CHARACTERIZATION OF EUKARYOTIC TRANSLATION INITIATION FACTOR 5A ISOFORMS (eIF-5A1 & eIF-5A2) USING HUMAN CELL LINES AS A MODEL SYSTEM

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Eukaryotic translation initiation factor 5A (eIF-5A) is the only known cellular protein that contains the post-translationally derived amino acid, hypusine. Initially, eIF-5A was named as a translation initiation factor because of its capability to stimulate the formation of methionyl-puromycin, which mimics the first peptide bond formation during protein synthesis, under *in vitro* conditions. Subsequently, however, this proposed function of eIF-5A has been questioned because a similar effect on translation was not observed *in situ*. Moreover, eIF-5A appears not to be required for general protein synthesis. Rather, there is evidence that it facilitates the translation of specific subsets of mRNAs required for cell proliferation as well as apoptosis.

There are two isoforms of eIF-5A in the human genome which have designated eIF-5A1 and eIF-5A2. The objective of the present study was to gain an increased understanding of the roles of eIF-5A1 and eIF-5A2 during apoptosis and cell proliferation using human cell lines as a model system. Apoptosis was induced by treating the cells with Actinomycin D or sodium nitroprusside (SNP), which initiate programmed cell death by different mechanisms. It was observed for both normal and cancer cells that eIF-5A1 protein is up-regulated during apoptosis induced by Actinomycin D or SNP, whereas eIF-5A1 mRNA is constitutively expressed and does not change in abundance during this treatment.. The up-regulation of eIF-5A1 protein levels in the absence of a corresponding up-regulation in eIF-5A1 mRNA suggests that eIF-5A1 may be post-transcriptionally regulated. Moreover, eIF-5A1 protein up-regulation was stronger in normal cells than in cancer cells. By contrast, eIF-5A2 protein was below detection levels during apoptosis in both normal and cancer cells, although the

corresponding transcript was detectable by semi-quantitative RT-PCR. This is attributable to inefficient translation of eIF-5A2 mRNA.

The effects of eIF-5A1 and eIF-5A2 on cell proliferation were examined by modulating the levels of serum in cultures of UACC-1598 cells, which are ovarian cancer cells that express high levels of both isoforms of eIF-5A. Serum starvation, which induces cell cycle arrest and ensuing apoptosis, followed by the re-addition of serum had no effect on the transcript levels of either eIF-5A1 or eIF-5A2. However, eIF-5A1 and eIF-5A2 proteins were both up-regulated within 24 hours of the initiation of serum starvation, and this coincided temporally with the onset of apoptosis as measured by TUNEL and a subsequent decline in viable cells.

The data indicate that eIF-5A1 and eIF-5A2 are both post-transcriptionally regulated and that they have functionally redundant roles in apoptosis.

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Introduction

1.1 Apoptosis

The term "apoptosis" was coined by Kerr et al. (1972) to describe the "falling off" of leaves from trees or petals from flowers. This meaning coincides with the particular morphology of apoptosis or programmed cell death.

Apoptosis can be differentiated from necrosis, which is another form of cell death. Necrosis occurs in response to injury by toxins, physical stimuli, or ischemia. Swelling of cells, disruption of membranes and lysis of nuclear chromatin are the hallmarks of necrosis, whereas shrinkage of cells, blebbing of membrane, condensation of the nucleus and formation of apoptotic bodies are the characteristic features of apoptosis (Saikumar et al., 1999). Apoptosis has significant importance in various processes such as development, morphogenesis, homeostasis, and deletion of damaged cells. Dysfunction or dysregulation of the apoptotic program is associated with various pathological conditions (Fadeel et al., 1999). Tumor formation and metastasis are consequences of a defective apoptotic pathway (Jhonstone et al., 2002). Indeed, there is strong evidence that tumorigenesis is not only the result of excessive proliferation due to the activation of oncogenes, but also impairment of apoptosis pathways (Hanahan and Weinberg, 2000).

1.2 Transformation of normal cells into cancer cells

The development of a cancer cell from a normal cell is a multistep process driven by a series of somatic mutations. This has been demonstrated in human colon cancer, which progresses in a series of well-defined morphologic stages. Figure 1 illustrates a model of

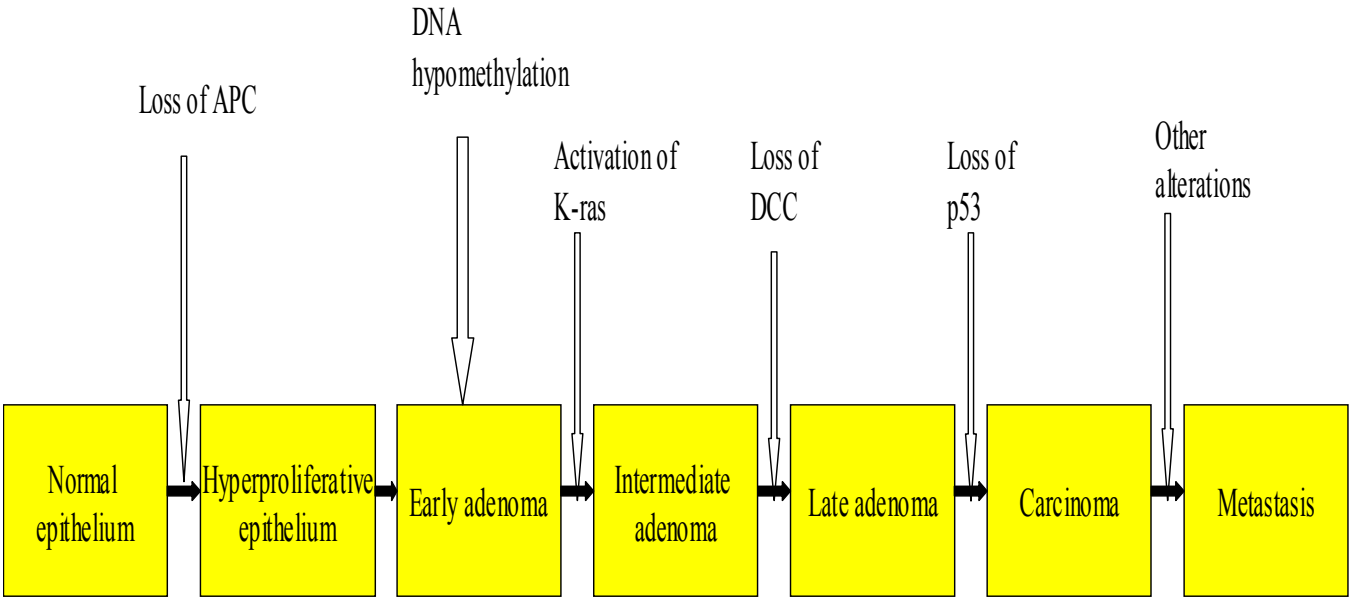
colon cancer where a sequence of gene changes occurs including inactivation or loss of three tumor-suppressor genes (APC, DCC and p53) and activation of an oncogene (K-ras). Various chemicals, such as DNA-damaging agents, and physical agents including ultraviolet light are able to induce mutations. Induction of malignant transformation with chemical or physical carcinogens entails at least two distinct phases: initiation and promotion. Initiation involves changes in the genome, but does not in itself lead to a malignant phenotype. Rather, after initiation, promoters stimulate cell division leading to the malignant phenotype (Goldsby et al., 2003).

1.3 Apoptotic pathways in normal cells:

1.3.1 Extrinsic apoptotic pathways

Extrinsic apoptotic signaling is initiated by the activation of death receptors that transmit apoptotic signals upon ligation with specific ligands including TNFR1, Fas, and TRAIL (Ashkenazi, 2002) (See Figure 2). Signals originating from death receptors activate the Death Inducing Signaling Complex (DISC), which in turn mediates activation of the initiator caspase-8. Caspase-8 initiates a caspase cascade by activating the effector caspases-3, 6, and 7, which results in cleavage of caspase substrates. Finally, cleavage of caspase substrates leads to the morphological and biochemical features of apoptosis. Cells able to induce this mainly caspase-dependent apoptosis are termed type I cells (Scaffidi et al., 1998). In some cells, the incoming signal from the DISC must be amplified via mitochondria-dependent apoptotic pathways, which is termed type 2 signaling. The link between the

Figure 1. A model for colon cancer depicting transformation of a normal cell into a cancer cell through genetic alterations (Goldsby et al., 2003).

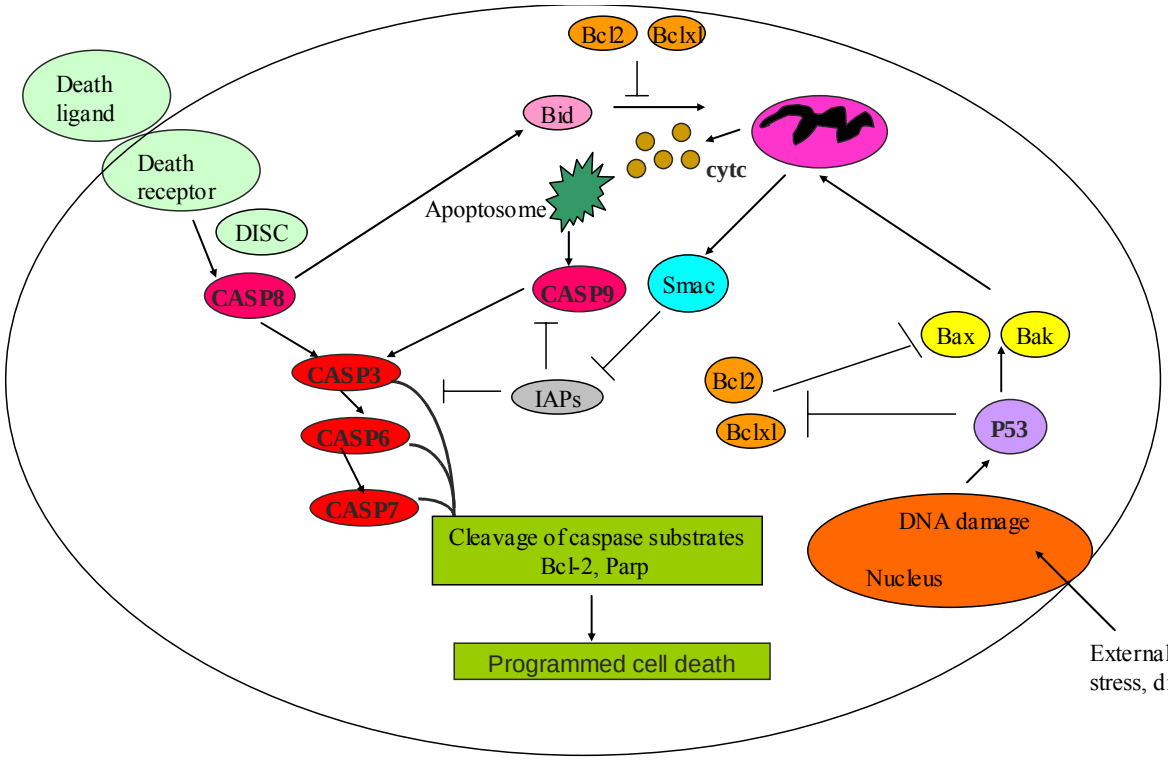

DNA
hypomethylation
Loss of APC
Activation of
K-ras
Loss of
DCC
Loss of
p53
Other
alterations
Normal
epithelium
Hyperproliferative
epithelium
Early adenoma
Intermediate
adenoma
Late adenoma
Carcinoma
Metastasis

caspase signaling cascade and mitochondria is provided by the Bcl-2 family member, Bid. Bid is cleaved by caspase-8, and in this truncated form is translocated to the mitochondria. In mitochondria, Bid acts in association with the proapoptotic Bcl-2 family members, Bax and Bak, to release cytochrome c into the cytosol (Luo et al., 1998). Cytochrome C initiates formation of the apoptosome, which triggers activation of caspase-9 (Acehan et al., 2002). Finally, activated caspase-9 activates caspase-3, caspase-6 and caspase-7, which results in apoptosis (Denault and Salvesen, 2002).

1.3.2 Intrinsic apoptotic pathways

Intrinsic stresses such as DNA damage can activate the intrinsic apoptotic pathway (See Figure 2). As a sensor of cellular stress, p53 is a critical initiator of this pathway (Lowe and Lin, 2000). p53 can initiate apoptosis by transcriptionally activating proapoptotic Bcl-2 family members, such as Bax and Bak, and by repressing antiapoptotic Bcl-2 family members such as Bcl-2, Bcl-XL (Bartke et al. 2001). However, p53 can also transactivate other genes that may contribute to apoptosis such as PTEN (Hwang et al. 2001). Moreover, p53 can transcriptionally activate various death receptors and sensitize cells to death-receptor-mediated apoptosis (Herr and Debatin 2001). Therefore, it appears that p53 is capable of coordinating the apoptotic program at multiple levels via several mechanisms. Still, p53 is not the only activator of the intrinsic apoptotic pathway. Some studies suggest that p73 (related family member) might substitute for p53 in some cases (Yang and McKeon, 2000).

Figure 2. Apoptotic pathways in normal cells


Bcl2
Bclxl
Death ligand
Death receptor
DISC
Bid
cytc
Apoptosome
CASP8
CASP9
Smac
CASP3
CASP6
CASP7
IAPs
Bax
Bak
Bcl2
Bclxl
P53
DNA damage
Nucleus
Cleavage of caspase substrates Bcl-2, Parp
Programmed cell death
External insults such as irradiation, stress, drugs

1.3.3 Role of mitochondria in the apoptotic pathway:

Mitochondrial membrane permeabilization in an important factor in apoptosis induction, which is regulated by the opposing actions of pro- and antiapoptotic Bcl-2 family members. The BH3-only protein, Bid, can directly interact with proapoptotic Bcl-2 proteins (e.g., Bax and Bak) and activate them. Alternatively, binding of other BH3-only proteins (e.g., Noxa, Puma, Bad, and Bim) to antiapoptotic Bcl-2 proteins (e.g., Bcl-2 and Bcl-X_L) results in activation of Bax and Bak (Adams and Cory 1998 and Huang and Strasser 2000). It is not clear whether Bcl-2 proteins control mitochondrial membrane permeability by directly forming pores in the outer membrane, or by regulating the opening and closing of the permeability transition pore (Martinou and Green, 2001). However, the consequence is regulated release of proapoptotic factors from the mitochondria, induction of downstream caspases, and loss of mitochondrial function. Another important mitochondrial proapoptotic factor is Smac, which is released from mitochondria and acts by inhibiting the antiapoptotic proteins termed IAPs (inhibitors of apoptotic proteins). IAPs can directly inhibit caspases. Smac inhibits IAPs from blocking caspase activity (Du, 2000).

1.4 Dysregulated apoptotic pathways in cancer cells

1.4.1 Dysregulation of the intrinsic apoptotic pathway:

Disruption of the intrinsic apoptotic pathway is extremely common in cancer cells. The p53 tumour suppressor gene is the most frequently mutated gene in human tumors. Loss of p53 function impairs apoptosis and accelerates tumor development in transgenic mice (Attardi and Jacks 1999 and Ryan et al. 2001). In addition, mutations of p53 downstream

effectors, such as PTEN, Bax, Bak, and Apaf-1, are common in human tumors. Therefore, the presence of wild-type p53 does not necessarily indicate that the pathway is intact (Schmitt et al., 1999).

Bcl-2 family members, which also regulate the intrinsic pathway, have been shown to be altered in tumor samples as well. In fact, Bcl-2, which is a negative regulator of apoptosis, is over-expressed in a number of cancers (Reed, 1999). Moreover, Bcl-2 over-expression can accelerate tumor formation in transgenic mice (Adams et al. 1999). Conversely, proapoptotic Bcl-2 proteins are inactivated in certain cancers, and dysregulation of these proteins promotes tumor formation in mice (Datta et al., 1999).

1.4.2 Dysregulation of the postmitochondrial death process:

Mutation in apoptotic proteins that function downstream of mitochondrial cytochrome c has also been reported. For example, over-expression of IAPs, which inhibit caspase 9 activation, is commonly observed in human tumors (Deveraux and Reed 1999). However, post-mitochondrial mutation is less frequent than those mutations which target upstream components of the apoptotic pathway (Johnstone et al., 2002).

1.4.3 Dysregulation of the death receptor pathway:

Dysregulation in the death-receptor pathway occurs less frequently than dysregulation in the intrinsic pathway (Jhonstone et al. 2002). However, it is not uncommon for tumor cells to be resistant to death-receptor-mediated apoptosis, largely because of mutations in CD95, TRAIL receptors, and downstream signalling pathways activated by ligand association with death receptors. Mutations in CD95, for example, result in inappropriate survival of activated

T lymphocytes, which causes Autoimmune LymphoProliferative Syndrome (ALPS). ALPS patients have an increased incidence of lymphoma, probably due to the large population of apoptosis-resistant T cells (Straus et al., 2001). It is to be expected that dysregulation of the death receptor pathway would provide a survival advantage to developing tumor cells. This contention is supported by the finding that loss of CD95L or TRAIL function promotes tumor growth and metastasis (Rosen et al. 2000 and Takeda et al. 2001).

1.5 Cancer producing mutations

Cancer producing mutations give rise to oncogenes and also commonly occur in tumor-suppressor genes.

1.5.1 Oncogenes

Dominant mutations can generate oncogenes that exhibit abnormal activity or produce an excessive amount of protein. The wild type gene that becomes an oncogene is known as a protooncogene. Oncogenes typically cause abnormal cell proliferation, which may lead to the accumulation of more mutations eventually leading to cancer (Hartwell et al., 2004). Oncogenes can be activated by point mutations that enhance the function of the oncoprotein (Downward et al., 2003). Other mechanisms of oncogene activation include chromosomal translocation, where a new fusion gene is transcribed into a protein with enhanced function. Oncogenes promote carcinogenesis through amplification and over-expression. Amplification may include short chromosomal regions of chromosomal arms involving hundreds of genes or entire chromosomes (Osborne et al, 2004).

1.5.2 Tumor-suppressor genes

Mutations of tumor-suppressor genes are typically recessive, resulting in expression of normal protein when they are heterozygous with the wild type allele and mutant protein when they are homozygous (Hartwell et al., 2004). Tumor suppressor genes are often inactivated by chromosomal deletions (Kohno and Yokota, 1999). In some cases there is not a mutation in the tumor suppressor gene; rather some other factor interferes with its expression or the function. For example, methylation of the gene promoter which suppresses its transcription, an increased rate of proteasomal degradation, or abnormalities in other proteins that interact with the tumor-suppressor gene product are all factors that have been shown to disrupt tumor suppressor gene function (Osborne et al., 2004).

1.6 Division of normal cells

A number of genes and their cognate proteins control the cell cycle. These genes and proteins enable progression of the cell cycle from one stage to the next under normal conditions, and inhibit progression through the cell cycle in the event the cell is damaged and there is a need for repair (Hartwell et al., 2004).

1.6.1 The Cell cycle

The cell cycle has four phases: G1, S, G2, and M. G1 is the period between the end of mitosis and the initiation of DNA synthesis that precedes the next mitosis. S is the period of DNA synthesis, whereas G2 is the gap between DNA synthesis and mitosis. M is the period when the breakdown of the nuclear membrane, condensation of the chromosomes, attachment of the chromosomes to the mitotic spindle, and segregation of chromosomes to

the two poles occur. At the end of mitosis, the cell divides (Hartwell et al., 2004). Entrance into the cell cycle and subsequent proliferation are tightly regulated, in part by cyclin-dependent kinases (CDKs). Upon activation, CDKs promote phosphorylation of other proteins, especially retinoblastoma protein (Rb), which allows the transition from a resting state into mitosis. Cyclin D1 and cyclin E play a key role in progression of the cell cycle from the G1 to S phase, and their expression levels oscillate as the cell cycle progresses (Vermeulen et al., 2003). When cyclin D1 is complexed with its respective CDK partner, the Rb protein is phosphorylated, which in turn releases the transcription factor, E2F, and induces proteins required for DNA synthesis (Loden et al., 2002). Additional controls in the cell cycle are known as checkpoints because they check the cell cycle machinery before allowing the cell to continue to the next phase of cell cycle (Hartwell et al., 2004).

1.6.2 The G_1-to-S Checkpoint

DNA damage during G_1 causes postponement of DNA replication, which allows time for DNA repair before the cell proceeds to DNA synthesis. In the event of DNA damage, G_1 delays entry into S phase by activating p53, which induces expression of DNA repair genes (Hartwell et al., 2004).

1.6.3 The G_2-to-M Checkpoint

DNA damage during G2 delays mitosis, which allows time for repair before chromosome segregation (Hartwell et al., 2004).

1.6.4 A checkpoint in M

This checkpoint oversees formation of the mitotic spindle and proper engagement of all pairs of sister chromatids. As chromosomes condense and attach to the spindle, sometimes a single chromosome fails to attach at the expected time. In this case, the cell does not initiate sister chromatid separation until the lagging chromosome attaches to the spindle (Hartwell et al., 2004).

1.7 Dysregulated cell cycle in cancer cells

Many genes that contribute to the normal control of cell division are subject to mutations. Such mutations cause malfunctions in the CDK-cyclin complexes or in the checkpoints. Table 1 lists some cell-division genes in which mutations contribute to cancer (Hartwell et al., 2004).

The gene encoding cyclin D1 has been found to be over-expressed in 40%–50% of breast cancers (Steeg and Zhou, 1998), and high cyclin D1 expression is positively associated with an increased proliferative index (Loden et al., 2002). Like cyclin D1, over-expressed cyclin E in cancer cells results in hyperphosphorylation of Rb protein and increased proliferative activity. However, high cyclin E- tumors are able to induce DNA synthesis independently of Rb phosphorylation and E2F activation. The consequence is a marked reduction in cell cycle control and a significant dysregulation of proliferation (Loden et al., 2002 and Keyomarsi and Pardee, 2003). Furthermore, mutations in the tumor suppressor gene, p53, disrupt the G_1-to-S checkpoint. Faulty p53 allows cells carrying damaged DNA to replicate, which leads to chromosome rearrangements (Hartwell et al., 2004).

Table 1. Cell cycle genes which, when mutated, contribute to cancer

Genes	Gene products and their functions
CDK4	A CDK of mammalian cells important for G_1 to S transition
CDK2	A CDK of mammalian cells important for G_1 to S transition
Cyclin D1	A Cyclin of mammalian cells important for G_1 to S transition
Cyclin E	A Cyclin of mammalian cells important for G_1 to S transition
Cyclin A	A Cyclin of mammalian cells important for S phase
Cyclin B	A Cyclin of mammalian cells important for the G_2 to M transition
E2F	A transcription factor of mammalian cells important for the G_1 to S transition
RB	A mammalian protein that inhibits E2F
P^{21}	A protein of mammalian cells that inhibits CDK activity
P^{16}	A protein of mammalian cells that inhibits CDK activity
P^{53}	A transcription factor of mammalian cells that activates transcription of DNA repair genes as well as transcription of P^{21}

(Hartwell et al., 2004)

1.8 Eukaryotic translation initiation factor 5A (eIF-5A)

1.8.1 Post-translational modification of eIF-5A

Eukaryotic translation initiation factor 5A (eIF-5A) is the only known cellular protein that contains the post-translationally derived amino acid, hypusine (Park *et al*., 1982). The synthesis of hypusine within eIF5A is a two-step posttranslational modification process. The first modification step is catalyzed by deoxyhypusine synthase (DHS), which mediates the transfer of a butylamine moiety from spermidine to the ε-amino group of a specific lysine residue, generating deoxyhypusine (Park *et al*., 1982). This intermediate does not accumulate in cells; rather, it is immediately hydroxylated at the C-2 of the incoming 4-aminobutyl moiety by deoxyhypusine hydroxylase (DHH), generating hypusine (Abbruzzese *et al*., 1985 ; Abbruzzese *et al*., 1986, 1988 a, b).

1.8.2 Proposed function of eIF-5A

Hypusine plays a key role in the regulation of eIF-5A function based on the finding that hypusine-deficient eIF-5A precursors have little or no activity (Park *et al*., 1991). Initially, hypusine-modified eIF-5A was isolated from immature red blood cells and named as a translation initiation factor because of its capability to stimulate the formation of methionyl-puromycin, which mimics the first peptide bond formation during protein synthesis, under *in vitro* conditions (Kemper *et al*., 1976). However, this proposed function of eIF-5A has been questioned because a similar effect on translation was not observed *in situ* (Zuk and Jacobson, 1998). Moreover, eIF-5A appears not to be required for general protein synthesis. For example, only marginal changes in total protein synthesis were observed when both isoforms of eIF-5A in yeast were mutated. This finding led to the

proposal that eIF-5A may be selectively required for translation of certain mRNAs (Kang and Hershey, 1994). In support of this, recent research reported the identification of mRNA species that bind specifically to eIF-5A (Xu et al., 2004). It has been further suggested that eIF-5A may facilitate the translation of mRNAs required for cell proliferation (Lipowsky et al., 2000). This is based on the finding that hypusine modified eIF-5A is essential for sustained proliferation of mammalian cells (Park et al., 1994), yeast (Schnier et al., 1991), and archea (Jansson et al., 2000). When the levels of hypusine-modified eIF-5A were depleted through direct inhibition of hypusination in vivo, by biochemical means or genetic means, or through depletion of the polyamine spermidine, cell cycle arrest was seen at the G1/S boundary, and the treated cells ultimately died (Park et al.,1994 ; Jansson et al., 2000). The antiproliferative agent, deoxyspergualin, has also been shown to inhibit active eIF-5A formation (Nishimura et al., 2002). Furthermore, when mammalian cells were treated with the anticancer agent, IFN-α, a reduction in hypusine synthesis occurred in parallel with induction of apoptosis (Caraglia et al., 2003). These observations suggest that hypusine-modified eIF-5A is required for cell proliferation and that blocking cell division indirectly triggers the onset of apoptosis (Thompson et al., 2004).

Work with plants has provided evidence for the direct involvement of eIF-5A in cell death. Wang et al. (2001) found that both DHS and eIF-5A transcripts and their proteins increased in senescing tomato flowers, senescing tomato cotyledons, and senescing tomato fruit. They argued that if eIF-5A were only required for cell division, eIF-5A transcript would not be expected to be present at high levels in a dying tissue (Wang et al., 2001). These observations led to the proposal that separate isoforms of eIF-5A are involved in cell

division and cell death (Thompson et al., 2004). In plant systems, transgenic upregulation of a putative cell division isoform of eIF-5A gave rise to flowers with more petals and larger seeds (Thompson et al., 2004).

Recent evidence suggests that eIF-5A functions as a nucleocytoplasmic shuttle protein, translocating specific mRNAs from the nucleus to the cytoplasm (Rosorius *et al*., 1999). The involvement of eIF-5A in mRNA translocation is further supported by the finding that it serves as a cellular cofactor of HIV-1 Rev and HTLV-1 Rex transactivator proteins (Ruhl *et al*., 1993; Elfgang *et al*., 1999). HIV-1 Rev and HTLV-1 Rex are also nucloecytoplasmic shuttle proteins that mediate nuclear export of incompletely spliced and unspliced viral mRNAs (Smith and Greene, 1991; Pollard and Malim, 1998).

1.8.3 Isoforms of eIF-5A

Two isoforms of eIF-5A have been identified for many mammals, although three or more isoforms have been found in various plant species. Plant eIF-5A sequences are highly conserved, but they have only 50-60% amino acid identity with human, yeast and fungal eIF-5A. However, the lysine modification site is flanked by highly conserved residues across the kingdoms (Wang et al., 2001). The human eIF-5A1 cDNA was first isolated in 1989 (Smit-McBride et al., 1989), and the EIF5A1 gene was identified in 1995 (Koettnitz et al., 1995). Later, the human EIF5A2 gene was identified, which encodes a protein with 84% identity to eIF5A1 (Jenkins et al., 2001). The eIF-5A1 protein is abundantly expressed in all mammalian cells and human cells. Although eIF-5A2 mRNA is detectable in most cancer and human cell lines, eIF-5A2 protein expression is very low, which can be attributed to inefficient

translation of eIF-5A2 mRNAs (Clement et al., 2003). It has been proposed that eIF-5A1 and eIF-5A2 are involved in apoptosis and cell proliferation, respectively (Thompson et al., 2004).

1.8.4 eIF-5A1 as a novel pro-apoptotic protein

The possible role of eIF-5A1 in apoptosis has been demonstrated using tumor necrosis factor (TNF-α) in synergy with camptothecin. eIF-5A1 was upregulated in lamina cribrosa (LC) cells in response to TNF-α and camptothecin, and there was a coincident induction of apoptosis (Taylor et al., 2004). Moreover, small interfering RNA (siRNA) against eIF-5A1 protected LC cells from apoptosis in response to TNF-α and camptothecin, suggesting that eIF5A1 is a pro-apoptotic protein involved in the death receptor pathway (Taylor et al., 2004). Recent research indicates that eIF-5A1 may function as a regulator of p53. Over-expression of eIF-5A1 induced apoptosis in human cells and resulted in up-regulation of p53 (Li et al., 2004). Furthermore, silencing eIF-5A1 expression by siRNA reduced the p53 protein level (Li et al., 2004). Further analysis by reverse transcription PCR showed that eIF-5A1 activated p53 transcription. Moreover, the effect of eIF-5A1 on p53 transcriptional activity was further observed by the increasing expressions of p21 and Bax, which are well known target genes of p53 (Li et al., 2004). In contrast, a point mutant of eIF-5A1, which was unable to be hypusinated, was found to be functionally defective in p53 up-regulation (Li et al., 2004). However, Taylor and colleagues have shown that eIF-5A1 is also able to induce apoptosis independently of p53 using RKO-E6 cells which lack functional p53 (Catherine Taylor, unpublished results). Therefore, it can be concluded that eIF-5A1 may be an important regulator of both p53-dependent and –independent apoptotic pathways.

1.8.5 The role of eIF-5A2 as an oncogene

The eIF-5A2 gene is located at 3q26.2, which is a region of frequent chromosomal alterations in many solid tumors, including ovarian, lung, oesophageal, prostate, breast, and nasopharyngeal cancers. In a recent study with the ovarian cancer cell line, UACC 1598, it was found that cell growth was inhibited when the eIF-5A2 expression level was decreased (Guan et al., 2004). In addition, treatment of UACC-1598 cells with antisense EIF-5A2 decreased the cell growth (Guan et al.,2004). Moreover, anchorage-independent growth in soft agar has been observed in eIF-5A2-transfected NIH3T3 and LO2 cells (Guan et al.,2004). Furthermore, tumor formation in nude mice was observed after the injection of eIF-5A2-transfected LO2 cells, and over-expression of EIF-5A2 appears to be correlated with the advanced stages of ovarian cancer (Guan et al., 2004).

1.8.6 Subcellular localization of eIF-5A

Although the subcellular distribution of eIF-5A was studied to gain insight into the role of this protein, conflicting results were obtained. Shi et al. (1997) reported that eIF-5A is localized in the cytoplasm, and that treatment with the protein synthesis inhibitor, puromycin altered its subcellular distribution from an endoplasmic reticulum network like structure to a patched dotted pattern dispersed throughout the cytoplasm. They concluded that eIF-5A is associated with the endoplasmic reticulum through ribosomes and plays a role in protein synthesis (Shi et al., 1997). Li-En and Yu (2002) reported that eIF-5A is localized in both the cytoplasm and the nucleus as determined by indirect immunofluorescent staining and by direct visualization of green fluorescent protein-tagged eIF-5A. They concluded that eIF-5A enters the nucleus via passive diffusion, but it does not undergo active nucleocytoplasmic

shuttling (Li-En and Yu, 2002). Recently, Taylor et al. (2006) reported that eIF-5A was predominantly localized in the cytoplasm, but that treatment with apoptotic agents including actinomycin D or INF-γ and TNF-α caused a dramatic shift in localization from the cytoplasm to the nucleus. This indicates that eIF-5A may function as a nucleocytoplasmic shuttle protein during apoptosis and that re-localization of eIF-5A may play a role in the regulation of apoptosis.

1.9 Objectives of proposed research

There is very little information on the expression of eIF-5A1 and eIF-5A2 in response to apoptotic and proliferative stimuli. Thus, the overall objective of this research was to determine whether the expression of eIF-5A1 and eIF-5A2 correlates with the functions that have been tentatively ascribed to them, viz., a role for eIF-5A1 in apoptosis and a role for eIF-5A2 in proliferation. To this end, the temporal expression patterns of both eIF-5A1 and eIF-5A2 at the transcriptional and translational levels were assessed in CCD-112CoN, a normal human colon fibroblast cell line, and in RKO, a human colon adenocarcinoma cell line, in response to apoptosis induced by treatment with Actinomycin D (ActD) or sodium nitroprusside (SNP). In addition, the temporal expression patterns of eIF-5A1 and eIF-5A2 were studied at the transcriptional and translational levels in UACC-1598, a human ovarian cancer cell line, in response to changes in cell proliferation induced by modulation of serum levels in the culture medium.

Methods and Materials

2.1 Cell culture

Three cell lines were used for experiments reported herein . A normal human colon fibroblast cell line (CCD-112CoN), and a human colon adenocarcinoma cell line (RKO) were obtained from ATCC (American Type Culture Collection, Rockville, MD). They were cultured in minimum essential medium (Eagle) containing 2 mM L-glutamine and Earle's BSS (Sigma, Oakville, ON) , 0.1 mM non-essential amino acids (Sigma), 1.0 mM sodium pyruvate (Sigma) and 10% fetal bovine serum (FBS) (Sigma) .The ovarian cancer cell line (UACC-1598) was kindly provided by Anita Antes of the University of Medicine and Dentistry , New Jersey. This was cultured in 90% RPMI (Sigma) supplemented with 10% FBS (Sigma). All three cell lines were supplemented with 100 units/ml penicillin/streptomycin (Sigma) and kept at 37^0 C in a humidified atmosphere of 5% CO_2 .

2.2 Apoptosis

2.2.1 Induction of apoptosis

Cells were treated with agents known to induce apoptosis for periods of up to 24 hours by which time they had reached about 90% confluence. Specifically, apoptosis was induced by adding Actinomycin D [0.5 μg/ml (w/v) dissolved in DMSO] or sodium nitroprusside (3mM) to the culture medium.

2.2.2 Deadend™ Fluorometric TUNEL assay to detect apoptosis

The DeadEnd™ Fluorometric TdT-mediated dUTP Nick-End Labelling (TUNEL) assay was performed according to the DeadEnd™ Fluorometric TUNEL kit (Promega, Madison, WI). This assay measures nuclear DNA fragmentation by catalytically incorporating fluorescein-12-dUTP at the 3'-OH ends of DNA using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Following the induction of apoptosis, cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS (w/v). The cells were then permeabilized by treatment with 0.2% Triton X-100 (Bioshop, Burlington, ON) for 5 minutes and equilibrated with equilibration Buffer provided in the kit. Following equilibration, cells were incubated with incubation buffer containing equilibration buffer, nucleotide Mix and rTdT Enzyme at 37°C for 1 hour. Subsequently,the cells were immersed in 2X SSC to terminate the reaction. Hoescht 33258 (blue) was used to stain the nuclei of all cells and TUNEL (green) was used to stain apoptotic cells only. Cells were visualized directly by fluorescence microscope (Carl Zeiss Meditec, Oberkochen,Germany) with a UV filter (UV-G 365, filter set 487902) to count nuclei of all cells and with a fluorescein filter (Green H546, filter set 48915) to count apoptotic cells only. The percentage of apoptotic cells in any field of view was calculated by dividing the number of apoptotic cells by the total number of cells.

2.3 Cell proliferation

2.3.1 Serum starvation and serum re-addition

The effects of serum starvation and serum re-addition on cell proliferation and apoptosis were determined using the ovarian cancer cell line, UACC -1598. The cells were

cultured to about 75% confluence and then serum-starved for 3 days by replacement of the normal medium with medium that did not contain serum. After an additional 3 days, the serum-free medium was replaced with serum-containing medium and the cells were harvested at various intervals up to 8 hours.

2.3.2 XTT cell proliferation assay

Cell proliferation was measured using the XTT assay according to the Manufacturer's protocol (Roche Applied Science, Mississauga, ON). This assay measures cell proliferation by determining the metabolic activity of viable cells. UACC-1598 cells were cultured in 96-well microplates until they reached about 75% confluence, and serum- starved for 3 days. The time points for XTT assay were as follows: 0 day, 1 day, 2 days and 3 days after serum starvation. After day 3, serum was added again. The time points for XTT analysis after serum re-addition were as follows: 1 hour, 4 hours and 8 hours. The assay entailed incubating cells in XTT solution for 4 hours so that viable cells could convert XTT to a water soluble formazan dye. Following solubilization of the formazan dye in the microplate, the dye was quantitated using a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, CA) set at 490 nm and 650 nm.The absorbance indirectly indicates the number of viable cells.

2.3.3 BrdU ELISA assay of cell proliferation

Cell proliferation was also measured using the BrdU ELISA assay according to the Manufacturer's protocol (Roche Applied Science). UACC-1598 cells were cultured in 96-well microplates until they reached about 75% confluence, and then serum-starved for 3

days. The time points for BrdU ELISA assay were as follows: 0 day, 1 day, 2 days, 3 days. After day 3, serum was added again. The time points for BrdU ELISA assay after serum re-addition were as follows: 1 hour, 4 hours and 8 hours. The assay entailed labeling adherent cells with BrdU labeling solution for 2 hours at 37^0 C. Following labeling, the cells were fixed with FixDenat solution for 30 minutes at 15-25^0 C and then incubated in Peroxidase-conjugated anti-BrdU antibody for 90 minutes at 15-25^0 C. The cells were then rinsed three times with washing solution to remove antibody conjugate. Finally, the cells were incubated with substrate solution at 15-25^0 C for 5-30 minutes or until color development was observed. The absorbance was measured at 370 nm and 492 nm (reference wavelength) using a Versamax Tunable microplate reader (Molecular Devices).

2.4 Protein study

2.4.1 Protein extraction

Following the induction of apoptosis for RKO or CCD-112CoN cells, they were washed in Dubelco's phosphate buffered saline (Sigma) and lysed with boiling cell lysis buffer containing 2% SDS (w/v) and 50 mM Tris-HCl (pH 7.4). The cell lysate was collected in a microcentrifuge tube, boiled for 3 minutes and stored at -20^0 C until required for analysis. Following serum starvation and serum re-addition for UACC-1598 cells, they were washed with Dubelco's phosphate buffered saline (Sigma) and lysed with boiling cell lysis buffer containing 2% SDS (w/v) and 50 mM Tris-HCl (pH 7.4). Each sample was then sonicated for 5 seconds using a Sonic Dismembrator Model 100 (Fisher Scientific, Napean , ON). All samples were centrifuged at 14 000 rpm for 10 minutes at 4 ^{0} C in a microcentrifuge

(Heraeus Instruments, Hanau , Germany). After centrifugation, supernatants were removed by pipetting, transferred to new Eppendorf tubes and stored at -20^{0} C until required for analysis.

2.4.2 Protein Quantitation

Protein concentration was determined by the Bicinchoninic Acid protein assay kit (Sigma). Dilutions of bovine serum albumin (BSA, Sigma) ranging from 0 to 1000 µg/ml were prepared in lysis buffer from a 1 mg/ml stock of BSA. Aliquots of isolated protein samples were diluted 1:5, 1:10 and 1:20 in lysis buffer. Twenty five µl of each BSA standard was pipetted onto a polystyrene 96 well assay plate in duplicate. An equal volume of each diluted protein sample was also pipetted onto the plate in duplicate. A 50:1 mixture of BCA reagent A to reagent B (Sigma) was prepared, and 200 µl of the mixture was added to each well. The plate was covered with aluminium foil and incubated at 37^{0} C for 30 min .The plate was read using a Versamax Tunable microplate reader (Molecular Devices) at 562 nm. The BSA standards were used to construct a standard curve that was used to determine the concentration of each protein sample.

2.4.3 SDS PAGE & Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini Protean II gel apparatus (BioRad, Mississaugua, ON). A 15 % separating gel was overlayed with 1 ml of 50% 1-butanol for 40 minutes, by which time the separating gel had polymerized. The 1-butanol layer was then discarded, and the stacking gel was layered on the top of separating gel and allowed to polymerize for 40 minutes. For

western blot analysis, 5 µg (for RKO and CCD-112CoN cells) or 10 µg (for UACC-1598 cells) of lysate protein was suspended in lysis buffer [2% SDS (w/v) , 50 mM Tris-HCl (pH 7.4)]. The resultant suspensions were then mixed 1:1 (v/v) with , loading buffer [2% SDS (w/v) , 10% BME (v/v) , 250 mM Tris (w/v), 30% glycerol (v/v), 0.002% bromophenol blue(w/v)] and boiled for 10 minutes and centrifuged in a microcentrifuge before loading into the wells. The tank was filled with 1 X running buffer [25 mM Tris (w/v), 0.2 M glycine (w/v), 1 mM SDS (w/v), pH 8.3)], samples were loaded and electrophoresed at 0.06 amps until the dye front reached the bottom of the gel. Before transferring the proteins to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ), the membrane was activated with methanol for 30 seconds, washed with water, and then soaked in cold transfer buffer [25 mM Tris (w/v), 192 mM glycine (w/v), 20% methanol (v/v)] for 30 minutes. Following electrophoresis, the gels were also soaked in transfer buffer for 10-15 min. Proteins were transferred to the PVDF membrane using a Trans-Blot Semi-dry Transfer Cell (BioRad) at 15 Volts for 36 minutes. The membrane was incubated for 1 hour in blocking solution of 5% skim milk in PBS-T [137 mM NaCl (w/v), 2.7 mM KCl (w/v) , 4.3 mM $Na_2HPO_4 . 7H_2O$ (w/v) , 1.4 mM KH_2PO_4 (w/v) , pH 7.3, 0.1% Tween-20(v/v)] . The primary antibody was diluted in a solution of 5% milk in PBS-1/2 T [137 mM NaCl (w/v), 2.7 mM KCl (w/v) , 4.3 mM $Na_2HPO_4 . 7H_2O$ (w/v) , 1.4 mM KH_2PO_4 (w/v) , pH 7.3, 0.05% Tween-20(v/v)] and incubated with the membrane for 1 hour. The primary antibodies used were anti-eIF5A1 (BD Transduction Laboratories), anti-ß-actin (Calbiochem), and anti-eIF-5A2 (Novus) at a dilution of 1:20,000, 1:10,000, 1:1000, respectively. The membranes were washed three times in PBS-T and incubated for 1 hour with the appropriate secondary

antibodies diluted in 1% milk in PBS- 1/2 T. The secondary antibodies used for eIF-5A1, ß-actin, and eIF-5A2 were diluted 1:5000, 1:5000, and 1:3000, respectively. The membrane was washed and analyzed for bound antibodies.

2.4.4 Antibody Detection

An ECL plus western blot analysis detection kit was used according to Manufacturer's protocol (Amersham Biosciences) to discern bound antibody. Detection solution A and B were mixed in a ratio of 40:1, and the membrane was incubated with detection solution for 5 minutes. After 5 minutes, detection solution was drained off, membrane was placed in a cassette wrapped with SeranWrap, and a sheet of autoradiography film (Fuji RX film) was put on the top of the membrane. The film was exposed for 15 seconds. The film was developed and fixed immediately as described in section 2.5.2.4. Depending on the intensity of the band, the second film was exposed either for a longer or shorter period of time.

2.4.5 Densitometry Analysis

Densitometry analysis was performed using a Fluorchem 8000 Chemiluminescence and Visible Imaging System equipped with AlphaEaseFC software (Alpha Innotech, San Leandro, CA). Densitometry analyses were mostly performed in triplicate and in some cases in duplicate. The data obtained were analyzed statistically using GraphPad software.

2.5 RNA Isolation and Analysis

2.5.1 RNA Extraction from Mammalian Cells

Total RNA was extracted from mammalian cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). Following the induction of apoptosis or cell proliferation, confluent cells were lysed using lysis solution/2-Mercaptoethanol mixture. Lysed cells were pipetted into the genelute filtration column and centrifuged at maximum speed (13,000 x g) in a microfuge for 2 minutes to remove cellular debris. An equal volume of 70% ethanol (v/v) was added to the filtered lysate and pipetted thoroughly to mix. Then the ethanol/lysate mixture was transferred into the gene-elute binding column and centrifuged at maximum speed in a microfuge for 15 seconds. The flow-through liquid was discarded. Five hundred µl of wash soultion1 was pipetted into the column, and the column was again centrifuged at maximum speed in a microfuge for 15 seconds. The flow-through liquid was discarded again. Then, 500 µl of ethanol containing wash solution 2 was pipetted into the column, and the column was again centrifuged at maximum speed in a microfuge for 15 seconds. The flow-through liquid was discarded. Again, 500 µl of wash solution 2 was pipetted into the column and the column centrifuged at maximum speed in a microfuge for 2 minutes to dry the binding column. The flow-through liquid was discarded again. Finally, 50 µl of elution solution was pipetted into binding column and the column was centrifuged at maximum speed for 1 minute to collect RNA.

2.5.2 Northern Blotting

2.5.2.1 RNA gel electrophoresis & transfer

The isolated RNA was fractionated on a gel of 1.2% agarose containing formaldehyde . To this end, 5 µg of RNA from each sample was mixed with 20 ul of denaturing solution (56% formamide [v/v], 18% formaldehyde [v/v] , 10x MOPS [0.2 M 3-(N-morpholino)propanesulfonic acid {MOPS}, 50 mM sodium acetate, 10 mM EDTA], 10 % DEPC-treated water [v/v], 1 % bromophenol blue [w/v]). In addition, 0.05 µg/µl ethidium bromide was included in the samples to enable visualization of RNA. Electrophoresis was performed at 75 volts for 4 hours in 1x MOPS buffer. The fractionated RNA was then transferred onto a Hybond-N^+ membrane (Amersham biosciences). To achieve this, the gel was soaked in 0.05 N NaOH for 20 min at room temperature to partially hydrolyze the RNA. The gel was then rinsed with DEPC-treated water and soaked for 45 minutes in 20 X SSC buffer [3 M sodium chloride, 300 mM sodium citrate (pH 7.0)]. A piece of Whatman paper was placed on a glass plate to form a support longer and wider than the gel. The gel was then put on the top of support in an inverted position and surrounded with parafilm to prevent short-circuiting. The membrane was soaked in deionized water and then 20 x SSC, each for 5 minutes, and placed on the top of the gel. Two pieces of Whatman paper soaked in 2 x SSC were placed on top of the wet membrane. On the top of the Whatman papers, a stack of paper towels (5-8 cm high) was placed. Finally, a glass plate was put on the top of the stack and weighed down with a 500-g weight. The transfer was allowed to proceed overnight. Following transfer, the paper towel and blotting paper layers were discarded, and the correct side of the membrane was marked with pencil. After transfer, the

RNA was cross-linked to the damp membrane using a Gene Linker UV Chamber set at C3 (cross-linking setting) (Biorad, Mississaugua, ON).

2.5.2.2 Preparation of radiolabelled cDNA probe

The transferred membrane was probed with ^{32}P-labelled human eIF-5A1 (3' UTR) and eIF-5A2 (3' UTR) cDNAs. The ^{32}P was incorporated into the DNA using a Hexalabel DNA labelling Kit according to the Manufacturer's instructions (Fermentas Lifesciences, Hanover, MD). To this end, 100 μg of cDNA template, hexanucleotide (in 5X reaction buffer) and nuclease- free water were mixed in a tube, incubated in a boiling water bath for 5-10 minutes and cooled on ice. Subsequently, dCTP, dTTP, dGTP mixture, [α^{32}P] dATP, and Klenow enzyme were added to the reaction mixture, and the solution was incubated for 10 minutes at 37^0 C. Then 2 μL of dNTP mix was added to the reaction mixture, and it was incubated for 5 minutes at 37^0 C. Finally, the reaction was halted by adding 0.5 M EDTA (pH 8.0).

2.5.2.3 Hybridization

Before the addition of ^{32}P-labelled cDNA, the membrane was prehybridized for 2 hours at 42^0 C in a hybridization oven (model 2720, VWR Scientific, West Chester, PA) with a pre-hybridization solution [40% formamide (v/v), 20x SSC (w/v), 50x Denhart's solution (w/v), 10% SDS (w/v), 7.5 mg/ml denatured salmon sperm DNA, DEPC water]. Subsequently, the [α^{32}P] labeled cDNA probe was added to the membrane. Hybridization was allowed to proceed overnight at 42°C. After hybridization, the membrane was washed with 2x SSC + 0.1% SDS at 42°C for 2 x 15 minutes and with 1 x SSC + 0.1% SDS at 42°C

for 2 x 15 minutes. The membrane was wrapped in seran wrap, placed in a cassette and exposed to film (Fuji RX film) at –80°C for 1 to 7 days depending on the intensity of the bands.

2.5.2.4 Membrane development

The membrane and the film were allowed to thaw for a few hours at the room temperature before the film was developed. The development of the film was carried out in a dark room. The film was developed using commercial developer solution (Eastman Kodak Company, Rochester, NY). Following development, the film was washed with deionized water and fixed using commercial fixer solution (Eastman Kodak Company). Finally, the film was washed again with deionized water and bands were examined.

2.5.3 Semi-quantitative Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from human cell lines as described in section 2.5.1. cDNA synthesis was performed using the Omniscript Reverse Transcriptase System for RT-PCR (Qiagen). Briefly, 10x buffer RT, dNTP mix (5 mM of each), 10 μm Oligo-dt primer, 10 units/μl Rnase inhibitor, 1 μl omniscript reverse transcriptase were added to a tube for one reaction. If more than one RT reaction was desired, a master mix was made with the above reagents. Later, 8 μl of master mix was distributed to each tube and up to 2 μg RNA for each sample and Rnase-free water were added to each tube to make 20 μl of reaction mixture. The mixture was incubated at 37^{0} C for 60 minutes to make first strand cDNA. Subsequently, the cDNA was amplified by PCR reaction in an Eppendorf mastercycler PCR Instrument (Eppendorf, Westbury, NY). Briefly, each PCR cycle was composed of the following steps: denatuation at 95 °C for 1 minute (exception, denaturation for 5 minutes at 95 °C for the first

cycle only) , annealing at 55 °C for 1 minute and extension at 72 °C for 2 minutes (exception, extension for 10 minutes at 72 º C for the final cycle). The PCR was performed using eIF-5A1-, eIF-5A2- and β-actin- specific primers (Sigma Genosys), which were designed by Catherine Taylor. The sequences of the primers are as follows: eIF-5A1 forward, 5' CGAGTTGGAATCGAAGCCTC 3' , eIF-5A1 reverse, 5'GGTTCAGAGGATCACTGCTG 3' ; eIF-5A2 forward , 5' AAACTACCATCTCCCCTGCC 3' ,

eIF-5A2 reverse, 5' CTTCACCAGTTTCTGTCAGC 3' ; β-actin forward 5'GATGATATCGCCGCGCTCGT3', β-actin reverse, 5' GTAGATGGGCACAGTGTGGGT 3' . The PCR products were electrophoretically separated on 1% agarose gels containing ethidium bromide and visualized using a Fluorchem 8000 Chemiluminescence and Visible Imaging System (Alpha Innotech).

2.6 Preparation of Probes for Northern Analysis

2.6.1 Human β-actin cDNA

pCMV-XL5 containing full-length human β-actin cDNA was obtained from Origene Technologies (Rockville, MD) and used as a source of β-actin probe. The vector was introduced into *Escherichia coli* DH5α cells (See Figure 3).

2.6.2 Cloning of the 3' UTR of Human eIF-5A1 and eIF-5A2

The 3' UTRs of human eIF-5A1 and eIF-5A2 were cloned by Catherine Taylor and Dave Powers (Dr. Thompson's lab). To generate the 3' UTR of eIF-5A1, total RNA was isolated from RKO cells and reverse-transcribed to cDNA. The cDNA was used as a

template for PCR using the following primers: forward, 5'GAGGAATTCGCTGTTGCAATCAAGGC 3'; reverse, 5'TTTAAGCTTTGTGTCGGGGAGAGAGC 3'. The resulting PCR fragment corresponding to the 3' UTR of eIF-5A1 was cloned into EcoR1/HindIII sites of pBluescript. Likewise, to generate 3' UTR of eIF-5A2 , the total RNA was isolated from RKO cells and reverse transcribed to cDNA. The cDNA was used as a template for PCR using the following primers: eIF-5A2 forward , 5' AAACTACCATCTCCCCTGCC 3' ; eIF-5A2 reverse, 3'TGCCCTACACAGGCTCAAAG 3'. The resulting PCR fragment was cloned into pGEM T easy vector. Following cloning, the insert was digested with EcoR1/Pst1 enzymes, which cut out the 400 bp fragment corresponding to the 3' UTR of eIF-5A2. The resulting 400 bp fragment or 3' UTR of eIF-5A2 was subcloned into EcoR1/Pst1 sites of pBluescript vector. The vectors containing 3' UTR cDNA of eIF-5A1 and eIF-5A2 were then transformed into *Escherichia coli* DH5α cells.

2.6.3 Transformation of *Escherichia coli*

An aliquot of 200 µl fresh competent *Escherichia coli* DH5α cells was transferred into a microcentrifuge tube. Then, pCMV-XL5 vector containing β-actin cDNA insert or pBluescript vectors containing the 3' UTR of eIF-5A1 or the 3'UTR of eIF-5A2 were added to the tube and the suspension incubated on ice for 30 minutes. The reaction mixture was then heated at 42 ^{0}C for 90 seconds. The cells were chilled on ice for 1-2 minutes, and 800 µl of LB broth was added to each tube. Finally, the cells were incubated at 37 ^{0}C for 45 minutes and plated onto LB plates containing ampicillin.

2.6.4 Isolation of plasmid DNA

Escherichia coli DH5α cells containing the vectors pCMV-XL5 or pBluescript were asceptically streaked on LB agar plates [1% (w/v) tryptone , 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar] (BioShop, Burlington, ON) supplemented with 50 μg/ml ampicillin (BioShop). The plates were incubated overnight at 37 ^{0}C. The following day, single colonies were asceptically transferred to 5 ml of LB broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] supplemented with 50 μg/ml ampicillin. Bacterial cultures were incubated overnight in a shaker at 37 ^{0}C. The following day, plasmids were isolated using FastPlasmid Mini kit (Eppendorf). To this end, 1.5 ml of fresh bacterial culture was centrifuged at 13,000 Xg for 1 minute. The supernatant was removed by decanting. Then, 400 μl of ice-cold complete lysis solution was added to the pellet and mixed properly by vortexing for 30 seconds. The lysate was incubated at room temperature for 3 minutes. The lysate was then transferred to a spin column assembly and centrifuged for 30-60 seconds at maximum speed in a microfuge. Subsequently, 400 μl of diluted wash buffer was added to the spin column assembly and centrifuged for 30-60 seconds at maximum speed in a microfuge. The spin column was then transferred into a collection tube. Finally, 50 μl of elution buffer was added to the centre of spin column membrane and centrifuged at 13,000 X g for 30-60 seconds in a microfuge. The eluted DNA was stored at -20 ^{0}C.

2.6.5 Restriction Enzyme Digestion

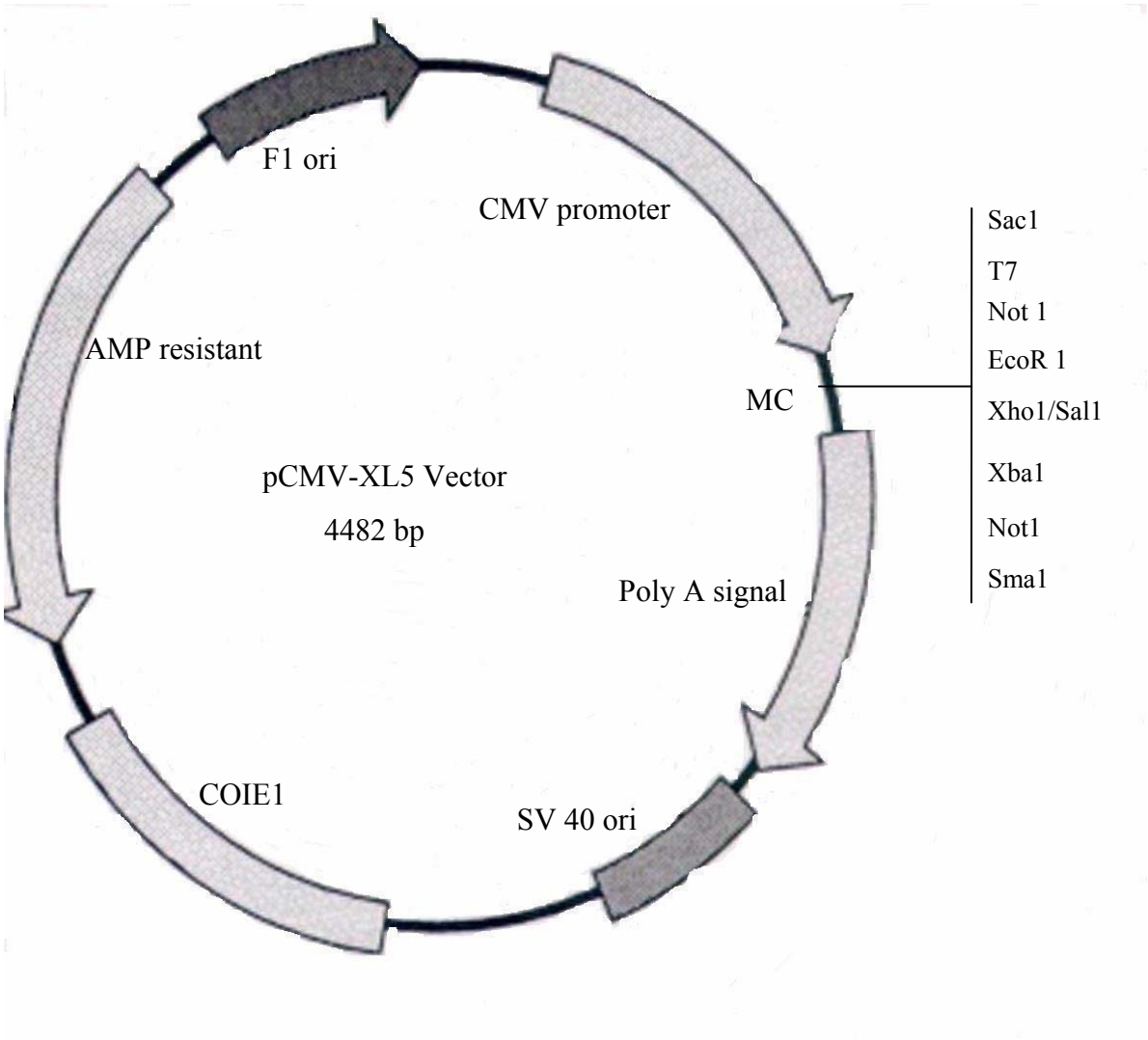
The 3' UTR of eIF-5A1 was cut from pBluescript using EcoR1 and HindIII restriction enzymes which generated a 637 bp product. The double digestion reaction was carried out using a common restriction buffer as it was compatible with both enzymes. The 3'

UTR of eIF-5A2 was cut from pBluescript using restriction enzymes EcoR1 and BamH1, which generated a 400 bp product. Again, the double digestion reaction was carried out using a common restriction buffer. The human β-actin cDNA insert was cut from pCMV6-XL5 using the flanking Not1 sites, which generated a 1200 bp product (see Figure 3).

2.6.6 Purification of cDNA Probes

Restriction enzyme digested cDNA probes were resolved by electrophoresis on a 1% agarose gel. The separated bands were viewed and compared with molecular weight marker X (Roche Diagnostics, laval, QC) to ensure that probes of the expected size were obtained. Then, bands were purified using a Perfectprep Gel Cleanup kit (Eppendorf). Briefly, the cDNA band was excised from the gel and weighed. Subsequently, 3 volumes of binding buffer was added for every 1 volume of gel slice, and the mixture was incubated at 50 ^{0}C for 5 to 10 minutes. Then, a volume of isopropanol equivalent to the volume of the gel slice was added and mixed by inversion or repeated pipetting. The sample was added to the spin column in a collection tube. The sample was spun at 10,000 x g for 1 minute, and the filtrate was discarded. Bound cDNA was washed by adding 750 µl of diluted wash buffer and centrifuging for 1 minute at 10,000 x g. Subsequently, the filtrate was removed and centrifuged for an additional 1 minute at 10,000 x g. Finally, the column was placed in a new 2 ml centrifuge tube, and 30 µl elution buffer was added and centrifuged for 1 minute at 10,000 x g. The purified cDNA was used as a probe for Northern analysis.

Figure 3. The Physical map of pCMV6-XL5. The human β-actin cDNA was directionally cloned between the EcoR1 and Sal1 sites. The Sal1 site was destroyed in the cloning process. The insert was cut from the plasmid using the flanking Not1 sites.


F1 ori
CMV promoter
AMP resistant
MC
pCMV-XL5 Vector
4482 bp
Poly A signal
COIE1
SV 40 ori
Sac1
T7
Not 1
EcoR 1
Xho1/Sal1
Xba1
Not1
Sma1

Results

3.1 Quantitation of Apoptotic Cells using TUNEL Assay

Apoptosis was induced in CCD-112CoN cells by treatment with the NO donor, sodium nitroprusside (SNP), which induces apoptosis by damaging DNA and by inhibiting DNA synthesis (Ozen et al., 2001). The cells were treated with 3mM SNP for 0, 2, 4, 8 and 24 hours, and apoptosis was measured using the TUNEL assay. At 24 hours, 35.7 % cells were undergoing apoptosis, whereas only 0.7 %, 1.2%, 3.2% and 4.5% of the cells were undergoing apoptosis at 0, 2, 4 and 8 hours, respectively (Figure 4).

RKO cells were also treated with 3mM SNP for 0, 2, 4, 8 and 24 hours in order to induce apoptosis, and apoptosis was again quantified by TUNEL. At 24 hours, 39.6 % cells were undergoing apoptosis, whereas no cells exhibited TUNEL staining reflecting apoptosis at 0, 2, 4 and 8 hours (Figure 5).

RKO cells were also treated with Actinomycin D (ActD), which induces apoptosis by inhibiting DNA-dependent RNA synthesis (Leclerc et al., 2002). Apoptosis was induced by treating cells with 0.5 μg/ml Actinomycin D for 2, 4, 8, and 24 hours. Treatment with DMSO, which was used as a vehicle control, was for 0 hours and 24 hours. At 24 hour, 23% cells were undergoing apoptosis, whereas 0.3%, 1.2% and 6.1% of the cells were undergoing apoptosis at 2, 4 and 8 hours, respectively (Figure 6).

3.2 Changes in eIF-5A1 and eIF-5A2 protein and mRNA levels in RKO cells during SNP induced apoptosis

In light of the proposed involvement of eIF-5A1 in apoptosis, levels of eIF-5A1 protein were examined in RKO cells at 0, 2, 4, 8 and 24 hours after SNP treatment. Moderate levels of eIF-5A1 protein were detectable after 0 hours and after 2 hours of SNP treatment, with a slight up-regulation after 4 and 8 hours (Figure 7). However, a decreased level of eIF-5A1 was observed after 24 hours of SNP treatment (Figure 7). Statistical analysis of ratios of eIF-5A1 protein to β-actin revealed that the up-regulation of eIF-5A1 after 4 and 8 hours of SNP treatment was not significantly different from the level of eIF-5A1 protein in the untreated control sample (0 hr SNP) (Figure 7).

To examine the effects of SNP-induced apoptosis on eIF-5A1 mRNA levels, RKO cells were treated with SNP for 0, 2, 4, 8 and 24 hours. Figure 8 shows that eIF-5A1 mRNA is constitutively expressed in RKO upto 8 hours of SNP treatment and that treatment with SNP has no effect on eIF-5A1 transcript abundance up to 8 hours. The transcript gets degraded within 24 hours of SNP treatment (Figure 8) . The loading control β-actin also shows constitutive mRNA expression until after 8 hours of SNP treatment, and thereafter that transcript gets degraded (Figure 8). The disappearance of both eIF-5A1 and β-actin transcripts within 24 hours is probably due to DNA damage and inhibition of DNA synthesis by SNP, which is a NO donor (Ozen et al., 2001).

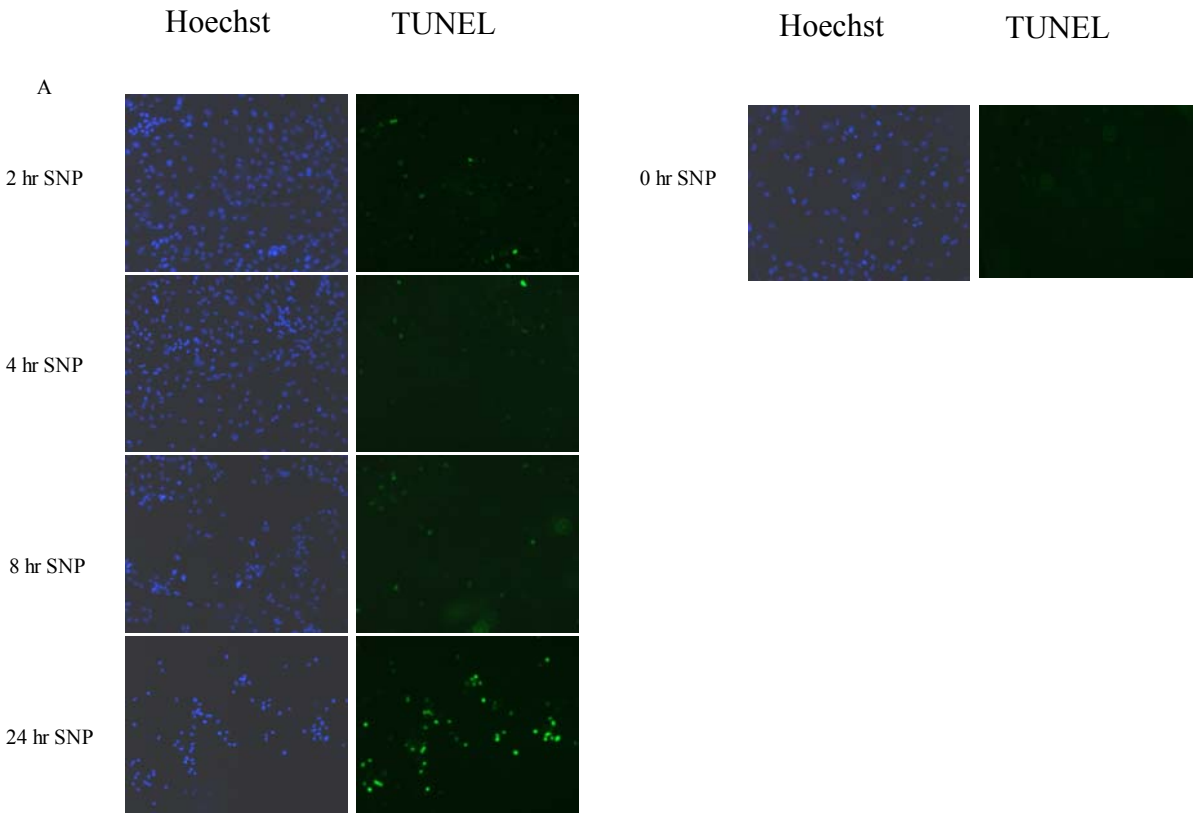
Although the up-regulation of eIF-5A1 protein during treatment with SNP is not statistically significant, the up-regulation in eIF-5A1 protein levels in the absence of a

corresponding up-regulation in eIF-5A1 mRNA suggests that eIF-5A1 may be post-transcriptionally regulated.

In order to examine eIF-5A2 protein expression during apoptosis, RKO cells were again treated with SNP for 0, 2, 4, 8 and 24 hours. However, eIF-5A2 protein was below detection levels in both control untreated cells (0 hours) and in the treated cells.

Changes in corresponding transcript levels in RKO cells during the same treatment were also measured. Initially, northern analysis was carried out using the 3' UTR of eIF-5A2 cDNA as a probe. However, the mRNA was below detection level due to its low abundance in RKO cells. As an alternative, RT-PCR was performed using eIF-5A2-specific primers in order to observe eIF-5A2 expression. Figure 9 shows that the eIF-5A2 mRNA is constitutively expressed in RKO cells up to 8 hours after SNP treatment. The housekeeping gene β-actin is also constitutively expressed during this time period (Figure 9).The disappearance of both eIF-5A2 and β-actin transcripts by 24 hours after SNP treatment is probably due to DNA damage and inhibition of DNA synthesis by NO donor SNP (Ozen et al., 2001). The undetectable eIF-5A2 protein in presence of eIF-5A2 mRNA is attributable to inefficient translation of eIF-5A2 mRNAs (Clement et al., 2003).

Figure 4. TUNEL-labeling of CCD-112CoN cells treated with Sodium nitroprusside (SNP). Panel A) For each sample, the same field was observed by fluorescence microscopy using two different filters. A UV filter was used to visualize Hoechst stained nuclei (blue), and a fluorescein filter was used to visualize TUNEL stained apoptotic cells (green). For each sample, the percentage of apoptotic cells was calculated by dividing the number of TUNEL stained apoptotic cells by the total number of Hoechst stained cells. Panel B) Graphical illustration of the percentage of apoptotic cells. The bars are means +/- standard deviation from five randomly chosen fields. *** $P < 0.001$, significantly different from control sample. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.


A
Hoechst
TUNEL
Hoechst
TUNEL
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
0 hr SNP

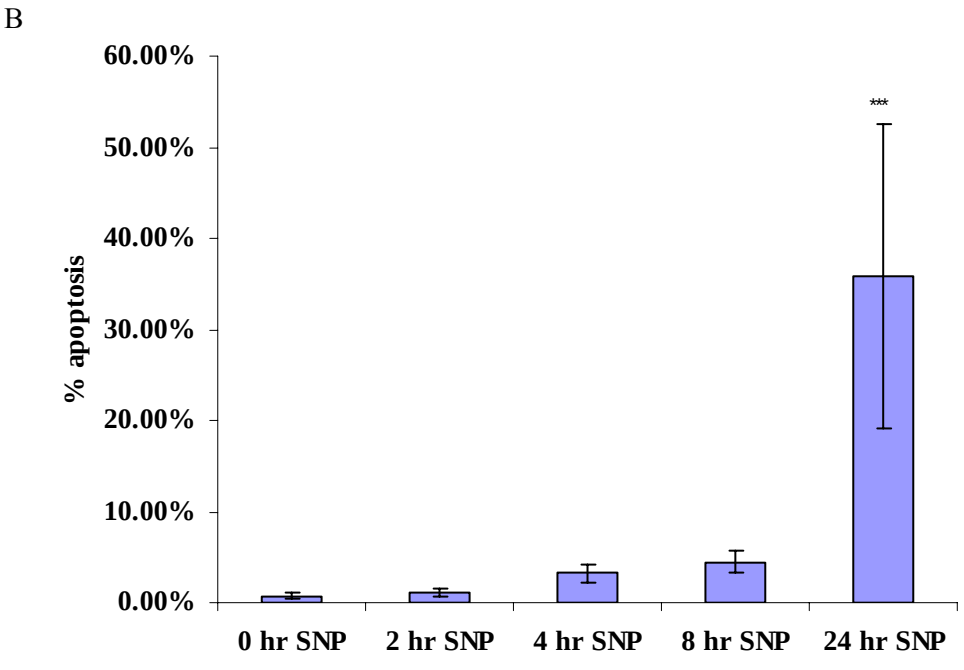
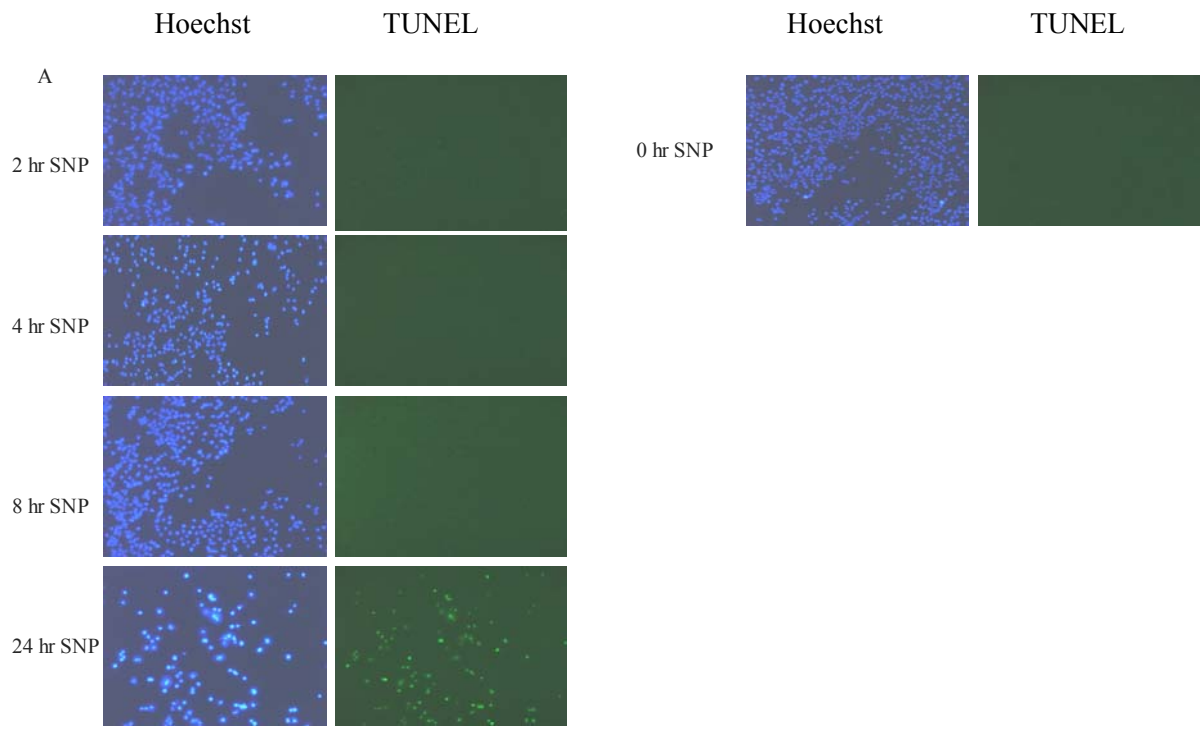

B
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% apoptosis
***
0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP

Figure 5. TUNEL-labeling of RKO cells treated with sodium nitroprusside (SNP). Panel A) For each sample, the same field was observed by fluorescence microscopy using two different filters. A UV filter was used to visualize Hoechst stained nuclei (blue), and a fluorescein filter was used to visualize TUNEL stained apoptotic cells (green). For each sample, the percentage of apoptotic cells was calculated by dividing the number of TUNEL stained apoptotic cells by the total number of Hoechst stained cells. Panel B) Graphical illustration of the percentage of apoptotic cells. The bars are means +/- standard deviation from five randomly chosen fields. *** $P < 0.001$, significantly different from control sample. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.


Hoechst
TUNEL
Hoechst
TUNEL
A
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
0 hr SNP

B

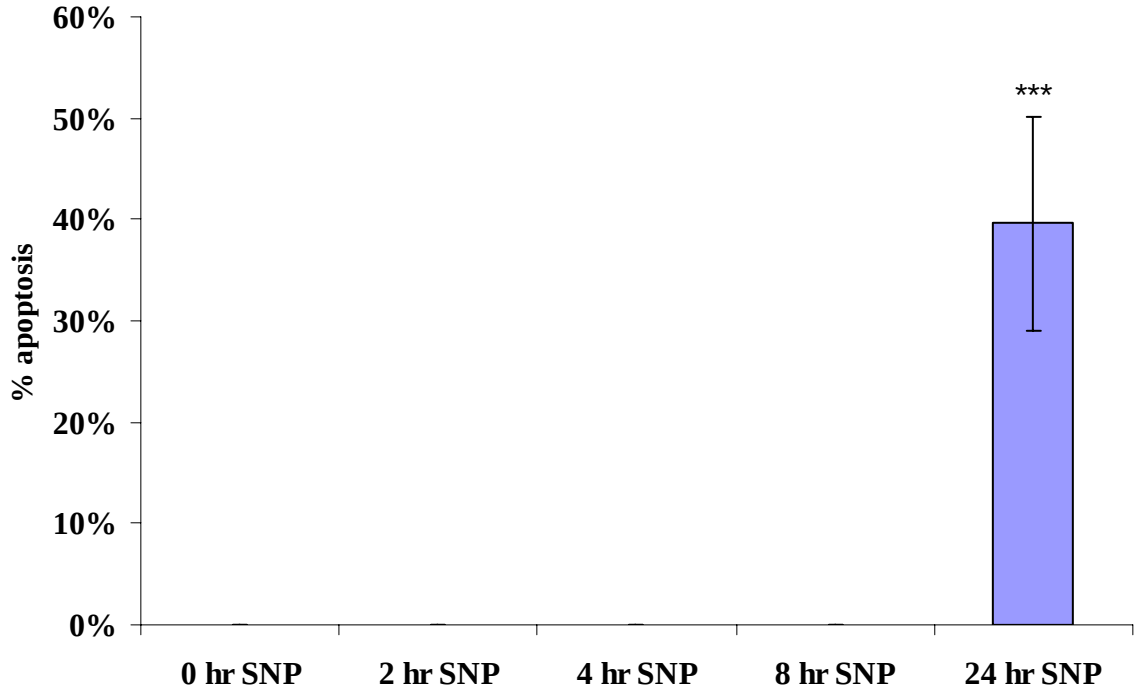
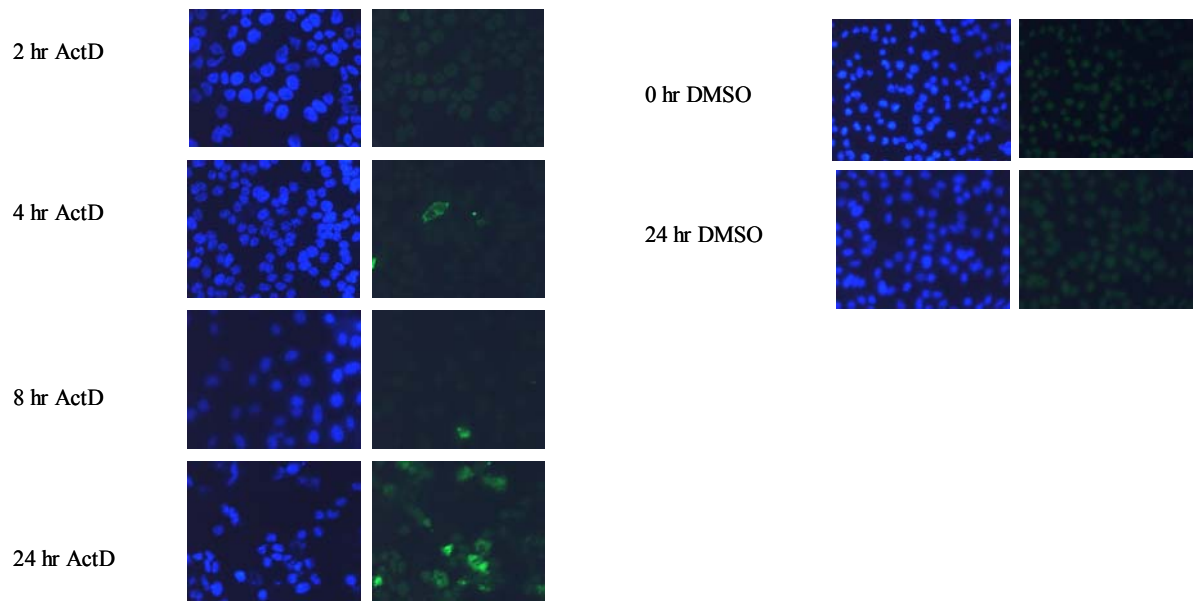

60%
50%
40%
30%
20%
10%
0%
% apoptosis
***
0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP

Figure 6. TUNEL-labeling of RKO cells treated with Actinomycin D (ActD). Panel A) For each sample, the same field was observed by fluorescence microscopy using two different filters. A UV filter was used to visualize Hoechst stained nuclei (blue), and a fluorescein filter was used to visualize TUNEL stained apoptotic cells (green). For each sample, the percentage of apoptotic cells was calculated by dividing the number of TUNEL stained apoptotic cells by the total number of Hoechst stained cells. Panel B) Graphical illustration of the percentage of apoptotic cells. The bars are means +/- standard deviation from five randomly chosen fields. *** $P < 0.001$, significantly different from control sample. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A


Hoechst
TUNEL
Hoechst
TUNEL
2 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
0 hr DMSO
24 hr DMSO

B

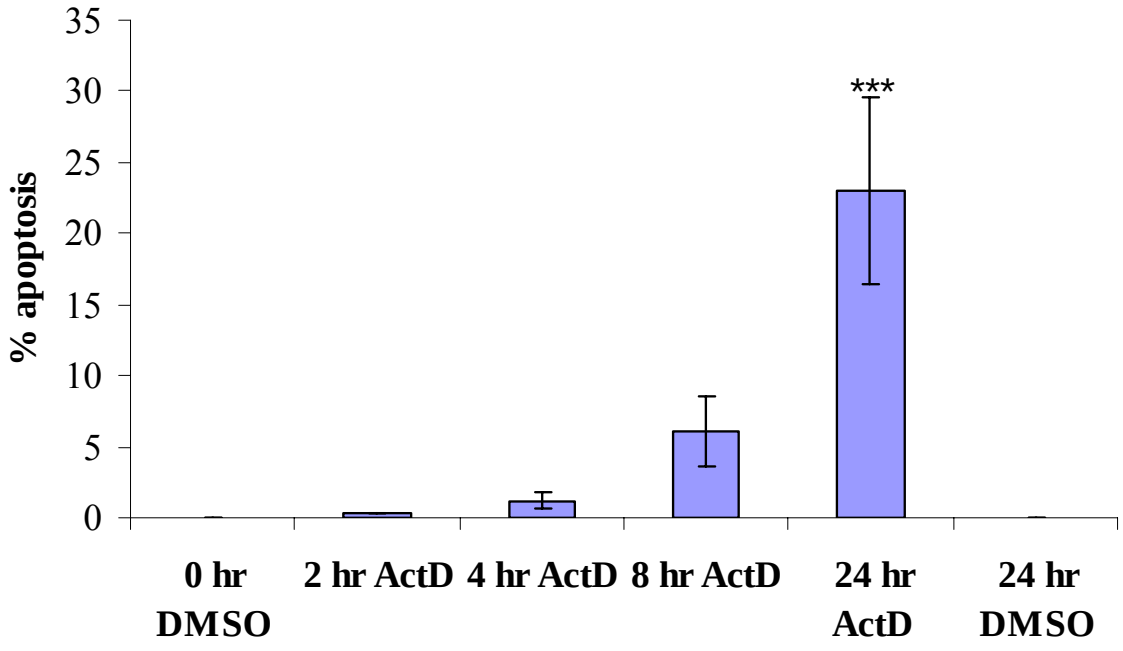
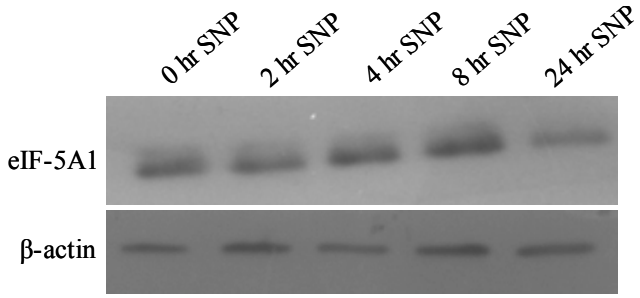

% apoptosis
35
30
25
20
15
10
5
0
***
0 hr DMSO
2 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
24 hr DMSO

Figure 7. The effect of SNP treatment on eIF-5A1 protein expression in RKO cells. RKO cells were treated with 3 mM SNP for 0, 2, 4, 8, and 24 hours . A) Western blot analysis of cell lysate from RKO cells treated with SNP. Each lane contained 5 μg of protein, and the membrane was probed with eIF-5A1 and β-actin antibodies. B) Plot of the relative intensities of eIF-5A1 bands normalized to β-actin. Graphed data are expressed as means +/- standard error from three different experiments. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A


0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
eIF-5A1
β-actin

B

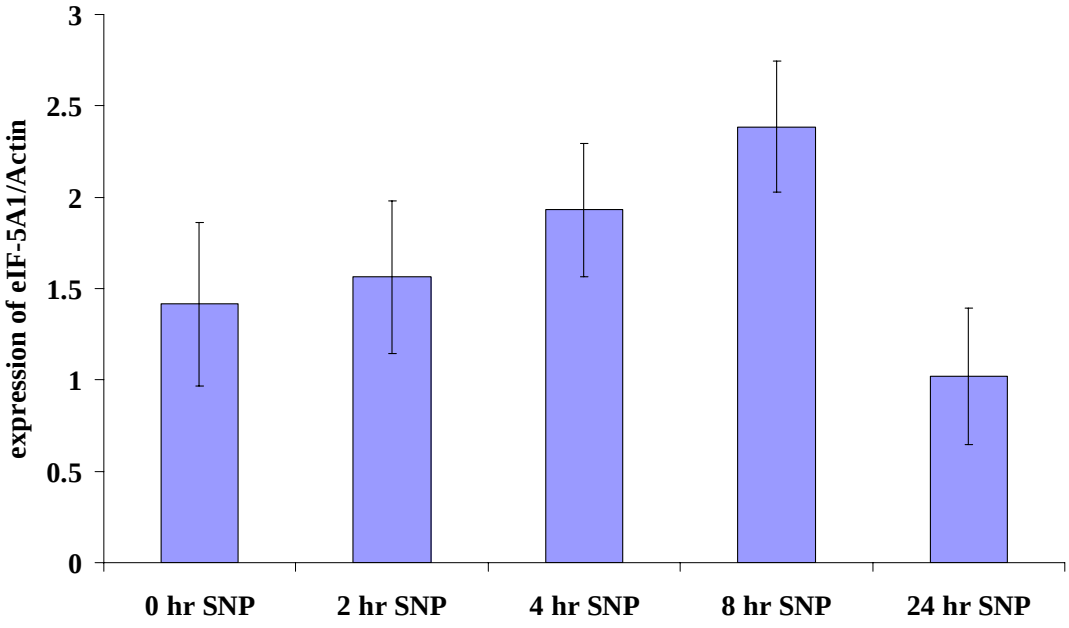

expression of eIF-5A1/Actin
3
2.5
2
1.5
1
0.5
0
0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP

Figure 8. The effect of SNP treatment on eIF-5A1 mRNA expression in RKO cells. RKO cells were treated with 3 mM SNP for 0, 2, 4, 8, and 24 hours. Northern blot analysis was performed using the 3' UTR of human eIF-5A1 cDNA as a probe, and human β-actin was used as a loading control. The corresponding agarose gel of fractionated RNA is also shown.

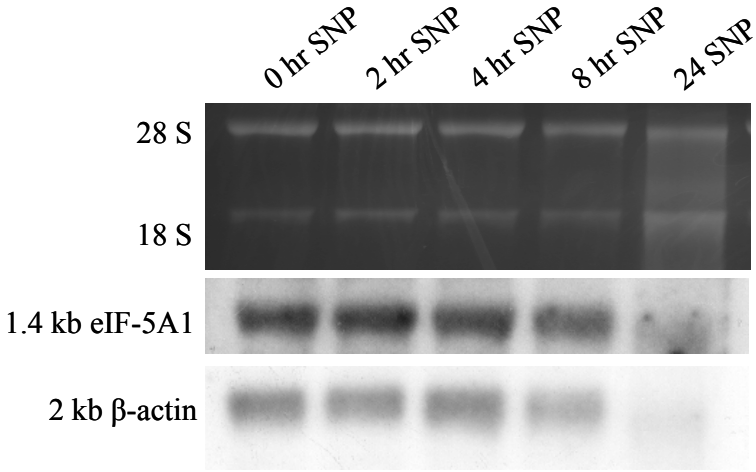
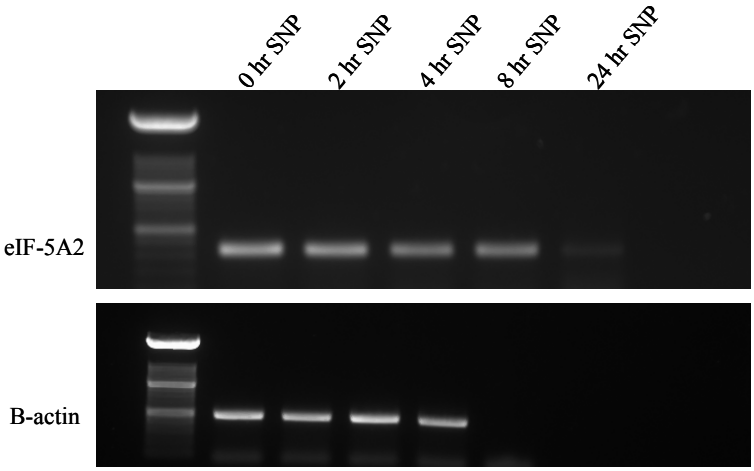

0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 SNP
28 S
18 S
1.4 kb eIF-5A1
2 kb β-actin

Figure 9. The effect of SNP treatment on eIF-5A2 mRNA expression in RKO cells. RKO cells were treated with 3 mM SNP for 0, 2, 4, 8, and 24 hours.. Transcript levels were determined by semi-quantitative RT-PCR. The size of PCR products corresponding to eIF-5A2 and β-actin transcripts were 345 bp and 500 bp, respectively.


0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
eIF-5A2
B-actin

3.3 Changes in eIF-5A1 and eIF-5A2 protein and mRNA levels in CCD-112CoN cells during SNP induced apoptosis

The role of eIF-5A1 protein in apoptosis was also examined in normal CCD-112CoN cells. Apoptosis was induced by treating the cells with sodium nitroprusside (SNP) for 0, 2, 4, 8 and 24 hours. A basal level of eIF-5A1 was detectable in control cells (0 hr SNP), and substantive up-regulation was evident after 2, 4, and 8 hours of SNP treatment. When the intensities of the eIF-5A1 bands in the Western blot were normalized to actin, it was clear that the increased expressions of eIF-5A1 at 2, 4, and 8 hours after SNP treatment were significantly different from control treatment (Figure 10). By 24 hours after treatment, eIF-5A1 protein was no longer detectable (Figure 10). This is consistent with the fact that SNP induces cell death and accompanying proteolysis. That eIF-5A1 had degraded within 24 hours of treatment of SNP, whereas actin had not, suggests that there is selective degradation of SNP once apoptosis is initiated (Figure 10).

Of particular interest is the finding that the increase in eIF-5A1 protein in normal CCD-112CoN cells following treatment with SNP was not accompanied by a corresponding increase in eIF-5A1 transcript. The Northern blot in Figure 11 shows that eIF-5A1 mRNA is constitutively expressed and does not increase in abundance following treatment with SNP for at least 8 hours. However, by 24 hours after the initiation of SNP treatment, levels of eIF-5A1 transcript decreased to undetectable levels in parallel with a corresponding decrease in the transcript for β-actin (Figure 11). The disappearance of both eIF-5A1 and β-actin transcripts after 24 hours of SNP treatment is probably due to DNA damage and inhibition of DNA synthesis by SNP. The up-regulation of eIF-5A1 protein in the absence of a

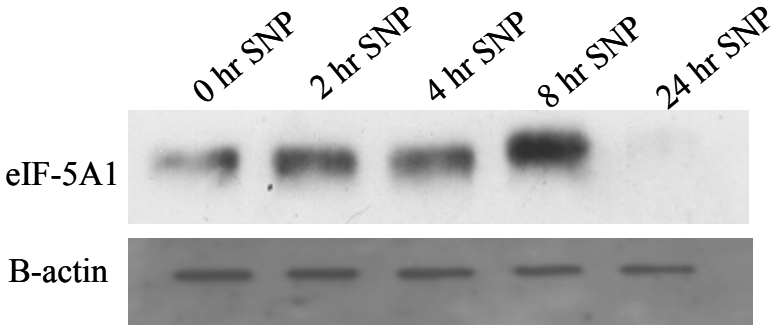
corresponding up-regulation in eIF-5A1 mRNA indicates that eIF-5A1 is post-transcriptionally regulated .

Unlike eIF-5A1 protein, eIF-5A2 protein was not detectable in untreated CCD-112CoN cells. Nor was it detectable following treatment with SNP for 0, 2, 4, 8 and 24 hours. Again, this presumably reflects the fact that eIF-5A2 mRNA is known to be inefficiently translated (Clement et al., 2003).

eIF-5A2 transcript levels in normal CCD-112CoN cells were examined by semi-quantitative RT-PCR during treatment with SNP for 0, 2, 4, 8 and 24 hours. Figure 12 illustrates that eIF-5A2 mRNA is constitutively expressed in CCD-112CoN in parallel with β-actin transcript, and does not increase in abundance for at least 8 hours following treatment with SNP. Thereafter, coincident with the progression of apoptosis, both eIF-5A2 and β-actin transcripts are undetectable by 24 hours after the initiation of SNP treatment . This presumably reflects DNA damage and inhibition of DNA synthesis, which are characteristic traits of apoptosis.

Figure 10. The effect of SNP treatment on eIF-5A1 protein expression in normal CCD-112CoN cells. CCD-112CoN cells were treated with 3 mM SNP for 0, 2, 4, 8, and 24 hours . A) Western blot analysis of cell lysate from CCD-112CoN cells treated with SNP. Each lane contained 5 μg protein. The membrane was probed with eIF-5A1-specific antibody and actin-specific antibody. B) Plot of the relative intensities of eIF-5A1 bands normalized to β-actin. Graphed data are expressed as means +/- standard error from three different experiments. ** $P < 0.01$, *** $P < 0.001$, significantly different from control treatment. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A


0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
eIF-5A1
B-actin

B

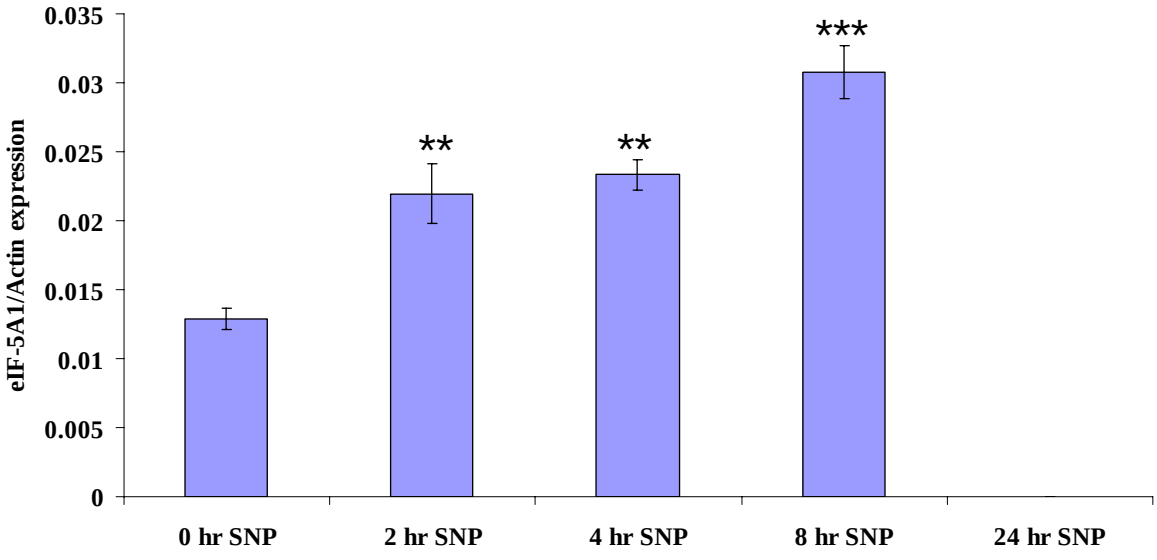

eIF-5A1/Actin expression
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
**
**
***
0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP

Figure 11. **The effect of SNP treatment on eIF-5A1 mRNA expression in CCD-112CoN cells.** CCD-112CoN cells were treated with 3 mM SNP for 0, 2, 4, 8, and 24 hours. Transcript levels were determined by semi-quantitative RT-PCR. The size of PCR products corresponding to eIF-5A1 and β-actin transcripts were 500 bp.

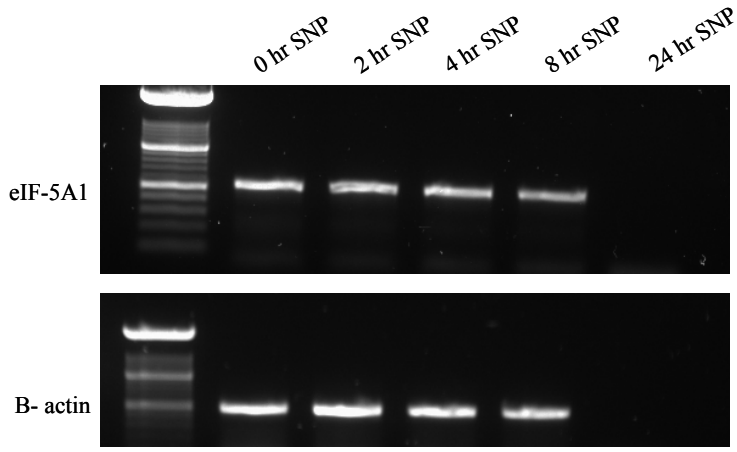
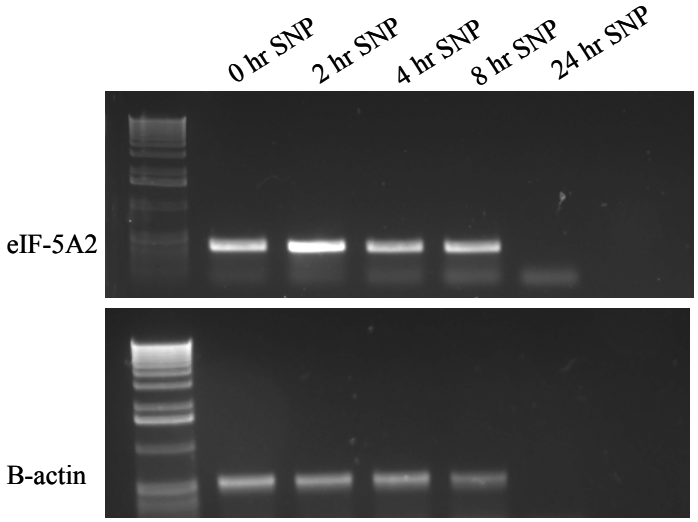

0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
eIF-5A1
B- actin

Figure 12. The effect of SNP treatment on eIF-5A2 mRNA expression in CCD-112CoN cells. CCD-112CoN cells were treated with 3 mM SNP for 0, 2, 4, 8, and 24 hours. Transcript levels were determined by semi-quantitative RT-PCR. The size of PCR products corresponding to eIF-5A2 and β-actin transcripts were 345 bp and 500 bp, respectively.


0 hr SNP
2 hr SNP
4 hr SNP
8 hr SNP
24 hr SNP
eIF-5A2
B-actin

3.4 Changes in eIF-5A1 and eIF-5A2 protein and mRNA levels following treatment of RKO cells with Actinomycin D

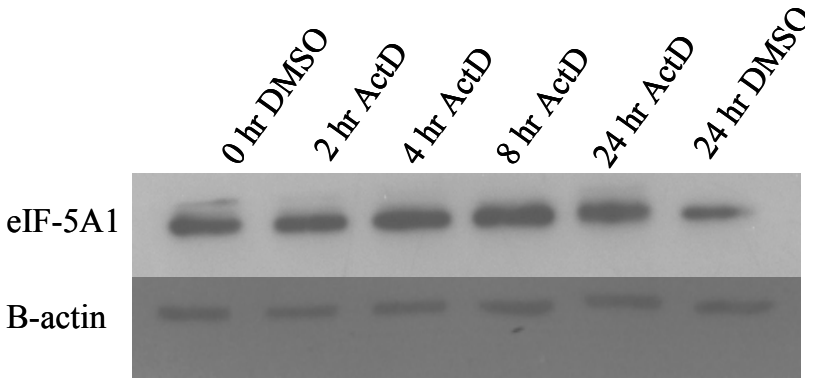
The effects of Actinomycin D treatment, which also induces apoptosis, on eIF-5A1 protein expression was examined in RKO cells. The cells were treated with Actinomycin D dissolved in DMSO for 2, 4, 8 and 24 hours, and treatment with DMSO (0 and 24 hours) was used as a vehicle control. A modest level of eIF-5A1 protein was detectable after 0 and 2 hours of Actinomycin D treatment, and slight up-regulation was observed after 4, 8 and 24 hours of treatment (Figure 13). However, when the level of eIF-5A1 expression was expressed as a ratio of corresponding levels of β-actin, the up-regulation relative to the DMSO control at 0 hours and 24 hours was not statistically significant (Figure 13).

Changes in eIF-5A1 transcript levels following treatment of RKO cells with Actinomycin D were also determined. The Northern blot in Figure 14 illustrates that eIF-5A1 mRNA is constitutively expressed until 8 hours after Actinomycin D treatment. Thereafter, the transcript apparently gets degraded and is not detectable by 24 hours after the initiation of Actinomycin D treatment (Figure 14). This reflects a general decline in transcription rather than selective inhibition of eIF-5A1 transcription is apparent from the finding that the β-actin transcript also decreases to undetectable levels by 24 hours after initiation of the Actinomycin D treatment (Figure 14). The disappearance of both eIF-5A1 and β-actin transcripts at 24 hour is probably due to the inhibition of transcription by Actinomycin D (Leclerc et al., 2002). The up-regulation in eIF-5A1 protein levels in the absence of a corresponding up-regulation in eIF-5A1 mRNA suggests that eIF-5A1 may be post-transcriptionally regulated.

In a similar vein, the effets of Actinomycin D – induced apoptosis in RKO cells on eIF-5A2 expression were also examined. In this case, the cells were again treated with Actinomycin D for 2, 4, 8 and 24 hours and with DMSO for 0 and 24 hours as a control. The eIF-5A2 protein level was below detection at all time points, again reflecting the very low levels of this protein in RKO cells. Furthermore, eIF-5A2 transcript was not detectable by Northern blotting. However, eIF-5A2 transcript was detectable by semi-quantitative RT-PCR using eIF-5A2 specific primers. The semi-quantitative RT-PCR data indicated that eIF-5A2 mRNA is detectable in untreated RKO cells (0 hr and 24 hours DMSO) and that its abundance remains essentially unchanged for at least 8 hours after the initiation of Actinomycin D treatment (Figure 15). The housekeeping gene β-actin is also constitutively expressed during this initial period of Actinomycin D treatment (Figure 15). However, both transcripts are undetectable after 24 hours of Actinomcyin D treatment (Figure 15), which presumably reflects the known inhibitory effect of Actinomycin D on transcription (Leclerc et al., 2002) .

Figure 13. The effect of Actinomycin D treatment on eIF-5A1 protein expression in RKO cells. RKO cells were treated with 0.5 μg/ml Actinomycin D for 2, 4, 8, and 24 hours. DMSO (0 and 24 hours) was used as a vehicle control . A) Western blot analysis of cell lysate from RKO cells treated with ActD. Each lane contained 5 μg protein. The membrane was probed with eIF-5A1-specific antibody and actin-specific antibody B) Plot of the relative intensities of eIF-5A1 bands normalized to β-actin. Graphed data are expressed as means +/- standard deviation from two different experiments. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A


0 hr DMSO
2 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
24 hr DMSO
eIF-5A1
B-actin

B

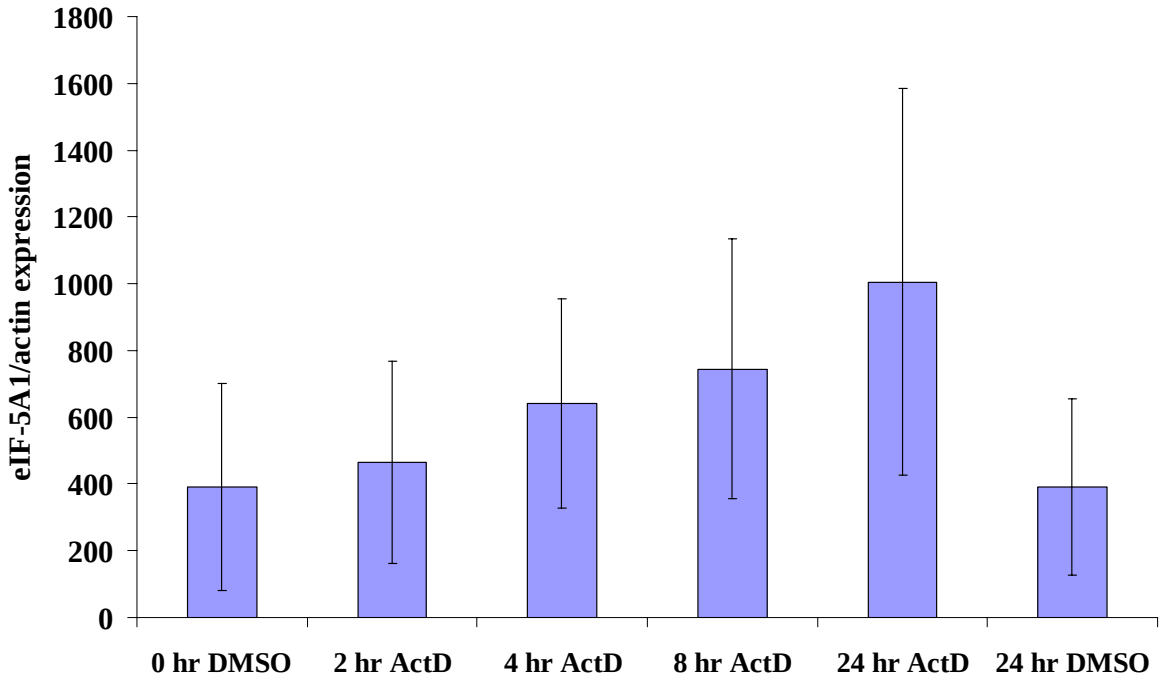

eIF-5A1/actin expression
1800
1600
1400
1200
1000
800
600
400
200
0
0 hr DMSO
2 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
24 hr DMSO

Figure 14. The effect of ActD treatment on eIF-5A1 mRNA expression in RKO cells.

RKO cells were treated with 0.5 μg/ml ActD for 2, 4, 8, and 24 hour. DMSO was used as a vehicle control for 0 and 24 hours. Northern blot analysis was performed using 3' UTR of human eIF-5A1 cDNA as a probe , and human β-actin was used as a loading control. The corresponding agarose gel of fractionated RNA is also shown.

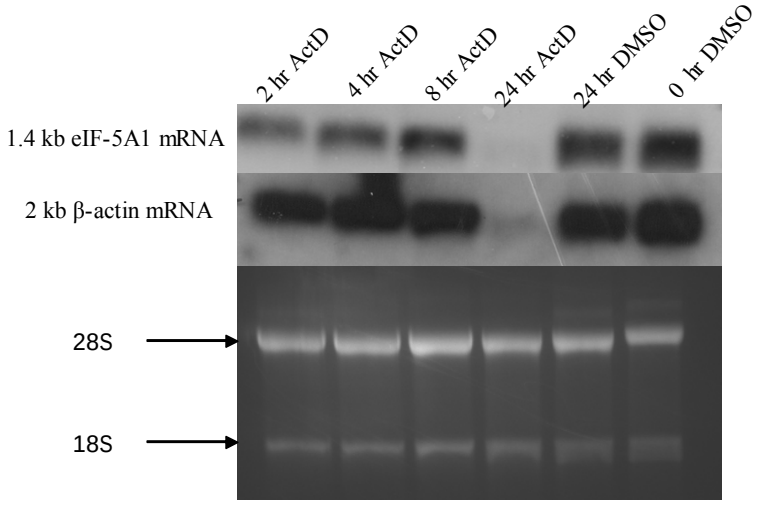
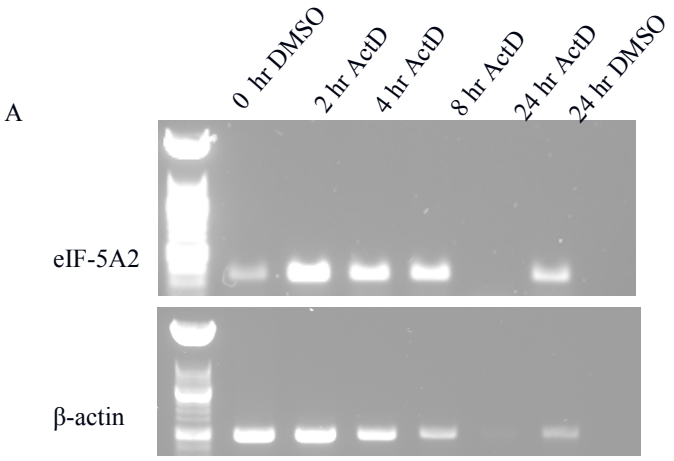

2 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
24 hr DMSO
0 hr DMSO
1.4 kb eIF-5A1 mRNA
2 kb β-actin mRNA
28S
18S

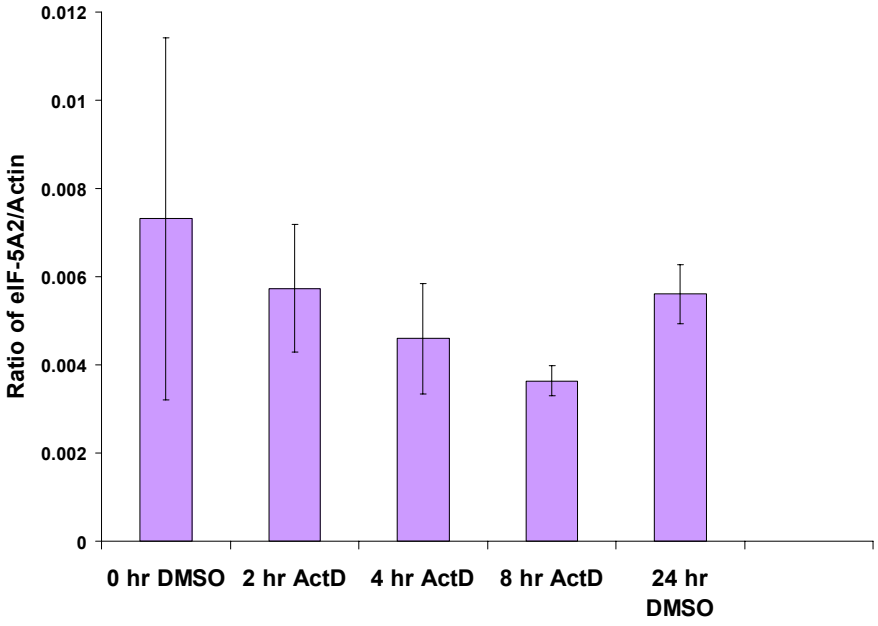
Figure 15. The effect of Actinomycin D treatment on eIF-5A2 mRNA expression in RKO cells. RKO cells were treated with 0.5 μg/ml of ActD for 2, 4, 8, and 24 hours. DMSO (0 and 24 hours) was used as a vehicle control. A) Transcript levels were determined by semi-quantitative RT-PCR. The size of PCR products corresponding to eIF-5A2 and β-actin transcripts were 345 bp and 500 bp, respectively. B) Densitometry analysis of eIF-5A2 mRNA expression relative to β-actin. Graphed data are expressed as means +/- standard error from three different experiments. The treated samples were not significantly different from control samples. The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A


0 hr DMSO
2 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
24 hr DMSO
eIF-5A2
β-actin

Cycle 30

B

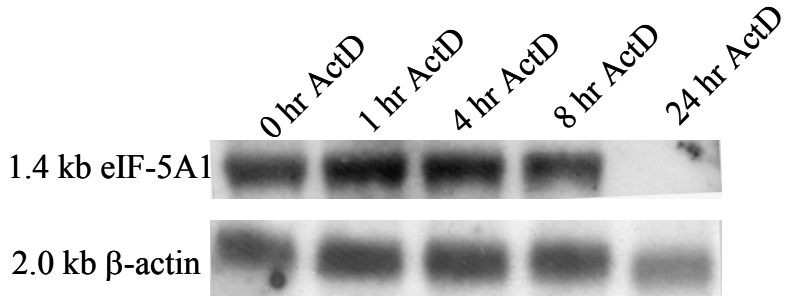

Ratio of eIF-5A2/Actin
0.012
0.01
0.008
0.006
0.004
0.002
0
0 hr DMSO
2 hr ActD
4 hr ActD
8 hr ActD
24 hr DMSO

3.5 Changes in eIF-5A1 and eIF-5A2 mRNA and protein levels in normal CCD-112CoN cells in response to Actinomycin D treatment

To examine the effects of apoptosis on eIF-5A1 mRNA levels in normal CCD-112CoN cells, apoptosis was induced by treatment with 0.5 μg/ml Actinomycin D for 0, 1, 4, 8 and 24 hours. Figure 16 shows that the eIF-5A1 mRNA is detectable in untreated cells and that its abundance does not change for at least 8 hours after the initiation of Actinomcyin D treatment. However, after 24 hours of Actinomycin D treatment, eIF-5A1 transcript was not detectable (Figure 16). Levels of β-actin transcript showed a similar pattern during the Actinomycin D treatment (Figure 16). The disappearance of both eIF-5A1 and β-actin transcripts at 24 hours is presumably due to the inhibition of transcription by Actinomycin D.

In subsequent experiments designed to determine the effects of Actinomycin D treatment on eIF-5A1 and eIF-5A2 protein levels and on eIF-5A2 mRNA level, technical problems were encountered. Specifically, CCD-112CoN cells did not undergo apoptosis in response to the application of Actinomycin D, even when the concentration was increased 0.5 μg/ml to 5 μg/ml. New CCD-112CoN cells were obtained from ATCC, but to no avail. Therefore, eIF-5A1 and eIF-5A2 protein data and eIF-5A2 mRNA data using this agent are not available.

Figure 16. The effect of Actinomycin D treatment on eIF-5A1 mRNA expression in CCD-112CoN cells. CCD-112CoN cells were treated with 0.5 μg/ml ActD for 0, 1, 4, 8, and 24 hours. Northern blot analysis was performed using 3' UTR of human eIF-5A1 cDNA as a probe , and human β-actin was used as a loading control.


0 hr ActD
1 hr ActD
4 hr ActD
8 hr ActD
24 hr ActD
1.4 kb eIF-5A1
2.0 kb β-actin

3.6 Effect of serum starvation and serum re-addition on cell proliferation and eIF-5A1 and eIF-5A2 expression utilizing UACC-1598 cells

In order to investigate the effects of serum starvation and serum-readdition on cell proliferation, an ovarian cancer cell line (UACC-1598), which contains a detectable level of eIF-5A2 was grown to about 70% confluence and then serum- starved for 3 days. Samples were analyzed using the BrdU ELISA assay to quantify cell proliferation after 0, 1, 2, and 3 days of serum- starvation. After day 3, serum was added again, and samples were analyzed using the BrdU ELISA after 1, 4, and 8 hours of serum re-addition. The BrdU ELISA revealed that cell proliferation increases at day 1 after serum starvation, and then starts decreasing gradually until day 3 of serum starvation (Figure 17). Moreover, cell proliferation continues to decrease even 8 hours after serum re-addition (Figure 17).

To confirm the results obtained by BrdU ELISA, the XTT proliferation assay was also performed. Figure 18 shows the same trend as obtained by BrdU ELISA. The highest cell proliferation was observed at day 1, and thereafter begins to decline. Even after serum re-addition, the cell proliferation continues to decrease (Figure 18).

In order to investigate whether decreased cell proliferation coincides with cell death, UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. After day 3, serum was added again, and apoptosis was measured using the TUNEL assay. The TUNEL assay indicated that approximately 6.5%, 10.2%, 10.6%, 22%, 22.5%, 23% and 25% of the cells were undergoing apoptosis at 0 day, 1 day, 2 days, and 3 days after serum starvation and at 1 hour, 4 hours and 8 hours after serum-readdition, respectively. Thus it would appear that in absence of serum, cells start undergoing apoptosis, and serum-

readdition does not reverse this process and re-initiate cell proliferation (Figure 19). The morphology of UACC-1598 cells following serum withdrawal and re-addition was also examined by microscopy using a halogen light source. Figure 19 reveals that initially the density of cell growth was very high with cells clustered together, but upon serum withdrawal the cells detach from each other and from the plate and become rounded. This morphology does not change even after serum re-addition, which is consistent with the TUNEL data indicating that the cells are undergoing apoptosis.

To examine the effect of cell proliferation on eIF-5A1 mRNA expression, UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. After day 3, serum was added back, and mRNA levels were determined 8 hours thereafter. Figure 20 illustrates that serum starvation and re-addition do not have any effect on eIF-5A1 mRNA expression as no change in transcript abundance was observed.

The effect of cell proliferation on eIF-5A1 protein expression was also investigated. UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. Protein samples were collected after 0, 1, 2, and 3 days of serum starvation. After day 3, serum was added back, and samples were collected 1, 4, and 8 hours thereafter. eIF-5A1 and eIF-5A2 proteins were both detectable after probing membrane with eIF-5A1 specific-antibody (Figure 21). The cross-reaction of eIF-5A1- specific antibody with eIF-5A2 protein is not surprising inasmuch as eIF-5A1 and eIF-5A2 are 84% identical at the amino acid level. Figure 21 shows that both isoforms respond in a similar manner to serum starvation and serum re-addition. Both are detectable until day 3 of serum starvation, but they disappear by day 3 and do not reappear even after the re-addition of serum. In addition, both seem to be

up-regulated at day 1 after serum starvation, which is temporally coincident with the onset of apoptosis. It was hypothesized that upon serum re-addition, cell proliferation would increase and the roles of eIF-5A1 and eIF-5A2 in response to cell proliferation could be clarified. Since serum re-addition did not increase cell proliferation, the role of eIF-5A1 and eIF-5A2 in cell proliferation still remains uncertain. The up-regulation of eIF-5A1 protein in absence of corresponding up-regulation of eIF-5A1 mRNA clearly indicates that eIF-5A1 may be post-transcriptionally regulated.

To examine the effect of cell proliferation on eIF-5A2 mRNA expression, UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. After day 3, serum was added back, and mRNA levels were determined 8 hours thereafter. Figure 22 illustrates that serum starvation and re-addition do not have any effect on eIF-5A2 mRNA expression as no change in transcript abundance was observed.The up-regulation of eIF-5A2 protein in absence of corresponding up-regulation of eIF-5A2 mRNA clearly indicates that eIF-5A2 may also be post-transcriptionally regulated.

.

Figure 17. BrdU ELISA assay of cell proliferation for UACC-1598 cells. UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. Samples were analyzed using the BrdU ELISA assay after 0, 1, 2, and 3 days of serum- starvation. After day 3, serum was added back, and samples were analyzed using the BrdU ELISA assay after 1, 4, and 8 hours of serum re-addition. Graphed data were expressed as means +/- standard deviation for two experiments performed in quadruplicate. *** $P < 0.001$, significantly different from control sample (0 day). The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

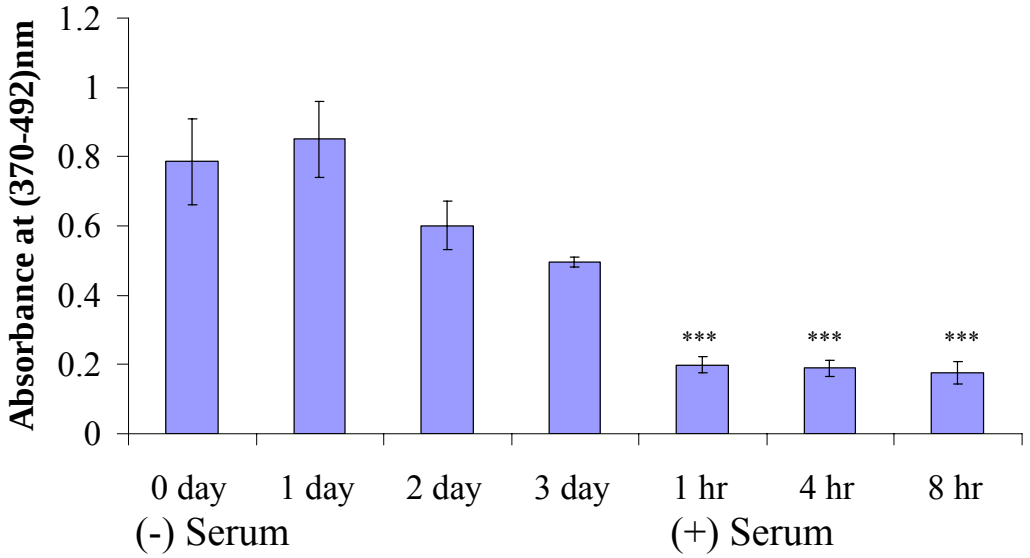

Absorbance at (370-492)nm
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
0 day
1 day
2 day
3 day
1 hr
4 hr
8 hr
(-) Serum
(+) Serum

Figure 18. XTT assay of cell proliferation for UACC-1598 cells. UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. Samples were analyzed using the XTT assay after 0, 1, 2, and 3 days of serum- starvation. After day 3, serum was added back, and samples were analyzed using the XTT assay after 1, 4, and 8 hours of serum re-addition. Graphed data were expressed as means +/- standard error for two experiments performed in quadruplicate. ** $P < 0.01$, significantly different from control sample (0 day). The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

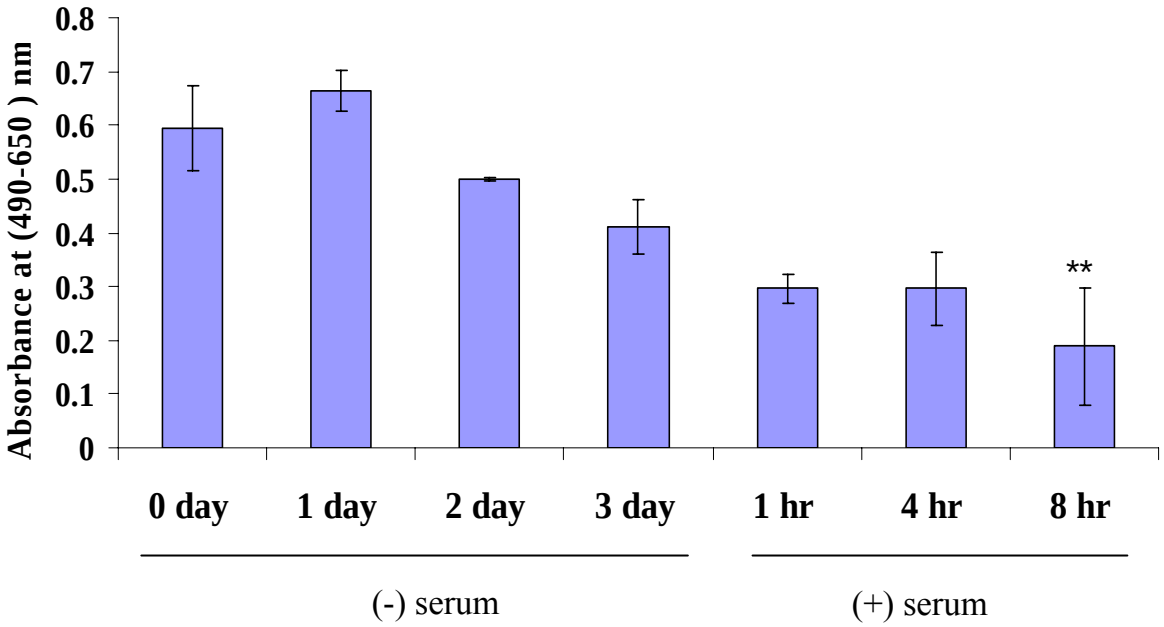
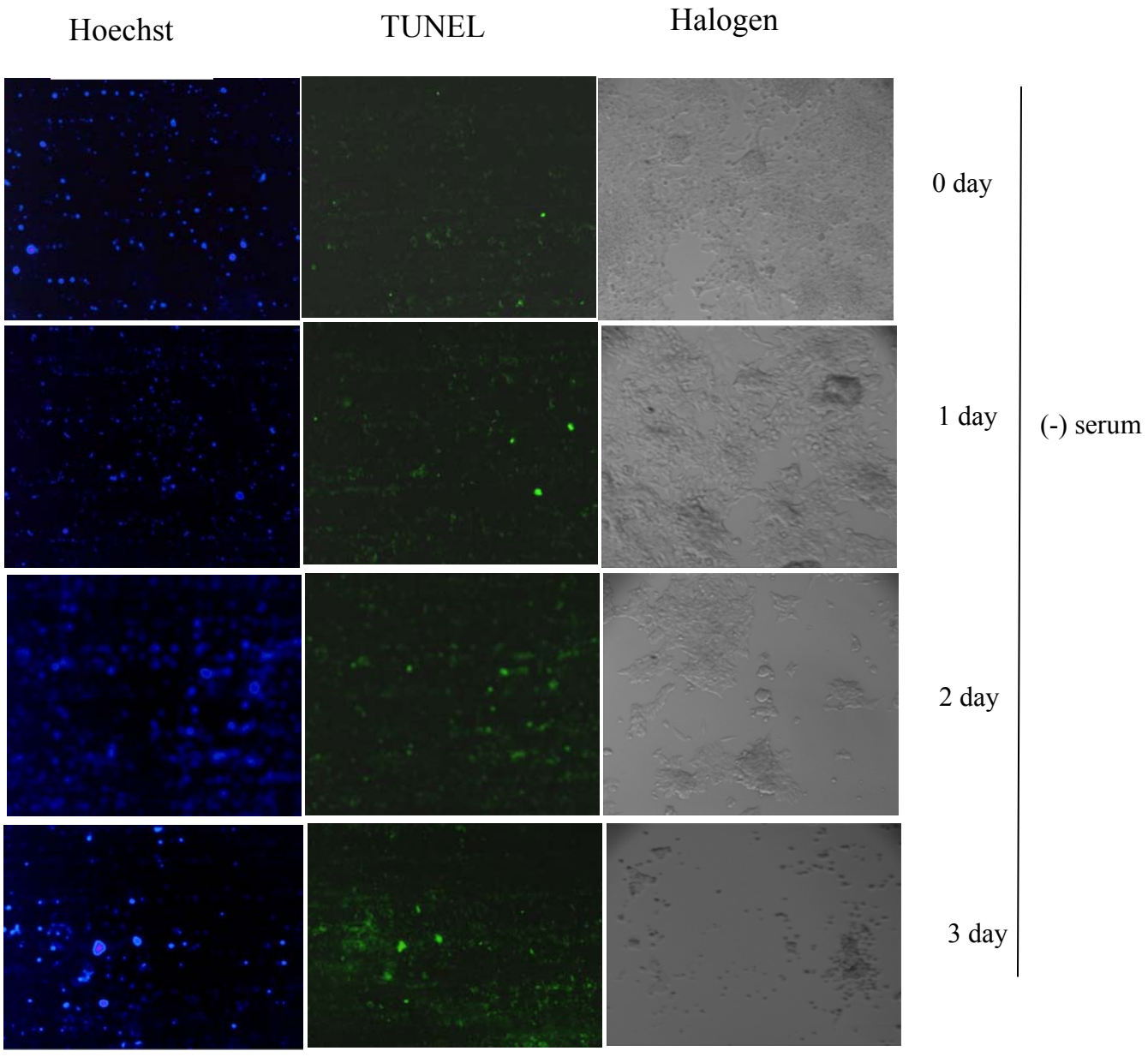
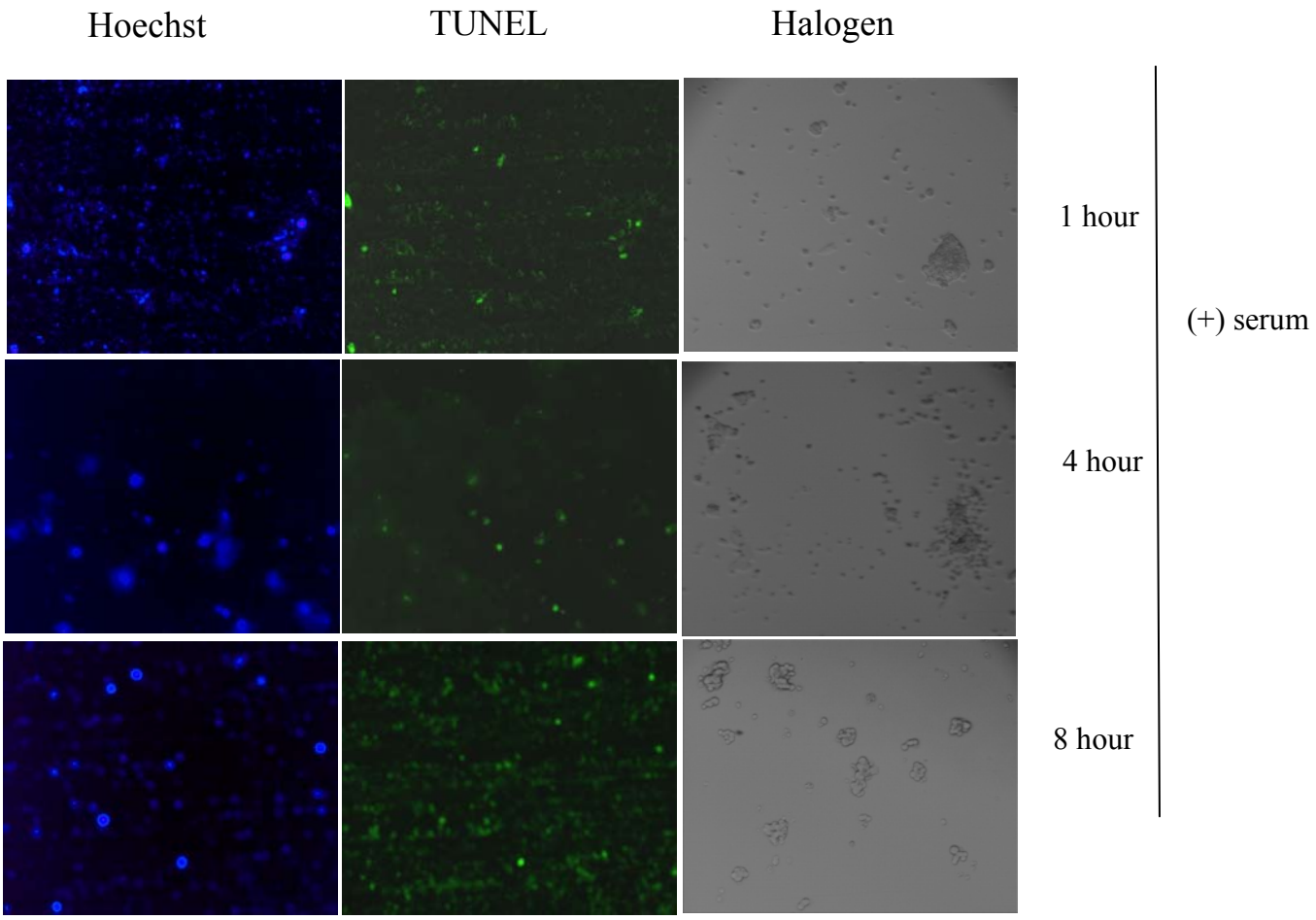

Absorbance at (490-650) nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
0 day
1 day
2 day
3 day
1 hr
4 hr
8 hr
(-) serum
(+) serum

Figure 19. TUNEL-labeling of UACC-1598 cells after serum- starvation and serum-re-addition. Panel A) For each sample, the same field was observed by two different filters using a fluorescence microscope. A UV filter was used to visualize Hoechst stained nuclei (blue), and a fluorescein filter was used to visualize TUNEL stained apoptotic cells (green). For each sample, the percentage of apoptotic cells was calculated by dividing the number of TUNEL- stained apoptotic cells by the total number of Hoechst- stained cells. The cell morphology was observed using a halogen light source. Images for halogen source are not from the same field as TUNEL and Hoechst. Panel B) Graphical representation of the percentage of apoptotic cells. Graphed data are expressed as means +/- standard deviation from five randomly chosen fields.** $P < 0.01$, * $P < 0.05$, significantly different from control sample (0 day). The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A




Hoechst
TUNEL
Halogen
1 hour
4 hour
8 hour
(+) serum

B

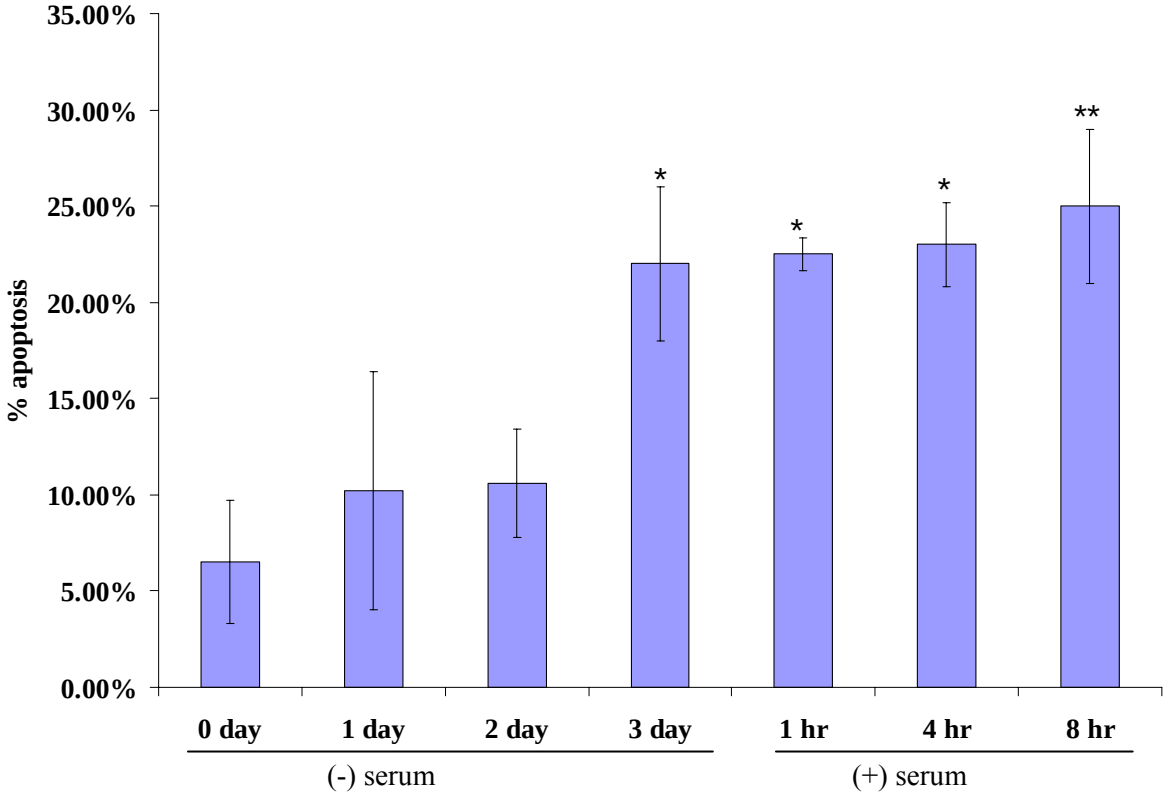

35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
% apoptosis
*
*
*
**
0 day
1 day
2 day
3 day
1 hr
4 hr
8 hr
(-) serum
(+) serum

Figure 20. The effect of serum- starvation and serum-re-addition on eIF-5A1 mRNA expression in UACC-1598 cells . UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. RNA samples were collected after 0, 1, 2, and 3 days of serum- starvation. After day 3, serum was added back, and samples were collected after 1, 4, and 8 hours of serum re-addition. Total RNA was isolated at each time point. Semi-quantitative RT-PCR was performed using specific primers. The size of the PCR products for both eIF-5A1 and β-actin was 500 bp.

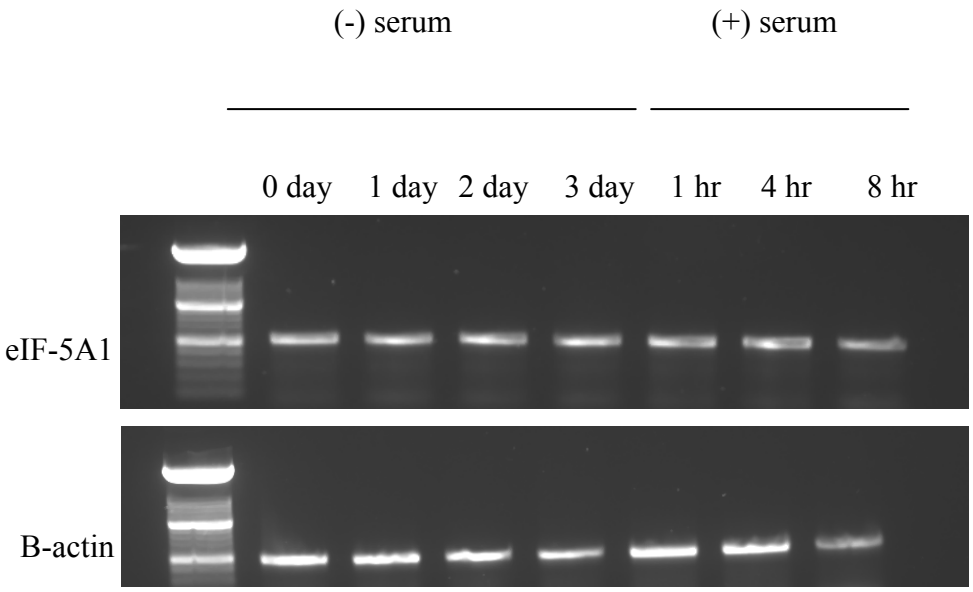
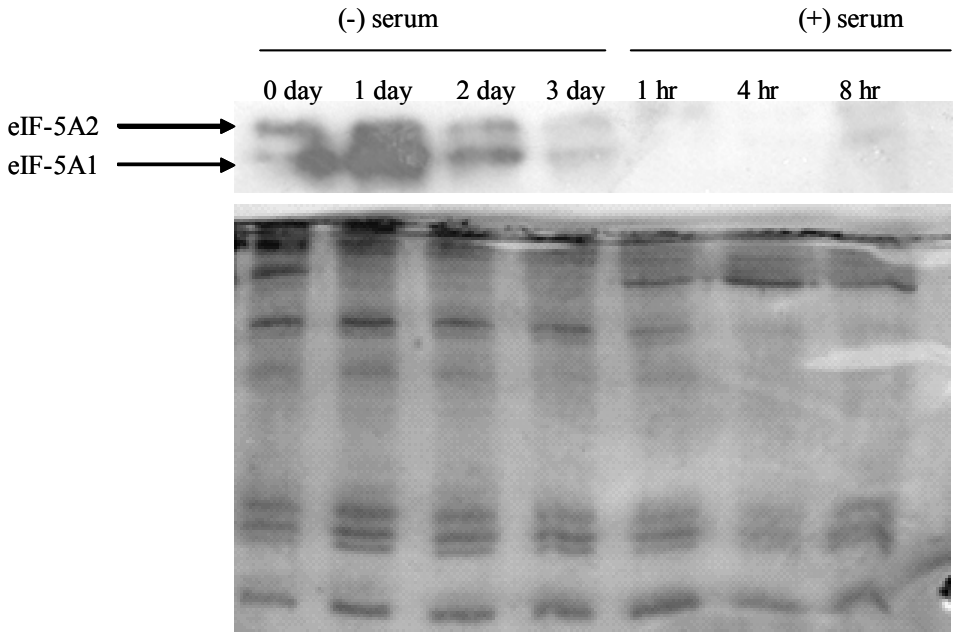

(-) serum
(+) serum
0 day
1 day
2 day
3 day
1 hr
4 hr
8 hr
eIF-5A1
B-actin

Figure 21. The effect of serum starvation and serum re-addition on eIF-5A1 and eIF-5A2 protein expression. UACC-1598 cells were grown to about 70% confluence and then serum- starved for 3 days. Protein samples were collected after 0, 1, 2, and 3 days of serum-starvation. After day 3, serum was added back and samples were collected after 1, 4, and 8 hours thereafter. Panel A) western analysis of UACC-1598 cell lysates. Each lane contained 10 μg of protein, and the gel was probed with eIF-5A1 antibody. The corresponding Comassie Blue- stained gel is also presented. The eIF-5A1 and eIF-5A2 proteins are 18 and 20 kDa in size, respectively. Panel B) Densitometry analysis of eIF-5A1 and eIF-5A2 expression. Graphed data were expressed as means +/- standard deviation for n=2. The treated samples were not significantly different from the control sample (0 day). The data was analyzed with two-way ANOVA followed by Bonferroni's post hoc test.

A


(-) serum
(+) serum
0 day
1 day
2 day
3 day
1 hr
4 hr
8 hr
eIF-5A2
eIF-5A1

B

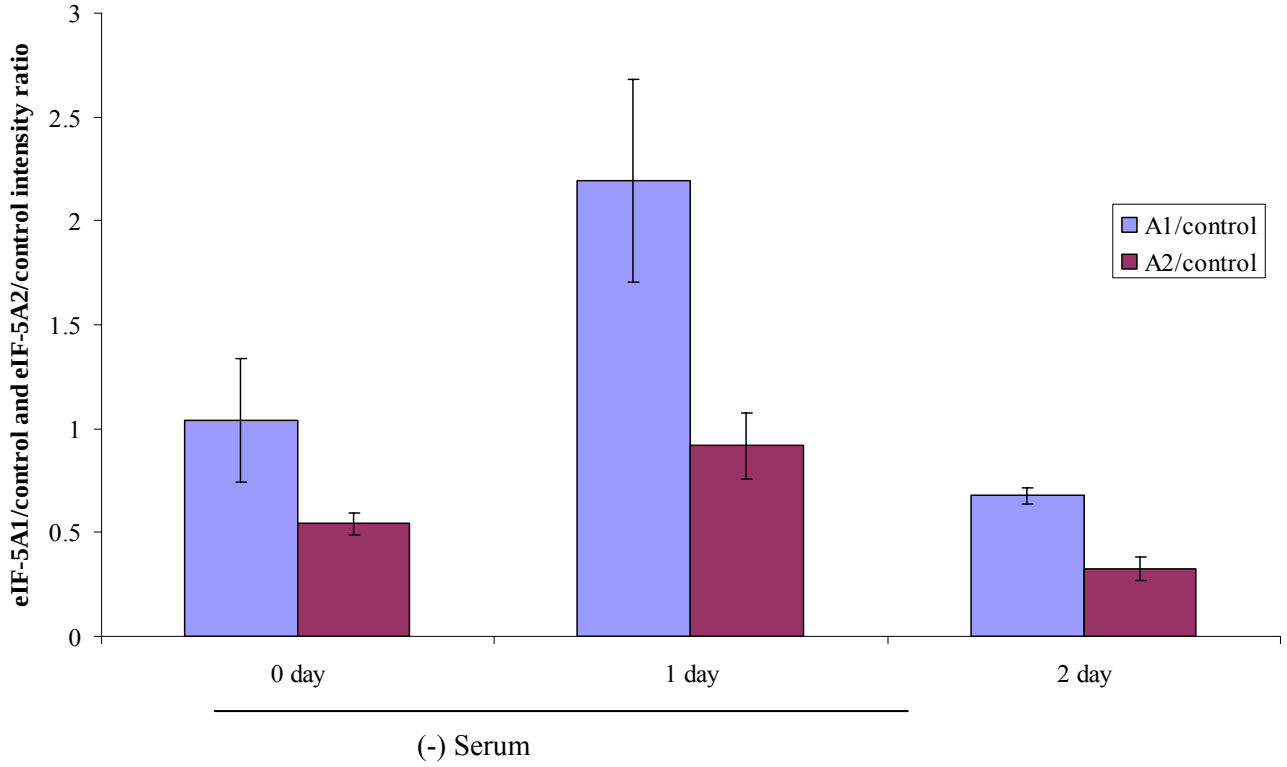
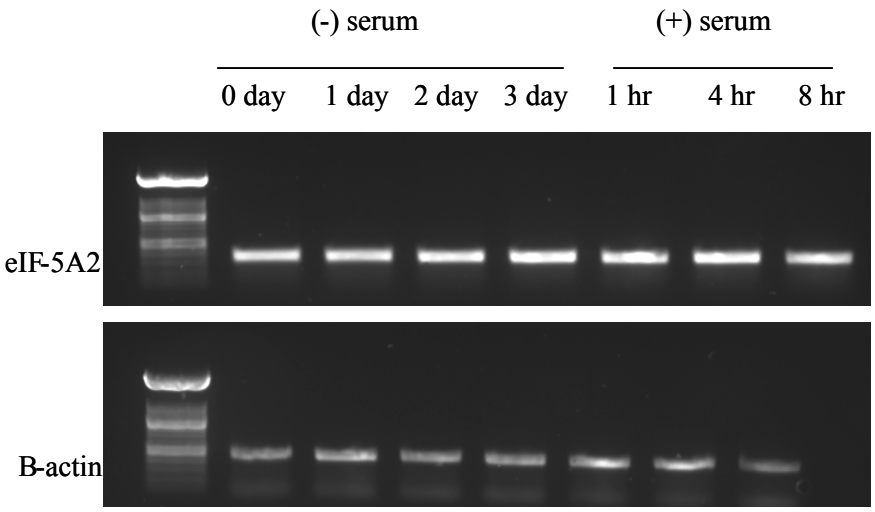

eIF-5A1/control and eIF-5A2/control intensity ratio
3
2.5
2
1.5
1
0.5
0
A1/control
A2/control
0 day
1 day
2 day
(-) Serum

Figure 22. The effect of serum starvation and serum re-addition on eIF-5A2 mRNA expression. Ovarian cancer cells (UACC-1598) were grown to about 70% confluence and then serum starved for 3 days. RNA samples were collected after 0, 1, 2, and 3 days of serum starvation. After day 3, serum was added back, and samples were collected 1, 4, and 8 hours thereafter. Semi-quantitative RT-PCR was performed using specific sets of primers as described earlier. The size of PCR products of eIF-5A2 and β-actin were 345 and 500 bp, respectively.


(-) serum
(+) serum
0 day
1 day
2 day
3 day
1 hr
4 hr
8 hr
eIF-5A2
B-actin

Discussion

The objective in this study was to elucidate the role of human eIF-5A in apoptosis and cell proliferation. There are two isoforms of eIF5A in the human genome, designated eIF-5A1 and eIF-5A2. Most of the studies to date have been with eIF-5A1, and roles in both apoptosis and cell proliferation have been ascribed to it (Taylor et al., 2004 ; Park et al., 1994). Little has been done with eIF-5A2, presumably in part because it is expressed at very low levels in most cells and tissues (Clement et al., 2003). There is evidence that eIF-5A2 is expressed at high levels in ovarian cancer cells and that it may be an oncogene (Guan et al., 2004). Of particular importance in interpreting these studies is that eIF5A is post-translationally modified through two reactions in which a conserved lysine in eIF-5A1 is converted to hypusine (Park et al., 1982). The first reaction mediated by deoxyhypusine synthase results in the formation of deoxyhypusinated eIF-5A1, and the second, mediated by deoxyhypusine hydroxylase, results in the formation of hypusinated eIF-5A1 (Park et al., 1982). Thus, there are three forms of eIF-5A1 protein present in cells, unhypusinated eIF-5A1, deoxyhypusinated eIF-5A1 and hypusinated eIF-5A1. Although it is generally assumed that hypusinated form of eIF-5A1 is physiologically active, there is at least one report indicating that unhypusinated eIF-5A1 may also be physiologically active (Jin et al., 2003).

In the present study, apoptosis was induced by treatment with Actinomycin D. The ability of Actinomycin D to induce apoptosis is well established, and it appears to do so by blocking the movement of RNA polymerase and interfering with DNA-dependent RNA synthesis (Leclerc et al., 2002). Apoptosis was also induced by NO donor sodium nitroprusside , which induces apoptosis by damaging DNA and inhibiting DNA synthesis (Gordana et al., 2004). Two human cell lines were used for apoptosis study, RKO cells which are colon adenocarcinoma cells and CCD-112CoN, which are normal colon fibroblast cells.

In response to Actinomycin D treatment, eIF-5A1 and β-actin transcripts were both detectable in RKO cells and CCD-112CoN cells and showed no change in abundance during first 8 hours of Actinomycin D treatment. However, 24 hours after the initiation of Actinomycin D treatment, by which time the extent of apoptosis had increased, transcripts for both the housekeeping gene, β-actin, and eIF-5A1 were undetectable. This may be attributable to the fact that Actinomycin D inhibits transcription. It does so by forming a stable complex with single-stranded DNA and blocking the movement of RNA polymerase, thereby interfering with DNA-dependent RNA synthesis (Leclerc et al., 2002). β-actin is a commonly used housekeeping gene because its cognate protein remains constant throughout the cell cycle (Leclerc et al., 2002). In light of this, it seems reasonable to conclude that the depletion of β-actin mRNA and eIF-5A1 mRNA within 24 hours of Actinomycin D treatment largely reflects inhibition of transcription. This contention is further supported by the finding that the half-life of β-actin mRNA following treatment with Actinomycin D has been reported to be 6.6 hours for human CCRF-CEM and 13.5 hours for human Nalm-6

cells (Leclerc et al., 2002). The finding in the present study that β-actin mRNA declined to undetectable levels within 24 hours of Actinomycin D treatment is consistent with these findings.

Of particular interest is the finding for both RKO cells and CCD-112CoN cells that, although there was no change in eIF-5A1 transcript during Actinomycin D –induced apoptosis, there was an up-regulation of eIF-5A1 protein. It was not possible to determine whether this was also the case for eIF-5A2 protein, because levels of this protein were below detection limits. Cellular levels of eIF-5A2 are generally low, and this has been attributed in part to inefficient translation of eIF-5A2 mRNA (Clement et al., 2006). Clement and his colleagues observed that eIF-5A2 precursor was synthesized at a level comparable to that for eIF-5A1 precursor, which led them to propose that negative cis elements retarding the translation of eIF-5A2 mRNA may reside in the 5' UTR or 3' UTR. Moreover, sucrose gradient fractionation of cytopalsmic RNA revealed that only a small proportion of total eIF-5A2 mRNA was associated with the polysomal fraction, compared to a much larger proportion of eIF-5A1 mRNA (Clement et al., 2006).

The effects of apoptosis on the expression of eIF-5A1 were also determined using SNP, which is a strong source of NO that serves as a powerful inducer of apoptosis. For both RKO (cancer) and CCD-112CoN (normal) cells, eIF-5A1 and β-actin transcripts were readily detectable in untreated cells and did not change in abundance within 8 hours of treatment with SNP. However, by 24 hours after the initiation of SNP treatment, the transcripts for both β-actin and eIF-5A1 had decreased to levels that were below detection. This presumably reflects DNA damage and inhibition of transcription, both of which are known to be caused

by SNP (Gordana et al., 2004). Likewise, β-actin and eIF-5A2 mRNA were found to be constitutively expressed for 8 hours, and they disappeared after 24 hours of SNP treatment during apoptosis in both cells. The disappearance of transcripts again attributable to the inhibition of DNA synthesis by SNP.

By contrast, eIF-5A1 protein was found to be up-regulated in both RKO and CCD-112CoN cells during apoptosis induced by SNP treatment. In fact, eIF-5A1 in normal cells (CCD-112CoN) was found to be up-regulated more than in cancer cells (RKO). It is possible that eIF-5A1may be mutated and hence non-functional in cancer cells. Relevant to this, Hong Ming sequenced the coding region of eIF-5A1 from mouse alveolar lung tumor and found that the coding region of eIF-5A1 was not mutated in the tumor samples (Dr. Thompson's lab, unpublished data). Although it is not possible to generalize from this observation to other types of cancer, it would appear that, at least in this mouse lung tumor, eIF-5A1 is functional. Recently, however, an interaction between eIF-5A1 and syntenin has been established (Li et al., 2004). Syntenin is found to be over-expressed in advanced metastatic melanomas (Helmke et al., 2004). Therefore, it is possible that in cancer cells, syntenin binds to eIF-5A1 and interferes with its function in inducing apoptosis. eIF-5A2 protein was again below detection levels in both RKO cells and CCD-112CoN cells following treatment with SNP.

It is noteworthy that in response to Actinomycin D-induced apoptosis and SNP induced apoptosis eIF-5A1 protein was up-regulated in the absence of a corresponding up-regulation of its transcript. These observations suggest that eIF-5A1 protein synthesis is post-transcritionally regulated.. The central concept of post-transcriptional regulation is that the

translation of a given mRNA occurs in the absence of a corresponding change in the steady-state level of that mRNA (Xi et al., 2006). Post-transcriptional regulation provides the cell with a more precise, immediate, and energy-efficient way of translating mRNAs without the need for transcriptional activation and subsequent mRNA processing steps (Xi et al., 2006). Moreover, post-transcriptional control provides the cell with greater flexibility in responding to various genotoxic stresses (Xi et al., 2006).

Expression of the tumor-suppressor gene, p53, is regulated through a post-transcriptional mechanism. Evidence supporting this includes the finding that there is increased expression of p53 protein in the absence of any change in the level of p53 mRNA during ultraviolet light radiation and gamma irradiation (Fu and Benchimol, 1997 ; Mazan-Mamczarz et al., 2003 ; Fu et al., 1999) . Moreover, the increase in p53 protein was accompanied by enhanced association of p53 mRNA with large polysomes, further supporting the contention that there was an increase in its translation (Fu and Benchimol,1997). There is also evidence that p53 post-transcriptional regulation entails association of the 3'UTR of p53 mRNA with the RNA binding protein, HuR, which enhances p53 translation in response to ultraviolet light irradiation (Mazan-Mamczarz et al., 2003). In addition, a cis acting element in the 3' UTR of p53 mRNA has been identified which appears to mediate translational repression of p53 mRNA. In response to gamma-radiation, the inhibitory effect of the repressor element on translation is relieved, and p53 protein is up-regulated without the up-regulation of p53 mRNA (Fu et al., 1999).

Post-transcriptional regulation of proteins required for apoptosis is advantageous because the process of programmed cell death can be initiated quickly. In the specific case of

eIF-5A1, cells can quickly translate eIF-5A1 without the need to activate transcription. Recent evidence suggests that post-transcriptional regulation of eIF-5A1 may be mediated by p53 (Xi et al., 2006). In addition to its function as a transcription factor, p53 also acts as an RNA-binding protein capable of regulating its own mRNA translation (Fu et al., 1996) as well as regulating the expression of other cellular mRNA transcripts at the post-transcriptional level (Miller et al., 2000). Xi et al. (2006) suggest that p53 affects gene expression at the post-transcriptional level either through miRNAs or its RNA-binding capability, although they have not discussed the detailed mechanism. It is possible that as an RNA binding protein, p53 binds to the 3' UTR of eIF-5A1 mRNA and enhances eIF-5A1 translation in response to apoptotic agents such as Actinomcyin D and sodium nitoprusside. Another possibility is that an RNA binding protein such as HuR may mediate eIF-5A1 post-transcriptional regulation through association with the 3' UTR of eIF-5A1 mRNA. It is also possible that the increased expression of eIF-5A1 protein in the absence of increased eIF-5A1 mRNA might occur through an enhanced association of eIF-5A1 mRNA with large polysomes. Like p53, there could be a cis acting element in the 3' UTR of eIF-5A1 mRNA which mediates translational repression of eIF-5A1 mRNA. In response to apoptotic agents such as Actinomycin D and Sodium nitoprusside, the inhibitory effect of this repressor element on translation may be relieved, allowing up-regulation of eIF-5A1 protein in the absence of a corresponding up-regulation of eIF-5A1 mRNA.

The serum- starvation and serum re-addition experiments were designed to examine the effects of cell proliferation on the expression of eIF-5A1 and eIF-5A2 protein and mRNA. In many cell types, serum- starvation leads to cell cycle arrest in the G0–G1 phase

and/or apoptosis after prolonged incubation (Yoshida and Beppu 1988; Fukami-Kobayashi and Mitsui 1999 ; Hasan et al. 1998). Serum re-addition causes arrested cells to re-enter the cell cycle and to synthesize DNA within a few hours (Campisi et al., 1984). In other words, serum re-addition re-induces cell proliferation within a few hours. Moreover, it has been shown that, upon serum re-addition, eIF-5A expression is induced and protein can be detected after a few hours of serum re-addition (Chen and Chen, 1997). Based on this finding, the serum- depletion and re-addition experiments conducted in the present study to clarify the relative roles of eIF-5A1 and eIF-5A2 in cell proliferation. The ovarian cancer cell line, UACC-1598, was used for these experiments as it was observed previously to contain detectable amounts of eIF-5A2 (Clement et al., 2003). Both BrdU and XTT data obtained using UACC-1598 cells revealed that cell proliferation decreases in response to serum starvation, and continues to decline even after 8 hours of serum re-addition. From TUNEL data, it was confirmed that the decrease in cell proliferation coincides with the induction of apoptosis.The reason for the discrepancy between the finding in this study that the cells did not re-initiate proliferation upon serum re-addition, whereas in the study of Chen and Chen (1997) re-initiation of cell proliferation was observed when serum was re-added, may reflect the fact that different cell lines were used in the two studies. In the present study, it is possible that the cells had reached a later stage of apoptosis, and serum re-addition was not able to reignite cell proliferation. Another possibility is that UACC-1598 cells require more than 8 hours after serum re-addition to re-enter the cell cycle and start DNA synthesis leading to proliferation.

Of particular interest is the finding that there was no change in the levels of either eIF-5A1 transcipt or eIF-5A2 transcript in UACC – 1598 cells in response to serum depletion or serum re-addition. Yet, eIF-5A1 protein and eIF-5A2 protein both increased in abundance within 1 day of serum starvation before declining again. Moreover, this increase in eIF-5A1 protein and eIF-5A2 protein coincided with the induction of apoptosis as measured by TUNEL and a subsequent decline in viable cells. The decrease in viable cells was evident from the finding that the cells detached and assumed a rounded phenotype, and that there was a decline in cell division measured by the BrdU ELISA assay and a decline in cell viability measured by the XTT assay. Moreover, there was no resumption of cell division and no increase in cell viability following re-addition of serum, and there was no increase in eIF-5A1 or eIF-5A2 protein levels.

There are several important findings that can be inferred from these data. First, levels of eIF-5A1 and eIF5A2 protein change in parallel in response to serum- depletion. This suggests that, at least in UACC – 1598 cells, their translation is commonly regulated. Second, eIF-5A1 and eIF-5A2 both exhibit up-regulation in response to serum- depletion in the absence of a corresponding increase in transcript. This suggests that, like eIF-5A1, eIF-5A2 is also post-transcriptionally regulated. Third, both proteins are up-regulated in parallel during serum- starvation just prior to the onset of apoptosis leading to loss of cell viability and cell death. This raises the possibility that they may both be involved in apoptosis. There is growing evidence from studies with cell lines that eIF-5A1 is able to induce apoptosis (Taylor et al., 2004 ; Taylor et al., 2006). Moreover, Hong Ming has shown that cells transfected with adenovirus constructs of eIF-5A1 or eIF-5A2 undergo apoptosis (Dr.

Thompson's lab, unpublished data). In addition, eIF-5A1 has been shown to be a strong and effective anti-cancer therapy in studies with tumorigenic mice (Songmu Jin, Hong Ming, and Catherine Taylor, unpublished data, Dr. Thompson's lab). The present results are consistent with the contention that eIF-5A2 is also involved in the induction of apoptosis and the two isoforms of human eIF5A may have redundant functions.

The roles of eIF-5A1 and eIF-5A2 in cell proliferation remain unclear. It was not possible to evaluate whether they play a role in cell proliferation in the present study because there was no resumption of cell proliferation by UACC – 1598 cells when serum was added back after a period of serum depletion. Rather, the cells continued to undergo apoptosis and lose viability. Several previous reports have implicated the role of eIF-5A1 in cell proliferation (Park et al., 1994 ; Jansson et al., 2000) . These studies mainly involved demonstrating that inhibitors of hypusination block cell division. However it was not made clear in these studies that the suppression of cell division observed was not an indirect effect of the inhibitors unrelated to their ability to inhibit hypusination of eIF5A (Taylor et al., 2006). The present study on the other hand supports previous reports (Taylor et al., 2004 ; Taylor et al., 2006) that eIF-5A1 is involved in the induction of apoptosis and, in addition , provides evidence that eIF-5A2 may also be involved in apoptosis.

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