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MOLECULAR GENETIC ANALYSIS OF GLUCOCORTICOID-INDUCED GENE 18

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Thomas David Leptich

entitled Molecular Genetic Analysis of Glucocorticoid-Induced Gene 18

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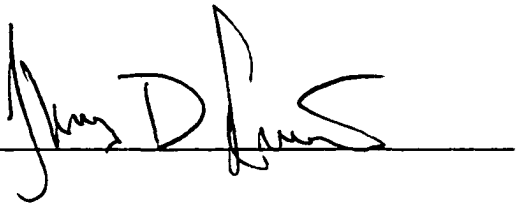
SIGNED: 

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ABSTRACT

In the course of thymocyte development, progenitor T cells from the bone marrow migrate to the thymus where they are positively or negatively selected depending on specific molecular interactions. This selection process takes place at a developmental stage where immature thymocytes are exquisitely sensitive to glucocorticoid exposure and they readily succumb in a process referred to as apoptosis. Glucocorticoid-induced thymocyte apoptosis has been shown to require RNA and protein synthesis, which indicates an active death-inducing process. One molecular event that occurs in immature thymocytes during this process is the rapid and high induction of a glucocorticoid-induced gene called GIG18. Within this dissertation, I identify a GIG18 open reading frame (ORF), that encodes a ~50KDa protein, that is 93% conserved between mouse and human. By expressing this ORF with a 6X-Histidine tag, I was able to purify the GIG18 protein on a nickel affinity column to generate a rabbit polyclonal antibody. The western blot showed glucocorticoid-regulated induction of two protein bands, a mildly induced band at the predicted size (50KDa) and a highly induced band at ~68KDa. Further analysis of mouse and human expressed sequence tags (ESTs) indicated that alternate transcripts produced from this gene, are likely account for the 68KDa band. The mouse GIG18 ORF identified in our lab maps to human chromosome 7 and contains 8 exons covering ~250 Kb. This region of human chromosome 7 corresponds to homologous sequence on mouse chromosomes 5 and 6. I also show that GIG18 belongs to family of

three genes encoded in the human genome with two extensive regions of conserved sequence. Expression analysis of the GIG18 ORF in thymocytes and HeLa cells did not reveal any obvious cellular morphological variations or functions induced by this gene. I conclude this dissertation with a discussion of potential roles that GIG18 may play within the context of thymocyte development.

CHAPTER I

INTRODUCTION

The Model Organisms

The word apoptosis is a descriptive term that refers to the morphological features that accompany programmed cell death. These features include chromatin and cytoplasmic condensation, nuclear fragmentation, degradation of genomic DNA and the formation of apoptotic bodies. This evolutionarily conserved process was first described in the early 1970s and aroused interest because of the fact that many different tissues and cell types shared these same morphologies suggesting an underlying cellular program may be directing the observed physical changes [1, 2].

The proposal that cells contain an inherent program to regulate apoptosis appears to be correct. Not only is there evidence for a genetically determined program, but components of the program appear to be functionally conserved between humans and yeast [3]. The genetic program of apoptosis has been discovered and dissected in several model organisms including mouse, fruit fly (*Drosophila melanogaster*) and nematode (*Caenorhabditis elegans*).

Genetic analysis in *C. elegans* has led to the identification of several genes that are essential for the control of cell death [4]. Two genes, *ced-3* and *ced-4*, are required for the killing process (Figure 1). The product of the *ced-3* gene is a caspase which is a member

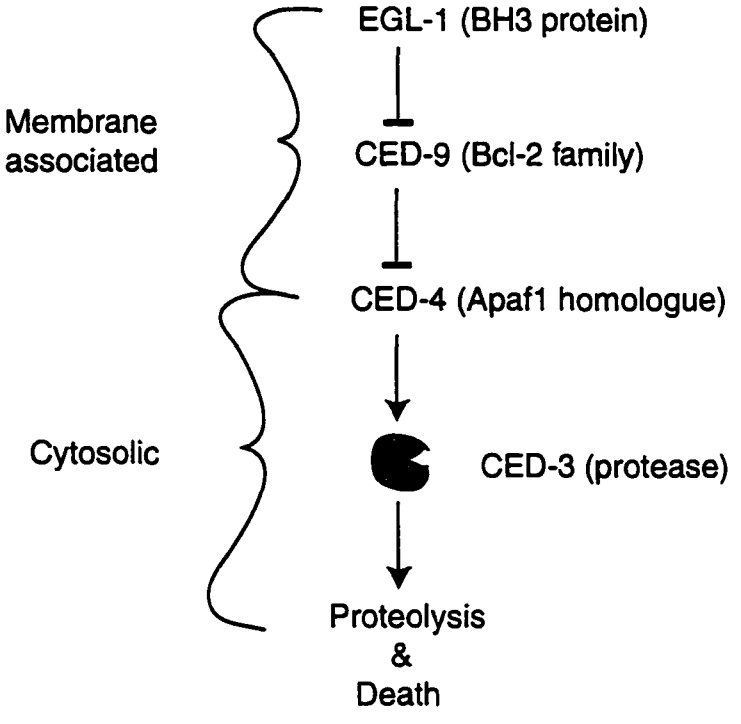


Figure 1. The genetic and physical relationship of the apoptotic components in C. elegans.

The activation of the CED-3 protease is thought to occur as a result of oligomerization with CED-4. CED-4 is held inactive by association with CED-9 in intracellular membranes. The association of CED-9 with CED-4 is disrupted by expression of EGL-1 via its BH3 domain, thus leading to release of CED-4 into the cytosolic fraction and CED-3 protease acivation.

of the cysteine aspartate protease family [5]. CED-3 and homologous caspases are synthesized as zymogens that require proteolytic processing at specific sites for activation [6]. Once CED-3 is activated it exerts its lethal effect by cleaving specific proteins in the condemned cell. The product of the *ced-4* gene is a positive regulator of CED-3 and is expressed primarily during embryogenesis when extensive programmed cell death is occurring [7]. In contrast, another gene called *ced-9*, which belongs to the Bcl-2 family, acts to oppose the activation of the CED-3 protease and protects cells from programmed cell death [8].

These three gene products have physical interactions that determine the fate of the host cell. Biochemical studies have shown that CED-4 binds to CED-3 and promotes its proteolytic and enzymatic activation [9], [10]. In contrast, CED-9 interacts with CED-4 and inhibits its ability to activate CED-3 [11], [12]. The precise mechanism of how CED-4 activates CED-3 is unknown but it is believed that oligomerization of CED-4 may cause CED-3 autoactivation due to the induced proximity [13].

Another gene in *C. elegans* called *egl-1*, has been shown to have an important role in the regulation of cell fate. It has been shown that EGL-1 is capable of interacting with CED-9 thus causing activation of the CED-3 protease. EGL-1 interacts with CED-9 via a BH3 domain (Bcl-2 homology) [14]. Gain of function mutants of *egl-1* result in the death of two hermaphrodite specific neurons whereas loss of function mutants prevent most of the somatic cell deaths normally associated with development [15].

A brief summary of the apoptotic components found in the fruitfly, *Drosophila melanogaster*, demonstrates the complexity of regulation of the cell death process that

has occurred during the course of evolution. As with *C. elegans*, the critical attribute is the activation of the executioner proteases, the difference is the increased number of components and their corresponding diversity. In contrast to the nematode, in which the three identified caspases have long prodomains that are removed in the activation process, the caspases in fruitfly have both long and short prodomains that allow them to be classified as either apical (upstream) or executioner (downstream). The apical long prodomain caspases in mammals all contain sequence motifs known as death effector domains (DED) and caspase activation recruitment domains (CARD). These domains are thought to be involved in procaspase recruitment to membrane receptors and activation by oligomerization, again by induced proximity [16]. The prodomain in apical caspases in the nematode and the fruitfly contain unique sequences and one of the fly prodomains has a CARD homologous sequence. Once activated, the apical caspases are thought to cleave and activate short prodomain executioner caspases.

Activation of the caspases occurs as a result of one of two pathways (Figure 2). One pathway is initiated at the cell membrane by binding of a ligand to a member of the tumor necrosis factor (TNF) family of receptors. Ligand dependent multimerization of these receptors, which have cytoplasmic domains known as "death domains" (DD), leads to recruitment of cytoplasmic DD containing adapter proteins. These adapter proteins also contain DED sequences that are responsible for recruiting procaspases. Although DDs have been found in *Drosophila*, these adapter genes are lacking any recognizable DED or CARD homology [17]. In fact, no DEDs were found in the *Drosophila* genome

suggesting that homology is either very low, or there are distinct sequences used as oligomerization motifs.

The second pathway of apical caspase activation involves components of the mitochondria that are released in response to cellular stress. The major factor released is cytochrome C which is released and binds to a cytosolic cofactor in *Drosophila* called dapaf-1 (*Drosophila* apaf-1 homologue), now known as Ark (apaf-1 related killer). Biochemical and genetic evidence suggests that the cyt C/Ark complex interacts with *Drosophila* caspases leading to their subsequent activation [18], [19].

Without activation stimuli, caspases are held in check by another family of proteins called the inhibitors of apoptosis proteins (IAP). This extra measure of regulation probably arose not only to prevent spurious cell death, but also to control apoptosis more tightly. In *Drosophila*, the IAPs are essential for cell survival and one mechanism for inducing cell death is the inhibition of IAP function [20], [21], [22]. IAPs were first identified as baculovirus-encoded cell death inhibitors and they contain amino-terminal repeats of ~70 amino acids called baculovirus IAP repeats (BIR, or BIR containing proteins (BIRPs)) and a carboxy-terminal RING finger domain [23]. BIR and RING finger domains are not restricted to proteins involved in apoptosis but are also found to be involved in cytokinesis and in conferring E3 ubiquitin protein ligase activity, respectively [24]. The *Drosophila* genome encodes four BIRPs, three of which have been tested and shown to act as cell death inhibitors]. BIRPs that are not cell death inhibitors are found in yeast and *C. elegans* where they help regulate cell division [26], [27].

The downstream apoptotic effects of nuclear condensation and DNA fragmentation in *Drosophila* are likely caused by functional homologues of caspase-activated DNases in mammals (CAD). The *Drosophila* genome encodes homologues to mammalian AIF (apoptosis inducing factor, which is released from the mitochondria) and acinus (a nuclear factor that is proteolytically activated by caspase 3) which are both involved in condensation and fragmentation of the nuclear material.

Key to the regulation of the apoptotic components in *Drosophila* are the proteins REAPER, HID and GRIM. An early genetic screen identified genomic region 75C as being responsible for nearly all normally occurring embryonic cell deaths, which is where these three genes are located [28]. The expression patterns of *reaper* and *grim* foreshadow cell death during development and there is evidence that these three gene products are responsible for integrating and transducing many different death signals eventually leading to caspase activation [29]. An example is demonstrated by ecdysone triggered apoptosis in *Drosophila* salivary glands. The ecdysone receptor, with bound ligand, binds to a response element upstream of the *reaper* and *hid* genes to directly induce their transcription [30]. Although REAPER, HID and GRIM have limited homology with each other, expression of each of these proteins induces apoptosis in mammalian cells demonstrating that this aspect of their function has been conserved. These gene products all bind to DIAP-1 (BIRP) through interactions that require their amino termini which is thought to relieve the inhibition of the caspases, thus leading to apoptosis [31], [32].

The apoptotic mechanism in the mammalian field is even more diverse with many more participants. The critical aspect is activation of the caspases, and fourteen have been identified to date. Ten of these fourteen have long prodomains (suggesting apical function) and the remaining four have short prodomains (suggesting executioner function). However, six of the ten long prodomain caspases are known to be involved in cytokine processing (caspases 1, 4, 5, 11, 12, 13). The remaining caspases, with the exception of caspase-14, can be activated by extrinsic or intrinsic apoptotic pathways.

In the extrinsic pathway (Figure 2), Fas ligand (FasL) binds to the receptor, Fas, which leads to trimerization of the receptor interaction of the DDs on the cytoplasmic side of the membrane. The DD containing adapter protein FADD is recruited to the membrane through homophilic DD interactions. FADD and related adapter proteins contain DEDs which are also found in the prodomains of caspase-8 and caspase-10. Homophilic interactions between DEDs leads to caspase oligomerization and autoactivation just inside the membrane in what is known as a death-inducing signaling complex (DISC).

There are three known mechanisms involved in death receptor regulation. The first one involves inhibition of procaspase recruitment and/or activation by competitive binding to FADD. One group of these inhibitory proteins belongs to a family of viral proteins called vFLIPs (for viral FADD-like inhibitory proteins) and they contain two DEDs that prevent recruitment of the caspases to the DISC [33]. A mammalian homologue of vFLIP has been identified (cFLIP) that has two isoforms as a result of alternate splicing, both of which bind to procaspase-8, procaspase-10 and FADD via DED interactions [34].

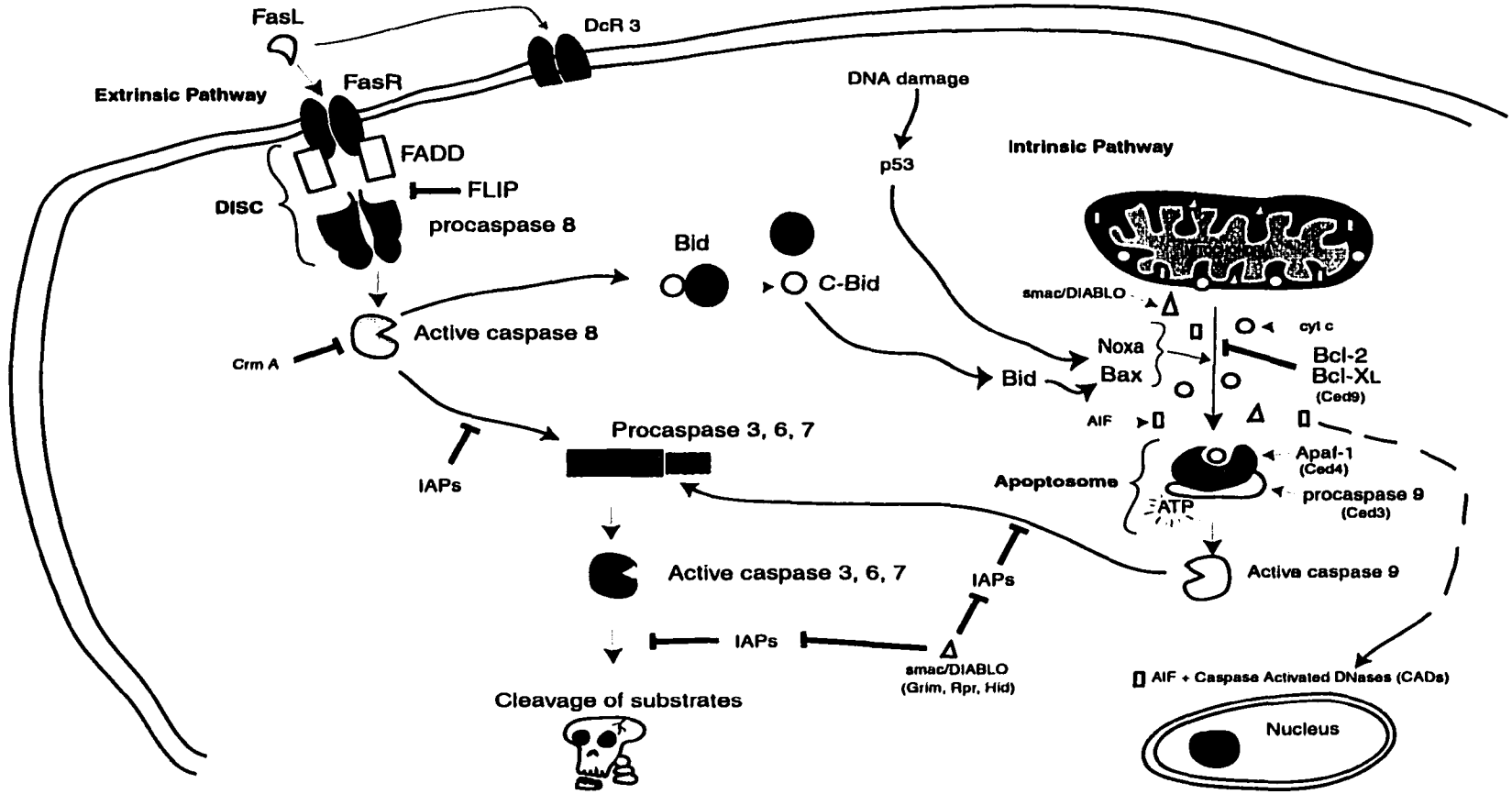


Figure 2. Summary of the basic components involved in the apoptotic process.

The activation of the caspases during apoptosis can be a result of signaling through the extrinsic pathway (membrane bound receptors), or the intrinsic pathway (through the mitochondria). There is crosstalk from the extrinsic pathway to the intrinsic pathway through proteolytic processing of Bid. Both pathways activate the effector caspases 3, 6, & 7. Inhibition of the extrinsic pathway occurs through decoy receptors for ligand (DcR3), innocuous death domain containing proteins imitating procaspase 8 (FLIP), and inhibitors of apoptosis (IAPs) which prevent caspase activation. The intrinsic pathway is activated by cell stress, for example, DNA damage and increased levels of reactive oxygen species.

A second mode of regulation at the membrane receptor level comes about as a result of decoy receptors being expressed which lack functional cytoplasmic domains and compete for binding of ligand thus diminishing the intact receptor's response. An example of this is the DcR3 receptor that has been found to be overexpressed in lung and colon cancer cells [35]. This receptor binds FasL and could partially explain how these tumor cells evade immune surveillance by cytotoxic lymphocytes which kill target cells by signaling through Fas.

The third mechanism for preventing death receptor-induced stimuli is to directly inhibit the proteolytic activation of the procaspases. An example of this is the viral protein crmA which is a member of the serpin family of protease inhibitors and potently inhibits procaspase-8. CrmA inhibits autoproteolysis and activation of procaspase-8 as well as blocking its ability to cleave Bid which would normally lead to release of cytochrome c and the activation of downstream caspases [36], [37].

Caspase activation can also be triggered through an intrinsic pathway by a number of stress stimuli that induce apoptosis via the mitochondria. Cytochrome c, after being released from the mitochondria, binds to Apaf-1 which also binds dATP leading to recruitment and activation of procaspase-9, and the eventual proteolytic activation of procaspase-3 [38]. Apaf-1 is able to bind to dATP by using its Walker A and B boxes which are the most conserved regions between Apaf-1 and CED-4 (the *C. elegans* homologue). Apaf-1 also has an amino-terminal domain that is proposed to act as a CARD domain in the recruitment of caspases 1, 2 and 9 which have a similar sequence in their prodomain. WD40 repeats in the carboxy-terminal half of Apaf-1 may also be

involved in caspase-9 recruitment [39]. Apaf-1, caspase-3 and caspase-9 gene knockout mice have highly similar phenotypes displaying excessive neuronal cells in their brains and dying one to two days postnatal. In Apaf-1 mice, caspase-9 and caspase-3 can't be activated in response to various stimuli and likewise caspase-3 activation is negated in caspase-9 knockout mice [40].

Regulation of the mitochondrial-mediated caspase activation pathway occurs at the level of cytochrome c release (amongst other proapoptotic factors). Cytochrome c is normally found in the intermembrane space of the mitochondria whereas Apaf-1 and procaspase-9 are cytosolic proteins. The release of cytochrome c is regulated by members of the Bcl-2 family. This family of proteins are divided into those that are antiapoptotic, as mentioned above, and those that are proapoptotic It is thought that the balance between the two opposing factors is what determines whether or not cytochrome c will be released. Release of cytochrome c is blocked by overexpression of Bcl-2 or Bcl-xL in response to a number of stimuli [41], [42]. Conversely, cytochrome c release is promoted by Bad, Bax and Bid which contain BH3 domains that confer these proteins with proapoptotic activity [43]. It has also been shown that high levels of BH3 containing peptides will induce cytochrome c release [44]. It is thought that the BH3 domain, which is a amphipathic helix, neutralizes the antiapoptotic function of Bcl-2 by binding to a hydrophobic groove [45].

The intrinsic and extrinsic apoptotic pathways are not completely independent since the receptor mediated induction of apoptosis is connected to the mitochondria by a proteolytically activated protein called Bid. Bid normally resides in the cytoplasm but

translocates to the mitochondria after being activated by caspase-8 mediated cleavage [46]. It is thought that mitochondrial targeting of Bid is aided by cardiolipin which is found in the outer membrane [47]. Once at the mitochondria, Bid promotes the recruitment of BH3 containing proteins which favors the release of cytochrome c.

In mammals, as in *Drosophila*, there are inhibitor of apoptosis molecules (IAPs) that all contain at least one baculovirus IAP repeat (BIR) domain. The IAPs have been shown to function by blocking the processing of procaspases, inhibiting active caspases and competing with procaspases in Apaf-1 recruitment via their CARDs (CARDs are found in cIAP-1 and cIAP-2). IAPs can block both the death receptor and mitochondrial pathways by inhibiting the activity of the executioner caspase-3 and caspase-7, and the initiator caspase-9 [25]. Also, as in *Drosophila*, these IAPs are subject to inhibition by a protein called Smac/DIABLO that functions similarly to rpr, grim and hid. Smac/DIABLO is a mitochondrial protein that is released with cytochrome c, acinus and AIF in response to apoptotic stimuli and promotes caspase activation by binding to and neutralizing the IAPs [48], [49]. Smac/DIABLO has been shown to promote the activity of initiator caspase-9 and the effectors, caspase-3 and caspase-7 [50].

Do yeast contain an apoptotic program? Yeast have cellular components that functionally interact with mammalian apoptotic proteins and can induce signature signs of apoptosis [51]. The cell death program in yeast is mediated by reactive oxygen species (ROS) which are produced by all aerobic organisms as a normal byproduct of respiration. In *S. cerevisiae*, low doses of H_2O_2 or accumulation of ROS by depletion of glutathione induces apoptosis. Also, expression of bax results in the accumulation of ROS whereas

depletion of oxygen radicals or hypoxic treatment prevents apoptosis [52]. "Petite" yeast, which lack mitochondria, survive bax expression which is consistent with an active respiratory chain being required for the suicide process [53]. These observations suggest that the basic components of apoptosis seem to have their roots deeply seeded in evolution with mitochondrial signaling being the ancient core.

Cell Death and Disease

The balance between cell death and cell survival is under tight genetic control. Many components are involved in extracellular and intracellular signaling and are integral to maintaining this balance. At a certain threshold, biochemical or biological injury leads to an imbalance causing a stress response, which leads to apoptosis. Dysregulation of the controlling mechanisms of this system are often responsible for causing disease. Deficient apoptosis is associated with cancer, auto-immunity and viral infections whereas excessive apoptosis is associated with ischaemic heart disease, stroke, neurodegenerative disease and sepsis [54], (Table 1A). One of the most well known and intensely studied human diseases is caused by HIV (human immunodeficiency virus) and is the result of inappropriate apoptosis.. HIV depletes the body of CD4+ helper T lymphocytes which are essentially the eyes and ears of immune surveillance. When the CD4+ cell count drops below a certain threshold, patients become susceptible to a myriad of opportunistic infections. Recent studies have suggested that CD4+ killing is a direct result of HIV infection [55]. The intrinsic mitochondrial pathway appears to be the primary mechanism that induces depletion of CD4+ T cells since cytochrome c release in HIV-1

Table 1A. Human diseases associated with dysregulated apoptosis.

Increased apoptosis	
Central Nervous System	Degenerative diseases (Alzheimer's and Parkinson's)
	Stroke (Cerebral ischaemia)
Myocardium	Heart attack
Lymphocytes	Sepsis and HIV infection
Decreased apoptosis	
Epithelial tissues	Carcinogenesis
Blood vessels	Hyperplasia
Lymphocytes	Autoimmune disorders
Haemopoietic system	Leukaemia, lymphoma

Table 1B. Differences between apoptosis and necrosis.

Apoptosis	**Necrosis**
Physiological or pathological	Always pathological
Asynchronous process in single cells	Occurs synchronously in multiple cells
Genetically controlled	Caused by overwhelming noxious stimuli
Late loss of membrane integrity	Early loss of membrane integrity
Cell shrinkage	Cellular and nuclear swelling
Condensation of nuclear contents	
Laddering of chromosomal DNA	Chromatin disintegration
No inflammatory reation	Inflammatory reaction

Table 1. Summary of human diseases brought about by dysregulated apoptosis and the difference between apoptosis and necrosis.

infected cells lead to caspase-9 and caspase-3 activation. These events are preceded by phosphorylation of p53 and subsequent up-regulation of Bax which causes release of cytochrome c [56].

Genetic mutations can also cause dysregulated apoptosis which can lead to cancer. The tumor suppressor gene, p53, prevents cell cycle progression due to DNA damage. Without functional p53, cells continue to divide without repairing DNA breaks or mutations. Failure to repair the DNA damage can kill the cell, or worse, may allow the cell to proliferate with a corrupted genome. The importance of p53 is underscored by the fact that p53 is deficient in over half of all human cancers [57]. The function of p53 is critical to the efficacy of many cancer treatments since radiotherapy and chemotherapy act in part to trigger apoptosis by causing DNA damage. Consequently, cancer patients that have a p53 deficiency, have a less favorable prognosis [55]. Part of the value of having a functional p53 resides in the fact that it has been shown to be able to induce the expression of the proapoptotic BH3-containing proteins, Bax and Noxa and the repression of Bcl-2 [58], [59], [60]. Another way in which failure it initiate apoptosis can lead to cancer is by overexpression of the antiapoptotic protein, Bcl-2. In human follicular lymphoma, translocation of chromosomes 14 and 18 leads to up-regulation of Bcl-2 expression [61]. Levels of Bcl-2 are elevated in various other cancers including lymphomas, leukemias, adenosarcomas, renal and lung cancers, neuroblastomas and melanomas [62], [63].

Cells can also die as a result of necrosis which is distinct from apoptosis in several regards. The most important difference is that necrosis is pathological in nature and leads

to an inflammatory response (Table 1B). Necrosis has traditionally been thought to be induced by physical damage or overwhelming noxious stimuli, but recent evidence suggest that it can be induced by signaling through Fas in caspase-8 deficient human lymphocytes which is dependent on the DED domain of FADD [64]. Necrotic cell death is associated with loss of the mitochondrial-transmembrane potential (MTMP), ROS generation and the conspicuous absence of cytochrome c release which could account for some of the phenotypic distinctions between these two modes of cell death.

T cell Selection and Glucocorticoid Effects

The development and function of the cells that comprise the immune system are subject to both intrinsic and extrinsic factors. A variety of ligands and receptors induce cells to become activated, to migrate, adhere, and to express effector functions. Cytokines, which are typically glycoproteins, are secreted in an inflammatory or immune response in order to modulate the intensity or nature of immune action. Another class of molecules that participate in the modulation of immune function belong to the neuroendocrine system. Neuroendocrine influences can and do participate in shaping the immune response.

Lymphocytes, derived from hemopoietic stem cells, migrate via the blood to the thymus where they mature (Figure 3A). Thymocytes undergo an ordered progression of phenotypic changes as they develop from early precursors into mature T cells. Immature thymocytes are double negative for expression of markers that will specify helper T cell

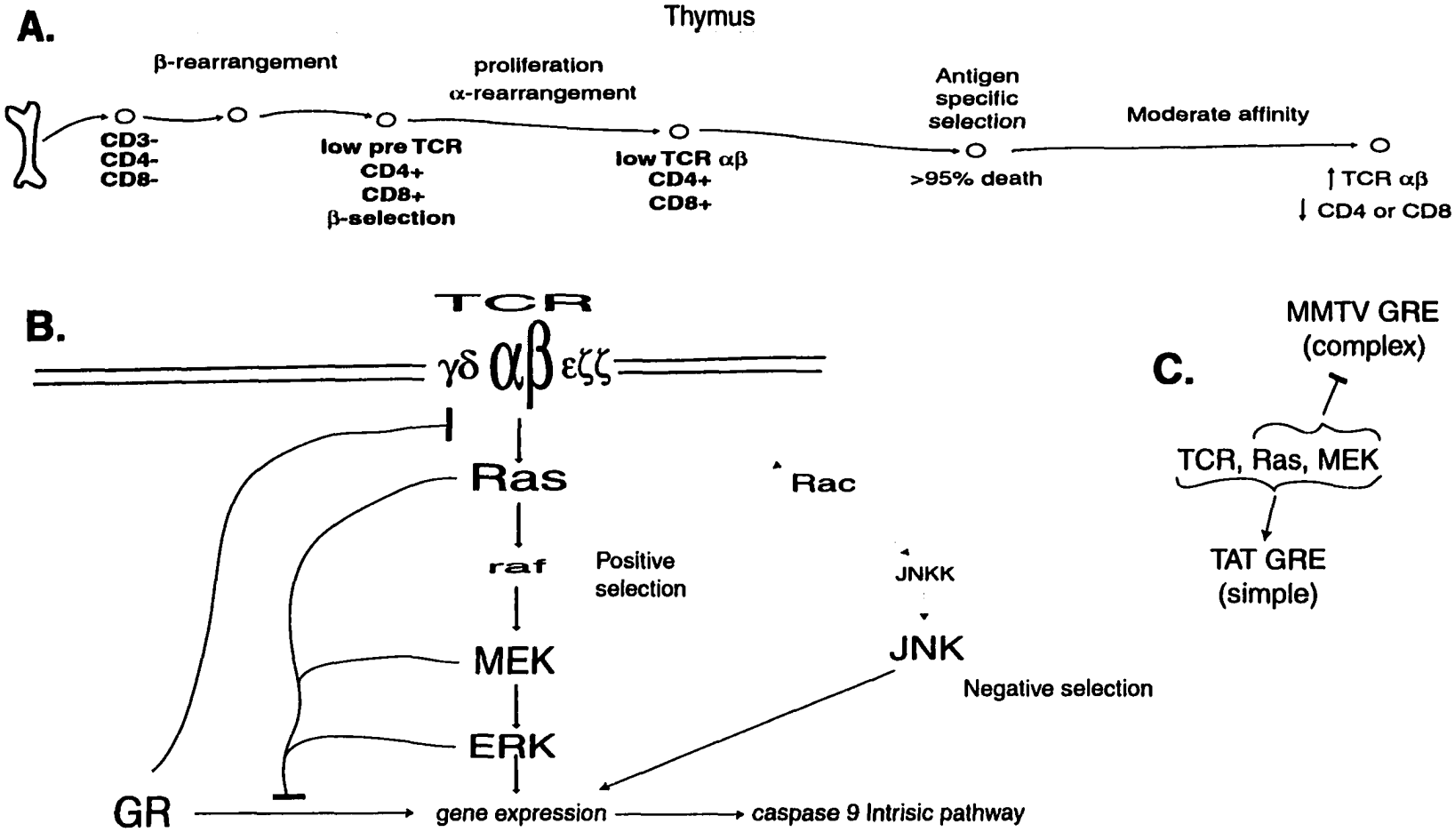


Figure 3. Thymocyte development and GR/TCR signaling involved in the selection process.
(A) Representation of the development of thymocytes. Pluripotent stem cells in the bone marrow give rise to precursor thymocytes which migrate to the thymus and mature. (B) Schematic representation of crosstalk between GR and TCR signaling. (C) The effect of TCR signaling components on the transcriptional activity of GR response elements (GREs).

(CD4-) and cytotoxic T cell function (CD8-). Thymocytes that productively rearrange the TCR β gene locus express a pre-TCR with subunits of γ, δ, ε, a ζ homodimer, and TCR β heterodimerized with the non-polymorphic pre-TCR α chain. Once the β chain is expressed as a component of the pre-TCR complex, signals associated with this turn off further rearrangement of the β chain (termed β selection), which leads to extensive proliferation and differentiation to double positive (DP, CD4+CD8+) thymocytes, while turning on the process of TCRα chain gene rearrangement [65]. Productive rearrangement of the TCRα locus results in low level expression of TCRαβ and antigen specific selection of favorably rearranged TCRs. TCR recognition of antigen requires concomitant recognition of major histocompatability complex proteins (MHC) via the CD4 or CD8 coreceptors. Thymocytes that don't recognize MHC proteins are eliminated (death by neglect) as are the thymocytes that have TCRs that bind to strongly to MHC/self-peptide complexes (autoreactive). This selection process eliminates >95% of all thymocytes developing in the thymus and preserves those with functional potential [66]. DP thymocytes that express receptors with a moderate avidity for the MHC/self-peptide undergo positive selection, which is accompanied by up-regulation of TCR levels and down-regulation of either CD4 or CD8 to give functional single positive (SP) cytotoxic or helper T cells.

There is increasing evidence that positive and negative selection signals diverge in the MAPK pathway (Figure 3B). Transgenic mice expressing dominant negative forms of Ras, Raf, MEK-1 or ERK have decreased positive but unaltered negative selection, whereas constitutively active v-Raf enhances the generation of TCR^{hi} SP cells [67], [68],

[69], [70]. Thymocytes of transgenic mice expressing a dominant negative JNK-1 were resistant to anti-TCR mediated deletion and modulation of p38 kinase activity implicated this kinase as another MAPK family member important for negative selection [71], [72]. These data support roles for the MAPK pathway in positive selection and the JNK/p38 pathway in negative selection, although recent evidence suggests a role for MEK in negative selection, since blocking MEK activity greatly reduced deletion of DP thymocytes [73].

Other signaling pathways modulate TCR effects due to the expression of many coreceptors, cytokine receptors, and adhesion molecules that can transmit intercellular signals that alter TCR-mediated signaling. Examples include CD2, CD5, and Thy-1 that act as negative regulators of TCR signaling and therefore could modulate thymocyte selection [74], [75], [76]. Mice lacking these molecules showed varied responses in thymocyte selection which may be attributable to the individual TCR transgenic systems used. Other accessory molecules including CD30 and CD40 transduce death signals independently of the TCR and may also have a role in negative selection [77], [78]. Soluble mediators, such as steroids, can also trigger signaling in thymocytes. Steroids are small lipophilic molecules that can participate in normal and pathological processes. One class of steroid, the glucocorticoids (GCs), have been long known to affect the thymus and have also been implicated in thymocyte differentiation and selection. It was observed as early as the 1920s that adrenalectomy, (the main source of glucocorticoids), resulted in thymic hypertrophy demonstrating that circulating levels of GCs can have a constitutive

repressive effect on the thymus and provided an early link between the neuroendocrine and immune systems [79].

The effects of GCs on lymphoid cells are dramatic and include G1 cell cycle arrest and apoptosis in immature thymocytes, pre-B lymphoma cells, mature peripheral T lymphocytes, and several leukemic cell lines [80], [81], [82], [83]. This has lead to their extensive use and efficacy in the treatment of corresponding leukemias and lymphomas. GCs have antiproliferative effects in many cell types and are widely used for their anti-inflammatory and immunosuppressive effects.

Glucocorticoid Receptor Biology and Immune Function

The effect of GC signaling is mediated by the glucocorticoid receptor (GR) which is a member of the steroid hormone receptor family of transcription factors that also includes the mineralocorticoid (MR), progesterone (PR), estrogen (ER), and the androgen receptors (AR) [84], [85]. These steroid receptors share a domain organization consisting of a highly homologous DNA-binding domain (DBD), a hormone-binding domain (HBD), and an N-terminal domain (NTB). The 65 amino acid DBD is composed of two zinc fingers that make specific contacts with nucleic acid sequences. This domain is also involved in homodimerization and interactions with other proteins. The C-terminal HBD is required for ligand binding, dimerization and also has a ligand dependent transactivation function that is also important for nuclear translocation (AF-2 region), [86]. The NTD contains a transcriptional activation function (AF-1) that works independently of ligand binding [87]. The overall activity of GR is controlled by the

HBD through interactions with cytosolic non-receptor proteins including hsp90, hsp70 and FKBP506 [88], [89]. This association is maintained until GCs diffuse into the cell and bind to GR which leads to nuclear translocation and binding to glucocorticoid response elements (GREs). GREs are comprised of two hexanucleotide repeats separated by a few nucleotides. After dimerized GR is bound to the GRE, interactions with cofactors and the basal transcription machinery, either directly or indirectly, leads to transcriptional activation or repression. Despite the vast knowledge of the effects of GCs on the tissues of the body, the molecular mechanisms leading to GC-induced apoptosis remain poorly understood. What does appear clear is that GR is required for the process, and loss of mitochondrial potential and the subsequent activation of caspases is the result [90], [91], [92], [93].

It has been hypothesized that GR may transmit the death signal by activation of death-inducing genes and/or repression of survival genes [94], [95], [96]. Several candidate genes, showing GR regulation, have been identified including the oncogene c-myc, which is down-regulated in multiple cell lines [97]. It has been proposed that down-regulation of c-myc may lead to inhibition of cell proliferation and induction of apoptosis in lymphocytes. GR induced genes include AP-1 family members c-jun [98] and c-fos [99], calmodulin [100], clusterin (TRPM-2) [101], and several other genes identified in murine thymocytes [102], [95]. Nevertheless, none of these glucocorticoid regulated genes have been shown to be necessary for the induction of apoptosis in lymphoid cells.

GR can stimulate or repress gene transcription through interaction with the c-jun homodimer [103] or a c-jun/c-fos heterodimer [103] at the AP-1 site, respectively. GR

can repress the function of NFκB by direct binding and also by increasing the expression of IκB which is the cytosolic protein responsible for maintaining NFκB in an inactive state [104]. Since AP-1 and NFκB are both involved in cytokine signaling, this may represent a possible mode of action for GC-induced apoptosis in lymphocytes. GR-mediated inhibition of NFκB is dependent on the catalytic subunit of PKA and GR functioning appears to be regulated by cytoplasmic PKA-associated signaling [105]. This is important because inhibition of NFκB by activated GR has been suggested to play a role in GR-mediated anti-inflammatory responses [106]. Other recent work has shown that calcitonin-related gene peptide (CRGP) is able to selectively inhibit NFκB (but not AP-1) mediated transcription in thymocytes *in vivo* and *in vitro* and this effect was correlated with apoptosis [107]. CRGP was found in a subpopulation of epithelial cells at the corticomedullary junction in the thymus and thymocytes were found to express functional receptors for CGRP [108] potentially implicating this neuropeptide in maturation and/or selection of T cells.

Pharmacological levels of GCs induce CD4+CD8+ thymocyte apoptosis, but death is not the only outcome. Signals delivered by the TCR or GR separately result in the death of thymocytes and T cell hybridomas. Unexpectedly, simultaneous exposure to both stimuli can result in cell survival [109], [110]. This is an example of mutual antagonism and has been proposed as an explanation for how a potentially negative TCR signal can be negated to allow survival (positive selection) [111]. In this model (Figure 4), biologically relevant TCR signaling alone, regardless of the avidity with which it is bound, is not a positive signal. Instead, these signals lead to apoptosis unless

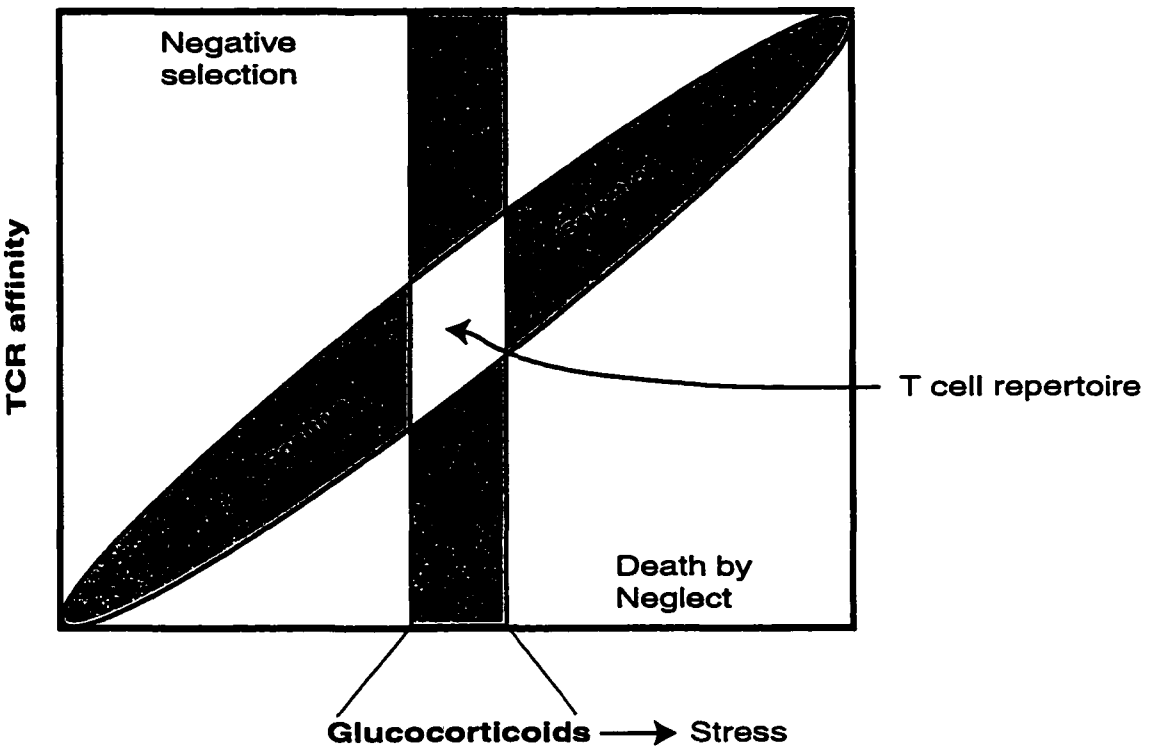


Figure 4. Model for thymocyte selection in the thymus.

Positive selection of thymocytes is based on balance signaling from GCs and also from the TCR. TCRs with high affinity to MHC/Antigen are negatively selected and those with low/no affinity die as a result of neglect. This model is supported by the observation that stage specific GR antisense expression in mice causes decreased thymic size and greatly reduced lymphoid mass (TCR+CD4-CD8- cells). Also consistent with this model is the observation that mice with spontaneous autoimmuntity and human multiple sclerosis patients have elevated circulating levels of GCs, which according to the model, would result from thymocytes being selected with higher affinity to self-antigen.

counteracted, and there is evidence that these two signaling pathways are engaged in crosstalk. The proposal for crosstalk between the TCR and GR signaling pathways is supported by the identification of two glucocorticoid-induced genes that appear to block TCR-mediated apoptosis in T cells [112]. Conversely, TCR-mediated activation of the mitogen-activated protein kinase kinase/extracellular signal regulated kinase (MEK/ERK) cascade via Ras is necessary and sufficient to inhibit GR-mediated death in thymocyte cell lines and primary T cells [113]. A contributing factor to this observed inhibition may reside in the fact that the signaling components including Ras, MEK and CD3, had differential effects on simple and complex GREs (Figure 3C). It appeared as though TCR signaling had an inductive effect on simple GREs, which only require GR for activation, and a repressive effect on complex GREs [113]. By this logic, TCR-mediated antagonism of GR-regulated apoptosis may suggest that GR-regulated death-inducing genes may be under the control of complex GREs and/or simple GREs may regulate the expression of survival genes.

Despite an immense effort to understand the role of GR in thymocyte selection and apoptosis, no clear connection between a GR-mediated effect and mitochondrial dysfunction has been established. GR is expressed in essentially all cell types which are also exposed to circulating levels of corticosteroids, yet cell types of varying lineages have unique responses to activated GR. The fact that GCs kill immortalized and primary thymocytes from many different genetic backgrounds, as evidenced by the immunosuppressive action of corticosteroids, indicates that a common mechanism is

likely responsible for this observation. It could be as simple as the induction of a gene or genes that stimulate the eventual activation of the caspases, or it could be a complex composition of transcriptional regulation combined with essential inhibitory/stimulatory protein-protein interactions that vary depending on the state of differentiation, and the local environment which determines the fate of the cell.

CHAPTER II

MATERIALS AND METHODS

Cell Culture and Transfection.

Human and mouse cell culture was performed at 37°C, in 8% CO_2 and 90% humidity. Cells were grown in Dubelco's Modified Eagle Medium (DMEM) with 10% charcoal stripped, heat-inactivated calf bovine serum (Hyclone), and 1% Pen/Strep. Exceptions were the immature thymocyte cell line 2B4.11 and HeLa cell lines which were both grown in the same media as above except the calf bovine serum was not charcoal stripped or heat inactivated. Suspension cells were typically maintained at a density between 100,000 and 700,000 cells/milliliter and harvested for experiments at a density between 400,000 and 600,000 cells/ml. For transient transfection assays via electroporation, cells were harvested and rinsed with 0.2μ filtered HBS (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM glucose at pH 7.05), at 37°, and resuspended in HBS at $10x10^6$ cells/ml. Cells were placed on ice for 10 minutes, aliquoted into 1ml cuvettes and electroporated at 300 volts and 1180μF after being mixed by inversion with the cesium pure plasmid DNA. Cells were allowed to recover for 10 minutes at room temperature, added back to 50% conditioned/50% fresh media and incubated until further analysis. Transfection into HeLa cell lines was done using a 5:1 lipofectin to DNA mix that was incubated in 200μl of DME at room temperature for at least 15 minutes to allow the

cationic lipid to bind the DNA. This solution was added to a minimal volume of DME (3mls for a 10cm plate) covering the cells to be transfected. The transfection proceeded for two hours, followed by aspiration of the transfection solution and the addition of 10mls of growth media. Finally, the cells were incubated until further analysis.

Northern Blot Analysis.

RNA was isolated using the guanidinium lysis procedure. Briefly, cells were homogenized in a denaturing solution (4M guanidine isothiocyanate, 25mM sodium citrate, 0.5% Sarkosyl and 0.1M βME), mixed sequentially with 2M sodium acetate (pH 4), phenol, extracted with chloroform/isoamyl alcohol and precipitated with isopropyl alcohol. The pellet was resuspended in denaturing solution, precipitated as before and resuspended in either H_2O or formamide. The total RNA was quantitated by UV absorption spectroscopy at A_{260} and frozen at -80°C (-20°C for formamide) or loaded on a 1% agarose formaldehyde gel after adding loading dye, denaturing at 65°C for five minutes and ice quenching. Total RNA gels were loaded using 10μg/lane (poly-A RNA 1μg/lane) and run at 150V for 1-3 hours for size separation in 1X running buffer (0.05M HEPES, 0.001M EDTA, 0.005M sodium acetate, 0.01M NaOH at pH7). After electrophoresis, the gel was soaked in 20X SSC for 30 minutes, and transferred to a nylon membrane (Duralon) over night using capillary action. RNA was then cross-linked to the membrane using a UV Stratalinker 2400 (Stratagene) and the auto cross-link option. The membrane was placed in pre-hybridization solution containing 50% formamide, 50 mM

KPO4, 5X SSC, 2X Denhardts, 0.2% SDS, pre-boiled 0.25mg/ml yeast tRNA and allowed to incubate with agitation at 42°C for 2-24 hours. Following the pre-hybridization step, a α P^{32} dCTP (Amersham) labeled DNA probe was synthesized (Prime-A-Gene Kit, Promega) that had been boiled for 10 minutes and ice-quenched was added to the prehybridization solution at $>1X10^6$ cpm/ml. The hybridization proceeded for at least 16 hours at 42°C with agitation after which the blot was rinsed briefly in low-stringency solution (1X SSC, 0.1% SDS), washed twice at 42°C for 15 minutes in low-stringency solution, washed twice at 55°C for 15 minutes in high-stringency solution (0.2X SSC, 0.1% SDS), wrapped in saran wrap and exposed to film at -80°C.

Library Screening.

Several libraries were screened including a random/oligo dT primed Dex induced WEHI 7.2 Lambda Zap II cDNA library [114], a GIG18/1/oligo dT primed 8 hour Dex induced WEHI 7.2 Lambda Zap II cDNA library [115], or a human placenta 5'-STRETCH PLUS Lambda gt10 cDNA library (Clontech). Typically, 30,000 to 50,000 plaques were plated on 12-15 cm plates, incubated at 37°C in a lawn of XL1-Blue bacteria that were grown in LB supplemented with 0.2% maltose and 10mM $MgSO_4$ to an A_{600} of 0.3 prior to plating. After plaque development, Protran® nitrocellulose filters (Schleicher and Schuell) were placed on top of the plaques for 1 minute, carefully removed, and inverted for a stepwise denaturation in 0.5M NaOH solution, neutralization in 1M Tris(pH7.5) and incubation in 2XSSC/0.2M Tris(pH7.5). Phage DNA was cross-linked to the membrane, probed with

labeled pGIG18.10 DNA fragments (the first library-derived GIG18 sequence) or fragments from previous rounds of library screening. Washes were performed as in the northern blot procedure except the high stringency wash was done at 65°C instead of 55°C for >30 minutes per wash. Plaques were picked and replated until purified phage were isolated (usually three rounds). DNA was rescued from the Lambda Zap II libraries by infecting SOLR bacterial cells with the purified phage in the presence of helper phage and plating the infection on 100μg/ml ampicillin plates that were < 25 days old. For the human placenta library, high titer stocks of the purified phage were obtained after infection of host strain C600 Hfl. The Lambda DNA was purified, digested with EcoRI restriction enzyme and the excised fragment was cloned into a convenient cloning/sequencing vector (pBSK-). Sequencing of the inserts was done using the University of Arizona LMSE core sequencing facility with either vector primers or specifically designed GIG18 primers (GIBCO BRL). Analysis and assembly of the clones was performed using AssemblyLIGN™ software (Oxford Molecular Group). ESTs that were purchased (Research Genetics) for northern blots were also sequenced through LMSE prior to being used as probes to confirm their identity.

Construction of Expression Plasmids.

All expression plasmids were constructed using standard molecular biological techniques [116]. For most plasmids, primers specific to either GIG18 or eGFP (Clontech) were designed with appropriate anchor and restriction sequences built into the 5' end of the

primers and ordered on-line (Gibco BRL, Life Technologies). PCR reactions were optimized to generate the desired products using PLATINUM® P*fx* Polymerase (Gibco BRL) which has higher fidelity than Taq polymerase and also possesses 3' to 5' proofreading activity. The resulting PCR products were purified on 1% L.M.P. Agarose (Gibco BRL) gels or on a QIAquick PCR purification column (Qiagen), digested with the appropriate restriction enzymes (along with the vector into which the fragments were to be cloned), EtOH precipitated, resuspended and ligated in molar ratio of 3:1, insert:vector. Ligations were performed at room temperature or 16°C depending on the particular cloning strategy. DH5α bacterial cells were transformed with a 1:10 dilution of a fraction of the ligation reaction, plated on agarose plates. Colonies were picked and inoculated into 2 mls of broth for subsequent minipreps and verification of correct insertions.

Plasmid Preparations.

Large scale pure plasmid DNA preparations were obtained by a double-cesium chloride gradient method. Briefly, 1 liter of Frank's super broth (12g tryptone, 24g yeast extract in 900mls of H_20, pH7.5, plus 100mls of KPO_4/glycerol stock), with the appropriate antibiotic, was inoculated with a 2 ml overnight culture of bacteria expressing the plasmid of interest. This was grown overnight at 37°C with shaking (220rpm) and the cells were harvested after 16-20 hours. The cells were lysed using freshly prepared 10mg/ml lysozyme solution in TE and the DNA was separated from the cellular debris by

centrifugation. This was followed by an EtOH precipitation step, 70% EtOH wash, resuspension in TE and a LiCl precipitation to remove the remaining debris. After an isopropanol precipitation, the DNA was resuspended in 3.8 mls of TE. Four grams of CsCl and 120µl of stock ethidium bromide were added to this solution followed by loading into two Quick-Seal centrifuge tubes (Beckman). The tubes were centrifuged overnight at 45,000 rpm in the vti65.2 rotor until there was deep-red plasmid band in the middle of the gradient (usually 12-16 hours). This band was extracted using a syringe and loaded onto a second gradient to complete the purification. The EtBr was extracted using 20X SSC saturated isopropanol followed by two more precipitations and finally resuspension of the plasmid in TE. Quantitation of the DNA was determined by UV spectroscopy.

Generation of GIG18 Protein for Rabbit Immunization.

The entire 1290 base pair GIG18 open reading frame was PCR amplified and cloned into the pET-20B(+) expression vector (Novagen) with restriction sites NdeI and XhoI engineered into the amino terminal primer and carboxy terminal primer, respectively. The pET-20B(+) expression vector adds a 6X-HisTag to the protein that is subsequently used for purification on a Ni-NTA column. This plasmid was transformed into BL21 DE3 cells (Qiagen) and three colonies were picked and tested for IPTG (1mM final concentration) induction of the fusion protein. The colony that showed the best induction for a protein of about 50kDa molecular weight was picked for large scale production. Briefly, a lawn of

this colony was grown on an ampicillin containing plate, the bacteria were scraped of the plate and inoculated into 1 liter of LB (with amp), grown to an A_{600} of ~0.5 at which point the IPTG induction began and proceeded for 5 hours. The culture was harvested and the cell pellet was frozen at -20°C. Next, the cell pellet was thawed on ice for 15 minutes and lysed in 8M urea; 0.1M NaH_2PO_4; 0.01M Tris-Cl, pH8 (at 5mls/g wet weight of the cell pellet). The cells were stirred for 60 minutes, until the lysis was complete and centrifuged at 10,000Xg for 30 minutes to pellet the cellular debris. One milliliter of the Ni-NTA slurry was added to the lysate and this was mixed at 200 rpm for 60 minutes at room temperature to allow binding of the HisTag to the Ni. This mixture was loaded onto a column and washed two times with 4 mls of a solution containing 8M urea; 0.1M NaH_2PO_4; 0.01M Tris-Cl, pH6.3. The bound protein was eluted from the column with 4 X 0.5mls of 8M urea; 0.1M NaH_2PO_4; 0.01M Tris-Cl, pH5.9 followed by 4 X 0.5mls of 8M urea; 0.1M NaH_2PO_4; 0.01M Tris-Cl, pH4.5. A PAGE was run to monitor the purification process, fractions that contained the eluted GIG18 protein were combined and concentrated using Centicon-30 column (Amicon). Three milliliters of 1mg/ml concentrated GIG18 protein was sent for immunization of two rabbits (HTI Bio-Products, Inc.).

Western Blotting.

Cell were collected, rinsed once with PBS and resuspended in 200µl PBSTDS/inhibitor solution which consisted of 1xPBS, 1% Triton X100, 0.5% sodium deoxycholate, 0.1%

SDS, 1mg/ml leupeptin, 1mg/ml aprotinin, 1mM EDTA and 0.5M PMSF. Lysates were centrifuged to remove cellular debris and the protein samples were quantitated using a BCA assay (Pierce). Protein samples was loaded on a denaturing SDS 12% polyacrylamide gel (10-40μg protein/lane), run at 10-50 mA for 1-2 hours and blotted onto a nitrocellulose membrane overnight (Trans-Blot Transfer Medium, BIO-RAD). The following day, the blot was incubated in TBST (10mM Tris-HCl pH8, 150mM NaCl, 0.05% Tween 20) plus 3% nonfat dry milk for 1 hour at room temperature and incubated with a 1:2000 dilution of 50% glycerol stock of GIG18 Ab for 1 hour in TBST plus 0.5% nonfat dry milk. After multiple washes in TBST at room temperature, the secondary antibody conjugated to horseradish peroxidase (HRP) was added at a dilution of 1:20,000 in TBST plus 0.5% milk and incubated at room temperature for 30 minutes. The HRP signal was detected by chemiluminescent substrate (Pierce) and 1-10 minute exposures to film.

Generation of HeLa On Stable Cell Lines and Growth Curve Analysis.

HeLa On cells (Clontech) were grown to 80% confluency on 15 cm tissue culture plates. transfected by lipofection (5:1, lipo:DNA) with the expression plasmid of interest and the tk-Hygro selection plasmid in a molar ratio of 13:1 in DMEM for two hours in the tissue culture incubator. The cells were rinsed with PBS, harvested after being trypsinized and plated on multiple 10 cm tissue culture plates at a predetermined density in the presence of 400μg/ml hygromycin B (Calbiochem). The selection process was allowed to proceed

for 2-4 weeks during which time colonies would be picked and expanded on new 10cm plates containing coverslips for +/- tet experiments. Using UV microscopy, individual picks would be scored for background level of GFP expression and a subjective analysis of level of induction. All colonies with low GFP background expression and good tet-regulated induction were more objectively analyzed using FACS (Becton-Dickenson FACS Scan with Lysis II software). Growth curve analysis was performed on several stable cell lines that showed minimal background GFP expression and good tet regulated induction. To do this, cells were plated with and without tet (1μg/ml), harvested and counted daily for three days using a hemacytometer (Fischer Scientific).

GIG18 Expression in *Saccharomyces Cerevisiae*.

The 1290 base pair GIG18 open reading frame fused to the amino terminal of GFP was cloned into the galactose-inducible yeast expression vector pYES2 (Invitrogen) using the BamHI/NotI restriction sites in the multiple cloning site. The pYES2 vector contains the URA3 auxotrophic marker that allows selection of this plasmid in *ura-* yeast strains (uracil deficient). After transformation and plating of the yeast, using standard methods [117], individual colonies were picked and re-streaked on ura- plates to ensure a homogeneous population. Yeast colonies from the second plating were streaked on *ura-* plates with and without galactose and monitored visually for growth effects and using UV-microcopy to look for morphological differences. The yeast strains used in these

studies were YRP841 (care of the Roy Parker lab) and AAY217 (care of the Allison Adams lab).

CHAPTER III

CHARACTERIZATION OF GIG18 cDNA SEQUENCES ISOLATED FROM WEHI 7.2 CELLS

INTRODUCTION

Glucocorticoid mediated modulation of the cellular components of the immune system is well established. The basis for this modulation is complex and is dependent on both intrinsic and extrinsic properties of the system being observed. The immortalized immature thymocyte cell line, WEHI 7.2, is highly sensitive to corticosteroids and they succumb through the highly complex, multi-component expiration process referred to as apoptosis. GC-induced apoptosis is mediated through GR and transcriptional modulation is the triggering event. Evidence in support of this idea include the following observations: 1) inhibition of RNA or protein synthesis blocks the induction of GC-induced apoptosis in thymocytes, 2) a point mutation in GR that rendered it unable to bind DNA, demonstrated that thymocyte apoptosis required DNA binding by GR since the transgenic thymocytes were resistant to GCs [118], 3) deletion of the N-terminal transactivation domain of GR, while not affecting DNA or hormone binding, was able to inhibit GC-mediated apoptosis [119] and 4) substitution of the GR transactivation domain

with viral transactivation domains allowed retention of reporter gene inducibility and induction of apoptosis [95].

Hormone induced, receptor-mediated apoptosis is a common biological phenomenon. In flies, ecdysone induces apoptosis of larval salivary glands which is mediated through *rpr* and *hid* [120]. Thyroid hormone induced mRNA and protein synthesis are required for the destruction of the tadpole tail during *Xenopus* metamorphosis [121]. Many mammalian tissues are subject to hormone regulated apoptosis including mammary glands, testes, the prostate, ovaries and the uterus [122]. Glucocorticoid mediated apoptosis of immature thymocytes is the best studied vertebrate model for hormone regulated cell death but the molecular mechanism remains obscure.

A number of glucocorticoid regulated genes have been identified in lymphoid cells, but none have proven sufficient to induce apoptosis on their own. Identification of the GC signaling events that are crucial to thymocyte apoptosis will come as a result of characterizing the molecular events that occur during the process. One event, is the rapid and high induction of GIG18 and this dissertation describes its characterization.

RESULTS

Identification of a Glucocorticoid Regulated Transcript.

GIG18 was first identified through the use of a differential display PCR (DDPCR) strategy by Mark Chapman [114]. Differentially expressed sequence tags (DEST) were derived from reverse transcriptase PCR reactions using an RNA template population

isolated at 0 and 8 hour timepoints of 1μM dexamethasone (dex, a synthetic corticosteroid) treatment in WEHI 7.2 and in the derivative cell line WBcl-2. The two cell lines are essentially identical except that the WBcl-2 cell line has the Bcl-2 gene stably integrated, rendering it insensitive to the effects of GCs. The WBcl-2 cell line was used as a control to minimize the identification of genes that may become inconsequentially up-regulated as a result of cellular events associated with apoptosis.

Initial screens of a custom dex-induced mouse cDNA library led to the identification of a 2 Kb clone (pGig18.10) that when manually sequenced, revealed no obvious open reading frames (ORFs). This single clone was identified 11 times in the custom library and it was the only clone identified by the GIG18 DEST probe.

Database searches with the GIG18 sequence revealed that human GIG18 was expressed in placenta. Since our custom dex-induced library was exhausted for any additional GIG18 clones, a human placenta cDNA library was purchased and screened side by side with a second dex-induced mouse cDNA library with probes derived from human EST sequence or the pGig18.10 clone, respectively.

GIG18 Requires GR for Dex Induction and is also Induced by Ca^{2+}.

In a second immature thymocyte cell line, S49, which does not express GR and is insensitive to GCs, dex treatment did not result in up-regulation of GIG18 as determined by northern analysis (Figure 5). In addition, treatment of S49 and Wehi7.2 with 1μM A23187 (calcium ionophore) resulted in up-regulation of the GIG18 transcript. GIG18 was also induced by the sphingolipid, ceramide, and the GR antagonist, RU486. When

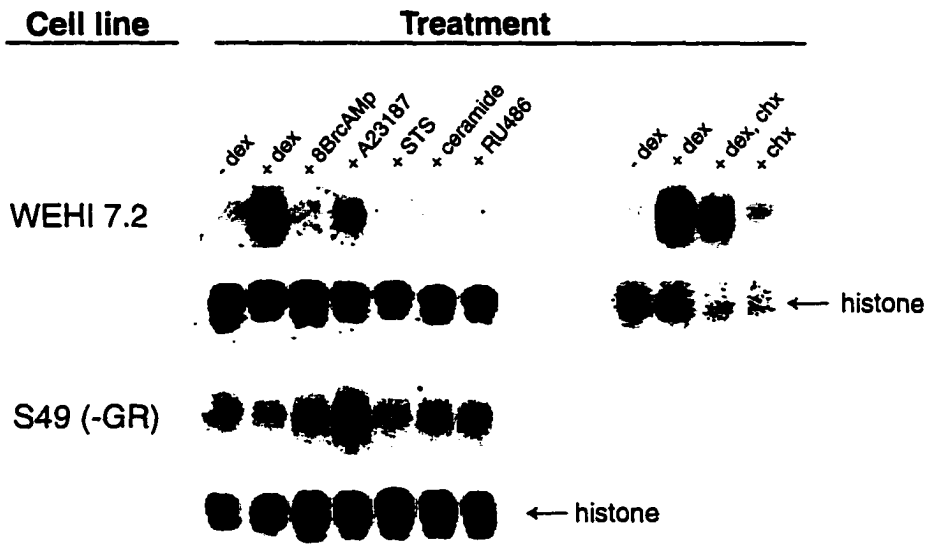


Figure 5. Regulation of GIG18 expression.

Northern analysis of GIG18 regulation under various conditions. Notice that dex induction of GIG18 is dependent on the glucocorticoid receptor as evidenced by the lack of expression of GIG18 in the GR- cell line S49. GIG18 appears also induced by calcium ionophore (A23187), ceramide and RU486 although it is less obvious in W7.2 because of the high background signal. GIG18 induction by dex appears to be partially inhibited by the protein synthesis inhibitor cycloheximide (chx), however, the histone loading control was also down-regulated in response to this treatment.

cycloheximide was added (10μg/ml final), simultaneously with dexamethasone to WEHI 7.2, the induction of GIG18 was cut approximately in half at 8 hours post-treatment. However, the cycloheximide also interfered with expression of the histone loading control (Figure 5).

GIG18 Induction is Greatly Reduced in WEHI 7.2 Mutant Cell Lines.

A chemical mutagenesis screen performed in our lab led to the acquisition of many GC resistant WEHI 7.2 derivative (Apt-) cell lines. These mutants have been characterized extensively in terms of their sensitivities to multiple apoptotic inducers including staurosporine, ceramide, A23187 and H_2O_2 [123], [93]. It was determined that basis for their resistance to dex was not due to GR mutations or lack of GR expression. rather, they all appeared to have mutations downstream of GR signaling. Several of these mutants were characterized by complementation assays which revealed that they had distinct mutations in GR mediated apoptotic pathway. Even though these Apt- cell lines were resistant to GCs, they were still sensitive to A23187. GIG18 was induced by A23187 in Apt3.8, Apt4.8, and Apt5.8 (Figure 6) in a correlative manner with the sensitivities of these mutants to A23187 [123]. Importantly, a highly diminished GC induction of GIG18 was seen in the same three mutants. A second GR+ immature thymocyte cell line (2B4.11), demonstrated that GIG18 regulation by dex was not a molecular event specific to WEHI 7.2. It was also found that GIG18 was induced by A23187 in a profile similar to WEHI 7.2 (Figure 6).

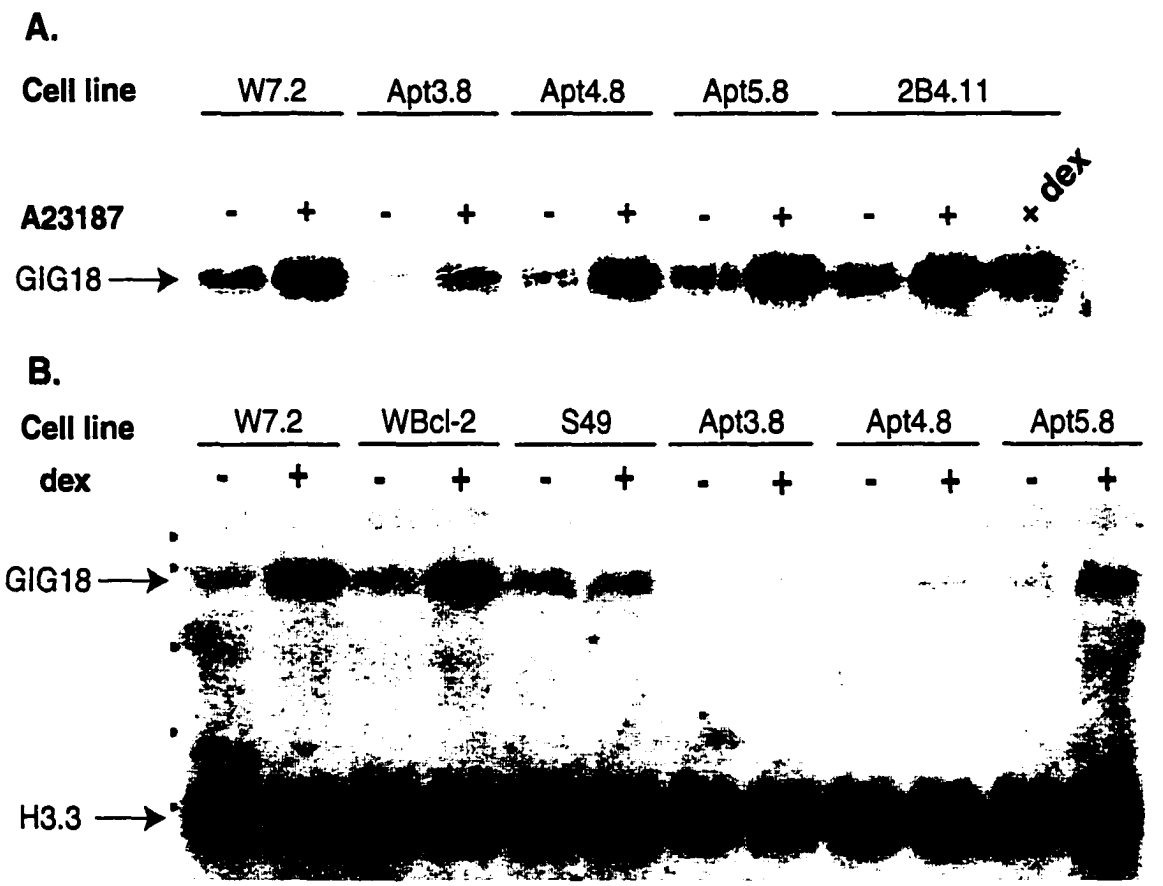


Figure 6. GIG18 expression in wildtype and mutant cell lines.

(A) Regulation of GIG18 expression by calcium ionophore in the parental cell line (WEHI7.2) and in three mutants that are deficient in glucocorticoid mediated apoptosis (Apt 3.8, 4.8, 5.8). The induction levels of GIG18 correspond to the relative sensitivities of the mutants to A23187. The T cell hybridoma cell line, 2B4.11, also shows GIG18 induction in response to dex and calcium ionophore. This latter observation supports the idea that GIG18 regulated expression by GR is not an idiosyncracy of WEHI7.2. (B) GC regulated expression of GIG18 is greatly reduced in the GC-insensitive mutant cell lines. Expression is normal in WBcl-2 which is a cell line that overexpresses the anti-apoptotic protein Bcl-2.

A GIG18 ORF was Identified in λ-clone Sequences.

Library clones obtained in each round of screening that extended the furthest in the desired direction were digested and used as probes on northerns to confirm that no ligation artifacts had introduced non-GIG18 sequence. The contiguous cDNA clones were assembled together with EST sequences obtained from database searches. Sequencing and assembly of approximately 6Kb of contiguous GIG18 cDNA sequence led to the identity of a 1290 base pair GIG18 ORF which corresponded to sequence found in the pGIG18.10 clone. A summary of just some of the clones that were used to identify the 1290 base pair ORF are diagrammatically illustrated in Figure 7.

Database Analysis Suggests Alternate Splicing and a GIG18 Gene Family.

The GIG18 ORF was used in multiple EST BLAST searches at the NCBI database which revealed that the same 1290 base pair ORF could be detected in minimally overlapping mouse ESTs, but many of the EST sequences demonstrated that alternately spliced GIG18 ORFs were also present. These alternately spliced products could be found in EST sequences from mice, cows, rats and humans (Figures 8 and 9). It was impossible to identify a complete alternate GIG18 ORF which could be due to the fact that the cDNA sequence became ~80% GC-rich. GC-rich sequences are known to be problematic for sequencing reactions and it appears that the reverse transcriptase reactions used to generate EST cDNAs also had difficulty processing this sequence. Repeated attempts to obtain the entire sequence of the alternate GIG18 ORF by means of library screens and database searches, provided ~200 base pairs of additional GC-rich

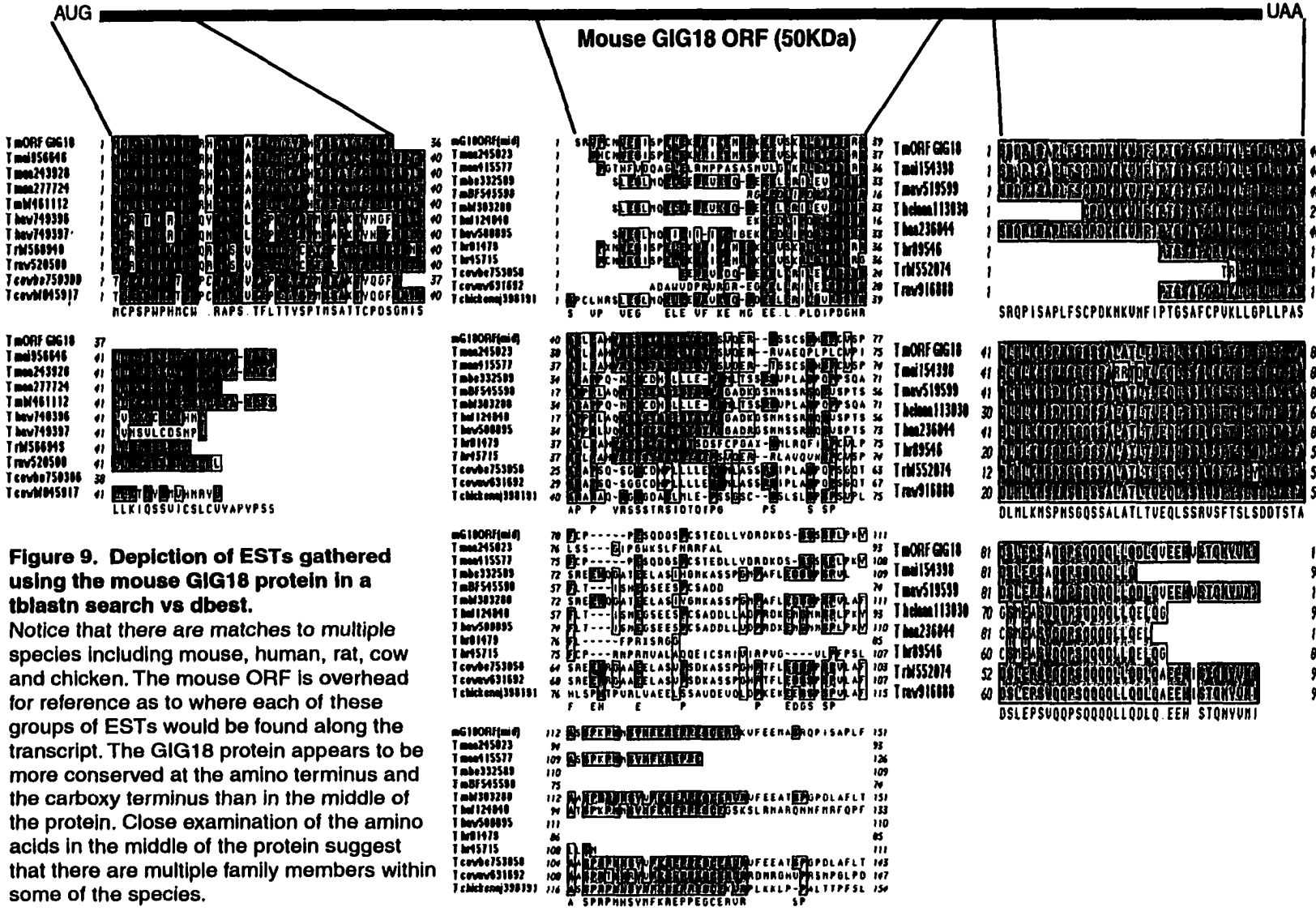


Figure 9. Depiction of ESTs gathered using the mouse GIG18 protein in a tblastn search vs dbest.

Notice that there are matches to multiple species including mouse, human, rat, cow and chicken. The mouse ORF is overhead for reference as to where each of these groups of ESTs would be found along the transcript. The GIG18 protein appears to be more conserved at the amino terminus and the carboxy terminus than in the middle of the protein. Close examination of the amino acids in the middle of the protein suggest that there are multiple family members within some of the species.

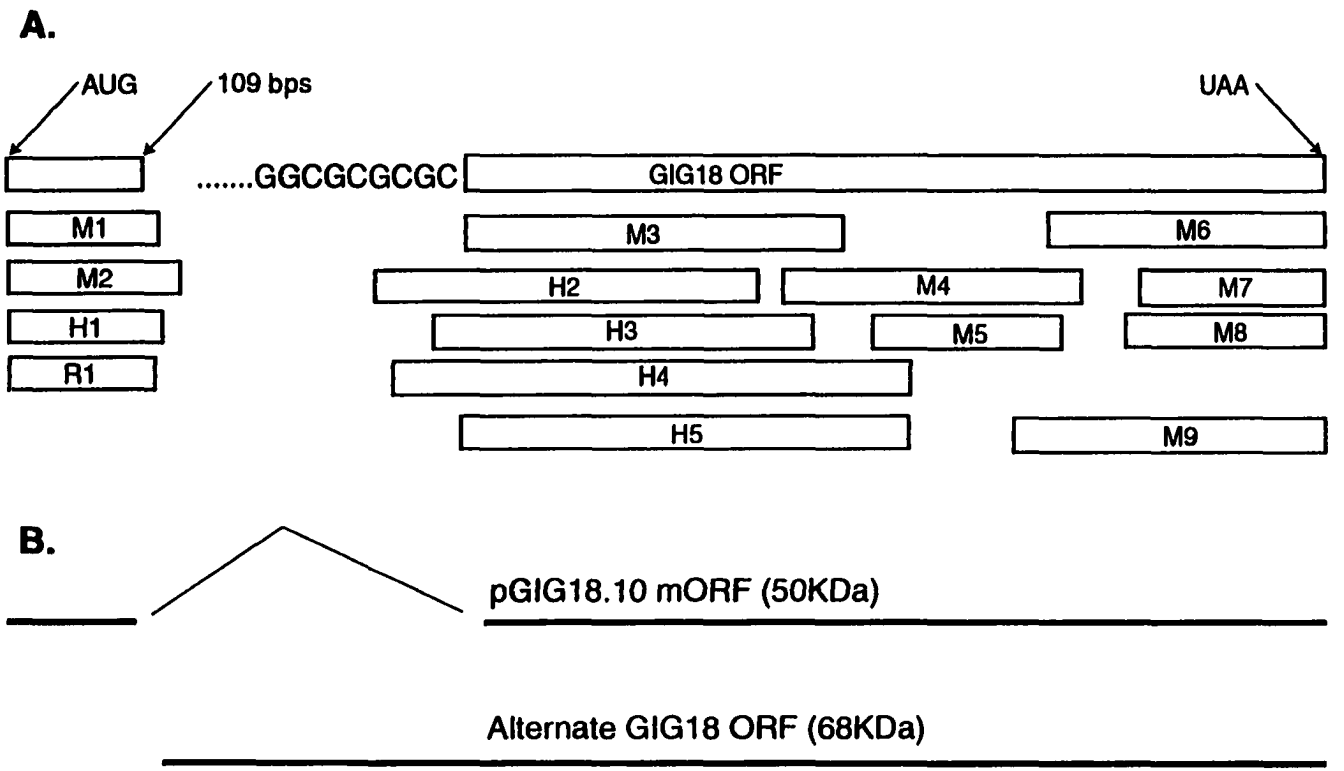


Figure 8. Relationship of GIG18 ORF to EST sequences and location of proposed alternate ORF.

(A) Notice that none of the ESTs traverse the entire GC rich area that was identified in several different clones although there are several that enter this region from both sides. The 3' side of the GC rich area is approximately 80% GC (based on 200 base pairs of sequence). Accession numbers are as follows: M1 (AA277724), M2 (AI956646), M3 (AI151800), M4 (AA245023), M5 (AA415577), M6 (AW519599), M7 (BE335749), M8 (AI286864), M9 (AA236844), H1 (AW749397), H2 (AI681256), H3 (AI936447), H4 (AW149532), H5 (AU122767), R1 (AW520500). M = mouse, H = human, R = rat. (B) Schematic representation of the ORF obtained from the pGIG18.10 clone and a proposed larger GIG18 that probably has its first exon(s) within the CpG island.

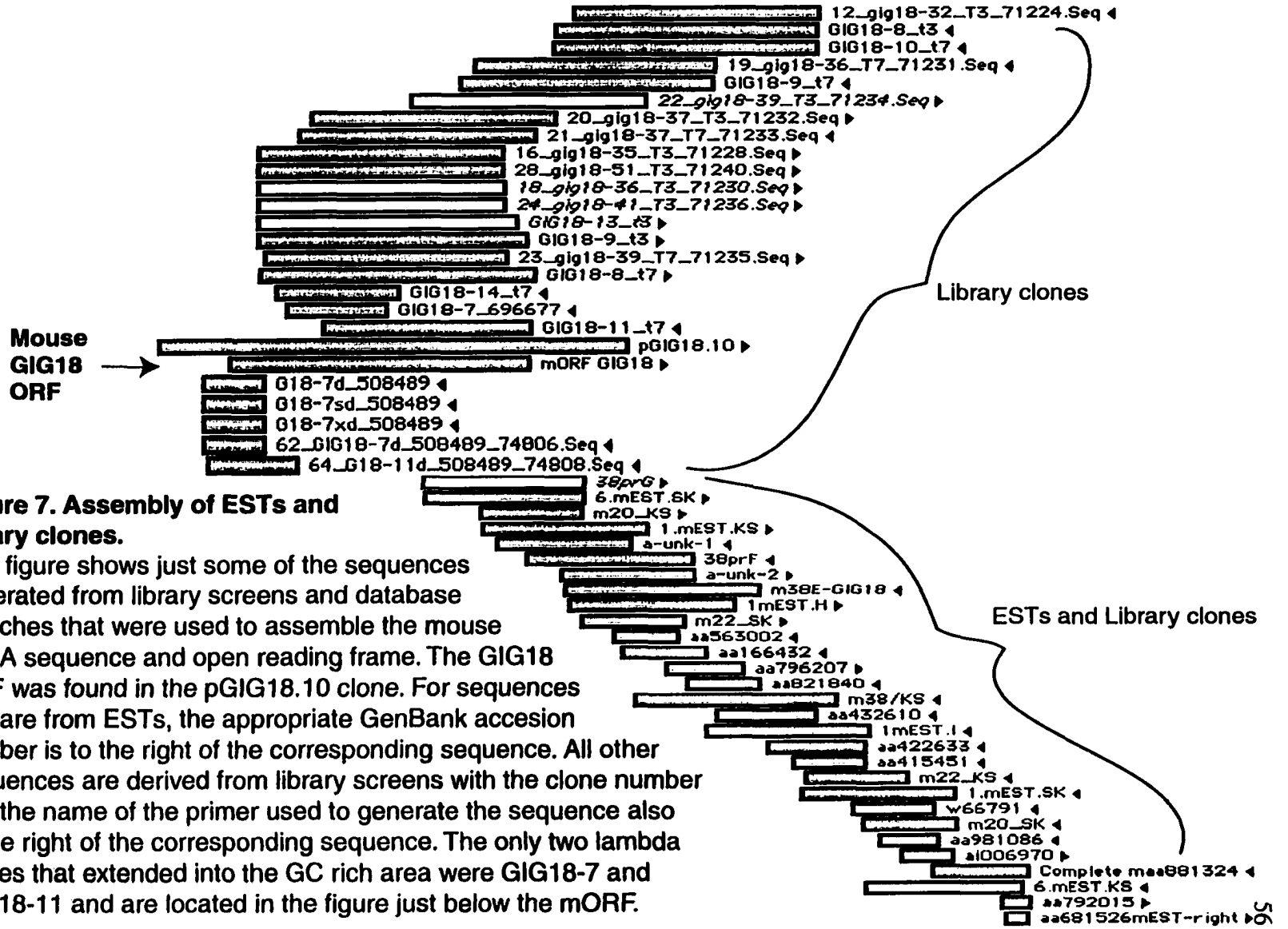


Figure 7. Assembly of ESTs and library clones.
This figure shows just some of the sequences generated from library screens and database searches that were used to assemble the mouse cDNA sequence and open reading frame. The GIG18 ORF was found in the pGIG18.10 clone. For sequences that are from ESTs, the appropriate GenBank accesion number is to the right of the corresponding sequence. All other sequences are derived from library screens with the clone number and the name of the primer used to generate the sequence also to the right of the corresponding sequence. The only two lambda clones that extended into the GC rich area were GIG18-7 and GIG18-11 and are located in the figure just below the mORF.

ORF, however, no cDNA clone contained the 5' end of the alternate ORF proposed in Figure 8. The GC-rich sequence may correspond to an alternate, downstream promoter that is not used by the GIG18 transcript contained in the pGIG18.10 clone.

Translation of EST sequences demonstrated that the amino and carboxy termini are more conserved than the central region when comparing multiple species (Figure 9). The presence of multiple GIG18 family members is indicated since highly-related, non-identical EST sequences were found within the same species (Figure 9). The existence of GIG18 family members was later confirmed by human genome sequence analysis (Chapter IV).

GIG18 Maps to the p21-p22 Region of Human Chromosome 7.

Using the mouse GIG18 ORF in a BLAST search against human genomic sequence, it was determined that the human homologue mapped to small arm of human chromosome 7 in the region of p21-p22. This region corresponds to homologous mouse sequences on chromosomes 5 and 6. The resolution of the mouse genomic sequence is not of high enough resolution at this point to accurately determine on which mouse chromosome GIG18 resides (Figure 10). The 15 Mb human chromosome 7 sequence depicted in Figure 10 shows homology to at least 4 separate mouse chromosomes suggesting a large degree of genomic shuffling since divergence from a common ancestor. The 250 Kb of human genomic sequence, through which GIG18 exons are found, is located between the ICA1 gene and the RPA3 gene. The GIG18 ORF is contained within two contiguous

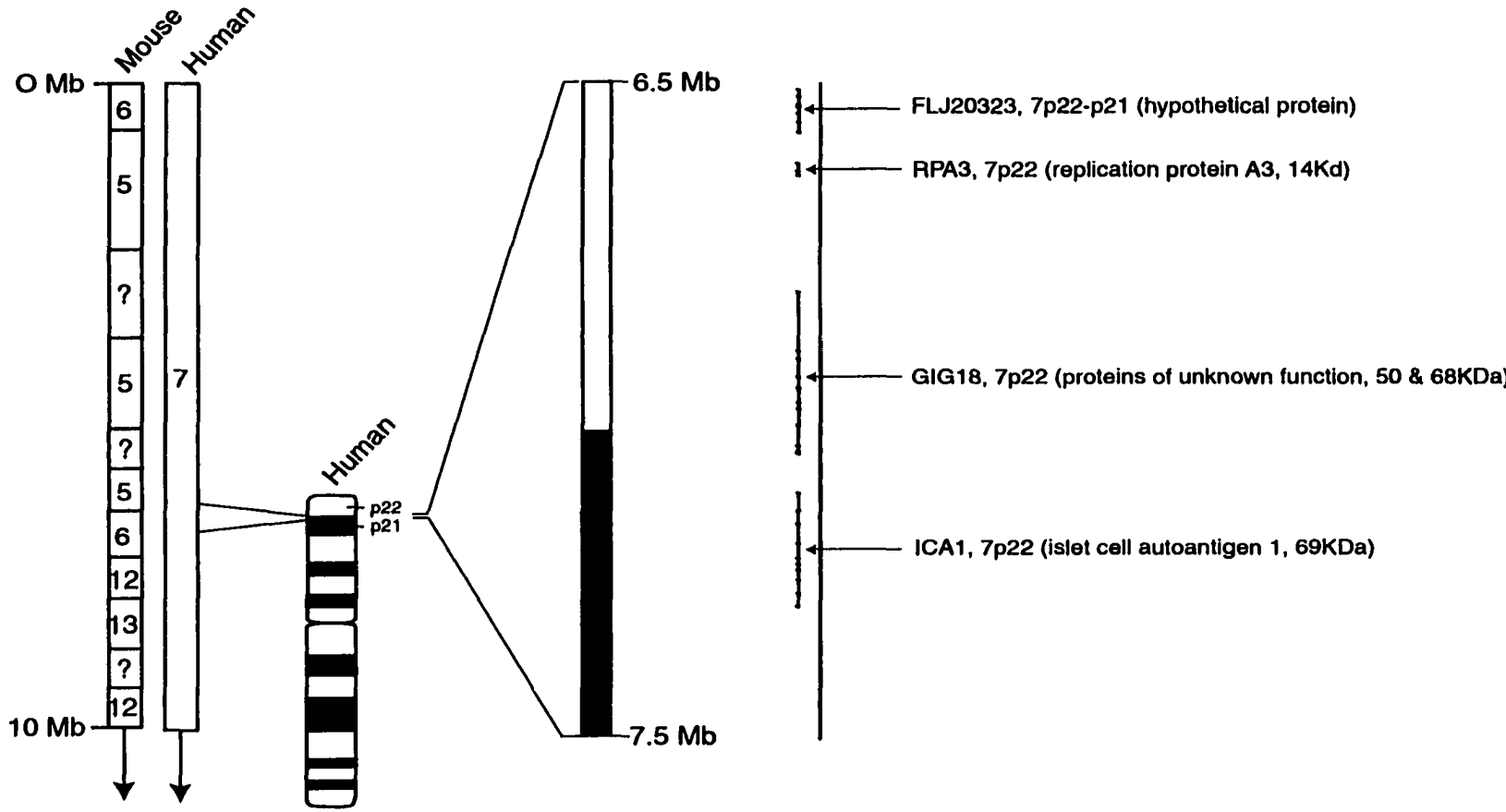


Figure 10. Chromosomal assignment of GIG18.

Depiction of the location of GIG18 on human chromosome 7 and and the mouse chromosomal fragments that are homologous to that region. mGIG18 is most likely to reside on mouse chromosome 6 within100 Kb of the ICA1 gene. The closest marker that was found in both human and mouse databases on the opposing side of GIG18 (towards telomere of p arm) was the PMS2 gene which was approximately 2.2 Mb distant from the ICA1gene. The PMS2 gene is located on mouse chromosome 5.

BAC clones that gave rise to the genomic sequence and allowed mapping of the intron/exon boundaries of the GIG18 ORF (Figure 11).

As suggested by the sequence analysis of clones obtained in previous library screens, there is a GC-rich sequence that is 1400 base pairs long and is identified as a CpG island in the human genomic database at NCBI. The identification of this CpG island is the basis for the alternate ORF that is proposed in Figure 8 and could potentially represent the location of an alternate GIG18 promoter (Figure 11). This speculation is consistent with the inability to generate RTPCR products that contain sequence different from the original GIG18 ORF, since the 5' primers used in these reactions would not find complementary sequence on a transcript initiated from the alternate 3' promoter. By this same reasoning, the GIG18 ORF identified in our lab would be generated from the 5' promoter. In fact, BLAST searches using the 5' end of the GIG18 ORF have revealed additional transcripts that contain 5' GIG18 sequence and 3' sequence that is different from the original GIG18 ORF. Taken together, there are variable transcripts generated from the GIG18 gene.

A GIG18 Family Member and Apoptosis Related Genes are Regulated by GCs.

To determine if genes with a similar but not identical sequence to GIG18 were also regulated by dex, ESTs were purchased (Research Genetics), verified by sequencing to be correct, and used as probes on northern blots. Figure 12 shows the region of the GIG18 ORF used to identify matching EST sequences and the corresponding sequences of the purchased ESTs. One of the GIG18 related ESTs was down-regulated on a northern blot

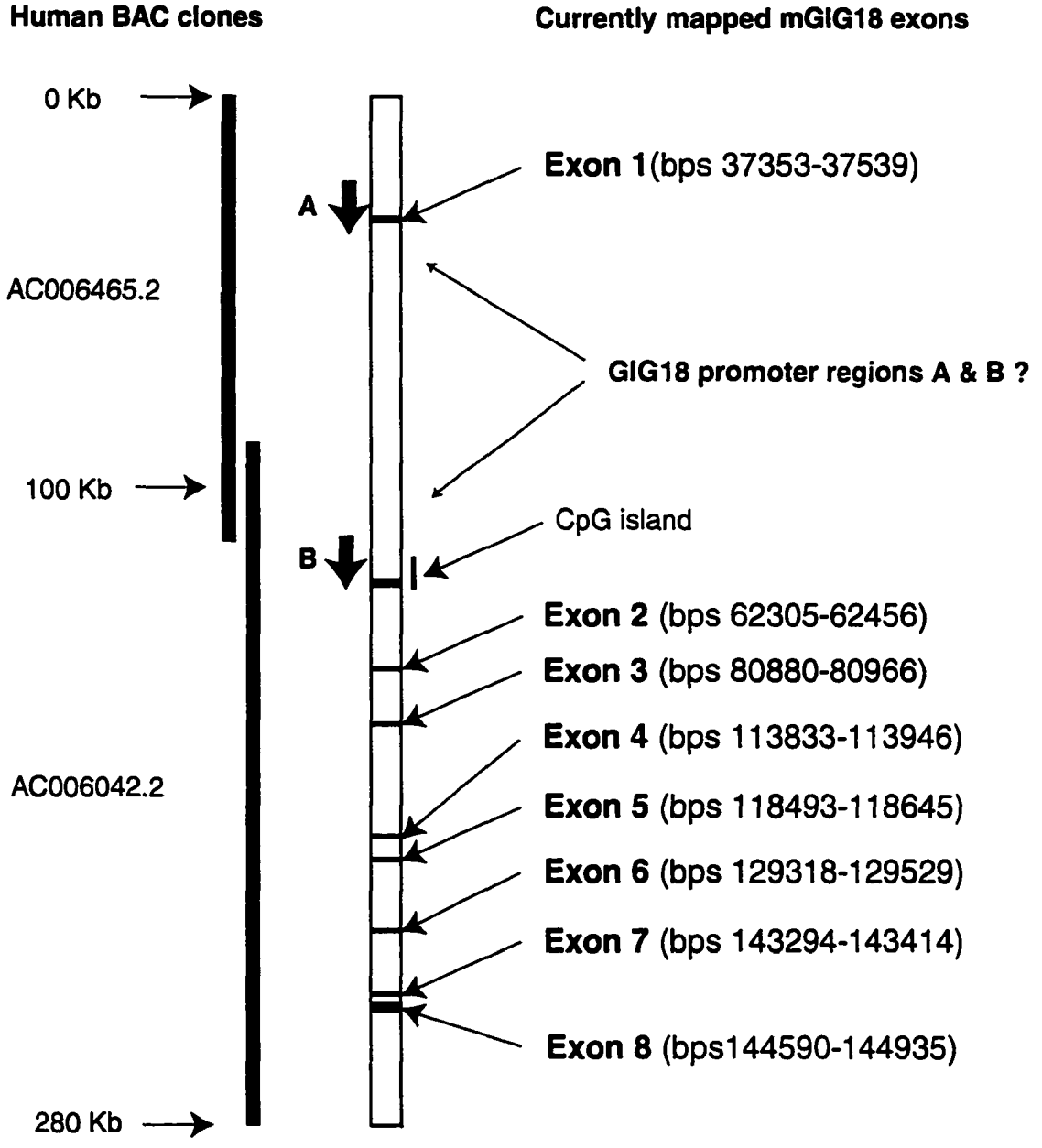


Figure 11. Location of mGIG18 (50 KDa) exons relative to hChromosome 7(p21-p22) and the BAC clones that gave rise to the human genomic sequence.

The CpG island contains at least one additional GIG18 exon corresponding to the larger GIG18 ORF. The annotation of the human sequence suggests that the region 5' of exon 2 contains a CpG island covering 1397 base pairs (%GC=73.1) and other small regions of CGG and GGA repeats. The location of the exons relative to the BAC clone on which they are found are indicated by the base pair numbers in parentheses.

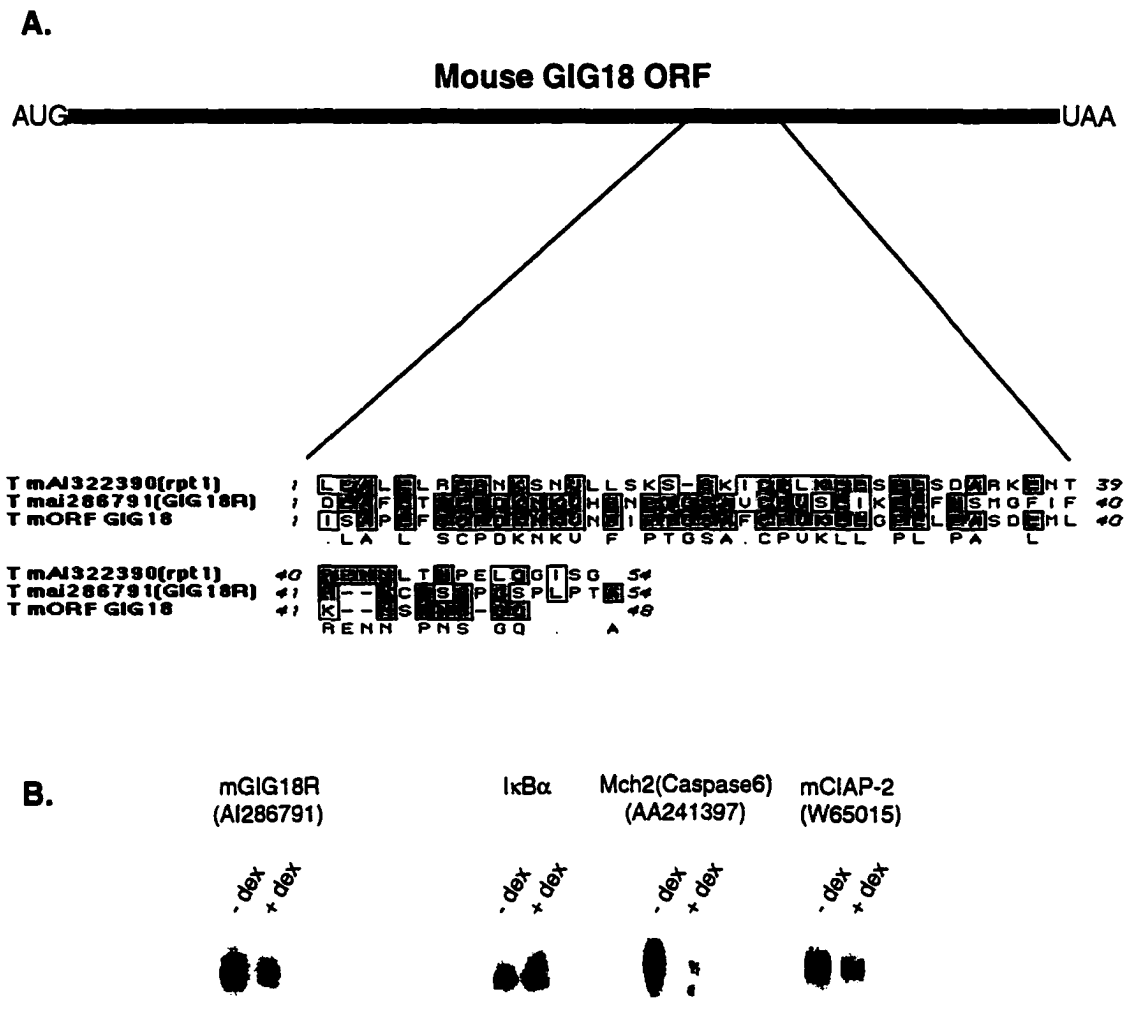


Figure 12. GIG18 and apoptosis-related mouse ESTs and the corresponding northern.

(A) Two ESTs that show homology to mGIG18, but not identity were selected for northern analysis to see if GIG18 family members would also be regulated by dex. mAI286791was down-regulated on the northern (see 12B) and mAI322290 was not expressed in WEHI7.2. The sequence of the EST for mAI286791 showed ~40% identity/conservation over a 100 amino acid region of mouse GIG18. (B) Northerns showing glucocorticoid mediated regulation of four genes after treatment with dexamethasone for 8 hours in WEHI7.2. Loading controls were not included so these results are preliminary.

and the other was not expressed in WEHI 7.2. ESTs of genes with potential roles in the apoptotic process were also inspected on northern blots. The IκBα transcript, whose protein product is an inhibitor of NFκB, is induced by dex, whereas the messages for caspase 6 and cIAP-2 appear to be repressed. The induction of IκBα is of interest due to the fact that active NFκB has been associated with positive selection and proliferation of thymocytes [124], [125]. Induction of IκBα could potentially negate the prosurvival signals mediated by NFκB and contribute to an increased susceptibility to apoptosis. The down-regulation of cIAP-2 could also contribute to the induction of apoptosis by relieving the inhibition of caspases. Auto-catalyzed degradation of IAPs via their RING domain has been shown to increase apoptosis in thymocytes where as RING domain mutants were effective in preventing apoptosis [126]. Thus, any decrease in the number of cellular IAPs could conceivably contribute to the induction of apoptosis.

GIG18 Expression Found in Frogs to Humans and in Multiple Tissues.

BLAST searches against the EST database suggested that GIG18 is widely expressed and in several organisms. It appears that expression of related sequences is found in animals as divergent as frog and chicken. There is also evidence from this analysis that GIG18 is expressed in multiple tissues in mammals (Table 2). RTPCR analysis in multiple mouse tissues confirmed that GIG18 is expressed widely. Also, GIG18 was expressed in all thymocyte subsets including CD4-CD8-, CD4+CD8+, CD4+CD8- and

Species	Tissues and cell types
Homo Sapiens (human)	Adult heart, infant brain, placenta, testis, liver/spleen, retina melanocytes, Daudi lymphoma, endometrial tumor, pancreas tumor, parathyroid tumor.
Mus Musculus (mouse)	Thymus, mammary gland, bronchial arches, embryo, kidney, B cells, unfertilized egg, **skeletal muscle, B220 spleen cells, liver, brain, intestine (large and small), lymph node and especially high (27X) in CD4+CD8+ thymocytes.**
Rattus Norvegicus (rat)	Mixed tissues.
Bos Taurus (bovine)	Mixed tissues, placenta.
Gallus Gallus (chicken)	Bursa of fabricius.
Sus Scrofa (swine)	Mixed tissues.
Xenopus Laevis (frog)	Egg.

Tabie 2. Tisue and species summary of GIG18 expression based on EST and RTPCR analysis.

The tissues in bold showed expression of GIG18 via semi-quantitative RTPCR (Frank Flomerfelt, personal communication), with expression being ~27X higher in the CD4+CD8+ thymocyte population than any other tissue. The regular font corresponds to information gathered from the EST database.

CD4-CD8+. Due to expression of GIG18 in all thymocyte subsets, it was possible that the RTPCR results showing multiple tissue expression was due to the fact that T cells are found in all tissues of the body. To eliminate this possibility, the analysis was performed on tissues derived from Rag-/- and nude mice. Rag-/- mice are unable to initiate the recombination process that gives rise to mature T and B cells and thymocytes that don't express a functional TCR die within 3-4 days. Nude mice have a genetic defect that render them with a vestigial thymus and as a consequence, thymocytes fail to mature. Tissues derived from these mice also displayed a wide tissue distribution of GIG18 expression indicating a potential role for GIG18 unrelated to T cells (Table 2, [127]).

Interestingly, it was found that GIG18 was found to be expressed at levels 27X higher in CD4+CD8+ cells than in any other tissue or cell type. Since this is the stage of thymocyte development when mature TCRs are emerging on the cell surface and antigen specific selection is taking place, it is possible that GIG18 has some role in thymocyte selection and/or maturation.

DISCUSSION

GIG18 was identified in a broad-based screen using DDPCR. This technique, in theory, can represent the entire differential transcriptional status of any cell type or tissue under the desired experimental conditions. At the time of its application, it was the state of the art for monitoring differential expression and was successfully applied in our lab to identify multiple GC regulated transcripts. Glucocorticoid mediated apoptosis of

immature thymocytes is dependent on transcriptional modulation of one or more genes that directly or indirectly lead to the release of proapoptotic factors from the mitochondria and the activation of the caspase cascade. Many genes are regulated by GCs in thymocytes, however, not one of them has proven to be able to induce apoptosis in lymphoid cells. It could be that the gene responsible for triggering apoptosis has not yet been identified or that multiple genes act in concert to deliver the death signal.

GIG18 is quickly and highly induced in thymocytes that are destined to die. The induction is reduced by about half when the cells are treated simultaneously with the protein synthesis inhibitor, cycloheximide, suggesting that full induction may be dependent on a primary gene product, or a simultaneously expressed product with a short half-life. This is a preliminary observation due to the fact that cycloheximide also reduced the expression of the histone loading control.

The GR deficient cell line, S49, indicated that GIG18 induction is fully dependent on GR signaling which suggests that glucocorticoid response elements may be present in the promoter region(s). GIG18 expression is greatly reduced in Apt- mutants that are insensitive to dex and the sensitivities of these same mutants to A23187, at 48 hours post-treatment, correlates closely with the relative induction of GIG18 [123]. The expression profiling of GIG18 with regard to thymocyte apoptotic and/or developmental processes outlined in this chapter indicates that the level of expression of GIG18 is consistently correlated with the degree of apoptosis induced in the various cell types. This is especially true for the CD4+CD8+ population of thymocytes where GIG18 expression is very high and 95% of this same population will eventually succumb to apoptosis.

The GIG18 ORF identified in the pGIG18.10 clone, which was affirmed by RTPCR analysis, contained 8 exons that mapped linearly along human chromosome 7. Careful consideration of EST sequences that were obtained through BLAST searches indicated additional exon sequence in an alternately spliced GIG18 transcript. This observation stimulated an effort that resulted in the identification of ~200 more base pairs of ORF sequence that may represent GIG18 transcription from an alternate promoter. Technical problems associated with the CpG island and the likelihood of multiple promoters prevented complete identification of the alternate GIG18 ORF sequence proposed in Figure 8. The EST analysis suggested that the difficulty in obtaining this alternate ORF is a bona fide technical hurdle since no EST sequence in the database could provide ORF sequence equal to or beyond the 5' end of a single library clone obtained in our lab (pGIG18-7). The reason for this could be due to the 80% GC-rich region encountered in that area which is a sequence composition that is notoriously difficult, sometimes impossible for a polymerase to traverse *in vitro*. All EST sequences and all library clones end within an approximate 200 base pair GC-rich region, more or less randomly. The sequences of the ESTs indicate that none of the clones arrived at a stop codon in the alternate ORF which allows the possibility for the eventual identification of additional coding sequence. This technical hurdle could potentially be side-stepped by designing primers in the region of the proposed alternate 3' promoter and using RTPCR conditions that would discourage problematic 2° and 3° nucleic acid structures. Proceeding in this way, it could be possible to generate and clone cDNA sequence that completes the sequence of the hypothesized downstream-initiated GIG18 ORF.

Finally, GIG18 related transcripts are found in frogs, chickens, rodents, cows and humans but no equivalent could be found in flies, worms, yeast or plants. This suggests that GIG18 is of relatively recent origin and that the family has arisen due to selection of traits specific to larger, more complex organisms. Conversely, GIG18 functional equivalents could exist in less complex organisms but may have been missed in our database analyses due to sequence divergence.

CHAPTER IV

EXPRESSION AND ANALYSIS OF THE GIG18 PROTEIN CODING SEQUENCE

INTRODUCTION

Expression profiling is quickly becoming a valuable means of characterizing the transcriptional status of a cell or tissue type. This seems to be especially promising in the field of cancer research where the eventual goal is to tailor chemotherapeutic regimens to malignancies based on this characterization [128]. Transcriptional readouts could dictate the current state of the tumor with regard to metastatic potential, antigen presentations and potential susceptibilities to treatment [129], [130]. In this way, patients with more severe disease could be treated more aggressively and those who have less threatening tumors could be spared the undesirable side-effects of the more toxic treatments. Regardless of what the expression profile is, the biological phenomenon being studied is usually dependent on the synthesis of proteins corresponding to the genes transcribed in the particular tissue or cell type. The expression profiling technique of DDPCR that was applied in our lab, identified GIG18 as a gene that was quickly and highly induced by GCs. Many GC-induced genes have been identified in immature thymocytes (Chapter I), and many more are likely to be discovered in the future. Identifying regulated transcripts

is the first step towards determining how the transcriptional status of a cell or tissue type relates to the resulting biological effect. Identification of the genes that are critical to the induction of apoptosis requires a determination of whether or not a protein is produced from that gene in the experimental model system. To determine if transcriptional induction of GIG18 results in the translational induction of a corresponding protein, antibodies against the GIG18 protein were needed.

RESULTS

Generation of a ~50KDa His-tagged GIG18 Protein.

In order to generate and purify the expected GIG18 protein, the ORF needed to be PCR amplified from the pGig18.10 plasmid and cloned into the pET 20b cloning/expression vector. By introducing engineered restriction sites into the PCR primers, the GIG18 ORF was cloned in frame with the carboxy-terminal 6X His Tag downstream of a T7 RNA polymerase promoter in the pET 20b vector. This vector was transformed into the BL21 bacterial strain which has a genomic copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter which makes the polymerase inducible with IPTG. Transformation of BL21 allowed expression of the fusion protein in liquid culture after the addition of IPTG (Figure 13). An induced band in the insoluble fraction of the appropriate size was visualized on an SDS PAGE gel. The volume of the

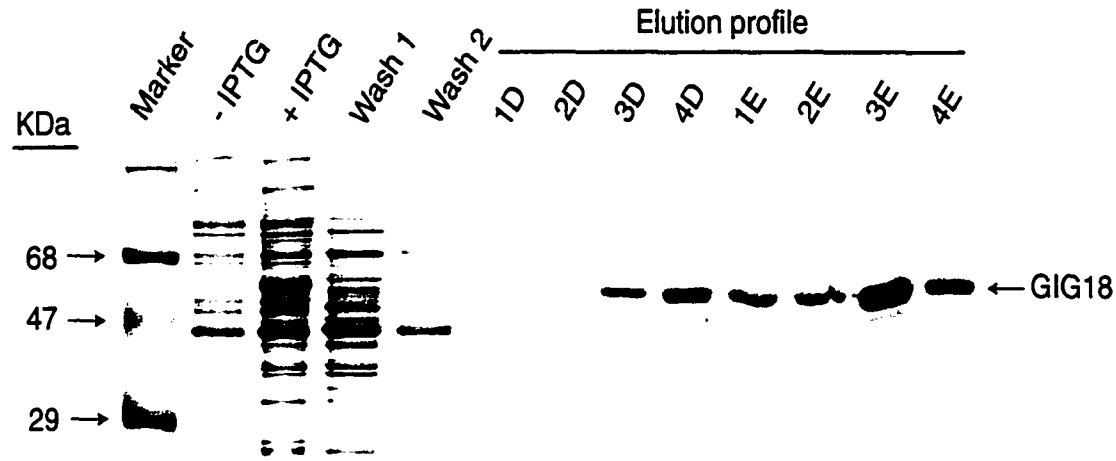


Figure 13. GIG18 protein expression and purification.

This gel shows IPTG induction of mGIG18 protein at ~55KDa and the subsequent purification of the 6X-His-tagged protein on a Ni-NTA (nickel-nitrilotriacetic acid) column.

induced culture was scaled up in order to generate milligram quantities of the protein needed for the two rabbit immunizations. The pH gradient elution profile of the fusion protein from the Ni-NTA resin showed that a major band of appropriate size was eluted from the column. Minor bands also appeared on the elution profile suggesting either that other proteins were bound to the column or perhaps other proteins had an affinity for the GIG18 protein (Figure 13). The GIG18 fractions were collected, combined, concentrated and sent for immunizations.

Rabbit Polyclonal Antibodies Identify Rapidly-induced 68 and 50 KDa Proteins.

Using protein extracts that were harvested at various time intervals from WEHI 7.2 cells treated with 1μM dex, a 68 KDa protein was found to be induced within two hours and appeared to reach maximal induction at approximately 8 hours. Background bands were recognized by the GIG18 polyclonal antibody on most of the gels, most likely due to shared protein epitopes or the minor contaminants in the protein samples sent for immunizations. There was, however, only one prominent band (68 KDa), and one minor band (50 KDa), that were induced in a correlative fashion with the transcriptional induction seen on the northern blot (Figure 14). The 50 KDa band corresponds in size to the original 1290 base pair ORF and probably is the translation product of this GIG18 cDNA. The prominent 68 KDa band is likely to be the translation product of the downstream initiated GIG18 ORF that has been recalcitrant to complete identification. Although efforts to extend this open reading frame from 3' to 5' though the GC-rich region have added ~200 base pairs, not enough additional ORF sequence has been

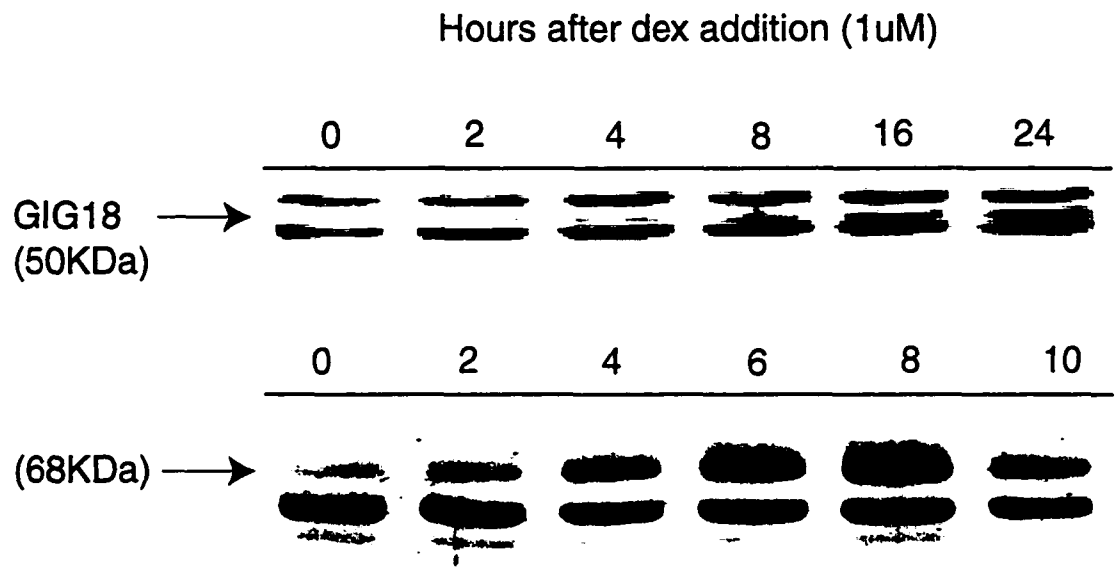


Figure 14. Western blots showing induction of mGIG18 proteins in WEHI7.2.

The upper blot shows the induction of an ~50KDa protein which starts to show induction at 4 hours while the lower blot shows induction of an ~68KDa protein that appears to be induced as soon as two hours.

obtained to account for the size of the 68 KDa induced protein observed on the western blot.

The protein coding sequence acquired thus far can account for 51KDa. The "missing" sequence, therefore, is likely to be no greater than 460 base pairs (17KDa). By using the most 5' ORF sequence to establish the correct reading frame in the human genomic sequence, it is possible to establish a continuous ORF that would yield a 60KDa protein. Without confirmation by a cDNA sequence, the existence of this projected ORF is only hypothetical. This ORF may correspond to the mouse 68KDa protein since it is possible that the equivalent human protein may be smaller. Alternatively, there may be additional exons in the human CpG sequence that are not readily identifiable by translating genomic sequence, thus leading to a smaller ORF. Finally, it may be possible that this *is* the desired ORF, and the discrepancy in size is due to post-translational modification of the encoded protein.

Mouse and Human GIG18 Proteins are Highly Conserved.

Comparison of protein coding region of the smaller ORF (50KDa protein), demonstrates 93% identity and 95% conservation (Figure 15). It is unclear from the human EST database whether humans express a counterpart to the 50KDa protein. None of the human ESTs (hESTs) in the database suggest that there is an equivalent 50KDa protein encoding ORF, rather, all hESTs in this region traverse into the CpG island, and stop more or less randomly. Immature mouse thymocytes produce this 50KDa ORF as it

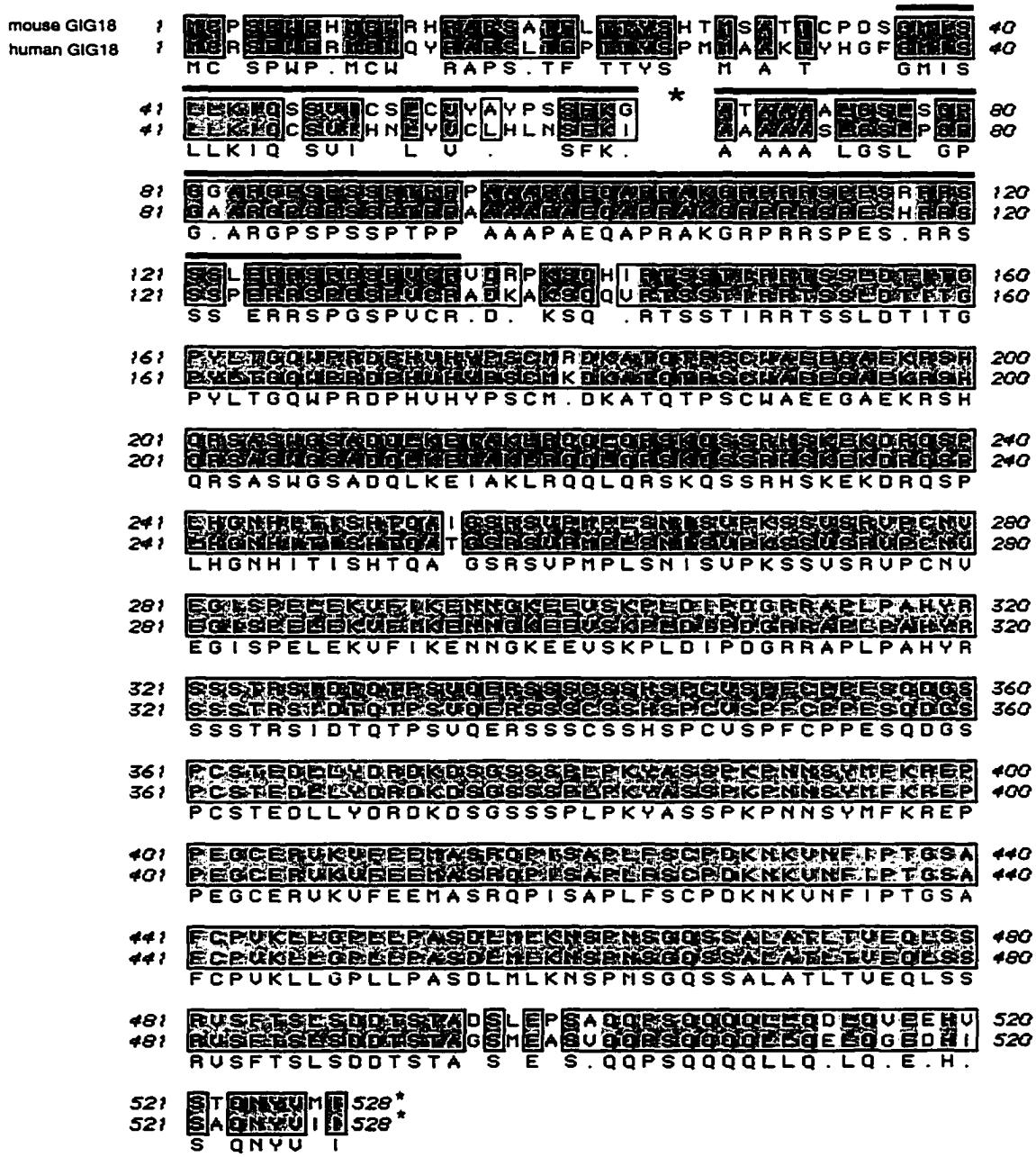


Figure 15. Comparison of the putative mouse and human GIG18 proteins.

The mouse 50KDa ORF is the entire top sequence except for the region that has the black bar over it. The sequences, minus the barred region, are 95% conserved with most of the non-identity residing at either the amino or carboxy termini. The sequence under the bar has been acquired from library clones and EST sequences and potentially represents some of the sequence of the larger 68KDa protein. The asterisk at position 68 indicates where future protein sequence will be added to complete the sequence of the 68KDa protein.

has been identified in λ-library clones, in RTPCR reactions from poly-A RNA and in overlapping EST sequences. Comparison of the entire amount of cDNA sequence acquired in attempt to get the larger GIG18 ORF, which is ~200 base pairs, suggests a translation product that would be 95% identical and 97% conserved between mouse and humans. The conservation of the additional 5' protein coding sequence indicates a selective pressure towards its maintenance and supports the idea that this alternate ORF may indeed be the one that encodes the 68KDa protein induced on the western blot.

A GIG18 Family of Genes is Encoded in the Human Genome.

As was suggested by highly-similar, non-identical EST sequences from several species, BLAST searches against the human genomic sequence verified that there are multiple GIG18 family members. Since the human genome sequence is essentially complete, it was possible to identify two other GIG18 related genes that are encoded on human chromosomes 2 and 17 (Figure 16). Therefore, GIG18 belongs to a gene family that has at least 3 members. These family members did not allow further scrutinizing of the genomic DNA for additional exon sequence corresponding to the 68 KDa protein due to the fact that the homology started downstream of the missing GC-rich sequence. The comparison of the conserved sequences found in the genome allowed identification of domains that may have roles with regard to GIG18 family member function. There are two extensive regions that are conserved in all three genes. The conservation of these domains indicates that not only have there been at least two gene duplications, but is highly likely that these duplications led to additional family members that are expressed

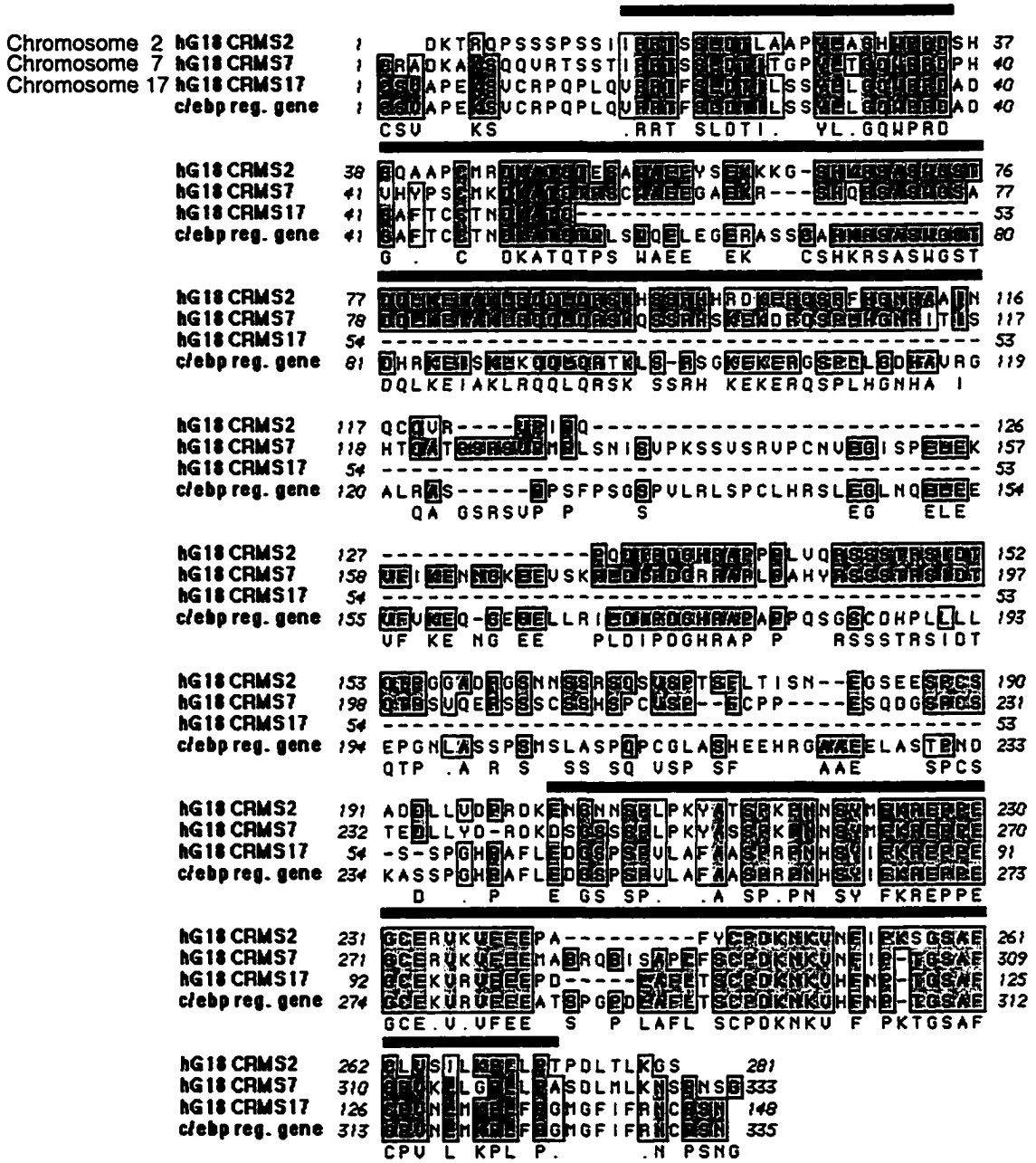


Figure 16. GIG18 gene family.

The original GIG18 gene identified in our lab had complementary sequence on human chromosome 7. Database searches have allowed the identification of GIG18 family members on two other chromosomes (2 and 17). The two regions of conserved sequence are indicated underneath the black bar. The sequence of the C/EBP regulated gene has been added to show that it is most related to the chromosome 17 GIG18 sequence.

and have important biological functions. If these gene duplications had occurred spuriously with no concomitant selective advantage, one would not expect particular regions to be any more conserved than other parts of the gene which is not the case in this situation.

In immature mouse thymocytes, one of the two other GIG18 related transcripts that were analyzed by northern blot analysis, was not expressed where as the other appeared to be down-regulated by GCs. This observation is important because it hints that the transcriptional regulatory regions of these separate genes do not respond similarly to GC treatment. The gene duplication events in the past may have left regulatory regions behind, leading to the observed differential expression. In the case of the GIG18 related transcript that is down-regulated in response to GCs, GR inhibition via a negatively-acting GRE and/or interference with transcription factors may be responsible for this observation. Likewise, the GIG18 related transcript that is not expressed in thymocytes may have lost its glucocorticoid-responsive promoter and acquired a different regulatory sequence.

GIG18 has a Novel Protein Sequence.

BLAST searches using the GIG18 protein sequence versus *Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster* and *Saccharomyces cerevisiae* at the respective web sites, revealed no significant matches. The matches that did come up with P-values < 0.01 were entirely the result of sequences that had high percentages of serine, proline, arginine and leucine. This greatly diminishes the significance of these hits since

41% of GIG18 is composed of these 4 amino acids. Attempts to characterize GIG18 with regard to domains, potential sites of modifications and cellular targeting showed that it has no recognizable domains (ScanProsite, SMART, databases), sites for potential glycosylation, phosphorylation, myristoylation, amidation (PROSITE-scan, ScanProsite, NetPhos databases), and a well distributed chance to end up in multiple locations (PSORT database). Specifically, the coding sequence had the following likelihood to end up in these regions: extracellular (35%), plasma membrane (30%), endoplasmic reticulum (22%), Golgi (13%), and a separate database search (MITOPROT) suggested a 5% chance of mitochondrial localization.

A recent cDNA sequence data submission from group studying cytokine regulation by C/EBP transcription factors, had high homology to all chromosomally identified GIG18 family members, especially the one on chromosome 17 (Figure 16). The cDNA was described as being a novel gene that is induced by C/EBP and is for now unpublished.

Using the GIG18 related EST sequences in a BLAST search, a significant match to the human rpt1 gene was discovered ($e = 1^{-65}$). Comparison of the entire human rpt1 protein coding region to the mouse GIG18 sequence revealed 43% conservation distributed through the entire sequence (Figure 17). The rpt1 gene has been implicated in the down-regulation of the IL-2 receptor in T cells [131], however, when the EST sequence that matched rpt1 was used on a northern in our lab it showed no expression in WEHI 7.2 with or without dex addition.

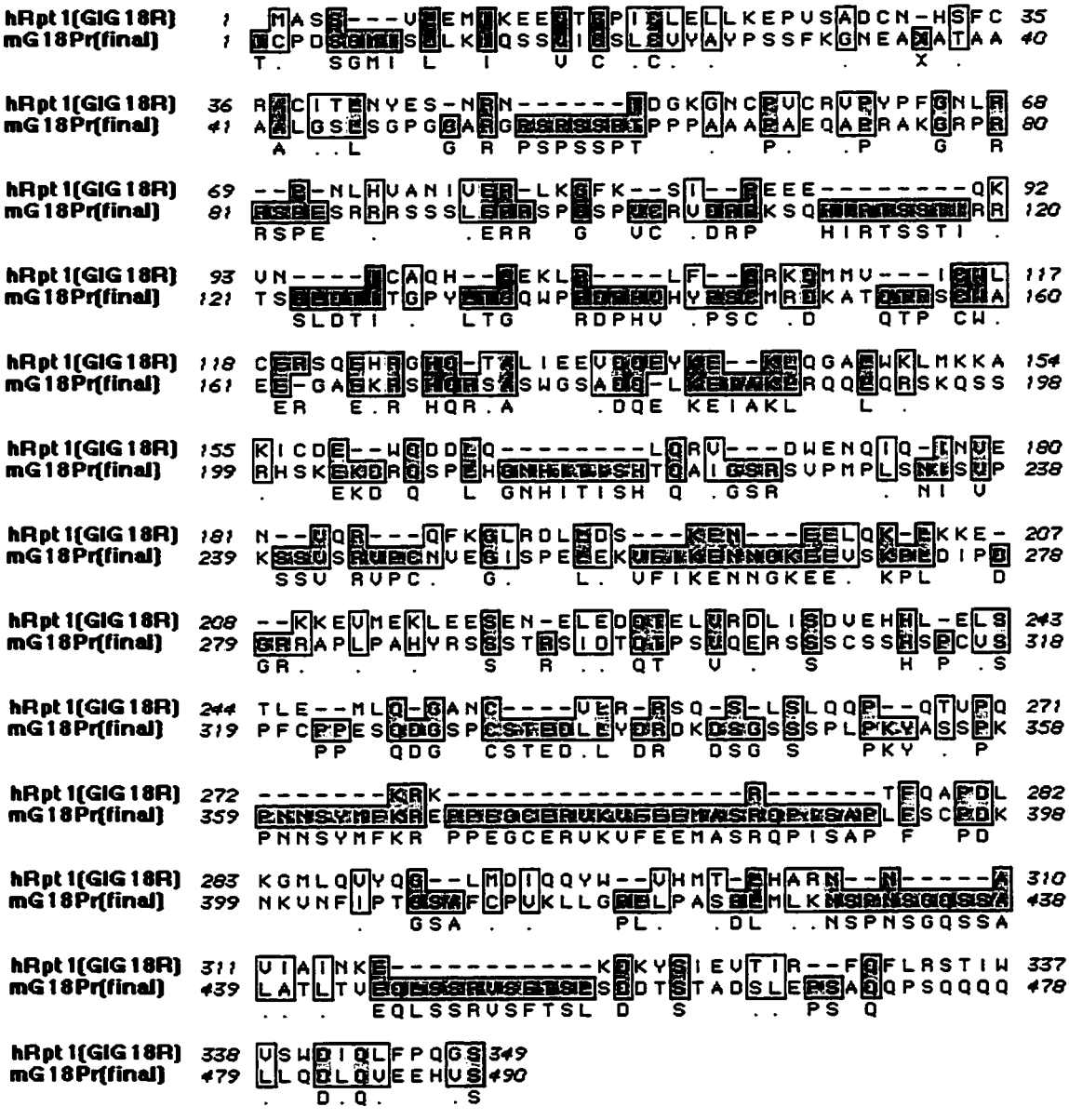


Figure 17. Mouse GIG18 ORF versus human Rpt1.

The mouse GIG18 protein shows ~45% conservation with the human Rpt1 gene at the protein level. The significance of this apparent homology is diminished due to the lack of conservation in the GIG18 domains indicated in Figure 16, the number of gaps included to generate the homolgy and the fact that the proteins are quite different in size.

DISCUSSION

It's not unusual for highly transcribed genes to produce correspondingly high quantities of protein. Examples of this are the many house-keeping genes expressed in virtually all cell types including histone, actin and tubulin. As a consequence, these same transcripts and their proteins are commonly used as loading controls on northern and western blot analyses, respectively. In other cases, low abundance transcripts can lead to a high quantity of corresponding protein and vice versa. The reasons for this include frequency of ribosome attachment to the transcript, protein turnover, RNA turnover and the metabolic state of the cell, tissue or organism. Although a highly induced GC-regulated transcript had been identified, which contained an ORF sequence, it was unknown if a protein could be expressed from this ORF, or if antibodies raised against the protein generated from this ORF would identify a protein induced in thymocytes. The HisTag fusion protein generated from this ORF gave rise to a polyclonal antibody, that on a subsequent western blots, showed the convincing induction of a 68 KDa band and a more subtle induction of a 50 KDa band. Based on sequences from the EST database, it is likely that these two bands are translation products of the 430 amino acid ORF that was identified in the pGIG18.10 clone and of an approximate 68KDa ORF that appears to start in a CpG island downstream of the smaller ORF.

There is a high degree of conservation between the human and mouse coding sequences in addition to there being multiple family members. The pattern of conservation between mouse and human suggests that the protein functions in a

biological process where mutations in the middle of the protein are selected against whereas mutations at the termini are more acceptable. On average, identity of protein sequences between human and mouse is on the order of 80% [132]. Therefore, the identity found for GIG18 is high at 93% and may relate to a well conserved role in mammals. The GIG18 family members identified in the human genome sequence revealed that gene duplication events allowed the generation of a gene family with significant divergence of its members.

Database analysis of the GIG18 protein coding sequence revealed a limited amount of information with regard to potential functional domains or cellular localization. However, there are at least two regions within the GIG18 family of genes that show retention of extensive homology. These regions are likely to possess functional significance due to the selective pressure towards their preservation. The database predictions of potential sites of phosphorylations, myristoylations and other post-translational modifications are of limited value for now since the consensus peptide sequences for these types of modifications are short and there are many types of protein modifications performed in the cell. Knowing this, there is a significant likelihood that these types of sequences could easily appear in protein sequences just at random.

The NCBI database, which was exploited in efforts to obtain the GIG18 ORF, information about tissue and species expression, other family members and intron/exon boundaries contains sequences that were created largely *en masse* without concern as to what specifically was being generated. The recent submission of a GIG18 related cDNA that is up-regulated under C/EBP control, demonstrates that a member of this gene family

has shown regulation under an alternate experimental condition. The cDNA was identified in murine B lymphoblast (P388) cells in which ectopic expression of C/EBP family members led to the identification of the induced transcript. One focus of their lab is to study myeloid differentiation and the ability of C/EBP family members to confer inducibility of IL-6 (promotes differentiation of myeloid stem cells and B cells) and MCP-1 (monocyte chemoattractant protein) by the bacterial toxin, LPS. Based on this information, it is possible that the GIG18 related gene, induced by C/EBP, is somehow involved in rearrangement of transcriptional complexes or modification of chromatin accessibility which leads to an altered transcriptional response of the B lymphoblasts to LPS. Expression analysis later revealed that this GIG18 related transcript is most highly expressed in liver and bone marrow [133]. The fact that this GIG18 family member is highly expressed in bone marrow is interesting since bone marrow is the site of B cell selection. This process is highly analogous to T cell selection, where immature B cells get positively or negatively selected based on their interactions with stromal cells and self antigens presented in the bone marrow. Thus, the expression of a GIG18 family member is associated with the priming of a naïve cell type for an antigen-induced, differentiation response to LPS. Likewise, the induction of GIG18 by glucocorticoids in thymocytes could be a priming event mediated through GR that modulates the response of thymocytes to TCR signaling. Hence, the expression profile of GIG18 family members is highly suggestive of a role in immune system development.

Finally, the 43% conservation of GIG18 to human rpt-1 is interesting especially in light of the fact that rpt-1 is involved in the down-regulation of the IL-2 receptor. This

receptor is typically associated with proliferation and differentiation of cytotoxic and helper T cells. Therefore, it is easy to speculate that down-regulation of the receptor could diminish both differentiation and proliferation. This is an attractive hypothesis but it is not without shortcomings. First of all, the homology between rpt-1 and GIG18 is not significantly similar in the more conserved regions identified as being potential functional domains. Second, GIG18 is much larger than rpt-1, and in order to get the homology, many gaps are inserted into the rpt-1 sequence which diminishes the significance of the homology. Most importantly, the gene corresponding to this related sequence is not expressed in our model cell line, WEHI 7.2.

CHAPTER V

TRANSIENT AND STABLE EXPRESSION OF GIG18

INTRODUCTION

The open-reading-frame identified in the pGIG18.10 clone that was obtained from a dex-induced mouse cDNA library was confirmed to exist as part of the RNA population in mouse thymocytes since RTPCR clones obtained from WEHI 7.2 poly-A RNA gave identical sequence. The mouse ORF maps colinearly to a highly conserved sequence on human chromosome 7 and the intron/exon boundaries can be clearly defined. Rabbit polyclonal antibodies raised against the corresponding 50KDa protein revealed two induced protein bands on the western blot. One band was the appropriate size (50 KDa) and induced to a relatively low level, the other band appeared to be ~18 KDa larger and was more highly and rapidly induced reaching maximal induction at 8 hours post-dex treatment. The reason for the two bands appears to be the result of using alternate promoters in the GIG18 gene region or perhaps alternate splicing. Regardless, further characterization of the smaller ORF, required expression studies to determine if it led to an induced phenotype or a specific cellular localization. Initial efforts were focused on transient expression in WEHI 7.2, since this was the cell line in which GIG18 was identified. Stable, tetracycline-regulated expression of GIG18 was performed in HeLa

cells. HeLa cells have intact apoptotic pathways including receptors of the TNF family [134], which are inhibitable by expression of Bcl-2 [135]. They are also susceptible to caspase-3 mediated apoptosis [136], apoptosis initiated through the intrinsic pathway and p53 [137], and thus have been a useful model cell line for the study of many proapoptotic and antiapoptotic proteins.

RESULTS

Expression of GIG18 in Immature Thymocyte Cell Lines.

To determine if expression of GIG18 led to any overt morphological changes, or in the case of fusion of GIG18 to GFP, whether there was an effect on the cellular localization of GFP, transient transfections were conducted via electroporation. Expression of all plasmids was driven by the cytomegalovirus (CMV) promoter except for the GFP plasmids used in the cotransfections which were driven by the SV40 promoter. Transfecting GFP, GFP/GIG18 fusions, and cotransfections of GFP on one plasmid and sense or antisense expression of GIG18 on a second plasmid showed similar kinetics in terms of loss of GFP positive cells over a timecourse of 48 hours in the two immature thymocyte cell lines tested (Figure 18). The loss of GFP positive cells was monitored by fluorescent-activated cell sorting (FACS) analysis. The cell lines chosen for this study, WEHI 7.2 and 2B4.11, had lost 90-95% of the GFP positive cells at 48 hours post-electroporation when GIG18 was fused to GFP. In contrast, when GIG18 was expressed from a separate plasmid, the loss of GFP positive cells did not vary appreciably from the

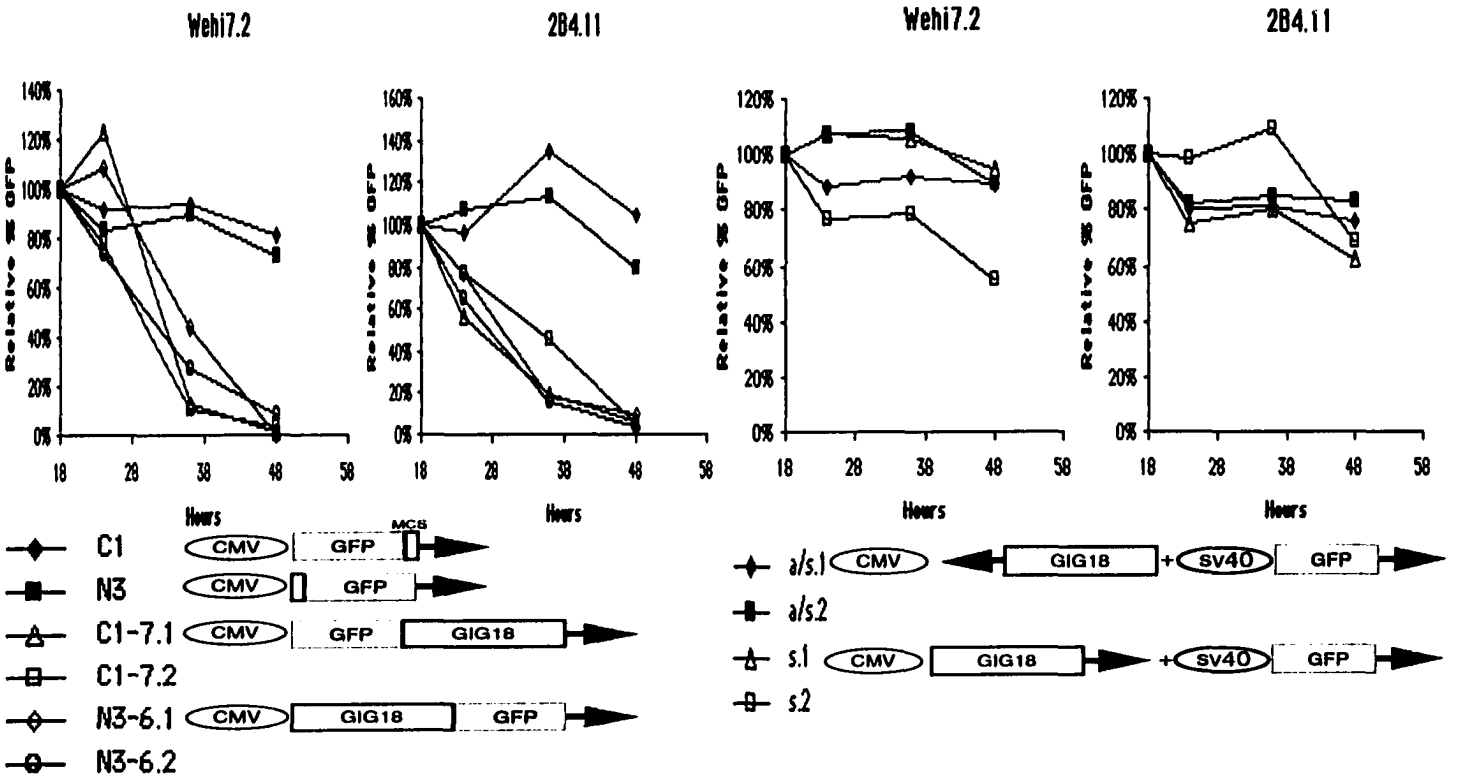


Figure 18. GIG18 expression in immature thymocytes.

Expression of GFP, GIG18 and GFP-GIG18 fusion proteins in WEHI 7.2 and 2B4.11, which are two different immature mouse thymocyte cell lines. The relative percent GFP in each cell type shows the same bacic profile over the same 48 hour timecourse. The two graphs on the top left show expresion of either GFP alone or expression of GIG18 fused to GFP. The two graphs on the top right show the effect of expressing GIG18 in the sense or antisense direction driven by the CMV promoter in a cotransfection with GFP expression being driven by the SV40 promoter.

antisense control. Although one of the data points did suggest that independent GIG18 expression may be contributing to the loss of GFP positive cells, overall, the difference between sense and antisense expression was not nearly as dramatic as expression of the GIG18/GFP fusions.

GIG18/GFP Fusion Protein Expression in the Dex Insensitive Cell Line Apt3.8.

Dex-insensitive, WEHI 7.2 derivative cell lines were previously obtained in the course of a chemical mutagenesis screen [123]. Of all the mutants that were isolated, one stood out as being resistant to several of the apoptotic inducers. This mutant line, (Apt 3.8), was chosen to study expression of the GIG18/GFP fusion plasmids to determine if the dex-resistant phenotype would have an effect on retention of GFP. In contrast to the parental cell line, in which GFP positive cell percentages drop to 5-10% at 48 hours post-transfection, the Apt 3.8 line allowed ~50% of the GFP positive cells to be maintained at this same timepoint. Only after 60 hours in Apt 3.8 was the GFP positive population reduced to 5-10% (Figure 19). Thus, the Apt 3.8 cell line had a kinetic loss of GFP positive cells that was significantly different from the parental cell line.

Transient Expression of the GIG18 ORF and Deletion Mutants in HeLa Cells.

The GFP expression profiles that had been observed in the thymocyte cell lines were encouraging, however, the transfection rates that are typically 1-5%, hindered the ability to be certain if a significant biological effect was resulting from the expression. The low transfection rate meant that data was being generated with a rather small population of

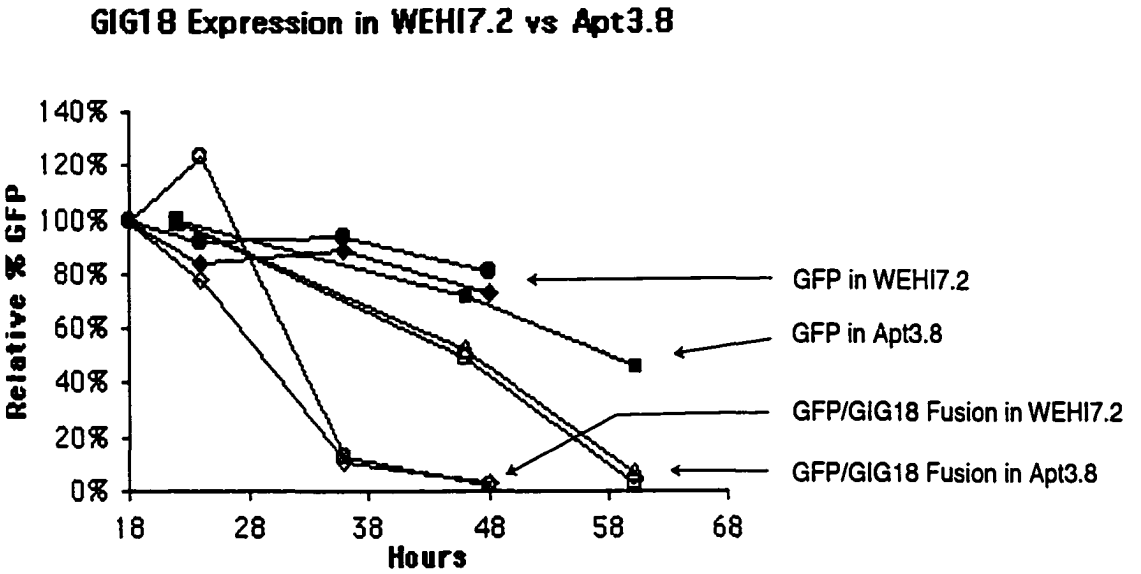


Figure 19. Expression profile of GIG18 in wildtype versus mutant cell line.

Comparison of GFP and GFP/GIG18 fusion protein expression in the wildtype cell line WEHI 7.2 and the dex insensitive cell line Apt 3.8. GFP expression alone follows the same timecourse in both cell types whereas the fusion protein expression in Apt 3.8 shows a profile that is intermediate between wildtype GFP expression and fusion protein expression in WEHI 7.2.

cells. In order to address this problem, stable expressing cell lines were generated in a HeLa On (Clontech) cell line that allows tetracycline-regulated expression of a gene of interest. Before generating stables, the GIG18-GFP fusion plasmid was tested the in lipofectamine-mediated transient transfections of HeLa On to monitor the loss of GFP positive cells as had been done in the thymocytes. In addition, a series of GIG18 deletion mutants were generated to identify the region of GIG18 that was responsible for the observed loss of green cells. Also included in these transient assays was a tet-regulated, bi-directional expression plasmid that either had GFP alone, or GFP and GIG18 which would be comparable to the cotransfections in thymocytes (Figure 20). Since two transcripts are produced from one plasmid, only one type of plasmid needs to be transfected. This would allow discrimination of the effect of GIG18 expression, independent of the fusion protein. For the transient transfections that included the tet-regulated plasmids, tetracycline was maintained in the growth media for the duration of the experiment. For the tet-regulated plasmid, no difference was observed in the loss of GFP positive cells whether or not the GIG18 ORF was cloned into the vector when equal molar amounts of plasmid was used. The GIG18-GFP fusion showed a similar loss of GFP positive cells although it took approximately twice as long (87 hours) to reach 10% of the initial values at 36 hours. Interestingly, GFP alone was retained in HeLa On cells over the timecourse of the experiment suggesting that expressed proteins may be more stable in this cell line than in WEHI 7.2. The deletion mutants suggested that the first 400 base pairs of the 1290 base pair ORF do not contribute much to the loss of GFP positive cells whereas the rest of the ORF seems to contribute significantly.

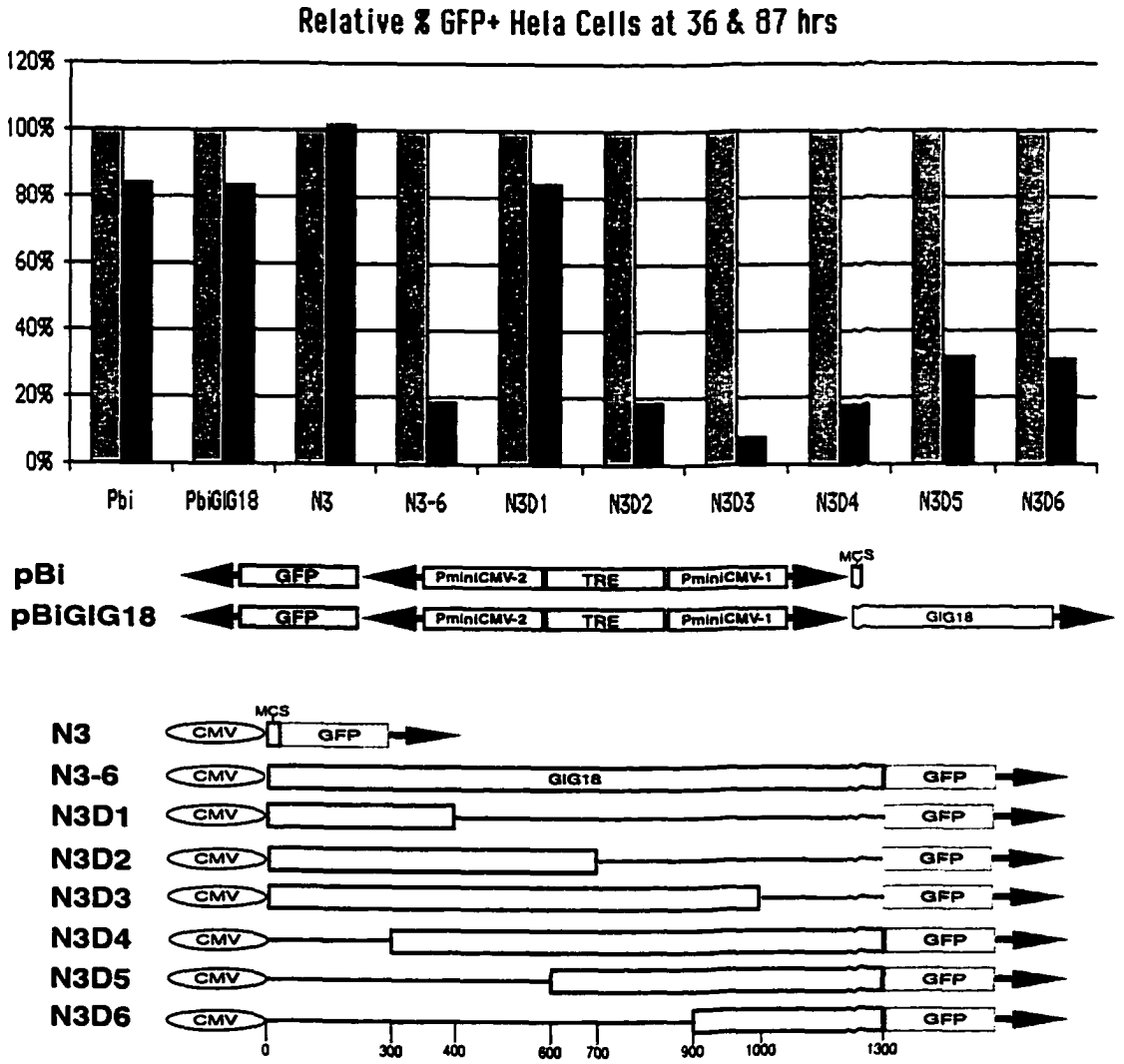


Figure 20. Expression profile of GIG18 and GFP in HeLa On.

The bar graph at the top of the page show the relative percentage of GFP positive Hela cells at 36 and 87 hours post transfection (light and dark, respectively). As shown in the diagram, the first two plasmids are tet-responsive which required that tetracycline be maintained in the media for the duration of the experiment (1µg/ml). The expression of GIG18-GFP fusion/truncation plasmids were driven by the constitutive CMV promoter.

Stable Tetracycline-regulated Expression of GIG18 in HeLa Cells.

In order to assess the effect of GIG18 expression in a large population of cells, stably integrated copies of several different tet-regulated plasmids were generated in the HeLa On cell line (Figure 21A). The p609 plasmid is a tet-regulated vector that was available in our lab and offered an alternative means to corroborate our data. The selection of stable cell lines is schematically represented in Figure 21B. Of the hundreds of colonies that were picked for further analysis, twenty-eight of the more promising clones are shown in Figure 21B. Interesting clones were selected based on low to no background GFP expression and a convincing induction of GFP when tet was added to the media. The clones expressing the pBiGIG18 plasmid consistently gave the best fold induction of GFP which continued to increase up until the final timepoint (90 hours). Interestingly, and in contrast to all the other stable cell lines generated, the GIG18-GFP fusions expressed from the p609 vector generally showed a similar loss of green positive cells over time as was seen in the transient assays. The observed loss of green cells was unexpected since these cell lines had the tet-regulated expression vectors stably integrated into their genome and therefore should continue to express the fusion protein as long as tet was present in the media. An encouraging aspect of this observation is that perhaps cells expressing the fusion protein were being selected against and thus eliminated from the population. If this were the case, it should be possible to induce the different stable cell lines with tet and monitor the effect of expression on their growth rate. The p609N3-6

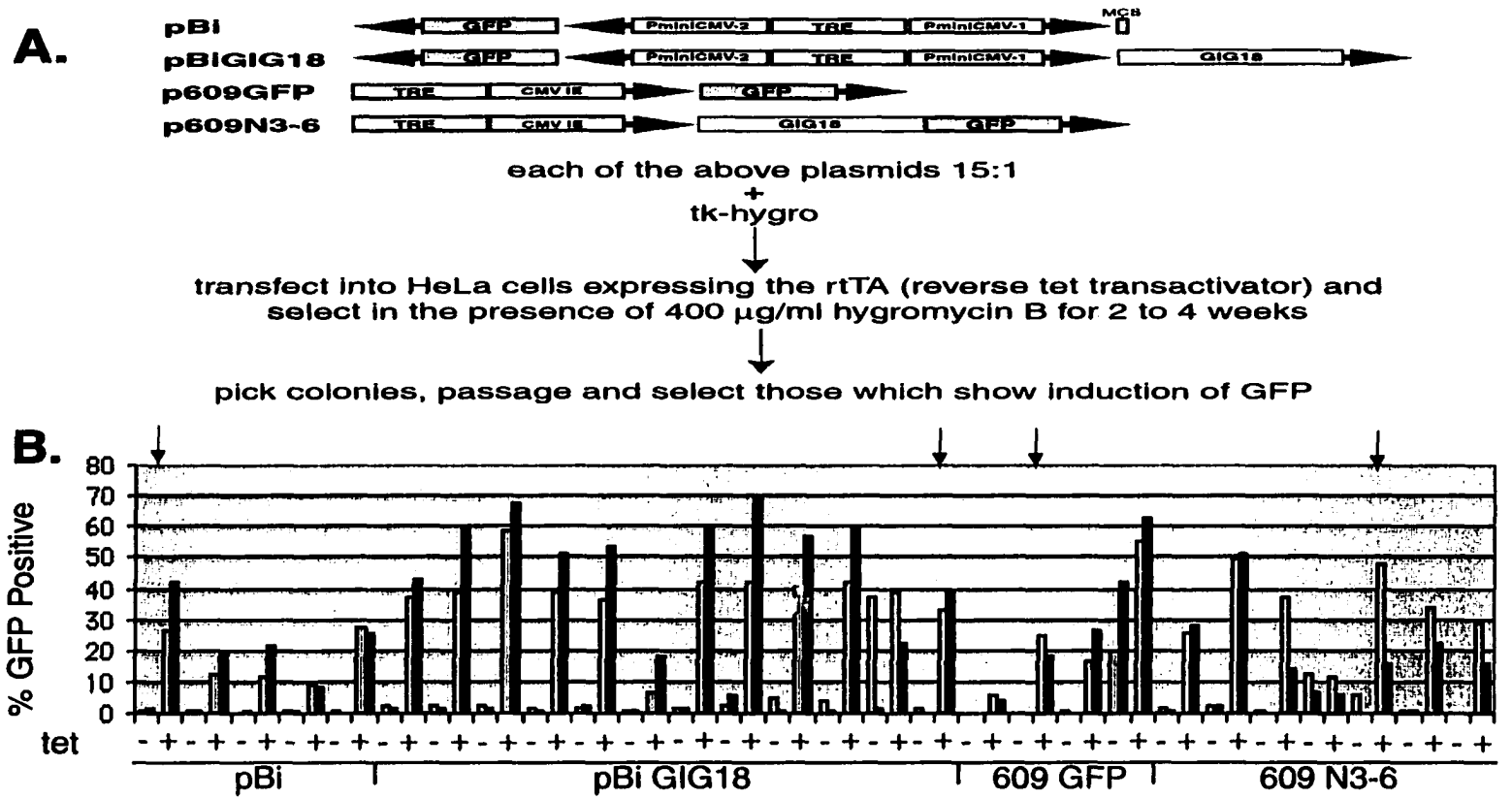


Figure 21. HeLa On stable cell line summary.

(A) At the top of this figure are the plasmids that were used in a cotransfection with the selection plasmid ptk-hygro to make stable HeLa On cell lines. The colonies that were picked after 2-4 weeks in Hygromycin B selection were first inspected visually for induction of GFP by using UV light microscopy. (B) Those colonies that showed zero to moderate background GFP expression were further studdied by FACS analysis for level and fold induction (shown above). The light shaded bar represents ~40 hours in the presence or absence of 1µg/ml tetracycline and the dark shaded bar represents ~ 90 hours. Cell lines with the arrow pointing to them were selected for growth curve analysis to determine if the plasmid being expressed had any effect on their viability.

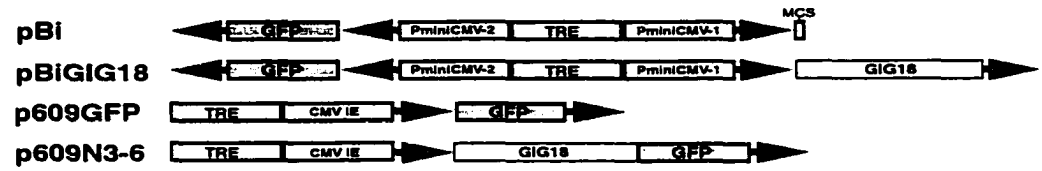
cell line expressing the fusion protein had GFP induction to a level of 50% at 40 hours. If these cells *were* being eliminated, the effect on growth rate should be readily observable. The arrows above individual cell lines in Figure 21B were chosen for growth curve analysis.

Growth Curve Analysis of Select HeLa On Stable Cell Lines.

To determine if tet-regulated expression of the different integrated constructs was having an effect on growth rate, the individual cell lines were plated at ~20 K cells/ml on 6cm plates. To each plate, tetracycline + vehicle, or just vehicle alone was added at time zero. At 24, 72, and 100 hours, the cells were harvested and counted using a hemacytometer (Figure 22B). The interesting aspect of the graphs in Figure 22B is the comparative growth of p609GFP versus p609N3-6. The ~30% reduction in GFP positive cells witnessed for p609N3-6 between 40 and 90 hours in Figure 21B is shown to have only a 6% difference in growth from 24 to 90 hours. This suggests that elimination of GFP positive cells due to expression of a toxic protein can account for only a fraction (~20%) of that loss.

Since there is no significant effect on the growth rate of the stables expressing the fusion protein, the loss of GFP seen in Figure 21B is not readily explainable since they should continue to express the protein in the presence of tet. Two of the six highest fusion protein expressing cell lines in Figure 21B maintain high level expression of GFP up until 90 hours. The reason for this could reside in the fact that stables arise from random integrations into the genome and perhaps the destabilizing sequence was removed from

A.



B.

Hours		pBi#8B		pBiGIG18#9		609GFP#8		609N3-6#9	
		- tet	+ tet	- tet	+ tet	- tet	+ tet	- tet	+ tet
24	(cells/ml)/1000	15.0	15.0	75.0	45.0	42.5	30.0	27.5	37.5
72		112.5	111.5	665.0	285.0	160.0	135.0	220.0	245.0
100		380.0	350.0	1260.0	1470.0	580.0	580.0	552.5	617.5
24	LOG 10	1.18	1.18	1.88	1.65	1.63	1.48	1.44	1.57
72		2.05	2.05	2.82	2.45	2.20	2.13	2.34	2.39
100		2.58	2.54	3.10	3.17	2.76	2.76	2.74	2.79
24	normalized	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
72		1.74	1.74	1.50	1.48	1.35	1.44	1.63	1.52
100		2.19	2.15	1.65	1.92	1.69	1.86	1.90	1.78

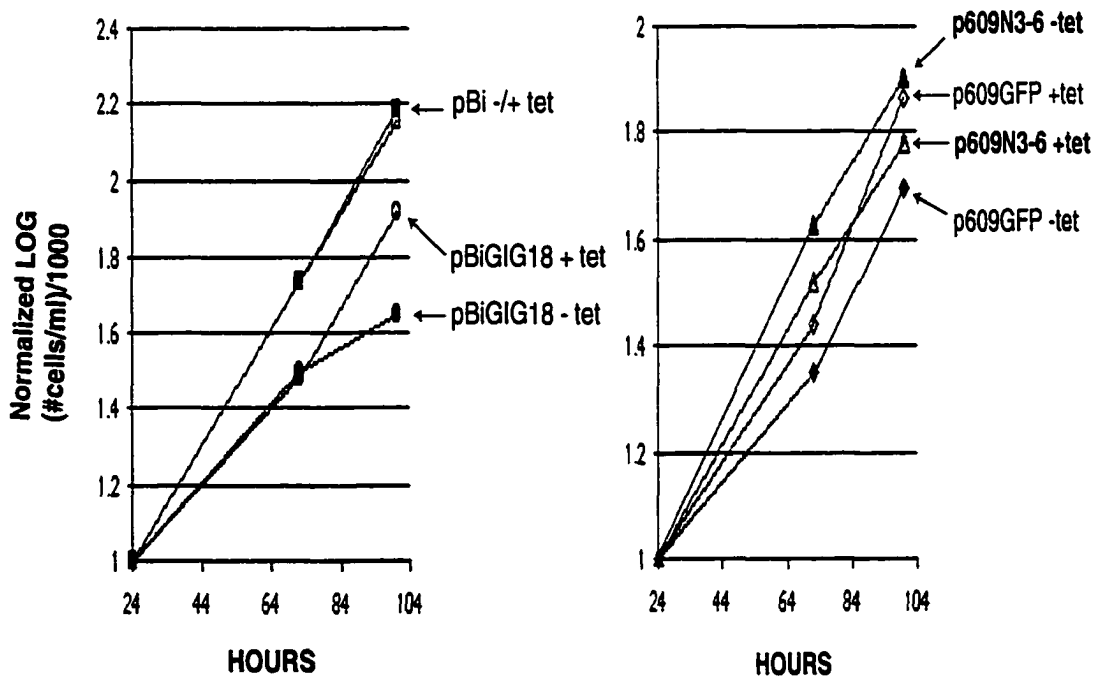


Figure 22. HeLa On growth curve.
(A) The four stable cell lines selected expressing the indicated plasmids, all displayed minimal backgound GFP expression by UV light microscopy (confirmed by FACS), and tet regulated induction of GFP. (B) The graphs show the normalized growth rates of each of the cell lines with and without 1µg/ml tetracycline.

the fusion protein during integration. This, however, is an unlikely scenario since the GIG18 ORF is between GFP and the tet-regulated promoter so one wouldn't expect stable integration events to remove intervening sequence. Conversely, these same two clones could be the "normal" ones in that they maintain expression of the fusion protein as expected and that the four others may lose expression of the fusion protein due to some unknown transcriptional repressing factor or perhaps by favored insertion into regions of genomic instability or transient accessibility.

Expression of GIG18 from Galactose-inducible Plasmids in Yeast.

Yeast are susceptible to induction of death by various apoptotic components including bax], caspase-1 and -3 [138] and CED4/Apaf-1 [139]. The dimerization-mediating BH3 domain of bax and subsequent targeting to the mitochondrial membrane are essential for induction of death in both mammalian and yeast cells [140], and bax induced death was inhibited by expression of Bcl-2. These observations suggest that functional components involved in apoptosis are present in yeast. *Saccharomyces cerevisiae* also displays several apoptotic markers due to expression of bax. Included are chromatin margination at the nuclear envelope, DNA fragmentation, membrane blebbing and externalization of phosphatidylserine at the plasma membrane [51]. Due to the phenotypic response of yeast to several inducers of apoptosis outlined above, expression of GIG18 was performed in *S. cerevisiae*. Figure 23 shows the expressed plasmids and the typical growth characteristics of transformed yeast after four days of growth $^{-}/_{+}$ galactose at 30°C. No effect on the growth rate of yeast was observed due to the expression of any of the plasmids. In

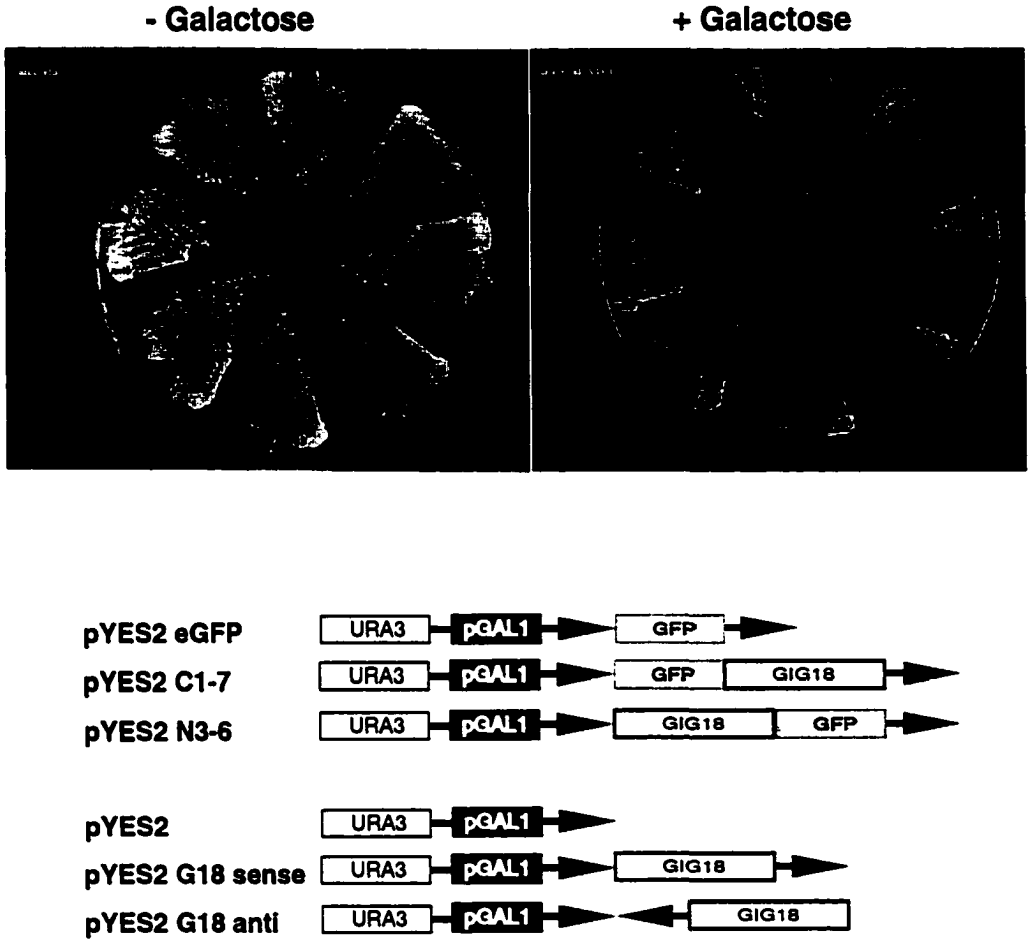


Figure 23. GIG18 expression in yeast.

The plasmids above were transformed into *S. cerevisiae* to monitor potential effects of GIG18 expression on growth rate and morphology. The plates above show typical results regardless of which of the above 6 vectors was transformed. Therefore, there appeared to be no effect on growth rate due to expression of GIG18. Only the yeast clones that expressed one of the three eGFP containing plasmids were studied by UV microscopy for morphological variations. There weren't any obvious morphological differences when viewed with either the FITC (fluoroisothiocyanate) filter or the DIC (differential interference contrast) filter.

contrast to mammalian cell types, the fusion protein was present in all the cells at four days (96 hours) post-induction. This can be partially explained by the selective pressure to maintain the transformed plasmid due to the URA3 auxotrophic marker or perhaps, as in HeLa cells, yeast provide a more stable environment for expressed proteins. Consistent with mammalian expression, there was no specific localization of the GFP fusion protein and no observable expression-induced morphological changes. Thus, it appeared as though yeast were perfectly tolerant and unaffected in any observable way by GIG18 expression.

DISCUSSION

The expression analysis of GIG18 was performed with attention to potentially induced morphological or growth effects, and whether or not there was a specific cellular localization of the GIG18-GFP fusion protein. Comparison of the fusion protein to GFP expression alone showed that the presence of GIG18 did not lead to any specific localization of GFP. The only difference was that GFP appeared everywhere in the cell, and the fusion protein appeared to be excluded from the nucleus. The basis for this observation could be that GFP (32KDa) alone is small enough to diffuse into the nucleus, however, when additional protein sequence is added in the form of GIG18, it becomes too large to permit passive diffusion. This idea is consistent with what is known about the kinetics and size exclusion of nuclear pore-mediated diffusion where a 40KDa protein

will equilibrate with the nuclear compartment in about 30 minutes and a 60KDa protein is hardly able to enter the nucleus at all [141].

The glucocorticoid insensitive cell line, Apt3.8, tolerated the presence of the fusion protein significantly longer than the parental cell line WEHI 7.2. The reason for this is not readily explainable, but perhaps the chemical mutagenesis used to generate this cell line disrupted some aspect of the proteolysis pathway rendering cellular proteins with a longer life. This idea is consistent with the insensitivity of Apt3.8 to GCs since it has been shown that inhibition of the 20S proteasome in thymocytes inhibits apoptosis induced by GCs, ionizing radiation and phorbol ester [142].

Finally, the HeLa On stables that expressed GFP and GIG18 in opposite directions from the bi-directional promoter, consistently had the lowest background and highest fold induction of all the cell lines generated. These stables were also generated at twice the frequency of any other stable cell type. Thus, it appears that stable expression of the pBiGIG18 plasmid allowed an increased transcriptional responsiveness to these particular cell lines.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Roles of Glucocorticoids in Thymocyte Development.

Glucocorticoid-induced thymocyte apoptosis is a classic model system that is well studied with regard to the involvement of multiple signaling pathways in the determination of the eventual fate of the cell. GC-induced thymocyte apoptosis is mediated through mitochondrial signaling as inhibition of caspase-8 function (in the extrinsic pathway) does not affect the sensitivity of thymocytes to GCs. However, caspase 9 and Apaf1 deficient thymocytes show greatly reduced sensitivity to dex [40], [143] as do thymocytes that overexpress Bcl-2. The mechanism of glucocorticoid-induced apoptosis is likely to be dependent on multiple genes being induced and/or repressed. The end result of this transcriptional modulation is the dissipation of the mitochondrial transmembrane potential and the release of proapoptotic factors including cytochrome c, which strongly suggests that the mechanism will involve members of the Bcl-2 family of proteins.

Not only do GCs induce thymocyte apoptosis, but at certain developmental stages, GCs appear to protect thymocytes from potentially deadly signals delivered through the

TCR. This observation has led to the proposal of mutually-antagonistic signaling pathways that are crucial to the selection of immature thymocytes [111]. Evidence in support of this mutually-antagonistic pathway includes the observations that antisense expression of GR, under control of a thymus specific promoter, rendered the thymocyte population exquisitely sensitive to TCR signaling. Likewise, inhibition of steroid synthesis in fetal thymic organ culture (FTOC) resulted in greatly increased sensitivity of thymocytes to TCR engagement [144]. Conversely, modulation of the MEK/ERK signaling pathway, downstream of TCR engagement, not only indicated roles in positive and negative selection, but also interfered with GR-mediated apoptosis [113]. More specifically, constitutively active forms of Ras, Raf, MEK or ERK were shown to block GR mediated apoptosis, whereas dominant negative forms of Ras, MEK or ERK blocked positive selection [145]. Thus, it appears as though the glucocorticoid and TCR-mediated signaling pathways are intimately integrated to deliver a complex yet concise verdict with regard to cell fate.

In this dissertation, the experimental procedure was aimed at the characterization of a highly-induced glucocorticoid-regulated gene whose expression precedes apoptosis. It is generally accepted that GC-induced apoptosis in immature thymocytes requires macromolecular synthesis. A similar observation, of an active death-inducing process, has been made in other organisms including distantly related, *S. cerevisiae*, where inhibition of protein synthesis prevented presentation of apoptotic markers in response to H_2O_2 treatment [52]. The actively induced gene, GIG18, fits this rather broad criterion and has demonstrated expression patterns that correlate with apoptosis. First, Ca^{2+}

ionophore induced GIG18 expression in several GC insensitive thymocyte cell lines is shown to be reduced in proportion to the relative sensitivities of these cell lines to this treatment (Figure 6). Second, GC-induced expression of GIG18 is essentially absent in these same cell lines which is not due to mutations in GR. Importantly, GIG18 transcriptional induction is followed by translational induction of two bands visible on western blots.

GIG18 was identified in the immature thymocyte cell line, WEHI 7.2, which is at an intermediate stage of development, where *in vivo*, it would be subjected to positive or negative selection occurring in the thymus. Combined with the fact that GCs have been shown to have pro and antiapoptotic effects on thymocytes, some of the molecular events occurring in response to GCs will have an effect on TCR signaling. In fact, two GC-regulated genes have been identified that can interfere with apoptosis induced by TCR signaling through modulation of NFkB and Fas/FasL signaling [112]. The B lymphoblast cell line, P388, in which a GIG18 family member was shown to be induced by C/EBP, provides an opportunity to include another potential role for GIG18 family members. Since expression of C/EBP in B lymphoblasts primes these cells for an inflammatory response to LPS, which is a scenario not totally unlike that of thymocyte selection, it could be that the GIG18 family member is involved in modulation of the transcriptional response to antigenic stimulation. In the case of thymocyte selection, the transcriptional modulation could either be pro or antiapoptotic depending on the nature of signaling through the TCR. The fact that GIG18 family members have shown the highest levels of expression in tissues where T and B cell are selected, thymus and bone marrow,

respectively, allows the hypothesis that this family of genes may be involved in the generation of the cell-mediated (T cell) and humoral (B cell) effector aspects of immune system function.

The HeLa stable cell lines that were generated in order to characterize the effect of GIG18 expression, led to an interesting observation. Not only were pBiGIG18 stable cell lines, which express GIG18 and GFP separately, obtained at twice the frequency of any other cell line, but the resulting stables had the highest fold tet-regulated induction of GFP, which was consistently enhanced with the passage of time (Figure 21). Although the outline of GIG18 expression patterns in WEHI cell lines was shown to correlate with the induction of apoptosis, the data just discussed are suggestive of a potential role in transcriptional modulation. This proposed modulation, in the context of thymocyte development, could guide the genomic response to TCR signaling and the decision of cell fate.

One of the effects of TCR signaling is the recruitment and activation of phospholipase C-γ, which leads to the mobilization of calcium from intracellular stores. In Chapter III, it was demonstrated that GIG18 is also induced by calcium mobilization mediated by A23187, so it is possible that TCR signaling can individually modulate expression of GIG18. Even though the role of calcium signaling in apoptosis is still being debated [146], [147], TCR signaling may still have an inductive or repressive effect on genes induced prior to apoptosis, including GIG18. Recent data has shown that the activity of simple and complex GREs are affected by TCR signaling components (Figure 3C), [113]. Transcription from simple GREs (requires only GR) appears to be favored by TCR

signaling, and transcription from complex GREs is disfavored (requires additional regulators). The nature of the GIG18 promoter(s) could thus dictate the transcriptional outcome, with a corresponding dependence on the strength of signal delivered by the TCR. Since the GIG18 ORF appeared to promote transcriptional induction when expressed from the pBiGIG18 plasmid, any effect of TCR signaling on GIG18 expression levels may be biologically important.

A conclusion from further analysis of the recently completed human genome sequence may be that mammals derive their complexity not only from more extensive shuffling of protein domains, but also from multiple transcripts being derived from "one" gene. There are already plenty of examples of this phenomenon, with clear differences between homologous mouse and human genes [148], [149]. Western blots suggested that two products with antigenic sequence recognized by our polyclonal GIG18 antibody were induced at the protein level. Another transcript identified in the EST database, in the 5' region of the GIG18 gene, has an ORF that potentially encodes a protein of ~10KDa which would have been missed on our western blots due to its small size. The GIG18 ORF that was characterized within this dissertation does not correspond to the most highly induced band on the western blot. The 68KDa band is more quickly and highly induced and therefore may have a more influential effect on thymocyte fate.

Obtaining the entire ORF from the transcript variant that encodes the 68KDa protein would be helpful since it could allow additional classification of GIG18 function. Database searches, GIG-specific library screens and RTPCR reactions did not allow complete identification of the corresponding ORF which hints that a technical challenge

needs to be overcome. Experimental evidence suggests that the reverse-transcriptase enzyme has trouble proceeding in the 5' direction on this transcript and the difficulty corresponds with an ~80% GC-rich area. This type of sequence could lead to problematic 2° and 3° RNA structures which can be very difficult for a polymerase to process [150]. This region may be the CpG-island within which the remaining ORF could be found. CpG-islands are commonly found in the promoter regions of housekeeping genes and are also associated with the expression of tissue-specific genes [141].

Even if the entire 68KDa ORF was obtained, much more effort would need to be put into characterization experiments like the ones described in this thesis. Perhaps the best possible characterization of GIG18 at this point would be to generate a GIG18-knockout in mouse. Cloned DNA available in the lab is adequate to screen libraries to obtain genomic DNA suitable for this purpose. A careful examination of the resultant mouse could give clear indications of GIG18 function. More specifically, analysis of the T-lymphocyte population for surface markers such as the TCR (CD3), CD4, CD8 and examination of their sensitivity to glucocorticoids and TCR signaling would be especially informative with regard to potential function of GIG18 in thymocyte development and apoptosis.

Final Remark.

I have described the molecular genetic analysis of glucocorticoid-induced gene 18 which was identified in our lab during a differential screening process. It belongs to a gene family with at least three human genomic members, each on separate chromosomes,

and they appear to have conserved regions that are likely critical to cellular function. GIG18 related ESTs have been found in several mammalian species and also in chicken and frog. No matches have been found in worm, flies, plants or yeast which could indicate that GIG18 is a relatively "new" gene having arisen to meet the demands of designing larger, more complex organisms. In contrast, there may be functional equivalents in these organisms but sequence divergence has prevented their identification. Lastly, during the process of thymocyte development and selection, many genes are known to be induced or repressed. The database analysis of the GIG18 ORF combined with the expression data suggested that this gene family may be involved in modulating transcriptional responsiveness of the cells in which it is expressed. It is possible that several of the variant GIG18 transcripts help contribute to the complex, multi-faceted process of thymocyte development (Figure 24).

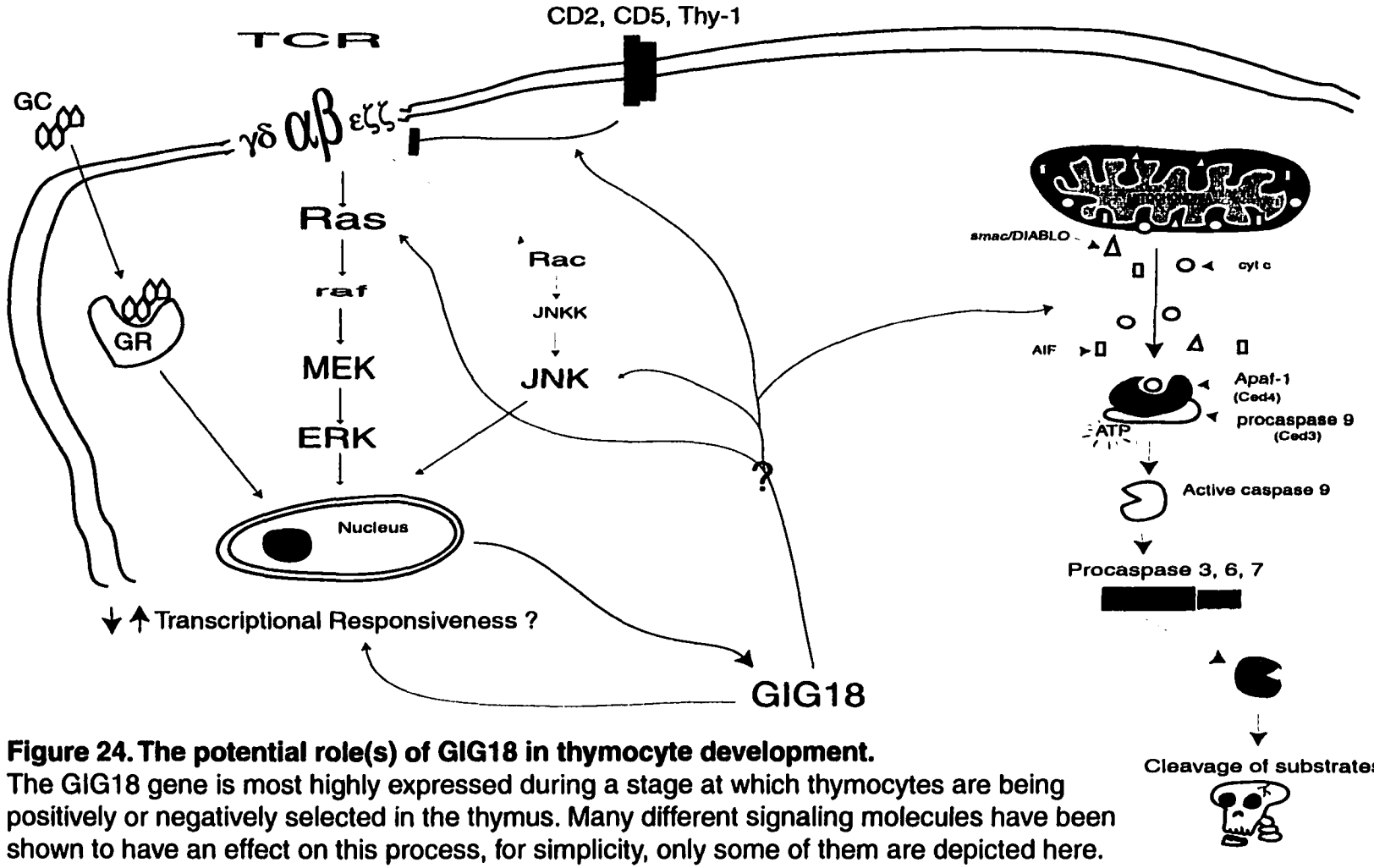


Figure 24. The potential role(s) of GIG18 in thymocyte development.
The GIG18 gene is most highly expressed during a stage at which thymocytes are being positively or negatively selected in the thymus. Many different signaling molecules have been shown to have an effect on this process, for simplicity, only some of them are depicted here. Expression analysis has suggested that GIG18 family members may have a positive effect on the transcriptional responsiveness of cells expressing these genes.

APPENDIX A:

NUCLEOTIDE SEQUENCE OF THE *mGIG18* cDNA

```
        10         20         30         40         50
CGTTGCTGTCGGGACCGTGGTGGGTGGCGAGCTGCGGGCTGCGGCGGAGT
GCAACGACAGCCCTGGCACCACCCACCGCTCGACGCCCGACGCCGCCTCA

        60         70         80         90        100
CTCAGATAGCAGCAGCAGCGGCGGCGGCGGCTATGTTTCACTTCTTCCGA
GAGTCTATCGTCGTCGTCGCCGCCGCCGCCGATACAAAGTGAAGAAGGCT

       110        120        130        140        150
AAGCCTGCAGAATCCAAAAAGCCCTCTGGGCCGGAACCGGAAGCAGATGG
TTCGGACGTCTTAGGTTTTTCGGGAGACCCGGCCTTGGCCTTCGTCTACC

       160        170        180        190        200
ATTTGTCCTCTTAGGAGCTACAGCAAATGAAGTGAGATGTAAAACTTCTG
TAAACAGGAGAATCCTCGATGTCGTTTACTTCACTCTACATTTTGAAGAC

       210        220        230        240        250
AAGCAGAAGGCAGCCAGGCTTTGGAGACCGGCAAAGAAGACACATCCAGC
TTCGTCTTCCGTCGGTCCGAAACCTCTGGCCGTTTCTTCTGTGTAGGTCG

       260        270        280        290        300
GTGACTGTATCAGGCCCCGAGACAGAGAATCAGACAGGCCAGACTCTGCA
CACTGACATAGTCCGGGGCTCTGTCTCTTAGTCTGTCCGGTCTGAGACGT

       310        320        330        340        350
GAACAACTCACTAACAGCTGAGCTCCTGAGCGATGTGCCCTTCACCCTGG
CTTGTTGAGTGATTGTCGACTCGAGGACTCGCTACACGGGAAGTGGGACC
                                    M  C  P  S  P  W>

       360        370        380        390        400
CCCCACATGTGCTGGCGGCACAGGGCACCATCAGCGACCTTCCTGACCAC
GGGGTGTACACGACCGCCGTGTCCCGTGGTAGTCGCTGGAAGGACTGGTG
 P  H  M  C  W  R  H  R  A  P  S  A  T  F  L  T  T>

       410        420        430        440        450
GTACTCTCATACGATGTCGGCGACAACTTGTCCCGATTCTGTGGACAGGC
CATGAGAGTATGCTACAGCCGCTGTTGAACAGGGCTAAGACACCTGTCCG
  Y  S  H  T  M  S  A  T  T  C  P  D  S  V  D  R>
```

```
       460        470        480        490        500
CAAAATCTCAACATATTCGAACCTCTAGTACAATAAGGCGAACCTCTTCT
GTTTTAGAGTTGTATAAGCTTGGAGATCATGTTATTCCGCTTGGAGAAGA
P  K  S  Q  H  I  R  T  S  S  T  I  R  R  T  S  S>

       510        520        530        540        550
CTGGATACGATAACAGGACCTTACCTCACAGGACAGTGGCCACGGGACCC
GACCTATGCTATTGTCCTGGAATGGAGTGTCCTGTCACCGGTGCCCTGGG
 L  D  T  I  T  G  P  Y  L  T  G  Q  W  P  R  D  P>

       560        570        580        590        600
TCATGTTCACTACCCTTCATGCATGAGAGATAAAGCCACTCAGACACCTA
AGTACAAGTGATGGGAAGTACGTACTCTCTATTTCGGTGAGTCTGTGGAT
  H  V  H  Y  P  S  C  M  R  D  K  A  T  Q  T  P>

       610        620        630        640        650
GTTGCTGGGCAGAAGAGGGAGCAGAAAAACGATCACATCAGCGCTCTGCA
CAACGACCCGTCTTCTCCCTCGTCTTTTTGCTAGTGTAGTCGCGAGACGT
S  C  W  A  E  E  G  A  E  K  R  S  H  Q  R  S  A>

       660        670        680        690        700
TCATGGGGAAGTGCTGATCAACTGAAAGAGATTGCCAAACTTAGGCAACA
AGTACCCCTTCACGACTAGTTGACTTTCTCTAACGGTTTGAATCCGTTGT
 S  W  G  S  A  D  Q  L  K  E  I  A  K  L  R  Q  Q>

       710        720        730        740        750
GCTACAACGCAGCAAGCAAAGTAGTCGGCATAGTAAAGAGAAGGACAGAC
CGATGTTGCGTCGTTCGTTTCATCAGCCGTATCATTTCTCTTCCTGTCTG
  L  Q  R  S  K  Q  S  S  R  H  S  K  E  K  D  R>

       760        770        780        790        800
AGTCACCTCTCCATGGCAACCACATAACAATCAGTCATACTCAGGCTATT
TCAGTGGAGAGGTACCGTTGGTGTATTGTTAGTCAGTATGAGTCCGATAA
Q  S  P  L  H  G  N  H  I  T  I  S  H  T  Q  A  I>

       810        820        830        840        850
GGATCAAGATCAGTCCCTATGCCTCTGTCAAACATATCAGTGCCAAAATC
CCTAGTTCTAGTCAGGGATACGGAGACAGTTTGTATAGTCACGGTTTTAG
 G  S  R  S  V  P  M  P  L  S  N  I  S  V  P  K  S>

       860        870        880        890        900
ATCTGTTTCCCGTGTACCCTGCAATGTAGAAGGAATAAGTCCTGAATTGG
TAGACAAAGGGCACATGGGACGTTACATCTTCCTTATTCAGGACTTAACC
  S  V  S  R  V  P  C  N  V  E  G  I  S  P  E  L>

       910        920        930        940        950
AAAAGGTATTCATCAAAGAAAACAATGGAAAGGAAGAAGTGTCCAAGCCA
TTTTCCATAAGTAGTTTCTTTTGTTACCTTTCCTTCTTCACAGGTTCGGT
E  K  V  F  I  K  E  N  N  G  K  E  E  V  S  K  P>
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960 970 980 990 1000
TTGGATATACCAGATGGTCGAAGAGCTCCGCTCCCTGCTCACTACAGGAG
AACCTATATGGTCTACCAGCTTCTCGAGGCGAGGGACGAGTGATGTCCTC
L D I P D G R R A P L P A H Y R S>

1010 1020 1030 1040 1050
CAGTAGTACTCGAAGCATAGATACCCAGACACCTTCTGTCCAAGAGCGCA
GTCATCATGAGCTTCGTATCTATGGGTCTGTGGAAGACAGGTTCTCGCGT
S S T R S I D T Q T P S V Q E R>

1060 1070 1080 1090 1100
GCAGTAGCTGCAGCAGCCACTCCCCTTGTGTGTCCCCATTTTGTCCTCCG
CGTCATCGACGTCGTCGGTGAGGGGAACACACAGGGGTAAAACAGGAGGC
S S S C S S H S P C V S P F C P P>

1110 1120 1130 1140 1150
GAATCCCAGGATGGAAGTCCTTGTTCAACAGAAGATTTGCTCTATGATCG
CTTAGGGTCCTACCTTCAGGAACAAGTTGTCTTCTAAACGAGATACTAGC
E S Q D G S P C S T E D L L Y D R>

1160 1170 1180 1190 1200
TGATAAAGACAGTGGGAGTAGCTCACCGTTACCCAAGTATGCTTCATCTC
ACTATTTCTGTCACCCTCATCGAGTGGCAATGGGTTCATACGAAGTAGAG
D K D S G S S S P L P K Y A S S>

1210 1220 1230 1240 1250
CCAAACCCAACAACAGCTACATGTTCAAACGGGAGCCCCCAGAGGGATGT
GGTTTGGGTTGTTGTCGATGTACAAGTTTGCCCTCGGGGGTCTCCCTACA
P K P N N S Y M F K R E P P E G C>

1260 1270 1280 1290 1300
GAGCGGGTGAAGGTCTTTGAGGAAATGGCGTCTCGTCAGCCTATCTCGGC
CTCGCCCACTTCCAGAAACTCCTTTACCGCAGAGCAGTCGGATAGAGCCG
E R V K V F E E M A S R Q P I S A>

1310 1320 1330 1340 1350
CCCTCTCTTTTCATGTCCTGACAAAAACAAGGTTAATTTCATCCCAACCG
GGGAGAGAAAAGTACAGGACTGTTTTTGTTCCAATTAAAGTAGGGTTGGC
P L F S C P D K N K V N F I P T>

1360 1370 1380 1390 1400
GATCAGCTTTCTGTCCTGTAAAACTTCTAGGCCCTCTCTTACCTGCCTCT
CTAGTCGAAAGACAGGACATTTTGAAGATCCGGGAGAGAATGGACGGAGA
G S A F C P V K L L G P L L P A S>

1410 1420 1430 1440 1450
GACCTGATGCTCAAGAACTCCCCTAATTCTGGCCAGAGCTCGGCTCTGGC
CTGGACTACGAGTTCTTGAGGGGATTAAGACCGGTCTCGAGCCGAGACCG
D L M L K N S P N S G Q S S A L A>

1460 1470 1480 1490 1500
AACACTGACCGTAGAGCAGCTTTCCTCCCGGGTTTCCTTCACATCTCTCT
TTGTGACTGGCATCTCGTCGAAAGGAGGGCCCAAAGGAAGTGTAGAGAGA
T L T V E Q L S S R V S F T S L>

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      1510       1520       1530       1540       1550
CTGATGACACCAGCACAGCCGACTCCCTGGAGCCCTCTGCCCAGCAGCCA
GACTACTGTGGTCGTGTCGGCTGAGGGACCTCGGGAGACGGGTCGTCGGT
S  D  D  T  S  T  A  D  S  L  E  P  S  A  Q  Q  P>

      1560       1570       1580       1590       1600
TCTCAGCAGCAGCAGCTCCTACAGGACTTGCAGGTGGAGGAACACGTCTC
AGAGTCGTCGTCGTCGAGGATGTCCTGAACGTCCACCTCCTTGTGCAGAG
 S  Q  Q  Q  Q  L  L  Q  D  L  Q  V  E  E  H  V  S>

      1610       1620       1630       1640       1650
CACTCAGAACTATGTGATGATCTAAAGCAGAGGGGGAGCTGGTCTCCACC
GTGAGTCTTGATACACTACTAGATTTCGTCTCCCCCTCGACCAGAGGTGG
  T  Q  N  Y  V  M  I  *

      1660       1670       1680       1690       1700
CATGGTCCATGGATTGGGAATGAGATCTCAGACATCTATCTGCATGGAGT
GTACCAGGTACCTAACCCTTACTCTAGAGTCTGTAGATAGACGTACCTCA

      1710       1720       1730       1740       1750
GACAAACTTTCCCAACATCACCAACAGCAAAGTACTTAGCATCACAAAAT
CTGTTTGAAAGGGTTGTAGTGGTTGTCGTTTCATGAATCGTAGTGTTTTA

      1760       1770       1780       1790       1800
AGCTATTAACACTGATCTTGGCAGGGACCGACTTATATTCAGCAGTTTTT
TCGATAATTGTGACTAGAACCGTCCCTGGCTGAATATAAGTCGTCAAAAA

      1810       1820       1830       1840       1850
GTGGAAAGCAGTAATGCTTGCAAAAATGTGTGTGTCATTCAGCATTTAAT
CACCTTTCGTCATTACGAACGTTTTTACACACACAGTAAGTCGTAAATTA

      1860       1870       1880       1890       1900
GGAGACTATGCATTTCATAGTATGTCTGACAGATTAGTACTGTGTCCTGT
CCTCTGATACGTAAAGTATCATACAGACTGTCTAATCATGACACAGGACA

      1910       1920       1930       1940       1950
GTTTTGTTCCAACATTTTCAGTATGAATAAGCTCTATTTCAAAAAGTTGC
CAAAACAAGGTTGTAAAAGTCATACTTATTCGAGATAAAGTTTTTCAACG

      1960       1970       1980       1990       2000
CTGTCTAAGTAGAAAATGTCTTGCTGTGTTTTGTCCTATGGAAAATACTG
GACAGATTCATCTTTTACAGAACGACACAAAACAGGATACCTTTTATGAC
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      2010       2020       2030       2040       2050
TACTTCAGGATTATGTTTACAATTGATCCAGGTGTTTGTTTCTAACTTCT
ATGAAGTCCTAATACAAATGTTAACTAGGTCCACAAACAAAGATTGAAGA

      2060       2070       2080       2090       2100
ATAATACATACAATGCMAAAAAAAAAAAAAAAATGGCCAACAACAGTTGCA
TATTATGTATGTTACGKTTTTTTTTTTTTTTTACCGGTTGTTGTCAACGT

      2110       2120       2130       2140       2150
CAGTGCCCACCCTATGGCCTAGCTTCAGGTACTTCAGTTGAAGTCTAAAC
GTCACGGGTGGGATACCGGATCGAAGTCCATGAAGTCAACTTCAGATTTG

      2160       2170       2180       2190       2200
TCAGGTAACTTGGAATGTATGTCATATTGGGATATTAAATATTTCACAGC
AGTCCATTGAACCTTACATACAGTATAACCCTATAATTTATAAAGTGTCG

      2210       2220       2230       2240       2250
TACAAAGCTAAAGAGGGAACATCACTCTTTTGCCTTTCCTTGTTTTATGC
ATGTTTCGATTTCTCCCTTGTAGTGAGAAAACGGAAAGGAACAAAATACG

      2260       2270       2280       2290       2300
ATTTCCTTTTCTTCATTCCATTCCACACTAGAATAAGAAGTGCATTGATC
TAAAGGAAAAGAAGTAAGGTAAGGTGTGATCTTATTCTTCACGTAACTAG

      2310       2320       2330       2340       2350
CCTAGGAGATGCTAATCCTGGGTATAGGGAACATGAGGAGAACCAGCAAA
GGATCCTCTACGATTAGGACCCATATCCCTTGTACTCCTCTTGGTCGTTT

      2360       2370       2380       2390       2400
TTTGTGGTGTGTGACATCACTTTTGTCATGTGGTTACCAGTAGAACAACT
AAACACCACACACTGTAGTGAAAACAGTACACCAATGGTCATCTTGTTGA

      2410       2420       2430       2440       2450
GTTGCATTCACTGTTTCAACATGTGTAAATGTGGCTTTTTTTTTTTAAAA
CAACGTAAGTGACAAAGTTGTACACATTTACACCGAAAAAAAAAAATTTT

      2460       2470       2480       2490       2500
AAAAAGAAAAAGAAAAAAGAAAAAACAAGTTGTGTTGCTCAGTGACTGAC
TTTTTCTTTTTCTTTTTTCTTTTTTGTTCAACACAACGAGTCACTGACTG

      2510       2520       2530       2540       2550
TGTTACAGTGTTGCAAATAAAGCCTCAGTAGTAGCCTACATAAACATTCC
ACAATGTCACAACGTTTATTTCGGAGTCATCATCGGATGTATTTGTAAGG
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      2560       2570       2580       2590       2600
CCATTGCGTGTGACGAAATTTAAATGCAGGGATGGCTGGAGTTCATTTCA
GGTAACGCACACTGCTTTAAATTTACGTCCCTACCGACCTCAAGTAAAGT

      2610       2620       2630       2640       2650
AAGCAAGTGGCGTTTTGCAGAAATAAATAAGGATTTTTCTTTATATTGGC
TTCGTTCACCGCAAAACGTCTTTATTTATTCCTAAAAAGAAATATAACCG

      2660       2670       2680       2690       2700
CATTGGGGTCATAATTGCTACTATTTTTATCTAAAGACATATTTATGTTT
GTAACCCCAGTATTAACGATGATAAAAATAGATTTCTGTATAAATACAAA

      2710       2720       2730       2740       2750
AGCTTCATTTTAGGAAATTGTAATTTTCTGGTTACAAAGTGAAACATTTT
TCGAAGTAAAATCCTTTAACATTAAAAGACCAATGTTTCACTTTGTAAAA

      2760       2770       2780       2790       2800
GGTTACTGTTTTCCTCAAGTATTTGGTAAATGAGTTGTATTCATGTAGAG
CCAATGACAAAAGGAGTTCATAAACCATTTACTCAACATAAGTACATCTC

      2810       2820       2830       2840       2850
AGAATGTGTATGTCTCAGAACCAGTGCAGCATAAGCAAGCAGATTTCTTG
TCTTACACATACAGAGTCTTGGTCACGTCGTATTCGTTCGTCTAAAGAAC

      2860       2870       2880       2890       2900
TTGACTTTCGAGAGCAATAGCTTAAAGCTGTCTCATTTAAGCCTCAGATA
AACTGAAAGCTCTCGTTATCGAATTTCGACAGAGTAAATTCGGAGTCTAT

      2910       2920       2930       2940       2950
ACACTATCATGAATAATTTTGTTAAGACGTAGAACGCATGGAGTGGAGCA
TGTGATAGTACTTATTAAAACAATTCTGCATCTTGCGTACCTCACCTCGT

      2960       2970       2980       2990       3000
GAACCCCGAGTAAATAGCCTTGAACTTGCAGACTTGACAGAAGTTCTCCA
CTTGGGGCTCATTTATCGGAACTTGAACGTCTGAACTGTCTTCAAGAGGT

      3010       3020       3030       3040       3050
CAAAGTGCATAGAAGAGAGCCATAGAGATTCCTGTTGCCACCTGGAGAGC
GTTTCACGTATCTTCTCTCGGTATCTCTAAGGACAACGGTGGACCTCTCG

      3060       3070       3080       3090       3100
CCGACTTCAGACTTTATTTTTTTGCAGCACAGCCTGTGCAAAAGTAAAGG
GGCTGAAGTCTGAAATAAAAAAACGTCGTGTCGGACACGTTTTCATTTCC
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      3110       3120       3130       3140       3150
GACGCTTTTGTATTGGTGTTCCGTTGCTCTCAGTTCAGTTAGAGCCTAGA
CTGCGAAAACATAACCACAAGGCAACGAGAGTCAAGTCAATCTCGGATCT

      3160       3170       3180       3190       3200
GTTCTCCCATCTCCACCTAACGTGTGTGGCACAATGTTTGGTTTGGTTGG
CAAGAGGGTAGAGGTGGATTGCACACACCGTGTTACAAACCAAACCAACC

      3210       3220       3230       3240       3250
TTTTTAGAGTCGGGAACCGCTGCCTACAGTTACTCTTCAGTTTTCCATAG
AAAAATCTCAGCCCTTGGCGACGGATGTCAATGAGAAGTCAAAAGGTATC

      3260       3270       3280       3290       3300
GTCATACCTGCGGTTTCCTTTGACTCCTCTAAGGAGTCAACGAGTAGAAG
CAGTATGGACGCCAAAGGAAACTGAGGAGATTCCTCAGTTGCTCATCTTC

      3310       3320       3330       3340       3350
GATGAGCTGATAAAGTGATGATACCGTGTTAATTTTTACAAAATGCTGTG
CTACTCGACTATTTCACTACTATGGCACAATTAAAAATGTTTTACGACAC

      3360       3370       3380       3390       3400
CGTGAGTGTGCATACACGTGCACATGGATGTGTGGATGTGTGTATTCTGT
GCACTCACACGTATGTGCACGTGTACCTACACACCTACACACATAAGACA

      3410       3420       3430       3440       3450
GTGCTATTTTGTCACCTTGATGTCTCCTTTCCACCAAAGTTTGCAGTAAT
CACGATAAAACAGTGGAACTACAGAGGAAAGGTGGTTTCAAACGTCATTA

      3460       3470       3480       3490       3500
ACTCTCTTCTGAAGGTACGCAGTCAGTGTTATAGAGACTGTCATGGAGAA
TGAGAGAAGACTTCCATGCGTCAGTCACAATATCTCTGACAGTACCTCTT

      3510       3520       3530       3540       3550
GAGGACTCAGTTGACTTTTGCCATTTTCATAGGGGAACCTTTTAAAACAA
CTCCTGAGTCAACTGAAAACGGTAAAAGTATCCCCTTGGAAAATTTTGTT

      3560       3570       3580       3590       3600
TCTTTTCAGCAGCAGACACCTTTAACCCTAATAATCTCAGGCCTTGATGA
AGAAAAGTCGTCGTCTGTGGAAATTGGGATTATTAGAGTCCGGAACTACT
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      3610       3620       3630       3640       3650
AAATACTATATTTTGTAGATTATGGTTAAAGGAAGAAAATTGCTAGTTCC
TTTATGATATAAAACATCTAATACCAATTTCCTTCTTTTAACGATCAAGG

      3660       3670       3680       3690       3700
GTAAGATAAATATGAGCTCCATTTAACTTCTTTTTTTTAAGCCTCTAACA
CATTCTATTTATACTCGAGGTAAATTGAAGAAAAAAAATTCGGAGATTGT

      3710       3720       3730       3740       3750
ATTTTATTGTAAGTAAACAAAATTATGTGTACTTGTTTTATTCGGCACGT
TAAAATAACATTCATTTGTTTTAATACACATGAACAAAATAAGCCGTGCA

      3760       3770       3780       3790       3800
GAAAACAGATATTTACAGATATAAAAAACTCGTATTGACAGACGTTTAAA
CTTTTGTCTATAAATGTCTATATTTTTTGAGCATAACTGTCTGCAAATTT

      3810       3820       3830       3840       3850
TGGACAGAGTCAAGTTGATAGAAGCATGACACAAATTTGGTAAAGCAAAT
ACCTGTCTCAGTTCAACTATCTTCGTACTGTGTTTAAACCATTTCGTTTA

      3860       3870       3880       3890       3900
CTCAGATACTGACACTGAAGCAAGTGCTCTCAGATCCATCAAACAGACCA
GAGTCTATGACTGTGACTTCGTTCACGAGAGTCTAGGTAGTTTGTCTGGT

      3910       3920       3930       3940       3950
CAGCCCTCCCTTGCCAAAACTCCAATCAATTCTATCTTAGTATCTAAGAT
GTCGGGAGGGAACGGTTTTGAGGTTAGTTAAGATAGAATCATAGATTCTA

      3960       3970       3980       3990       4000
AGACATCCTGTTTGTTTTTCATAAGTCAATCTTGGCACTTTTATGAACAA
TCTGTAGGACAAACAAAAAGTATTCAGTTAGAACCGTGAAAATACTTGTT

      4010       4020       4030       4040       4050
TTAGTTCCTCTATTCACAAAAGGCATCATAATTATTTTTACTTTCTTAAA
AATCAAGGAGATAAGTGTTTTCCGTAGTATTAATAAAAATGAAAGAATTT
  *

      4060       4070       4080       4090       4100
GTTTGAGGGTTTTTTGGTTTTTGTTTTAAGTAGCCAAGATCTACAGTTGA
CAAACTCCCAAAAAACCAAAAACAAAATTCATCGGTTCTAGATGTCAACT

      4110       4120       4130       4140       4150
TCTTYTAAAAGGTCACGATCTTAGTAAGTTCTTTTTAGTTACAGGCAAAG
AGAARATTTTCCAGTGCTAGAATCATTCAAGAAAAATCAATGTCCGTTTC
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      4160       4170       4180       4190       4200
GACCCACTTTACAGTTGGCTTTTAGGATCTTGCCATTCAAACAGTCTTKG
CTGGGTGAAATGTCAACCGAAAATCCTAGAACGGTAAGTTTGTCAGAAMC

      4210       4220       4230       4240       4250
TTGTTATGGTAAGCACACAGACACTTTGTACAATATTCAAATTGACAGCT
AACAATACCATTCGTGTGTCTGTGAAACATGTTATAAGTTTAACTGTCGA

      4260       4270       4280       4290       4300
TTCCCGTTGCAGAMTGCTCGCTCTAAATAGTGGTCATATTTGGCAGGGAA
AAGGGCAACGTCTKACGAGCGAGATTTATCACCAGTATAAACCGTCCCTT

      4310       4320       4330       4340       4350
ATTATAGCAAGCACAGGCTTGAGGCTTCCCTTGTTCTTCAATGTCTGCCA
TAATATCGTTCGTGTCCGAACTCCGAAGGGAACAAGAAGTTACAGACGGT

      4360       4370       4380       4390       4400
CYTCCACGAACTTGTTGTGCCAGCTGTAGGTAGAACCTTTCTGACCACGC
GRAGGTGCTTGAACAACACGGTCGACATCCATCTTGGAAAGACTGGTGCG

      4410       4420       4430       4440       4450
TGACTGGAGGACTTGACCTGAACATCTTTTTTTTGGGAGGTGCCCAGGTC
ACTGACCTCCTGAACTGGACTTGTAGAAAAAAAACCCTCCACGGGTCCAG

      4460       4470       4480       4490       4500
GACGATGACTTTGAACAGAAGTTAGTGCAGGTCTGCCCACAACATGTCYT
CTGCTACTGAAACTTGTCTTCAATCACGTCCAGACGGGTGTTGTACAGRA

      4510       4520       4530       4540       4550
CTCGTTGCAGCATGTAGTGACAACTTACTGCTTTCAATGACTCTGCATGG
GAGCAACGTCGTACATCACTGTTGAATGACGAAAGTTACTGAGACGTACC

      4560       4570       4580       4590       4600
TTTTGCTGTAGAGCTGGACCGCTCCCTTATTGTTTTCCAGTATCTTCTTC
AAAACGACATCTCGACCTGGCGAGGGAATAACAAAAGGTCATAGAAGAAG

      4610       4620       4630       4640       4650
CAAATTCTGCTCACTTTAGACAGGTTTATTAAGTCCGTGCCCCGAGCTTC
GTTTAAGACGAGTGAAATCTGTCCAAATAATTCAGGCACGGGGCTCGAAG

      4660       4670       4680       4690       4700
ATCACAATGTTAGCCAACAGGTGCACGAATCCCCTCCGGGAGAGCTCAGC
TAGTGTTACAATCGGTTGTCCACGTGCTTAGGGGAGGCCCTCTCGAGTCG
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      4710       4720       4730       4740       4750
CAGGAGATCTAGGTGTTCCAGGCCCATTTTCTTGCCAATTATACTTTGCA
GTCCTCTAGATCCACAAGGTCCGGGTAAAAGAACGGTTAATATGAAACGT

      4760       4770       4780       4790       4800
GTCTAAAGTTGCCGCTGGTGATAACTTCCTTCAGCATTTCTCGATCCACT
CAGATTTCAACGGCGACCACTATTGAAGGAAGTCGTAAAGAGCTAGGTGA

      4810       4820       4830       4840       4850
TTAGGGTTTCGCTTGCCATTCTTTTTTAGTGTTGAGCAAACCACTCTTTG
AATCCCAAAGCGAACGGTAAGAAAAAATCACAACTCGTTTGGTGAGAAAC

      4860       4870       4880       4890       4900
ACAATGCAGGACAGGCAGCAGGTTTTGTTGGGATGCTGGTCCAGGCTCTG
TGTTACGTCCTGTCCGTCGTCCAAAACAACCCTACGACCAGGTCCGAGAC

      4910       4920       4930       4940       4950
TGATGGCAGCAGGCGGGCCTGGGGACTGTTTCTGAAATTCTCCAGGATAA
ACTACCGTCGTCCGCCCGGACCCCTGACAAAGACTTTAAGAGGTCCTATT

      4960       4970       4980       4990       5000
GGATGCCGTCGTCACGGTCACTTTGTGTAAAAGATGAGTAGCCACTGTCC
CCTACGGCAGCAGTGCCAGTGAAACACATTTTCTACTCATCGGTGACAGG

      5010       5020       5030       5040       5050
TCATACAGTCTACTGGCCTCTAGCTCTTCTGTTTCGTTTGAACTATCTAG
AGTATGTCAGATGACCGGAGATCGAGAAGACAAAGCAAACTTGATAGATC

      5060       5070       5080       5090       5100
TGTGTTTTGTACACGTTGATTCTCCTTGCTATCATGACCCACAATTGGTG
ACACAAAACATGTGCAACTAAGAGGAACGATAGTACTGGGTGTTAACCAC

      5110       5120       5130       5140       5150
ACTCGATGAAAGACAACCTCTCATGGTTTCTAACACAGTCCTTACAGGAG
TGAGCTACTTTCTGTTGGAGAGTACCAAAGATTGTGTCAGGAATGTCCTC

      5160       5170       5180       5190       5200
GTTTCAAACACACGGGACTGTAGGAACCTTCTCTCCCACTGTCCTCAGGC
CAAAGTTTGTGTGCCCTGACATCCTTGGAAGAGAGGGTGACAGGAGTCCG
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      5210       5220       5230       5240       5250
TTCAACCATTTCAAGCTCAGAAAGATCAGGGTTGCAATTAAAACACTTCA
AAGTTGGTAAAGTTCGAGTCTTTCTAGTCCCAACGTTAATTTTGTGAAGT

      5260       5270       5280       5290       5300
TTGTAACAGAAAGAACAGGGCTTTCTTCTTTATGTCCACGGTCGTCCACG
AACATTGTCTTTCTTGTCCCGAAAGAAGAAATACAGGTGCCAGCAGGTGC

      5310       5320       5330       5340       5350
CGCCAAGACGGCAGGGACAGAACGACGGCCGGCGCAGATCACTGCAGGTG
GCGGTTCTGCCGTCCCTGTCTTGCTGCCGGCCGCGTCTAGTGACGTCCAC

      5360       5370       5380       5390       5400
TGCCTGCTCATGCGGGAAAACTCGGGCTCCAGTGCAGGGGAGGTGGCGGC
ACGGACGAGTACGCCCTTTTGAGCCCGAGGTCACGTCCCCTCCACCGCCG

      5410       5420       5430       5440       5450
GACCATAGGAAAGAGGTGGCCGGCCCTCCATTTAACTTCTAATATCTGGG
CTGGTATCCTTTCTCCACCGGCCGGGAGGTAAATTGAAGATTATAGACCC

      5460       5470       5480       5490       5500
TTAGCATTACATGATGTGTTGATCTTATGCTCAGCATTTGTCTAAGATGC
AATCGTAATGTACTACACAACTAGAATACGAGTCGTAAACAGATTCTACG

      5510       5520       5530       5540       5550
AATAGTATCATTATCAATTTCAGAAATATGGACTGAATATGCTTTTTTGG
TTATCATAGTAATAGTTAAAGTCTTTATACCTGACTTATACGAAAAAACC

      5560       5570       5580       5590       5600
TGATGAAATCTATGTACGATATTTATAGTGATGTGCTTTTATTTTCTCAT
ACTACTTTAGATACATGCTATAAATATCACTACACGAAAATAAAAGAGTA

      5610       5620       5630       5640       5650
GAGAAACTAAATATTGTGTTGTACATTTGTTTTTAGCATATATTAAAGTT
CTCTTTGATTTATAACACAACATGTAAACAAAAATCGTATATAATTTCAA

      5660       5670       5680       5690       5700
TTAAACCAAATGTGTTAAAGCTTATGCTTTGCCATGCAAATCCCCCAGAA
AATTTGGTTTACACAATTTCGAATACGAAACGGTACGTTTAGGGGGTCTT

      5710       5720       5730       5740       5750
GTTGGTGAGCTCAAATGTATCCTACATCCAGCTGTAGAAACTTGTCAGAA
CAACCACTCGAGTTTACATAGGATGTAGGTCGACATCTTTGAACAGTCTT
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      5760       5770       5780       5790       5800
ATTGTTTAAATTTTGTATATAGGTGACTGTTTAATTTTAGCCACTGCGCT
TAACAAATTTAAAACATATATCCACTGACAAATTAAAATCGGTGACGCGA

      5810       5820       5830       5840       5850
GAACAGTATTCAAGTTATATAATATGGCTTTACACAAGAAATGTGTGGCT
CTTGTCATAAGTTCAATATATTATACCGAAATGTGTTCTTTACACACCGA

TCT
AGA
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