjoining lane, which contains RNA from plants not treated with nitrate. The fact that this band is seen only in the nitrate treatment

lane, suggests that this signal is due to inducible nitrate reductase. The fact that a single band was observed, suggests that there is no cross hybridization between the probe and mRNA for the constitutive NR isoforms.

The low intensity of the band seen in this blot is probably due to the fact that the inducible NR mRNA is a low abundance message; [0.01% poly A+ RNA (46)], and only 16 ug of total RNA was used for this transfer, making the amount of inducible NR mRNA present in the sample in the approximately 1.6 pg range. This calculation is based on poly A+ RNA making up 1.0% of total RNA, and inducible NR mRNA comprising 0.01% of the poly A+ fraction.

Steady State NR mRNA Time Course Study

Quantities of inducible NR mRNA from plants given nitrate treatments were also determined at various time points after nitrate treatment. Poly (A+) RNA was isolated from 10 day soybean leaves harvested 0, 2, 4, 10, 24, 42, and 48 hours after treatment with either 50mM KNO_3 or 50mM KNO_3 and 10mM glutamine. The 42 hour harvest was during the dark portion of the photoperiod, and was performed to determine if any light specific controls might be affecting NR transcript levels. Figure 4 shows intact mRNA for each time point and treatment as

determined by gel electrophoresis. Five microgram amounts of poly (A+) RNA from each time point was slotted to nylon membrane and hybridized to the 1.2Kb cDNA probe as described. The results of this experiment are shown in Figure 5. Signals differing in intensity are seen under the differing nutrient treatments, and at the various time points after nutrient treatment.

Under 50mM nitrate treatment, the appearance of inducible NR specific mRNA was detected two hours after nitrate treatment. Its levels increased thereafter, reaching a peak 48 hours after nitrate treatment. A reduced signal was observed at the 42 hour time point, which was harvested after 6 hours darkness. No signal was observed at the zero hour time point, which agrees with with the result obtained in the dot blot experiment under zero nitrate treatment. Signals observed under combined nitrate and glutamine treatment were lower in intensity at every time point than the corresponding nitrate alone treatment time point. The signals observed in this hybridization were quantified by densitometry, and are shown in Table II.

Nuclei Isolation

Nuclei isolated by the procedure outlined in Materials and Methods are shown in Figure 6. This photograph was taken at 500X, under Normarski phase contrast. Nuclei appear intact and are free of cellular debris. Yields of nuclei from fresh tissue ranged from 0.5 to 1.0 X 10^8 nuclei per 5 grams tissue.

Total Incorporation Assays

Total incorporation assays were performed to measure the transcriptional activity of isolated nuclei. All nuclei samples isolated were assayed in duplicate for incorporation of ^3H UTP into trichloroacetic acid (TCA) precipitatable nucleic acid. Nuclei were considered transcriptionally competent if incorporation of label was observed to be linear over a 25 minute period. Graphical representation of these assays are shown in figures 7 through 9. Figure 7 shows the assays performed with nuclei isolated at various time points after treatment with nutrient media containing nitrate. Nuclei from all time points do show linear incorporation of label. Figure 8 shows similar results with nuclei isolated from plants treated with nitrate and glutamine. Note in these two figures that nuclei from the 42 hour harvest, which was

during the dark portion of the photoperiod, show the lowest levels of transcription of any of the nuclei samples in either treatment group. Control nuclei assay results were also linear and are displayed in figure 9. The rates of incorporation of [^{3}H UTP] of each nuclei sample, were calculated and are summarized in Table III.

Nuclear Runoff Transcription Assays

Nuclear runoff transcription assays were performed to assay for the presence of inducible NR nuclear RNA transcribed in the runoff assays. An autoradiogram of these hybridizations is shown in Figure 10. These assays were conducted with equal numbers of nuclei isolated at the times indicated from plants given nutrient treatment containing nitrate. No signal was observed at time 0, indicating that nuclear RNA specific for the inducible NR isoform is absent in plants not treated with nitrate. Signals increasing in intensity were observed from assays conducted with nuclei isolated 4, 24, and 48 hours after nitrate treatment. These results indicate the presence of inducible NR nuclear RNA in plants treated with nitrate in increasing amounts over a 48 hour period. The 42 hour time point which was during the dark portion of the photoperiod, showed no significant signal, indicating that low nuclear RNA levels of the inducible NR isoform

were present during darkness. Densitometer readings of the signals observed in this experiment are summarized in Table IV. These readings show that signals are several-fold higher for assays conducted with light harvested nuclei, as opposed to the assay conducted with dark harvested nuclei. This data also quantitates the increase in NR nuclear RNA levels over time, showing a two-fold increase in levels from 4 hours to 48 hours after treatment, and a 1.5-fold increase in levels from 24 to 48 hours after treatment.

TABLE I.

Data presented in this table lists the concentration and 260/280 absorbance readings of poly A+ RNA samples isolated from the four treatment groups. Concentrations are given in units of micrograms/microliter. The purity of nucleic acid samples are determined by a 260/280 absorbance ratio of greater than 1.8. These values are also listed for each sample.

TABLE I

CONCENTRATION AND 260/280 READINGS OF POLY A+ RNA ISOLATED FROM SOYBEAN PRIMARY LEAVES

TREATMENT	CONCENTRATION[a]	260/280[b]
NO NO_3^-	3.4	1.88
50 mM NO_3^-	4.0	2.0
50 mM NO_3^-/ 10 mM Gln	2.75	1.89
10 mM Gln	1.7	1.88

[a] ug/ul
[b] indicator of nucleic acid purity

FIGURE 1.

Agarose Gel of Poly A+ RNA Samples Isolated From Each Treatment Group

Glyoxal treated poly A+ RNA from each treatment group (5 ug) was loaded onto a 1% agarose gel, electrophoresed at 50 volts for 2.5 hours, stained with ethidium bromide and photographed under ultraviolet light. The approximate sizes of 28s and 18s rRNA are shown, and were calculated with RNA molecular weight markers (not shown). Lane assignments are as follows:

Lane 1 no nitrate
Lane 2 10 mM Glutamine
Lane 3 10 mM Glutamine/50 mM nitrate
Lane 4 50 mM nitrate

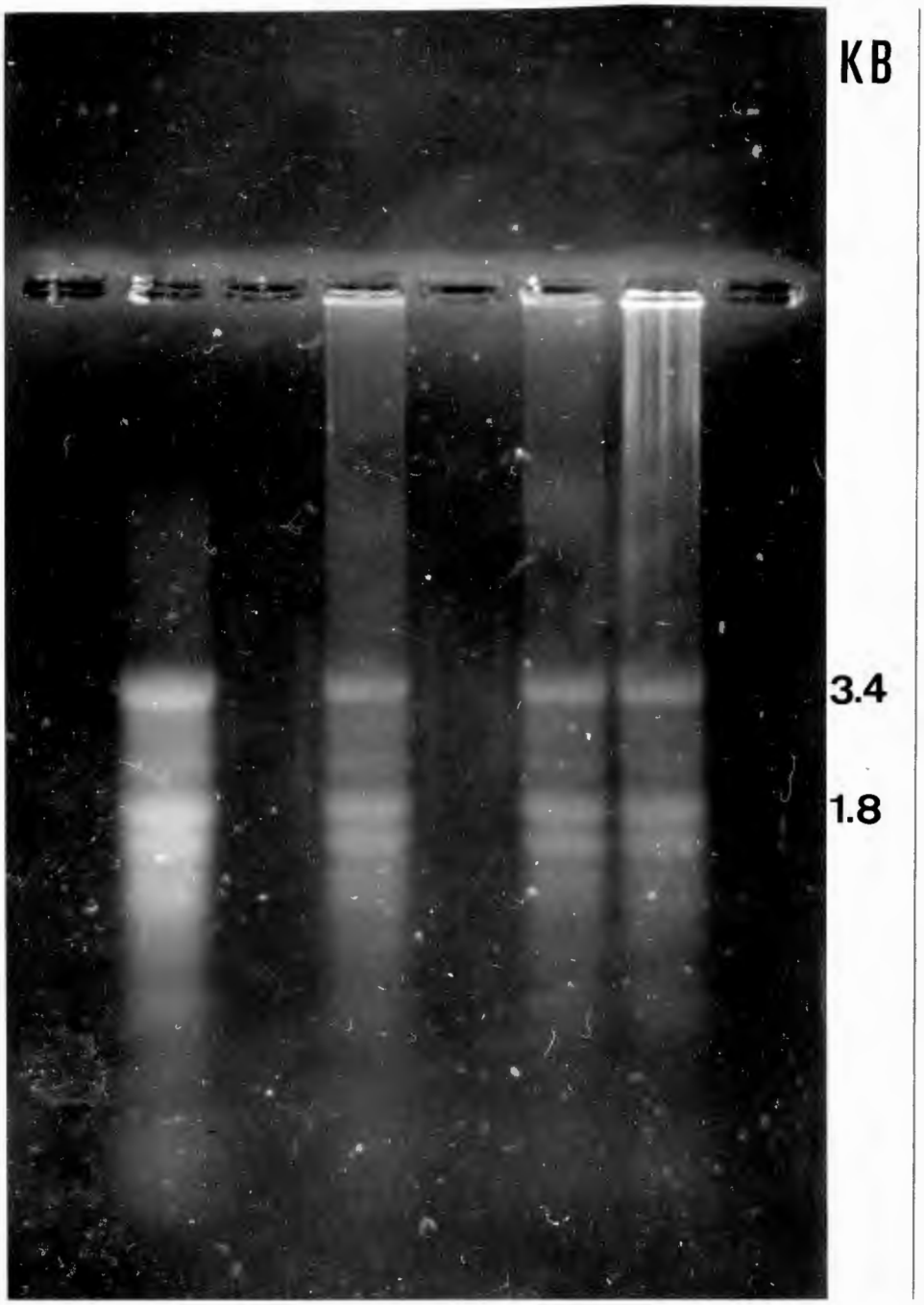

KB
3.4
1.8

FIGURE 2.

Dot Blots of Poly A+ RNA Isolated from Each Treatment Group

Glyoxal treated poly A+ RNA (15 ug) was dotted onto nylon membrane. The blot was hybridized to the 1.2 kb cDNA probe radioactively labled by nick translation. The control was unlabelled probe (0.01 ug)

Dot 1 control
Dot 2 no nitrate
Dot 3 10 mM Glutamine
Dot 4 10 mM Glutamine/ 50 mM nitrate
Dot 5 50 mM nitrate

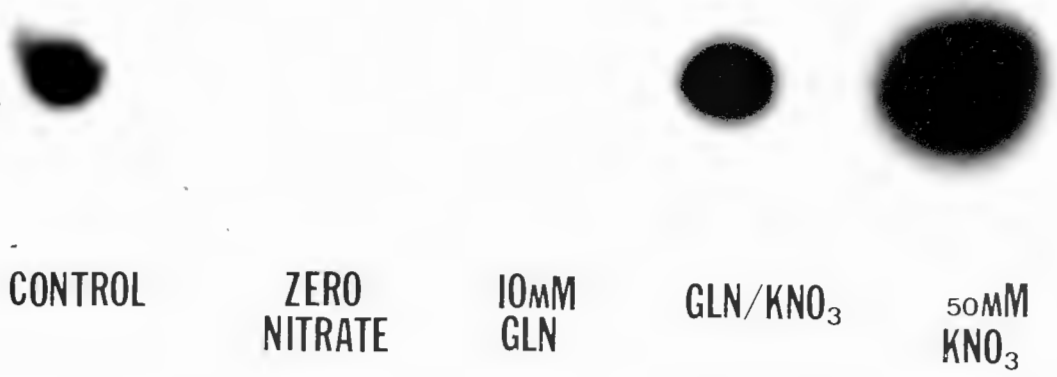

CONTROL
ZERO NITRATE
10mM GLN
GLN/KNO3
50mM KNO3

FIGURE 3.

Northern Blot of Total RNA from two treatment groups

Formamide/Formaldehyde denatured RNA (16 ug), was electrophoresed in a denaturing agarose gel, and transfered to a nylon membrane. The blot was hybridized to the 1.2 cDNA probe radioactively labeled by random priming. The membrane was exposed to x-ray film for 5 days. Sizes of RNA was determined by RNA markers (not shown). Lane assignments were as follows:

Lane 1 50 mM nitrate
Lane 2 no nitrate

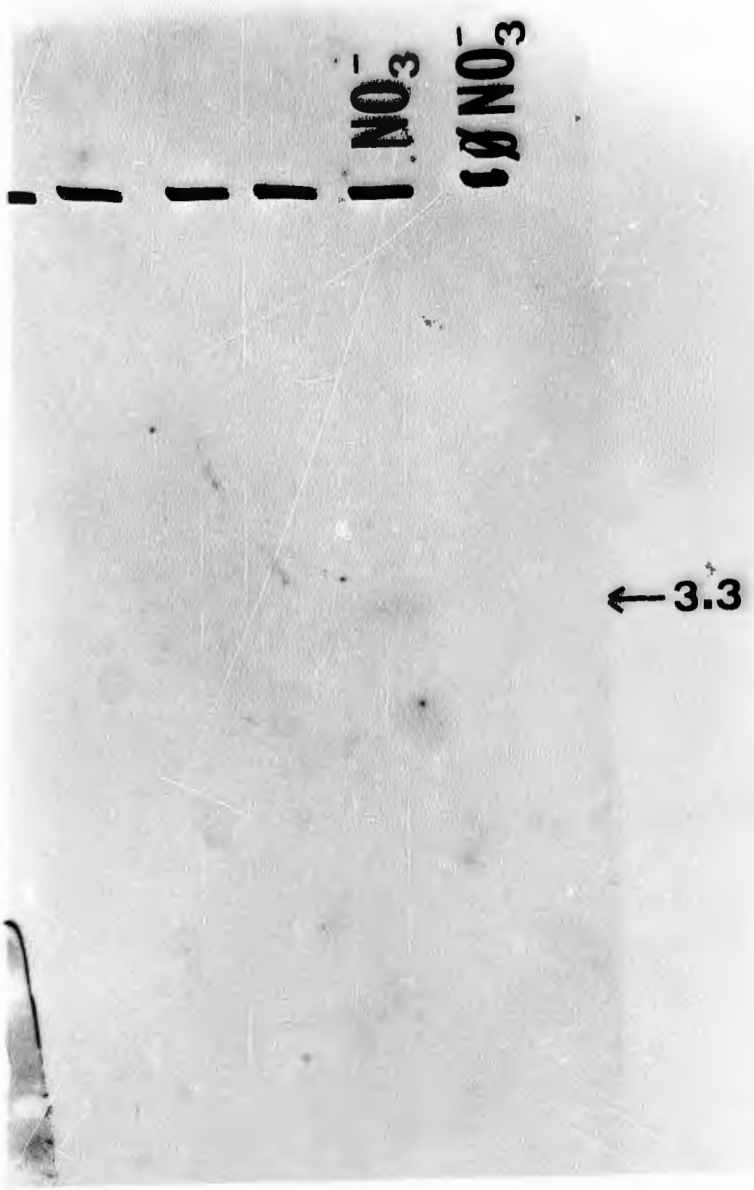

NO_3^-
$1\emptyset NO_3^-$
←3.3

FIGURE 4.

Agarose Gel of Poly A+ RNA from Time Course Study

Glyoxal treated poly A+ RNA from each time point in the time course study. RNA from the 50 mM nitrate and 50 mM nitrate/10 mM Glutamine treatments was used in this experiment.

LANE	TREATMENT	TIME (hrs)
1	nitrate/gln	0
2	nitrate	0
3	nitrate/gln	2
4	nitrate	2
5	nitrate/gln	4
6	nitrate	4
7	nitrate/gln	10
8	nitrate	10
9	nitrate/gln	24
10	nitrate	24
11	nitrate/gln	42
12	nitrate	42
13	nitrate/gln	48
14	nitrate	48


LANE
14
1

Figure 5.

Slot Blot of Poly A+ RNA Time Course Study

Five ug of glyoxal treated poly A+ RNA from the various treatments and time points used in the time course study were slotted onto nylon membrane, and hybridized to the ^{32}P labelled cDNA probe. The blot was exposed to x-ray film for 3 days. Treatments and times after treatment are shown on the autoradiogram.

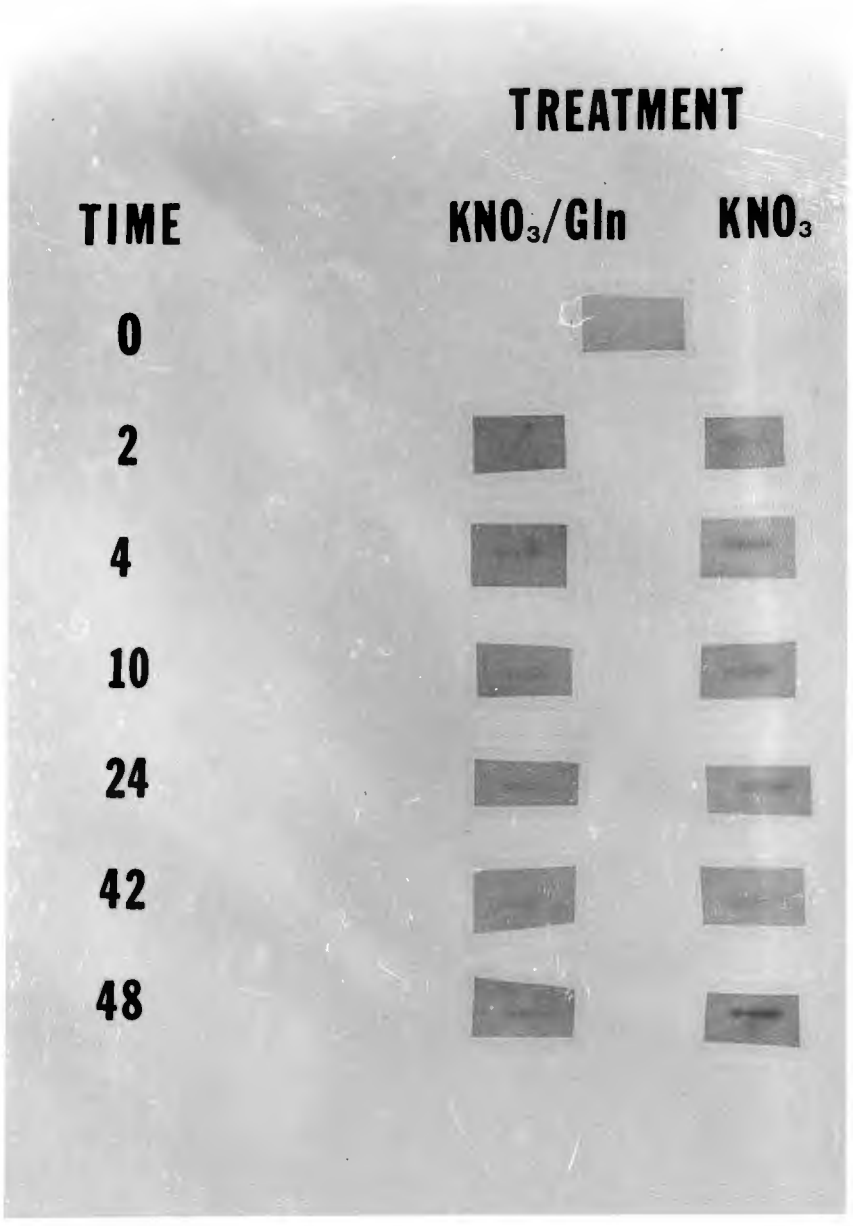

TREATMENT
TIME
KNO3/Gln
KNO3
0
2
4
10
24
42
48

TABLE II.

Data presented in this table lists the densitometer quantitation of the time course slot blot assay shown in figure 5. These numbers represent the peak height in cm of the densitometer tracing for signals seen at each time point.

TABLE II

DENSITOMETER READINGS OF TIME COURSE RNA SLOT BLOT HYBRIDIZATION

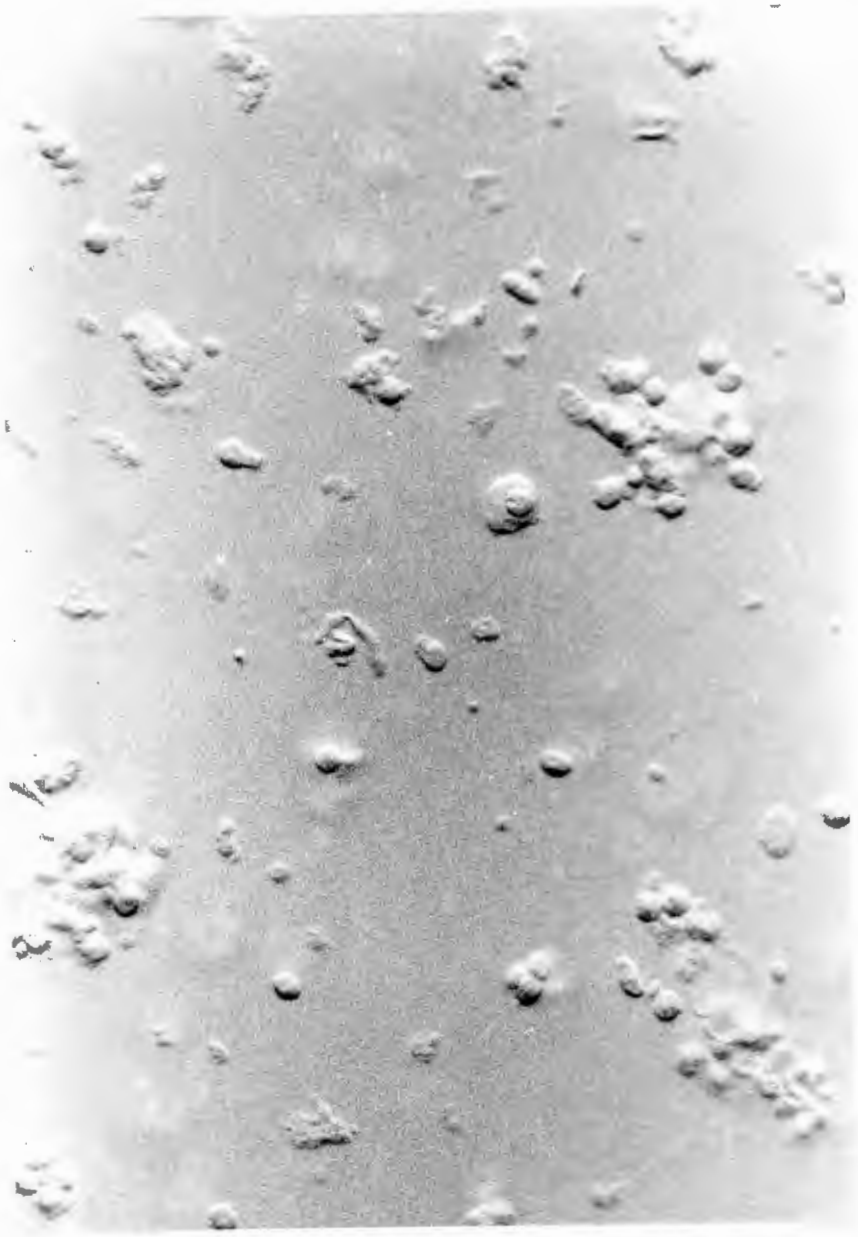
	PEAK HEIGHT (cm)		
	TRIAL I		TRIAL II
	TREATMENT		TREATMENT
TIME (hrs)	NITRATE	NITRATE + GLN	NITRATE
0	-	-	-
2	1.1	-	3.0
4	4.0	0.9	4.7
10	2.7	0.9	4.5
24	4.3	2.2	4.0
42[a]	1.7	1.3	3.0
48	7.5	2.3	10.0

[a]dark harvested

FIGURE 6.

Isolated Nuclei

This photograph of nuclei isolated for runoff transcription assays was taken at 500x under Normarsky phase contrast light microscopy. Nuclei are seen to be intact and essentially free of cellular debris.

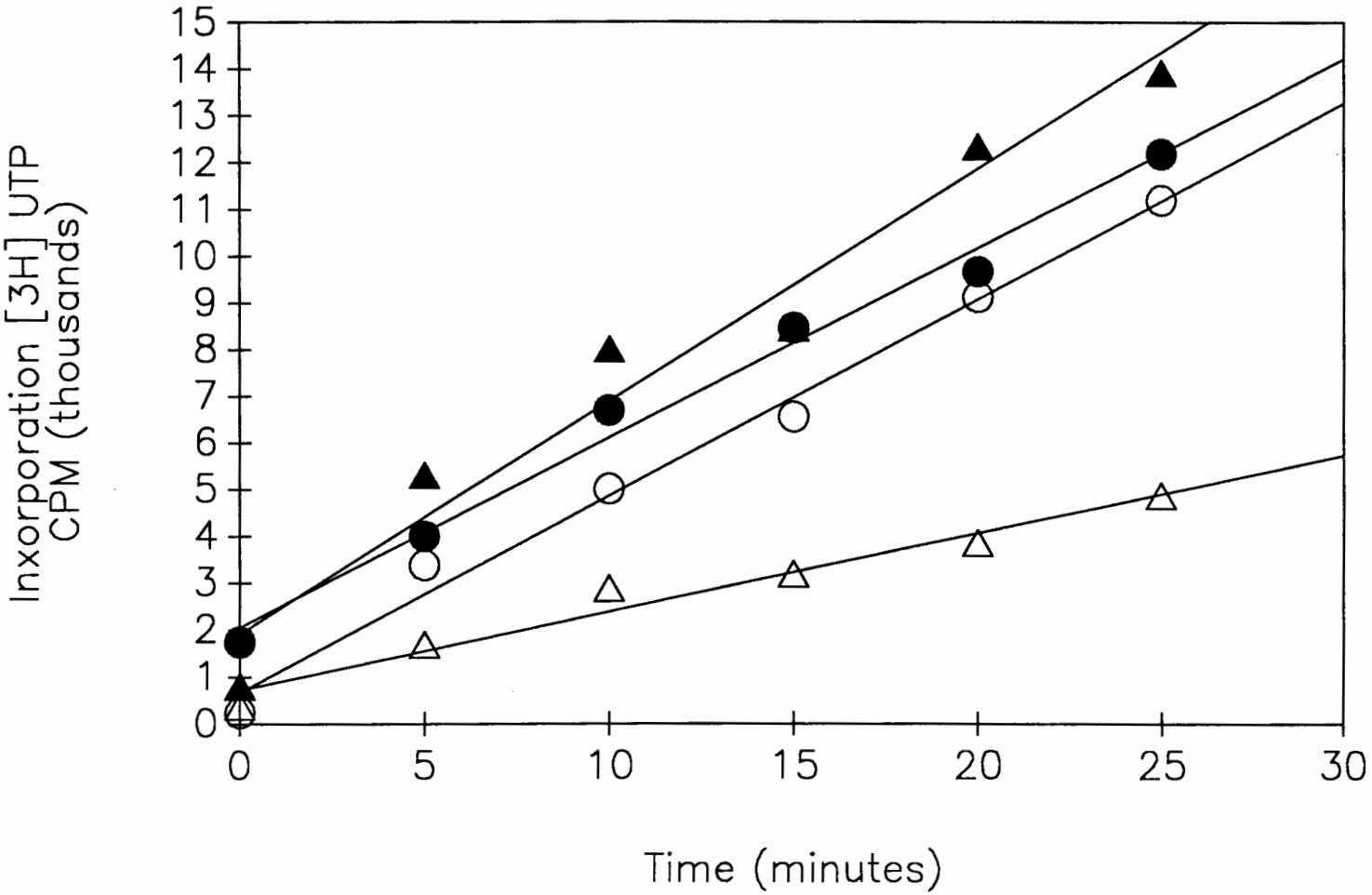


FIGURES 7,8 and 9.

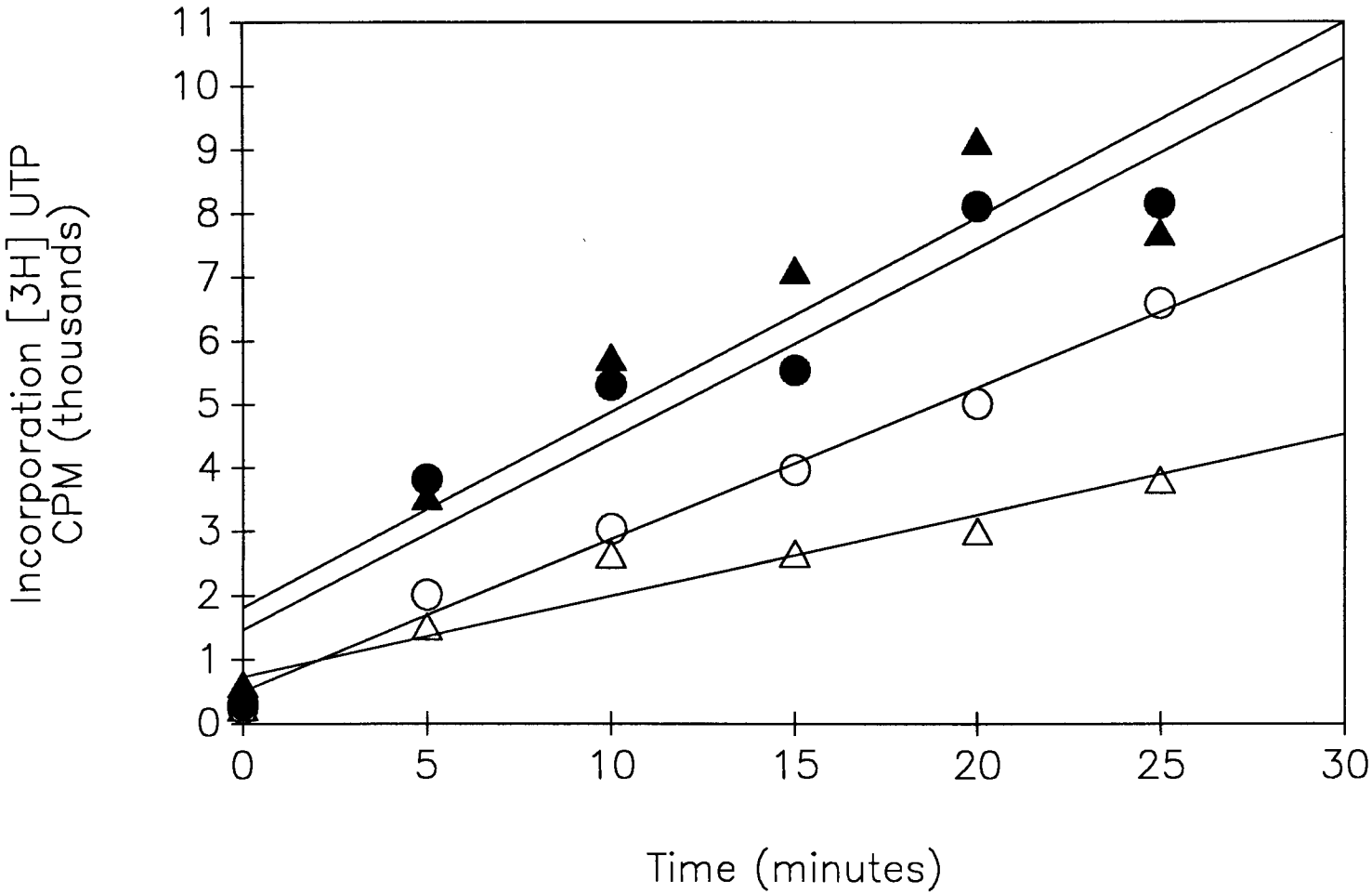
Graphs of Total Incorporation Assays

The following three figures are the graphical representation of total incorporation assays performed on isolated nuclei samples to chech the transcriptional competency of the samples. The graphs show incorporation of [^{3}H UTP] in CPM over a 25 minute time period of nitrate treated nuclei, nitrate/glutamine treated nuclei and control nuclei. Times of nuclei isolation are shown on the graphs.

Nitrate treated nuclei



Nitrate/Glutamine Treated Nuclei



Control Nuclei

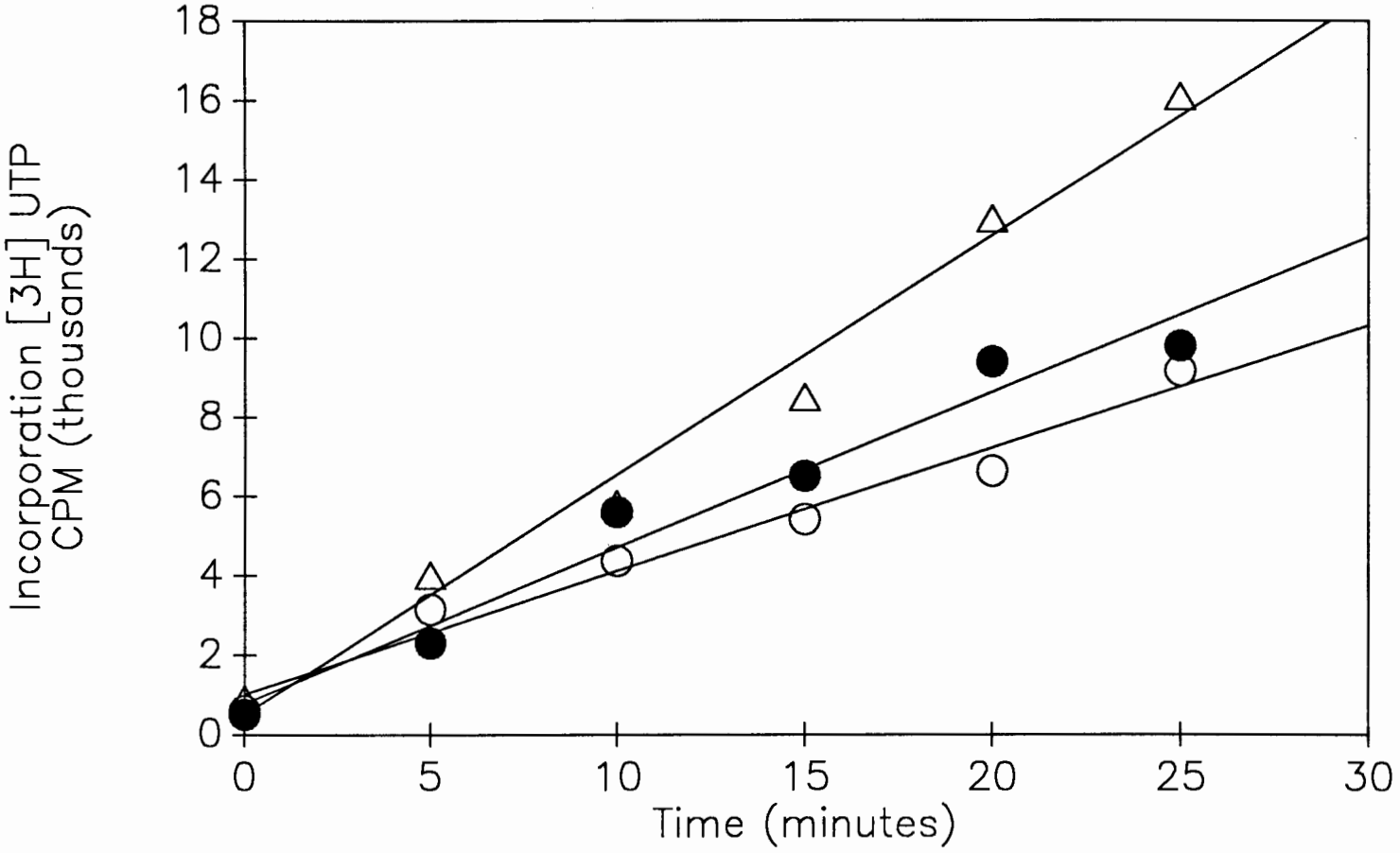


Table III.

Data presented in this table lists rates of incorporation of [^{3}H UTP] by isolated nuclei, in the total incorporation assays performed to asses transcriptional competency of the nuclei samples. Rates of incorporation in CPM/min. are listed for each assay performed as well as the standard error of the data compared to a linear regression.

TABLE III

RATES OF INCORPORATION OF [^{3}H] UTP
BY ISOLATED NUCLEI

	INCORPORATION RATE[a]			
	TREATMENT			
TIME (hrs)	NITRATE	NITRATE + GLN	CONTROL	r[b]
0	-	-	310.3	0.98
4	420.3	237.8	-	0.99,0.99
24	405.7	298.75	-	0.99,0.95
42[c]	167.1	126.5	-	0.98,0.95
48	497.8	363.3	604	0.97,0.98

[a]cpm/min.
[b]correlation coefficient
[c]dark harvested

FIGURE 10.

Slot Blots of Runoff Transcription Assays

Five micrograms of pCmc-1 was digested with EcoR 1, slotted onto nylon membrane and hybridized to the ^{32}P labelled transcripts from the runoff assays. Blots were exposed to x-ray film for five days. Treatment and time after treatment are shown on the autoradiogram.

NITRATE TREATED NUCLEI

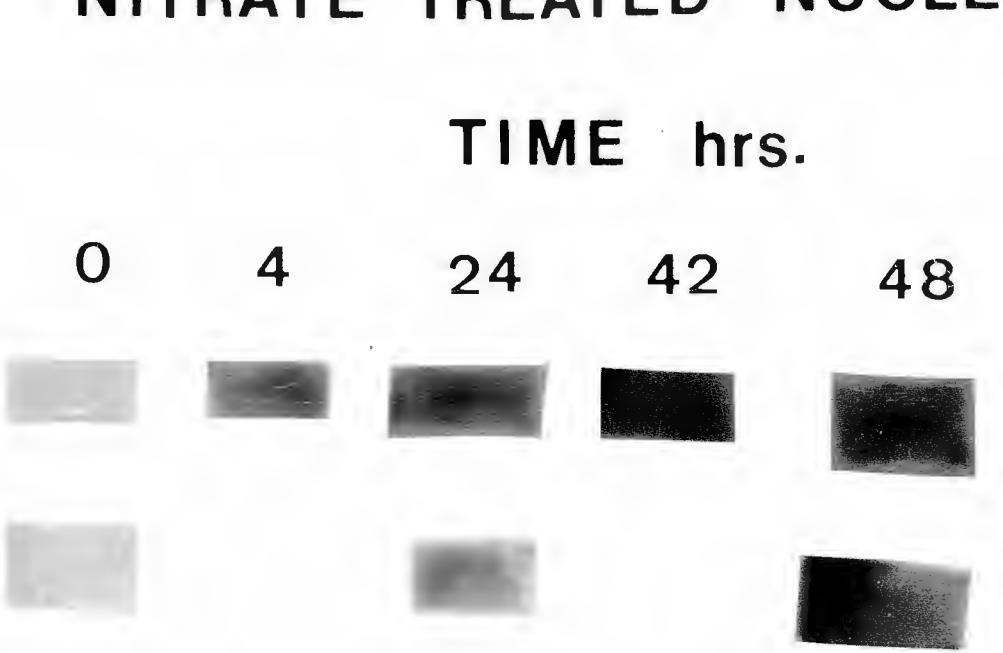


TABLE IV.

Data presented in this table lists the densitometer quantitation of the nuclear runoff transcription assays shown in figure 10. These numbers represent the peak height in cm of the densitometer tracing for signals seen at each time point.

TABLE IV

DENSITOMETER READINGS OF NUCLEAR RUNOFF TRANSCRIPTION ASSAY HYBRIDIZATIONS

PEAK HEIGHT (cm)	
	TREATMENT
TIME (hrs)	KNO_3
0	0.2
4	2.75
24	3.25
42[a]	0.3
48	5.3

[a]dark harvested

CHAPTER V

DISCUSSION

Nitrate is the major source of nitrogen for most higher plants. Nitrate assimilation involves two nitrogen reduction steps, which convert nitrate to ammonia, which is incorporated directly into the amino acid pool. The first enzyme of this nitrate reducing pathway, nitrate reductase, has been studied extensively in an effort to improve the efficiency of this process (5). This study has centered around the molecular events which regulate nitrate reductase synthesis in soybeans. This study attempts to answer the question of whether the synthesis of substrate inducible nitrate reductase is controlled at the level of transcription. To try to answer this question, experiments were performed to quantitate steady state, and nuclear RNA levels of the inducible NR isoform in soybean seedlings, under conditions were nitrate treatment, and time after treatment were varied.

Previous work performed in our laboratory involving metabolite control of nitrate reductase enzyme activity, laid the groundwork on which the work presented in this study is based. Curtis and Smarrelli (6), found peak levels of the pH 7.5 NADH-linked activity 48 hours after nitrate treatment. This peak in activity was 30 fold over

controls receiving no nitrate. Minimal activity was seen in seedlings receiving 10 mM glutamine as a nitrogen source . These finding led us to begin experiments designed to study the transcriptional regulation of this nitrate inducibe NR activity.

The first set of experiments examined steady state mRNA levels of the inducible NR isoform using three types of blot analyses: dot blot, Northern blot, and slot blot methods. The first question that needed to be answered was whether our cDNA clone for squash NR was specific for inducible NR mRNA in soybeans. We also wanted to observe whether the presense of inducible NR mRNA correlated with its activity 48 hours after treatment. Blotting equal amounts of poly A+ RNA from each treatment group, and hybridizing to the probe provided evidence to support the use of this probe to perform further blot analyses. Figure 2 shows that the mRNA for the constitutive isoforms do not cross hybridize to this probe under the non inducing conditions of zero nitrate and glutamine treatment. Hybridization is observed between the probe, and RNA samples from nitrate treated plants. In addition to its non inducing effect, glutamine is shown to repress NR mRNA synthesis, since RNA samples from plants treated with nitrate and glutamine, show less hybridization to the the probe, than samples from plants treated with nitrate alone.

This experiment confirms a comparable experiment performed by Smarrelli et al. (8). Several other studies also confirm this hybridization pattern. Crawford et al. (7), shows hybridization between this probe and 7.5 NADH:NR mRNA in squash, only under conditions of nitrate induction. Martino and Smarrelli (38), show an identical hybridization pattern using poly A+ RNA from squash under the same nutrient treatments used in this study. Thus from these studies we can conclude that mRNA for the inducible NR isoform is present only in samples from nitrate treated plants. RNA samples from plants given no nitrate , or glutamine as a nitrogen source, do not express measurable quantities of this isoform, relative to the sensitivity of the experiment performed. Plants treated with both nitrate and glutamine express levels of inducible NR mRNA intermediate between the nitrate only, and glutamine only treatment groups.

Northern blot analysis was used to determine the size of the transcript seen in the previous experiment, and to determine if the constitutive isoforms are contributing to the signal seen under nitrate inducing conditions. As seen in Figure 3, the probe hybridizes to a single mRNA species approximately 3.3 kb in length present only in RNA samples isolated from nitrate treated plants. This result suggests that only mRNA for the inducible NR isoform is hybridizing to the probe, and

that this transcript is approximately 3.3 kb in length. The size of this transcript is in agreement with the proposed length of a coding region needed for a protein of 110 kD (46). The size of this transcript is also in aggreement with several other studies which have used Northern blot analysis to determine the size of NR transcripts in other plant species. Crawford et al. observed 3.2 kb transcripts for inducible nitrate reductase from squash (7) and Arabidopsis thaliana (18). Cheng et al. (26), demonstrated a 3.5 kb message for inducible NR in barley.

The kinetics of inducible NR message were also examined in a time course study using slot blot analysis. Figure 5 shows the autoradiogram from this hybridization, and Table II gives the densitometer quantitation of these signals. This data suggests that the induction of NR by nitrate is a relatively slow process compared to the induction of gene expression observed for other "inducible" plant genes (13,43). The absence of signal at time zero after treatment agrees with prior observations that inducible NR mRNA is not present without nitrate treatment. The high levels of inducible NR mRNA seen at 24 and 48 hours after nitrate treatment, correlate with the high levels of activity of this isoform seen at the same times after treatment (6,8). While the increases in inducible NR mRNA levels correlate time wise with

increases in inducible NR activity, the levels of these increases do not correlate exactly. The significance this observation is not known.

Another interesting piece of data from this experiment is seen at the 42 hour dark harvested time point for each treatment. The signal at this time point is much lower in intensity than the signals at light harvested time points taken before, (24hrs) or after, (48hrs). This data seems to indicate a light effect on the level of expression of NR mRNA, since nitrate seems to trigger higher expression of the gene in the presence of light, than it does in darkness. This observation agrees with several other published reports that link high level NR gene expression with a light effect (23-25). These studies suggest that nitrate is the trigger for inducible NR gene expression, while light plays a major role in modulating the level of this expression. My data seems to fit well into this emerging picture of the control of expression of this inducible NR. The major effector of this light response seems to be the phytochrome system (25), although other effects of light other than that of phytochrome probably exist (25).

There is however, no unanimous agreement on the effects of light on NR transcription. Research published by Galangau et al. (45) suggests that light may actually prevent expression of nitrate reductase in tobacco and

tomato plants (45). This report states that nitrate reductase expression may be regulated by an internal clock mechanism, possibly a circadian rhythm (45), synchronized by the photoperiod the plants are grown under. It should be noted however, that these hypotheses were based on experiments where plants were given continuous nitrate treatment, differing from other experiments where plants were nitrogen starved and then supplied with nitrate.

To date no researchers have provided the evidence that nitrate reductase is controlled at the level of transcription. Nuclear runoff experiments were performed to shed light on this question by examining levels of inducible NR RNA transcribed <u>in vitro</u>. Isolation of intact nuclei from light grown leaves, proved to be a more difficult task, than was originally anticipated. Most studies have utilized etiolated tissue for nuclei isolations, because of the ease from which nuclei are extracted from such tissue, and the lack of chloroplast contamination such tissue provides (13). My experimental design did not allow for the use of such tissue, so much experimental manipulation was needed to modify existing procedures to extract intact nuclei from light grown leaves, free from cellular contaminants.

Once this task was accomplished, the next step was to check the transcriptional compentancy of the isolated

nuclei. The total incorporation assays performed with all isolated nuclei samples (figures 7-9), show the linear incorporation of labelled ribonucleotides characteristic of transcriptionally active nuclei (41). This data suggests that the nuclei were indeed isolated functionally intact. Since transcription of nuclei in these assays reflects completion of messages initiated in vivo, this seemed to be an ideal system to test if control of expression of a particular gene, is at the level of transcripion.

The data obtained from these assays (figure 10), allows us to make several observations about the transcriptional control of inducible NR. First is the fact that no inducible NR nuclear RNA is present at time zero after treatment. This indicates that inducible NR RNA is not transcribed when nitrate is not available to the plant. If inducible NR was controlled at a post-transcriptional level, one would expect that nuclear RNA specific for this isoform would be present at this time point. The fact that the message is not present, suggests transcriptional control. Signals are seen, in increasing intensity at 4, 24 and 48 hours after nutrient treatment. This data also suggests transcriptional control, since NR nuclear RNA seems to be present only after nitrate treatment. Nuclei isolated 42 hours (dark harvested), after nitrate treatment show no detectable amounts of NR

nuclear RNA. This indicates that the effect light has been shown to have over NR expression, may also be transcriptional. Several other studies citing transcriptional control of other plant genes (13,42,43), used data similar to that obtained in this study to make their determinations of transcriptional regulation.

The exploding human populations in many third world countries, with the accompaning food shortages that such populations cause, demand that science provide technology to meet these demands. Since inorganic nitrate fertilizer is a limiting factor in the production of many important crops, it makes sense to investigate possible ways to allow plants to use nitrate more efficiently. The reduction of nitrate to nitrite is generally considered to be the rate limiting step in nitrate assimilation (1,15). This might be explained by the fact that NR is a very low abundance protein, comprising only 0.01% of total cellular protein (46). Increasing NR levels in crop plants might be one way of increasing the efficiency of nitrate assimilation, thus lessening the dependance of farmers on costly nitrate fertilizer. Discovering how NR expression is regulated at the molecular level, seems to be an important first step in this process, and will hopefully lead to the developement of transgenic crop plants able to utilize nitrate more efficiently.

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The thesis is therefore accepted in partial fufillment of the requirements for the degree of Master of Science.

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