0273-2289/1559-0291 (Online)/07/141/349–360/$30.00

# Differential Display Identifies Genes in Chinese Hamster Ovary Cells Sensitive to Elevated Ammonium

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**Received July 27, 2006; Revised October 24, 2006; Accepted November 6, 2006**

## Abstract

Ammonium is a toxic waste product that has been reported to negatively inhibit cell growth and recombinant glycosylation in Chinese hamster ovary (CHO) cells; however, the effect of this toxicity on intracellular gene expression has received only limited investigation. We used a differential display method to identify genes in CHO cells that were affected by ammonium stress. Eight genes whose mRNA levels significantly changed in response to elevated ammonium were isolated and identified. Five of the genes were identified as having lower expression under the ammonium stress, whereas three genes were identified as having higher expression. Sequence homology with other mammalian organisms was used to attribute function to these newly identified genes. The identified ammonium-sensitive genes were grouped into three broad functional groups: cellular processes, energy metabolism, and genetic-information processing. The three cellular process–related genes had lower expression (anaphase-promoting complex subunit 5, eukaryotic initiation factor 5A II, KIAA1091 protein). The two energy-related genes had higher expression under ammonium stress (adenosine triphosphate synthase subunit C and mitofusin 1). Both of the genetic information-processing genes (endoplasmic reticulum [ER]–resident protein ERdj5 and structure-specific recognition protein 1) had lower expression under the ammonium stress, whereas the 26S proteasome subunit adenosine triphosphatase 3 gene had higher expression. These preliminary results indicate that ammonium stress lowers expression of genes controlling cell cycle, protein folding, and quality and raises genes that control energy metabolism and

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degradation. Our findings demonstrate the usefulness of mRNA differential-display techniques for the detection of CHO cell genes affected by ammonium stress.

**Index Entries:** Gene expression; bioreactor stress; ammonium; differential display; *Cricetulus griseus*.

## Introduction

The use of mammalian cell cultures for the production of therapeutic proteins has increased substantially in recent years. The culture environment has been shown to greatly influence the quality and productivity of therapeutic proteins, particularly for glycoproteins. The effect of environmental conditions, such as glucose, glutamine, dissolved oxygen, amino acids, serum, carbon dioxide, extracellular enzymes, temperature, and media pH, has been studied and demonstrated to impact the quality of the final protein product *(1)*. In addition, several cellular metabolic waste products have been reported to be inhibitory or toxic to cells *(2,3)*. Specifically, ammonium or ammonia has been demonstrated to be one of the most significant waste metabolic byproducts. Ammonium mainly comes from the catabolism and chemical degradation of glutamine in the media. By the end of a typical recombinant protein production campaign, the ammonium concentration can be as high as 5–10 m*M (4)*.

The effects of ammonium on cell growth, productivity, and glycosylation have been well investigated. It is known that elevated ammonium levels significantly inhibit cell growth *(5–8)* and recombinant protein productivity *(9,10)*. Additionally, elevated ammonium levels have been observed to decrease terminal sialylation levels as well as the tetraantennary and tetrasialylated oligosaccharide structures and to increase molecular heterogeneity *(5,11,12)*, all of which can negatively impact the efficacy of a therapeutic glycoprotein *(13)*.

Ammonium affects cells by rapidly reaching chemical equilibrium with ammonia in the media, and ammonia readily diffuses across the cellular membrane owing to its chemical potential *(14,15)*. Ammonium also can enter the cell by active transport via specific transport proteins, such as the $Na^+K^+$-ATPase complex, or by facilitated diffusion via the $Na^+K^+2Cl^-$ cotransporter and $Na^+/H^+$-exchanger proteins *(15,16)*. Once inside the cell, ammonia and ammonium both perturb the intracellular pH ($pH_i$) and electrochemical gradients. The effects of elevated ammonium on glycosylation-related gene expression have been studied *(5)*; however, the effects of ammonium on the cellular metabolic pathway in Chinese hamster ovary (CHO) cells, especially at gene-expression level, have not been examined.

CHO cells are widely used to produce recombinant glycoproteins; however, fewer than 700 *Cricetulus griseus* (CHO cell species) nucleotide entries are available in GenBank, compared to over 6 million entries for *Mus musculus* (mouse). Consequently, the tools necessary to study global gene expression in CHO cells, such as DNA microarrays, are not commercially

or publicly available. Differential display is a powerful method that allows the identification of differentially expressed genes between cellular populations and within a single cell type under altered conditions. Both known and unknown nucleotide sequences can be identified, and this method has been widely used to identify cancer genes, as well as unique plant genes *(17–21)*.

In the present study, a differential display technique was applied to explore how elevated ammonium might affect gene expression in CHO cells. Northern blots were used to confirm the differential gene expression. The differentially expressed cDNA sequences were queried against the GenBank database to identify sequence homologies with known nucleotide sequences. As expected, most of the ammonium-sensitive sequences identified had no known *C. griseus* sequence in the database. Genes from closely related species were used to assign function for the newly identified *C. griseus* nucleotide sequences, based on the sequence identity. These assigned functions were then examined in the context of an ammonium stress.

## Materials and Methods

### *Cells and Culture Conditions*

CHO cells (CRL-9606) were obtained from American Type Culture Collection. This cell line constitutively expresses tissue plasminogen activator. CHO cells were cultured in IS CHO-V™ (Irvine Scientific, Santa Ana, CA) with 0.1% fetal bovine serum (Invitrogen), 200 n*M* methotrexate, and 7 m*M* glutamine (Irvine Scientific) in 1-L spinner flasks in humidified incubators at 37°C and 5% $CO_2$. $NH_4$ Cl was used to simulate the ammonium stress. The $NH_4Cl$ stock solution was prepared in Milli-Q water and added to the fresh media prior to the addition of cells. Each spinner flask was inoculated from a common seed culture. The treatment cultures contained the basal media supplemented with 10 m*M* $NH_4Cl$. Milli-Q water was added to the control cultures to normalize culture volumes and media concentrations. Cell densities and viabilities were determined by the Trypan blue exclusion method. Harvested cells were centrifuged at 700*g* for 5 min at 4°C. The supernatant and pellets were saved at –20 and –80°C , respectively. The final ammonium concentration in the cell culture media was analyzed with a Dionex D-300 amino acid analyzer and a Dionex 2000i ion-exchange chromatography system with a dual wavelength detector (440 and 570 nm).™

### *Extraction of Total RNA*

Total RNA was isolated from CHO cells using RNAqueous-Midi™ kits (Ambion, Austin, TX) according to the kit's instructions except the DNase treatment was increased to 2 µL of DNase and 1 h. RNA concentrations were determined using a Ribogreen RNA quantitation kit (Molecular Probes, Eugene, OR) according to the kit's instructions.

Table 1
Anchor and Arbitrary Primers for RNAspectra Red 1 Differential Display Kit

| Category | Primer code | Sequence |
|---|---|---|
| One-base-anchored primers | H-$T_{11}$G | 5′-AAGCTTTTTTTTTTG-3′ |
| | H-$T_{11}$A | 5′-AAGCTTTTTTTTTTA-3′ |
| | H-$T_{11}$C | 5′-AAGCTTTTTTTTTTC-3′ |
| Arbitrary primers | H-AP1 | 5′-AAGCTTGATTGCC-3′ |
| | H-AP2 | 5′-AAGCTTCGACTGT-3′ |
| | H-AP3 | 5′-AAGCTTTGGTCAG-3′ |
| | H-AP4 | 5′-AAGCTTCTCAACG-3′ |
| | H-AP5 | 5′-AAGCTTAGTAGGC-3′ |
| | H-AP6 | 5′-AAGCTTGCACCAT-3′ |
| | H-AP7 | 5′-AAGCTTAACGAGG-3′ |
| | H-AP8 | 5′-AAGCTTTTACCGC-3′ |

## *Differential Display*

An RNAspectra™ Red 1 kit (GenHunter, Nashville, TN) was used for the differential display analysis. This kit has an estimated coverage of 28% of a genome *(22)*. The differential display process was performed according to the kit's instructions. Briefly, 0.2 µg of RNA was reverse transcribed and amplified with a total of 24 different combinations of primer sets using eight arbitrary primers and three one-base-anchored primers (Table 1). The reverse transcriptions were 65°C for 5 min, 37°C for 60 min, and 75°C for 5 min with a 4°C soak. After the tubes had been at 37°C for 10 min, the thermocycler was paused and 1 µL of Moloney murine leukemia virus reverse transcriptase was added to each tube. The polymerase chain reaction (PCR) conditions were 94°C for 30 s, 40°C for 2 min, and 72°C for 60 s for 40 cycles followed by 72°C for 5 min and a 4°C soak. The PCR products were run on 6% denaturing polyacrylamide gel in TBE buffer. After electrophoresis, gels were scanned using a Phosphoimager (Typhoon 9400; Molecular Dynamics) with a 585-nm filter. Differentially expressed cDNA bands were excised from the gels and transferred to 1.5-mL tubes containing 100 µL of distilled $H_2O$ ($dH_2O$). The tubes were incubated at room temperature for 10 min, then boiled for 15 min. The DNA was precipitated in with 10 µL of 3 *M* sodium acetate, 5 µL of glycogen, and 450 µL of 100% ethanol on dry ice for 30 min. DNA was collected by centrifuging for 10 min at 10,000*g* at 4°C. The pellet was rinsed with 200 µL of ice-cold 85% ethanol and recentrifuged. The DNA was then dissolved in 10 µL of $dH_2O$. The cDNA was reamplified in a 40-µL PCR reaction using the primer pairs used in the differential display reaction (except unlabeled). PCR reaction conditions were the same as those just described. The PCR products were purified using a QiaQuick PCR purification kit (Qiagen, Valencia, CA).

### *Sequencing of cDNA Fragments*

DNA sequences were obtained from the PCR products by the DNA laboratory, Arizona State University (Tempe, AZ) using an Applied Biosystems Sequencer. The sequences were obtained bidirectionally using the amplification primers.

### *Northern Blot Analysis*

Northern blots were used to confirm the differential expression. Briefly, 10 µg of total RNA from control and elevated ammonium–treated cultures was denatured with 2.2 *M* formaldehyde and separated on 1.2% agarose gels. The RNA samples were transferred by capillary blotting onto 10X saline sodium citrate (SSC)–saturated nylon (Immobilon Ny+; Millipore) membranes overnight and then fixed by ultraviolet crosslinking. The differentially expressed cDNA fragments were labeled with [$\alpha$-$^{32}$P] dCTP (MP Biomedicals) with a random-priming labeling kit. Sephadex columns were used to remove unincorporated nucleotides. The membranes were prehybridized for 30 min at 65°C in modified Church and Gilbert buffer. The labeled cDNA was added to the individual membrane and hybridized at 65°C overnight in the Church and Gilbert buffer. The membranes were washed twice with 2X SSC, 1% sodium dodecyl sulfate for 15 min. For detection and quantification of the radioactive signals, the membrane was exposed to a PhosphoImager (Amersham Biosciences) *(23)*.

### *Analysis of Sequences*

The homology search of genes against GenBank was queried on-line using a FASTA-based program *(24)*.

## Results

### *Culture Conditions and Sampling*

To study the impact of ammonium stress on gene expression, CHO cells were cultured under normal (control) and elevated ammonium conditions. As expected, the CHO cell growth rate of the ammonium-stressed cultures was significantly lower than that of the control cultures. Figure 1 shows the cell growth curves for control and ammonium-stressed cultures. To identify genes sensitive to ammonium, the 49-h time point was selected for RNA isolation and differential display analysis because both cultures were still in the exponential growth phase at that point. Additionally, the control and ammonium-stressed cultures still had sufficient glucose, glutamine, and amino acids for growth (data not shown). At 49 h, the ammonium concentrations in the control and ammonium-stressed cultures were 3.7 and 10.5 m*M*, respectively.

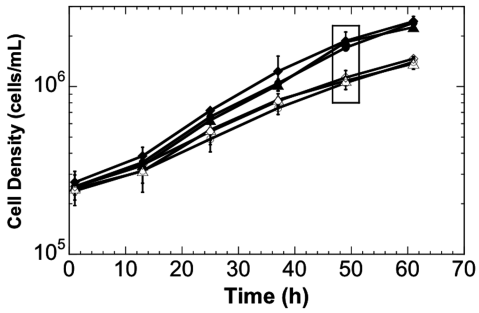


Fig. 1. CHO cell growth curves for triplicate control (▲,●,◆) and elevated ammonium–treated (△,○,◇) cultures. Samples taken for differential display analysis are boxed. Error bars represent 95% confidence intervals.

## *Differential Display Analysis*

The differential display kit RNAspectra Red 1 contains a total of 24 different combinations of primer sets obtained by pairing three 3′ composite one-base anchor primers (H-$T_{11}$G, H-$T_{11}$A, and H-$T_{11}$C) and eight arbitrary 13mers (Table 1). To minimize false-positive displays, duplicate samples were loaded onto the gels. In most of the lanes, 30–100 bands with sizes between 100 and 800 bp were visible. A total of 22 significantly differentially displayed bands were observed using all 24-primer pairs. All 22 bands were isolated for further PCR reamplification. Nineteen reamplification reactions produced reamplified products, whereas the remaining three bands were not reamplified. These three bands corresponded to large fragments (>700 bp), which are more difficult to reamplify. The 19 reamplified PCR products were directly sequenced. Direct sequencing prior to the Northern blot confirmations was conducted such that Northern blot conditions could be optimized based on the GC content of the sequences. Twelve of the 19 reamplified PCR products produced clean and readable sequences. As expected for sequences obtained by differential display, all of the cDNAs were partial gene coding sequences (cds) and were probably from the 3′ region because the sequences contained poly (A) tails. The cloned cDNA fragment lengths ranged from 57 to 70ø bp (Table 2).

## *Northern Blot Analysis*

To confirm that the pattern of bands obtained represented true differences in gene expression and not artifacts owing to the PCR amplification process, Northern blot analyses were performed. The 12 readable reamplified PCR products were analyzed. Eight of these sequences were confirmed to have differential expression by the Northern blots, as shown in Fig. 2. Five of these genes were observed to have lower expression for the ammonium-stressed cultures compared with the control cultures, whereas three genes were observed to have higher expression. Analysis confirmed that the four

Table 2
GenBank Analysis of Ammonium-Sensitive cDNAs in *C. griseus* Isolated by Differential Display and Confirmed by Northern Blot Analysis

| Code | New GenBank accession no. | Size (bp) | Expression in $NH_4^+$ | Best match function | E Value | Best match species | Best match accession no. |
|---|---|---|---|---|---|---|---|
| DD1 | AY574577 | 285 | – | APC5 | $3e^{-37}$ | *M. musculus* | BC010339 |
| DD2 | AY788841 | 700 | – | ERdj5 | $5e^{-69}$ | *R. norvegicus* | XM_215751 |
| DD3 | AY788842 | 216 | – | Eif5a2 | $2e^{-47}$ | *M. musculus* | AY205265 |
| DD4 | AY788843 | 306 | – | Similar to KIAA1091 protein (KIAA1091) | $4e^{-77}$ | *R. norvegicus* | XM_345308 |
| DD5 | AY788844 | 172 | – | Ssrp1 | $4e^{-54}$ | *R. norvegicus* | BC083588 |
| DD6 | AY535011 | 176 | + | ATP synthase | $4e^{-57}$ | *M. musculus* | NM_175015 |
| DD7 | AY574576 | 461 | + | Proteasome (prosome, macropain) 26S subunit, ATPase 3 (Proteasome ATPase) | $6e^{-151}$ | *R. norvegicus* | BC062019 |
| DD8 | AY788840 | 57 | + | Mfn1 | $3e^{-11}$ | *M. musculus* | BC008137 |
| DD9 | AY829234 | 355 | 0 | Unknown mRNA, partial cds | $3e^{-161}$ | *Cricetulus sp.* | AF08114 |
| DD10 | AY829235 | 291 | 0 | Mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal, transduction (Magmas), mRNA, partial cds | $7e^{-88}$ | *M. musculus* | NM_025571.1 |

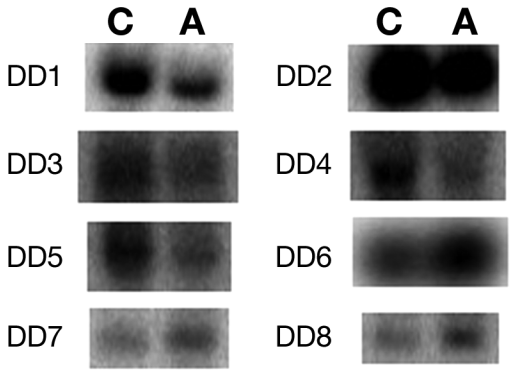


Fig. 2. Northern blot analysis of differentially expressed CHO cell genes for control (C) and ammonium-stressed (A) cultures. DD1, APC5; DD2, ERdj5; DD3, Eif5a2; DD4, KIAA1091; DD5, Ssrp1; DD6, ATP synthase; DD7, Proteasome ATPase; DD8, Mfn 1.

cDNA sequences were not differentially expressed by the Northern blot analysis. Isolation of nondifferentially expressed genes often occurs owing to comigration in the gel *(25)*, thus the need for cloning and confirmation by Northern blots. Because *C. griseus* has relatively few gene sequences in Genbank, all 12 genes were queried against GenBank. The differentially expressed sequences of the eight cDNAs had a high degree of identity with known *M. musculus* and *Rattus norvegicus* genes and, thus, were identified as new putative *C. griseus* genes. Two of the ammonium-insensitive sequences matched known *C. griseus*genes: mitochondrial benzodiazepine receptor gene (accession no. U12420) and intracisternal A-Particle gene corresponding to 5NLTR-GAG (accession no. X07947). The other two ammonium-insensitive sequences did not have *C. griseus* matches. These two ammonium-insensitive sequences were identified as new putative *C. griseus* genes based on homology. Ten new putative *C. griseus* nucleotide sequences were identified, and eight of these sequences were confirmed by Northern blots to be ammonium sensitive. All 10 new gene sequences were deposited in GenBank. These 10 newly identified putative *C. griseus* genes are given in Table 2 along with their assigned GenBank accession nos., size, probable functions, E values, closest species matches, and the GenBank accession nos. of the closest matches.

## Discussion

Any eight arbitrary primers paired with the three-anchor primer have an estimated coverage of 28% of a genome *(22)*. Thus, the ammonium-sensitive *C. griseus* genes identified in the present study are likely to be only a subset of the ammonium-sensitive genes in CHO cells. In this study, 10 of 12 genes identified were new *C. griseus* entries. This high ratio of new genes suggests that differential display is an appropriate technique to detect genes sensitive to a particular environmental condition for a highly undocumented species such as *C. griseus*.

The eight putative *C. griseus* genes identified as sensitive to ammonium can be classified into three main functional groups *(26,27)*: cellular processes, metabolism, and genetic information processing. More specifically, the cellular-processing genes included cell growth and death, cell motility, and cell communication genes. The metabolism genes included energy metabolism genes. The genetic information–processing genes included folding, sorting, and degradation genes as well as DNA replication and repair genes. The cellular-process genes identified in the present study were anaphase-promoting complex subunit 5 (APC5), eukaryotic initiation factor 5A II (Eif5a2), and KIAA1091 protein (KIAA1091). The energy metabolism genes identified were adenosine triphosphate (ATP) synthase subunit C and mitofusin 1 (Mfn 1). The genetic information-processing genes identified were 26S proteasome subunit ATPase 3 (Proteasome ATPase), endoplasmic reticulum (ER) resident protein ERdj5 (ERdj5), and structure-specific recognition protein 1 (Ssrp1). The effects of ammonium stress on these genes are discussed next according to functional groups.

Cellular processes are essential for cell survival. It is well known that ammonium stress significantly inhibits cell growth rates *(8)*. In the present study, the three cellular process–related genes were all downregulated by ammonium stress. Specifically, APC is a large ubiquitin-protein ligase complex responsible for the initiation of anaphase and, thus, plays a critical role in cell-cycle progression *(28)*. Eif5a2 is hypothesized to be responsible for sensing intracellular polyamine levels, which are essential for mammalian cell survival and correct differentiation *(29)*. It has been observed that the intracellular depletion of Eif5a2 results in inhibition of cell growth *(30)*. In addition, KIAA1091 is involved in cell motility and cell communication *(31)*. Thus, the downregulation of these cellular-process genes, such as was observed, is consistent with decreased cell growth under ammonium stress.

All cellular activity is energy dependent. It has been reported that elevated ammonium causes a high rate of glycolysis and lactate production in CHO cells and other mammalian cell culture systems *(5,32–35)*. Several different hypotheses have been proposed to explain why ammonium stress increases energy requirements. For instance, elevated ammonium might drive higher ATP requirement via futile cycles to transport ammonium across membrane *(33)*, via glutamine synthesis *(36)*, or via the synthesis of α-ketoglutarate from glutamate and $NAD^+$ *(35)*. In the present study, two energy metabolism–related genes were observed to be higher under ammonium stress. Both proteins have critical roles in maintaining ATP levels in the cell. Specifically, ATP synthase subunit C is a component of the ATP synthase complex, which is responsible for ATP synthesis *(37)*. Mfn 1 regulates mitochondrial fusion and morphology in mammalian cells *(38)*. The mitochondria are responsible for energy synthesis (ATP generation) in eukaryotic cells, where the overexpression of Mfn 1 in cell culture has been observed to result in a large mitochondrion with abnormal internal struc-tures *(38–41)*. Therefore, the higher levels of Mfn 1 under ammonium stress may indicate an increased energy production capacity. The high

levels of both the ATP synthase subunit C and the Mfn 1 genes observed owing to ammonium stress is consistent with a need for increased energy metabolism.

Genetic information processing includes DNA replication, RNA transcription, protein translation, and protein posttranslational modification. Elevated ammonium has been observed to negatively impact protein activity and posttranslational modifications (i.e., glycosylation) *(32)*. In the present study, the genes *ERdj5* and *Ssrp1* were lower under ammonium stress, whereas the gene Proteasome ATPase was higher. Specifically, the ERdj5 protein assists protein folding and quality control in the *(43,44)*. Ssrp1 is involved in transcription initiation, elongation, DNA replication, and DNA repair *(45,46)*. Ssrp1 has also been observed to play essential roles in cellular metabolism and viability *(47–49)*. Proteasome ATPase is a component of the 26S proteasome *(50)*, which accounts for a majority of cellular proteolysis *(51)*. This ATP-dependent protease is respon-sible for selective degradation of abnormal proteins owing to improper folding or assembling *(52,53)*. The higher levels of the proteasome ATPase gene and lower levels of the *ERdj5* and *Ssrp1* genes owing to ammonium stress could be examples that indicate that ammonium significantly affects posttranslational modification and degradation. Additionally, the increased energy demands owing to the proteasome ATPase function are also consistent with the higher ATP synthase gene expression levels.

## Conclusion

The results of this study show that elevated ammonium may have a pleiotropic effect by enhancing or repressing the expression of various genes. The differential display technique appears to be a very useful tool to identify genes in highly undocumented species such as *C. griseus* and is an effective method to identify genes sensitive to bioreactor conditions.

## Acknowledgments

This work was supported by the National Science Foundation (grant no. 0303782) and by the National Institutes of Health, National Center for Research Resources (grant no. P20 RR-016461).

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