DISTINCT MECHANISMS REGULATE INDUCTION
OF STRESS EFFECTOR GADD45B

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Temple University Graduate Board

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Requirements for the Degree
DOCTOR OF PHILOSOPHY

By
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The GADD45 family of proteins consists of three small nuclear proteins, GADD45A, GADD45B, and GADD45G, which are implicated in modulating the cellular response to various types of genotoxic/physiological stress. This family of proteins has been shown to interact with and modulate the function of cell-cycle control proteins, such as p21 and cdc2/cyclin B1, the DNA repair protein, PCNA, key stress response MAP kinases, including MEKK4 (an upstream regulator of JNK kinase), and p38 kinase. Despite similarities in amino acid sequence, structure and function, each gadd45 gene is induced differentially, depending on the type of stress stimuli. For example, the alkylating agent, methylmethane sulfonate (MMS), rapidly induces all three genes, whereas hydrogen peroxide and sorbitol preferentially induce gadd45a and gadd45b, respectively. Studies of the mechanisms of the stress-mediated induction of the
gadd45 genes have predominantly focused on gadd45a, with knowledge of gadd45b and gadd45g regulation lacking. Thus, in order to generate a more complete understanding of the collective regulation of the gadd45 genes, a comprehensive analysis of the stress-mediated induction of gadd45b has been carried out. Towards this end, a gadd45b promoter-reporter construct was generated, consisting of 3897bp sequence upstream of the transcription start site of gadd45b, fused to a luciferase reporter. In a human colorectal carcinoma cell line (RKO), in which gadd45b mRNA levels profoundly increase by various stress stimuli, we observe similar, high levels of induction of the gadd45b-luciferase construct with MMS or UVC treatments, but surprisingly not with sorbitol or anisomycin. Linker-scanning mutagenesis of the gadd45b promoter reveals several important MMS and UVC cis-acting responsive elements contained within the proximal promoter, including a GC-rich region and the CCAAT box. Furthermore, we have identified three constitutively bound transcription factors, Sp1, MZF1, and NFY, and one inducible factor, Egr1, which bind to these regions and which contribute to MMS-responsiveness. In contrast, a post-transcriptional mechanism appears to regulate gadd45b induction upon sorbitol treatment, as
this treatment increases the \textit{gadd45b} mRNA half-life, compared to MMS treatment. Interestingly, with the exception of a common cis-element, the stress-mediated induction of \textit{gadd45b} appears to be mechanistically distinct from \textit{gadd45a}. In conclusion, this study provides novel evidence that \textit{gadd45b} induction by distinct stress agents, MMS and sorbitol, is regulated differentially at the level of mRNA transcription or mRNA stability, respectively.
ACKNOWLEDGEMENTS

The completion of this dissertation would not have been possible without the help of several key people. To begin with I would like to recognize my advisor, Dan Liebermann, who allowed me to take on this project as an offshoot of my rotation work in the spring of 2003. Dan has been both a friend and a mentor since I joined his lab and from whom I’ve learned a great deal about science. I sincerely appreciate the opportunity he has given me to work in his lab, his guidance, the freedom he has given me in my project, and his patience. Next, I’d like to acknowledge my “co-advisor” Barbara Hoffman. Barbara has been as much a mentor to me as Dan. During our weekly Friday afternoon meetings, in which we sit with Dan and Barbara to discuss our results, formulate plans of action, troubleshoot technical matters, gripe about lab matters, or just talk about dogs, Barbara has always been a voice of reason. Her constructive suggestions and good ideas have undoubtedly been a big factor in the success of my project.

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Xavier, provided valuable constructive criticisms and input into the direction of my project. In addition to helping troubleshoot specific technical issues, they would provide informative suggestions related to experimental design and approach to address specific questions. Besides the regular formal committee meetings, Scott, Dale, and Xavier always had their doors open and were available to discuss experimental or graduate-student related issues. I felt grateful for this and frequently sought out their input and advice on technical issues, data interpretation, and the like.

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cohesiveness of a small to moderate size institute, the good resources available, the good working environment, and the nice selection of projects to work on.

I would like to thank the many lab-mates that I’ve worked with over the years, for their friendship, moral support, and help with my project in one way or another.

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DEDICATION

This work is dedicated to my family, who give real meaning to my life; to my loving wife Beth, to my son Ethan, and to my daughter Valerie.

Cape May Zoo, May 2008
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ABBREVIATIONS

MMS – Methylmethane Sulfonate (DNA alkylating agent)

RKO – Human Colorectal Carcinoma cell line

Egr1 – Early growth response gene 1 (transcription factor)

Sp1 – Specificity protein 1 (transcription factor)

MZF1 – Myeloid Zinc Finger 1 (transcription factor)

NFY – Nuclear Factor 1 (transcription factor complex – NFY= NFYA/B/C)

EMSA – Electrophoretic Mobility Shift Assay

ChiP – Chromatin immunoprecipitation assay

siRNA – Small interfering RNA

RNAi – RNA interference

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

SNP – Single Nucleotide Polymorphism
CHAPTER 1

INTRODUCTION

1.1 DNA Damage Response

During their life cycle, cells are constantly subjected to diverse stressors, from exogenous sources such as changes in temperature, exposure to chemicals, or solar radiation, to endogenous stresses such as changes in pH, or exposure to damaging reactive oxygen species (ROS) generated from metabolic reactions. These diverse cellular insults primarily damage DNA and proteins, and in general cause DNA strand breakage and/or base damage, and protein unfolding and/or amino-acid cross-linking, respectively. For DNA, it has been estimated that in each cell of the human body, endogenous DNA damage from ROS generated from normal metabolic reactions alone, occurs at a rate of ~10,000 damage events per cell per day (Loft S, et al. 1996). This equates to approximately $1 \times 10^{18}$ DNA damage events per day for all cells of the average-size adult human body, solely from normal endogenous sources! Yet, in normal human cells the mutation rate of DNA is astoundingly low, occurring at a rate of $\sim 10^{-10}$ mutations.
per nucleotide per cell per generation (Jackson AL, et al. 2001). Thus, the DNA
damage response network that has evolved to maintain genomic fidelity is
exquisitely efficient and robust.

Much has been learned about the DNA damage response network in the last two
decades (Lou Z, et al. 2005). During this time, an overall signaling scheme has
been elucidated that begins with the sensing of DNA damage and normally ends
with one of two cellular outcomes: 1). apoptosis (if the damage is too great), or
2.) cell cycle arrest and DNA repair (Figure 1; Smith J, et al. 2001). When
components of this network are missing or altered, such as in individuals with
Ataxia Telangiectasia or Li-Fraumenia, who carry mutations in the “stress sensor”
ATM (*Ataxia Telangiectasia Mutated*) or the “stress transducer” p53, respectively,
individuals are more susceptible to developing various cancers (Zhou T, et al.
2007; Bakkenist CJ, et al. 2003). Likewise, it has been reported that in over 50% of
human tumors, the p53 gene is mutated, rendering it non-functional (Soussi T,
et al. 2003). A tumor initiating event occurs when some sort of genotoxic insult
generates uncorrected mutations in either tumor suppressor genes, oncogenes,
or DNA repair genes, that confers a selective growth advantage to the cell (MacDonald, 2004).

![Diagram of DNA damage response scheme]

**Figure 1.** DNA damage response scheme. Smith J, et al. 2001

Depending on the type and extent of damage, the orchestrated response can involve the altered expression of over 1800 genes to modulate the G1/S or G2/M cell cycle checkpoints, DNA repair, and/or apoptosis (Zhou T, et al, 2007). One
of these genes, which in recent years, has been found to play an important role in the DNA damage response, is \textit{gadd45b}.

\textbf{1.2 GADD45B in the DNA Damage Response}

\textit{gadd45b} (\textit{gadd45β}/\textit{MyD118}) is a member of a family of structurally and functionally related genes, that includes \textit{gadd45a} (\textit{gadd45α}/\textit{gadd45}) and \textit{gadd45g} (\textit{gadd45γ}/CR6), which encode small (18kD), evolutionarily conserved proteins, sharing high homology (55-57\%, Figure 2), high acidity (pI 4.0-4.2), and which are primarily localized to the nucleus (Liebermann, 2002). \textit{gadd45b} was first identified in 1991 as a primary response gene in the myeloid differentiation program, when leukemic myeloblasts (M1) were induced to differentiate with IL-6 (Abdollahi, et al. 1991). Shortly thereafter, it was observed that \textit{gadd45b} and other \textit{gadd45} gene members are also coordinately induced by a wide variety of genotoxic stress stimuli (Zhan, et al. 1994 and \textit{Table 6}), in addition to being ubiquitously expressed in essentially all tissues (Takekawa M, et al, 1998). With the DNA damage response recognized as an important mechanism in limiting tumor initiation events, there was great interest in elucidating other important
components of the DNA damage response network, besides the well studied p53 and ATM proteins.

**Figure 2.** Amino acid sequence alignment of GADD45 family of proteins: GADD45A, GADD45B, GADD45G. These three proteins have 55%-58% amino acid sequence identity. Tornatore L, et al. 2008.

For the past decade, a great deal of insight has been gained into the pleiotropic functions of GADD45B, and other GADD45 protein counterparts, in the context of the DNA damage response. As a protein with no known enzymatic activity,
GADD45B exerts its function in response to genotoxic stress by interacting with protein partners to modulate their activity and maintain cellular homeostasis (Figure 3). Specifically, GADD45B-protein partner interactions can influence cell cycle arrest at the G1/S and G2/M checkpoints, DNA repair, and paradoxically, both apoptosis and survival, depending on the specific cell type and/or context.

Figure 3. A number of proteins interact with GADD45B. Numbers on the arrows reference the specific interaction. See Table 1, next page, for the corresponding reference.
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<td>Papa S, et al. 2007</td>
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<td>4</td>
<td>Smith ML, et al. 1994</td>
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<td>5</td>
<td>Hall PA, et al. 1995</td>
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<td>Azam N, et al. 2001</td>
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<td>7</td>
<td>Vairapandi M, et al. 1996</td>
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<td>Takekawa M, et al. 1998</td>
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</tr>
<tr>
<td>11</td>
<td>Zhan Q, et al. 1999</td>
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<td>12</td>
<td>Vairapandi M, et al. 2002</td>
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<td>13</td>
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<td>14</td>
<td>Fan W, et al. 1999</td>
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<td>Nokayama K, et al. 1999</td>
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<td>16</td>
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*Table 1.* Reference list for Figure 1.
1.3 GADD45B Structure

Recently, two separate laboratories deduced the tertiary structure of GADD45B using a combination of methods including native gel electrophoresis, gel filtration, LC-MS, circular dichroism (CD), limited proteolysis and alkylation analyses, and computer modeling (Papa S, et al. 2007 and Tornatore L, et al. 2008). The tertiary structure that emerged from these studies was similar to the recently elucidated crystal structure of GADD45G (Shakre, et al. 2007). This data shows, as predicted from the amino acid sequence, that GADD45B, like GADD45A and GADD45G, is a small protein, consisting of a central four-stranded $\beta$-sheet surrounded by five $\alpha$-helices with two acidic loops that extend outward from the structure proper (Figure 4).
From these studies, it was further determined that GADD45B exists in solution as a dimer (Figure 5), but is also capable of hetero-dimerizing with either GADD45A or GADD45G (Tornatore L, et al 2008 and Kovalski O, et al 2001). It is clear that this homo-dimerization is essential for GADD45B to functionally interact with and modulate the activity of other proteins. In contrast, GADD45A exists primarily as a tetramer in solution (Kovalski O, et al 2001). Knowledge of the structure of
GADD45B and other GADD45 members is important in understanding the interactions with other proteins.

Figure 5. GADD45B dimerization ribbon diagram (Tornatore L, 2008). GADD45B functions as a dimer by interacting with and modulating the activity of other proteins.
1.4 GADD45B Function

Following the discovery of the involvement of \textit{gadd45b} in the DNA damage response, many groups sought to define its importance and role in the process. Initial studies suggested that GADD45B primarily functions in a growth suppression capacity in cells subjected to genotoxic stress. The first evidence of this was the observation that GADD45A and GADD45B synergize to suppress colony formation in several human tumor cell lines (Zhan, et al, 1994). Similar observations were made shortly thereafter, when GADD45B, GADD45A and p21 were shown to synergize to suppress colony formation in NIH3T3 cells (Vairapandi M, et al 1996). GADD45A/B/G associates with CRIF1 to contribute to growth suppression in NIH3T3 cells through modulation of the cdc2/cyclin B1 complex (Chung HK, et al. 2003). Two independent studies demonstrate that GADD45B can function to mediate G2/M cell cycle arrest, by disrupting the cdc2/cyclinB1 complex, when cells are subjected to DNA damage agents (UV, MMS) (Wang XW, et al. 1999; Vairapandi M, et al 2002). Cell cycle arrest at the G1/S checkpoint was demonstrated to occur through a GADD45B-p21 interaction when GADD45B is over expressed in mammalian cells (Fan W, et al 1999).
Finally, it was shown that overexpression of GADD45B can induce apoptosis in mammalian cells by activation of JNK and p38 MAP kinases through the MEKK4/MTK1 MAP kinase kinase (Takekawa M, et al. 1998).

Paradoxically, more and more evidence is accumulating that GADD45B is a survival factor that plays an important role in blocking apoptosis when certain genotoxic- and non-genotoxic-mediated apoptotic signals are present. For example, in hematopoietic cells, GADD45B promotes survival following UV treatment by activating NF-κB signaling and blocking MKK4 activity to inhibit JNK- and p38-mediated apoptosis (Gupta M, et al. 2006). In stress- or TNFα-mediated apoptosis signaling, NF-κB blocks JNK induced apoptosis through GADD45B-mediated inactivation of MKK7/JNKK2 (Papa S, et al. 2004). In B lymphocytes, GADD45B mediates the protective effects of CD40 co-stimulation against FAS-induced apoptosis (De Smaile E, et al. 2003). Most strikingly, endogenous gadd45b over expression is associated with tumorigenesis and stress-resistant tumors in mice (Engelmann A, et al. 2008). Finally, apoptosis is blocked in human cells infected with cytomegalovirus through synergistic interactions between GADD45A/B/G proteins with cellular Bcl-XL and the viral protein, vMIA (Smith GB, et al. 2005).
Interestingly, whereas the apoptotic and cell cycle arrest roles of GADD45B are similarly shared with GADD45A and GADD45G, there is much less evidence for a similar function between all three proteins for survival. Only two studies (Gupta M, et al. 2006, and Smith GB, et al. 2005), indicate that Gadd45 members other than GADD45B are involved in cell survival. This is an important difference in defining GADD45B specifically as a chemotherapeutic target. This is further elaborated on in section 1.5 below.

The apparent paradox in function for GADD45B, appears to be dependent on stress stimulus, cell type, physiological state, and genetic makeup. (Liebermann DA, et al. 2002). GADD45B is a small protein with no known activity itself, which interacts with other proteins to modulate their function to mediate contrasting cellular outcomes -- growth arrest, cell survival, or apoptotic cell death.

### 1.5 GADD45B and Cancer

The importance of GADD45B to organisms as a whole is clearly demonstrated by the wide variety of cancers, diseases and ailments in which de-regulated
gadd45b expression is associated (Table 2). Given that GADD45B functions in such diverse and opposing cellular processes such as cell cycle arrest, apoptosis and survival, it is not surprising that both up-regulated and down-regulated gadd45b expression is associated with different types of cancer. For example gadd45b up-regulation is observed in prostate cancer (Tenta R, et al. 2007) and inflammatory breast cancer (Lerebours F, et al. 2008). On the other hand, a recent study involving the global expression analysis of 25 human breast tumors identified gadd45b as being significantly down-regulated 100% of the time (Hu, 2004). Likewise, there is consistent and clear evidence demonstrating a causative role of down-regulated gadd45b expression on hepatocellular carcinoma progression (Jiang C, et al 2007; Chung C, et al. 2007; Qiu W, et al 2004; Qiu W, et al. 2003).

Therefore, GADD45B can be considered a tumor suppressor protein or an oncoprotein depending on cell context. For example, if GADD45B promotes apoptosis, cell cycle arrest, and/or DNA repair (as is reported), then gadd45b is a tumor suppressor; if GADD45B promotes survival (as is reported), then gadd45b
is an oncogene. In either case, it would be useful for us to know the mechanism by which stress stimuli induce \textit{gadd45b}. 
<table>
<thead>
<tr>
<th>Disease/condition</th>
<th>classification</th>
<th>Gadd45b expression</th>
<th>Source method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoarthritis</td>
<td>autoimmune</td>
<td>DOWN</td>
<td>RT-PCR</td>
<td>Ijiri K, 2008</td>
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<tr>
<td>Cardiac hypertrophy</td>
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<td>DOWN</td>
<td>gene array</td>
<td>Wang J, 2008</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>autoimmune</td>
<td>OVER</td>
<td>other</td>
<td>Du F, 2008</td>
</tr>
<tr>
<td>Thrombocythemia</td>
<td>development</td>
<td>OVER</td>
<td>gene array</td>
<td>Puigdecanet E, 2008</td>
</tr>
<tr>
<td>autism</td>
<td>development</td>
<td>OVER</td>
<td>gene array</td>
<td>Garbett K, 2008</td>
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<td>pancreatic neuroendocrine tumors</td>
<td>cancer</td>
<td>OVER</td>
<td>gene array</td>
<td>Duerr EM, 2008</td>
</tr>
<tr>
<td>hepatocellular carcinoma</td>
<td>cancer</td>
<td>DOWN</td>
<td>gene array, other</td>
<td>Jiang C, 2007</td>
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<td></td>
<td></td>
<td></td>
<td>Chung C, 2007</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Qiu W, 2004</td>
</tr>
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<td></td>
<td>Qiu W, 2003</td>
</tr>
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<td>prostate cancer</td>
<td>cancer</td>
<td>OVER</td>
<td>gene array</td>
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<td>autoimmune</td>
<td>DOWN</td>
<td>other</td>
<td>Lu Q, 2007</td>
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<td>cancer</td>
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<td>other</td>
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<td>Liu L, 2005</td>
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<td>Xie Q, 2005</td>
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<td>mammary cancer</td>
<td>cancer</td>
<td>DOWN</td>
<td>SAGE</td>
<td>Hu Y, 2004</td>
</tr>
<tr>
<td>ovarian endometriosis</td>
<td>development</td>
<td>DOWN</td>
<td>Gene array</td>
<td>Arimoto T, 2003</td>
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<td>Alzheimer's disease</td>
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<td>OVER</td>
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<td>DOWN</td>
<td>RT-PCR</td>
<td>Larsen CM, 2006</td>
</tr>
</tbody>
</table>

Table 2. Conditions/Ailments associated with de-regulated *gadd45b*
1.6 Previous studies – *gadd45b* induction

Very little is known about the mechanism by which stress induces *gadd45b*. To date, only one study has been carried out to define the signaling pathway leading to *gadd45b* induction following genotoxic stress (Table 3; Thyss, et al. 2005). This study has several differences from ours: 1.) Use of UVC and not MMS as stress agent, 2.) Approach to Identify factors is not comprehensive, 3.) cis element not identified. While this study is a useful start, a full and comprehensive dissection of the mechanism will provide additional information about the required components. Two other studies investigate non-stress mediated *gadd45b* induction, as in TNF-alpha, cytokine, and inflammatory molecule signaling through TGF-β/SMAD3,4 and NF-κB (Major MB, et al, 2004 and Jin R, et al 2002).

<table>
<thead>
<tr>
<th>Stress</th>
<th>Regulatory protein</th>
<th>Function</th>
<th>Binding site</th>
<th>Binding after damage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB</td>
<td>Egr1</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Thyss R,</td>
</tr>
</tbody>
</table>

*Table 3. gadd45b* DNA/RNA binding proteins involved in stress-mediated expression.
As a comparison, much more work has been carried out on the stress-mediated induction of *gadd45a* (Table 4). While the gadd45 family of genes themselves have very similar sequence homology, their promoters are very different (Figure 6). Differences in these promoter regions between the gadd45 genes explain the stark differences in induction levels and kinetics with various genotoxic stress agents and in different tissues (Figure 1). Nonetheless, previous studies of *gadd45a* regulatory elements (Table 4) can be compared with those identified by *gadd45b*. Interestingly, no studies have been carried out on the stress-mediated regulation of *gadd45g*. 
<table>
<thead>
<tr>
<th>Regulatory protein</th>
<th>Function</th>
<th>Binding site</th>
<th>Binding after damage</th>
<th>Reference</th>
</tr>
</thead>
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<td>Myc</td>
<td>Repression</td>
<td>DNA promoter</td>
<td>Decreased</td>
<td>Marhin WW, et al. 1997;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Anumdson, SA, et al. 1998;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tan W, et al. 2004</td>
</tr>
<tr>
<td>ZBRK1</td>
<td>Repression</td>
<td>DNA Intron 3</td>
<td>Decreased</td>
<td>Zheng L, et al. 2000;</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Tan W, et al. 2004</td>
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<td></td>
<td>Kastan MD, et al. 1992</td>
</tr>
<tr>
<td>P53</td>
<td>Induction</td>
<td>DNA Intron 3</td>
<td>Increased</td>
<td>Jin Q, et al. 1998</td>
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<tr>
<td>WT1</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Jin S, et al. 2001;</td>
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<td>Takahashi S, et al. 2001;</td>
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<td></td>
<td></td>
<td>Hirose T, et al. 2003</td>
</tr>
<tr>
<td>OCT1</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Jin S, et al. 2001;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Takahashi S, et al. 2001;</td>
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<td></td>
<td></td>
<td></td>
<td>Hirose T, et al. 2003</td>
</tr>
<tr>
<td>NF-Y</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Tran H, et al. 2002</td>
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<tr>
<td>FoxO3a</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Thyss R, et al. 2005</td>
</tr>
<tr>
<td>Egr1</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Constance CM, et al. 1996</td>
</tr>
<tr>
<td>CEBPα</td>
<td>Induction</td>
<td>DNA promoter</td>
<td>Increased</td>
<td>Lal A, et al. 2006</td>
</tr>
<tr>
<td>AUF1</td>
<td>mRNA degradation</td>
<td>mRNA 3’ UTR</td>
<td>Decreased</td>
<td>Lal A, et al. 2006</td>
</tr>
<tr>
<td>TIAR</td>
<td>mRNA degradation</td>
<td>mRNA 3’ UTR</td>
<td>Decreased</td>
<td>Lal A, et al. 2006</td>
</tr>
</tbody>
</table>

Figure 6. Gene/Promoter map of gadd45a and gadd45b indicating key transcription factor binding sites that are potentially regulatory in nature. Balliet A, et al. 2001.
1.7 Rationale

Elucidating the stress mediated mechanism for \textit{gadd45b} induction would be useful to us for many reasons. Knowledge of the mechanism of \textit{gadd45b} induction, when it functions in the context of a tumor suppressor gene, would potentially allow us to include \textit{gadd45b}, as one of several cancer-related genes, in a cancer-propensity screen. Sequencing specific sets of cancer genes from a patient would identify specific polymorphisms within the stress-responsive cis elements of the \textit{gadd45b} promoter, known to regulate \textit{gadd45b} stress-responsiveness. Likewise, important polymorphisms of upstream genes could be similarly determined, which would indicate levels of \textit{gadd45b} stress-responsiveness and thus cancer propensity. Additionally, it would be conceivable to develop patient-specific gene therapy intervention regimens whereby pathway modulation would permit the increased induction of \textit{gadd45b} to suppress tumor growth. For example, stress-responsive pathway branch points upstream of \textit{gadd45b} could be targeted to increase \textit{gadd45b} induction in addition to other tumor suppressor targets. Similarly, upstream suppressors of \textit{gadd45b} could be targeted to increase \textit{gadd45b} induction.
On the other hand, knowledge of the mechanism of \textit{gadd45b} induction in the context of an oncogene, would allow us to identify how \textit{gadd45b} can become up-regulated in cancer. We could then intervene to suppress \textit{gadd45b} induction by chemical inhibitors, by RNAi, or other strategies. This is particularly efficacious in the context of traditional chemotherapeutic applications to eliminate or reduce progression of growing tumors. Reducing GADD45B levels during chemotherapy treatment would presumably lead to decreased cell survival and promote tumor cell apoptosis and ultimately result in tumor size reduction.

\textbf{1.8 Experimental Strategy}

Elucidation of the mechanism of stress-mediated induction of \textit{gadd45b} was accomplished in three phases. First, using a human colon carcinoma cell line (RKO), a cell line known to be particularly \textit{gadd45b}-responsive by a variety of stressors, we identified specific stressors with which to study, and determined the induction time courses for each. Next, the stress-responsive cis elements in the \textit{gadd45b} promoter were determined using a promoter-luciferase reporter
strategy. Finally, the transcription factors that are required, and that act through the cis elements of *gadd45b* were identified by mobility shift assay and chromatin immunoprecipitation analysis. From this information we will have established the immediate upstream components required for stress-mediated induction, from which we have determined how this pathway fits into the overall stress-responsive network.
CHAPTER 2

MATERIALS AND METHODS
CHAPTER 2
MATERIALS AND METHODS

2.1 Reagents

All reagents used were made from chemicals purchased from Sigma Aldrich, Inc. unless indicated otherwise.

2.2 Northern Blot

To determine the levels of *gadd45B* mRNA in mammalian cells following treatment with various stress stimuli, a Northern Blot was carried out. $2.0 \times 10^6$ RKO cells were plated in 10mL DMEM containing 10% FBS on 10-cm gas plasma-treated tissue culture plates (Falcon), and grown for 24 hours to ~70% confluency before treatment. For treatment, plates were washed once with room temperature 1X PBS, then DMEM media containing a stress agent was added back to the plate (For UVB/C, cells were exposed to radiation prior to adding DMEM with FBS). At the desired time point, the plate was washed with PBS and 3mL Trizol (Invitrogen) was added at room temperature for five minutes. The Trizol-cell slurry was collected in 15-mL round bottom polypropylene tubes
(Falcon) and total RNA extraction carried out according to the Trizol product insert. The final RNA pellet was resuspended in 30ul Rnase/Dnase-free water (Invitrogen) and quantified spectrophotometrically. 10ug total RNA (in 9.5ul) in 36.5ul loading buffer (8.7% (v/v) 10X MOPS, 43% (v/v) de-ionized formamide, 14% (v/v) 37% formaldehyde, 52ng/ul ethidium bromide, 10% 10X RNA loading buffer) from each sample was run for 3 hours at 5V/cm on a denaturing 1% agarose gel (10% (v/v) 10X MOPS, 16.5% (v/v) 37% formaldehyde) in denaturing running buffer (10% (v/v) 10X MOPS, 7.5% (v/v) 37% formaldehyde). RNA quality in the gel was assessed by UV imaging of the 18S and 28S ribosomal bands for RNA integrity. RNA was transferred to a Duralon UV (Stratagene) membrane by conventional capillary transfer for 16 hours at room temperature. RNA was fixed to the membrane by UV irradiation at 1200J/m² (Stratagene Stratalinker). 25ng of a 1.3kb gadd45b cDNA fragment was radio-labeled with the DNA radprime labeling system (Invitrogen) according to the product insert. The membrane was pre-hybridized in 12mL buffer (50% (v/v) formamide, 1M NaCl, 1% (v/v) SDS, 1% (v/v) H₂O, 10% (v/v) dextran sulfate, 100ug/ml denatured sheared salmon sperm DNA) at 42° C for 2.5hrs, and hybridized
overnight (16hrs) at 42°C in fresh buffer with hot probe. The membrane was washed twice at room temperature in 2X SSC, followed by two washes at 65°C (0.1X SSC, 0.1% SDS), before being exposed to film wrapped in saran wrap.

2.3 Nuclear Run-On Assay

To determine the rate of transcription initiation of *gadd45b* in mammalian cells following treatment with various stress stimuli, the nuclear run-on assay was performed. To prepare nuclei for the nuclear run-on transcription reaction, 1.4 x 10^7 RKO cells were seeded into 15-cm tissue culture dishes (30mL) and treated 24 hours later. At the desired time-point, nuclei were isolated according to the protocol by Greenberg, et al. in the red *Current Protocols in Molecular Biology* Manual (Ausubel, et al 2008). Nonidet P-40 (NP-40) lysis buffer A (10mM Tris-Cl, pH 7.4, 10mM NaCl, 3mM MgCl_2, 0.5% (v/v) NP-40) was used and the protocol was followed verbatim, with the final nuclei pellet stored in 200ul chilled glycerol storage buffer (50mM Tris-Cl, pH 8.3, 40% (w/v) glycerol, 5mM MgCl_2, 0.1mM EDTA) in a liquid nitrogen tank until ready for the run-on transcription reaction. The nuclear run-on transcription reaction was carried out in 15-ml
polypropylene tubes (Falcon) according to the protocol by Greenberg, et al. in the red *Current Protocols in Molecular Biology* Manual (Ausubel, et al, 2008). It was imperative that fresh [α-\(^{32}\)P]UTP (10mCi/mL) be used in the reaction to obtain enough signal in the hybridization. Following the run-on reaction at 30°C for 30 min., the labeled RNA transcripts were extracted and purified according to a modified protocol by Schubeler, et al, 1996, as follows. 41ul Dnase I (Promega, 1U/ul) was added to each tube followed by an additional 15 min. incubation at 30°C. 1350ul Trizol LS (Invitrogen) was added to each tube and the reaction was homogenized by passing through a pipet tip several times, followed by a 5min. room temperature incubation. 360ul chloroform was added and the tube was vortexed vigorously for 15 sec. within a closed 50-ml conical tube (Falcon), followed by a 10 min. room temperature incubation. The 15-ml reaction tube was centrifuged 12,000xg, 15min., 4°C, and the top layer was transferred to a fresh 15-ml tube containing 900ul isopropanol, which was mixed and incubated at room temperature for 10 min. The 15-ml reaction tube was centrifuged a second time at 12,000xg, 10min., 4°C, the supernatant was removed, and 1.8mL 75% Ethanol was added and mixed by vortexing briefly. The tube was again
centrifuged at 7,500xg, 5 min., 4°C to pellet the RNA, the supernatant was
removed and the pellet air dried 5-10 min. at room temperature. The RNA pellet
was resuspended in 100ul Rnase-free H2O (Invitrogen), and incubated at 60°C
for 10 min to dissolve the pellet. 3x10^6 CPM radiolabeled transcript was
hybridized to genescreen plus nylon membranes (NEN) containing 10ug
linearized target cDNAs prepared in a Schleicher & Schuell vacuum slot-blot
apparatus, kindly provided by Scott Shore. A previously prepared DNA slot blot
membrane (described below) was placed, DNA side in, into a 20mL glass vial to
which 2mL hybridization buffer (1% SDS, 10% Dextran sulfate, 1.4M NaCl, 325
ug/ml sheared salmon sperm DNA, 325ug/ml e. coli tRNA) was added. The
membrane was pre-hybridized at 60°C for 2 hours with rotation. To the tube,
13uL Rnasin (Promega 40U/ul), plus 80ul 1M DTT was added, and incubated at
60°C for 10 min. 3x10^6 CPM radio labeled transcript was then added and allowed
to hybridize at 60°C for 24 hours. The washing protocol was derived from
Shubeler D, et al. 1996 as follows. The membrane was transferred to a series of
nine 50mL conical tubes containing the following wash solutions and incubated at
the following temperatures, for the following times. 1- 25mL 2X SSC, 5 min. RT;
DNA slot-blot membranes were prepared using Gene Screen Plus II Nylon membranes (NEN). Membrane was soaked for 5 min in 0.4M Tris, pH 7.5 and assembled in the slot blot apparatus (Schleicher & Schell vacuum slot-blot apparatus) according to the manufacturer’s recommendations. 10ug of linearized cDNAs in 198ul H2O was denatured for 10 min at RT by adding to each tube: 6.25uL 10N NaOH, 25ul 5M NaCl, and 20.75uL H2O. Each sample was diluted to 787.5ul with 0.1X SSC, 0.125N NaOH. 175uL of each sample was loaded per slot and allowed to incubate for 30 min. at RT without suction. Light suction was then applied until the solution was drawn through. The membrane was then neutralized in a solution of 0.5N NaCl, 0.5M Tris-Cl, pH 7.5 and cross-linked by UV (Stratagene Stratalinker) at 1200J/m² while still moist. The
membrane was allowed to dry at RT, then cut up into strips, rolled up and placed in 20mL glass vials and stored under vacuum in a desiccator until ready for hybridization.

2.4 \textit{gadd45b} mRNA Half-Life Determination

2.6x10^6 RKO cells were seeded into 10-cm plates and after 24 hours, were treated with 2.5\mu M Actinomycin D. After 4 hours, cells were either treated or left untreated and then harvested with Trizol (see Northern section 2.2 above) every hour to analyze \textit{gadd45b} mRNA levels by Northern blot analysis. Absolute Gadd45b mRNA half-life was determined as follows. Densitometry values of \textit{gadd45b} mRNA bands were determined using the MacBas program. These values were plotted and the mRNA half-life was identified as the time point at which \textit{gadd45b} mRNA levels equaled 50\% of the value at time-point zero.

An alternative method used was the addition of Actinomycin D following treatment with a stress agent. In this experiment, 2.6x10^6 RKO cells were seeded into 10-cm plates and after 24 hours, were treated (100\mu g/ml MMS or
0.3M sorbitol) or untreated for 4 hours before addition of 2.5uM Actinomycin D.

Next, cells were harvested over time as described above and analyzed by Northern Blot analysis.

### 2.5 Stress Stimuli Used to Induce *gadd45b* mRNA

The various stress stimuli used to induce *gadd45b* mRNA levels in mammalian cells are listed in the table below. Included in the table is 1.) Name of the chemical/stimuli, 2.) Concentration used, and 3.) Type of stress elicited on the cell.

<table>
<thead>
<tr>
<th>Chemical/Stimuli</th>
<th>Concentration</th>
<th>Type of Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylmethane Sulfonate (MMS)</td>
<td>100ug/ml</td>
<td>DNA damage – Base alkylation</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.3M</td>
<td>Hyperosmotic stress</td>
</tr>
<tr>
<td>Ultraviolet radiation (UVB/UVC)</td>
<td>50-500J/m²</td>
<td>DNA damage – Base photoadducts</td>
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<tr>
<td>Hydrogen peroxide (H₂O₂)</td>
<td>500uM</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>10ug/ml</td>
<td>Protein synthesis inhibitor</td>
</tr>
</tbody>
</table>

*Table 5. Stress Stimuli.*
2.6 Isolation and Cloning of \textit{gadd45B} Gene/Promoter from Human Genomic Library

To study the \textit{gadd45b} promoter, a wild-type 8.1kb \textit{gadd45b} gene/promoter fragment was isolated from a human genomic lambda library (Clontech).

A 8172 bp \textit{gadd45b} promoter DNA fragment isolated from the lambda library was cloned into pBluescript II SK + as a blunted FspI (\textit{gadd45b})-EcoRV (pBluescript) and BamHI (pBluescript)-BglII (\textit{gadd45b}) fusion.

2.7 Cloning of \textit{gadd45B} Promoter Luciferase Reporter Construct

To study the \textit{gadd45b} promoter activity, the isolated 4kb \textit{gadd45b} promoter was cloned into the pGL3-basic (Promega) luciferase vector (Figure 7). A ~8.5kb \textit{gadd45b} promoter-luciferase construct was generated by a BglII-BglII and Ncol-Ncol fusion between \textit{gadd45b}-pBluescript and pG3-basic (Promega). This construct contains 3877bp of \textit{gadd45b} promoter sequence upstream from the transcription start site and 219bp of sequence downstream from the transcription start site, up to the native \textit{gadd45b} AUG translation start site codon.
Figure 7. pGL3-basic vector. www.Promega.com
2.8 Cloning of gadd45B Promoter 5’-Gross Deletion Luciferase Constructs

To identify broad regions of the gadd45b promoter required for stress-mediated activity, a series of 5’-gross deletion constructs were produced on the gadd45b promoter in the pGL3-basic luciferase backbone. Restriction sites were used within the gadd45b promoter to generate successively shorter gadd45b promoter fragments, from the 5’ end, in the pGL3-basic backbone. A total of eight, 5’-gross deletion gadd45b promoter-luciferase constructs were generated using the following restriction sites: 1. EcoR1, gadd45b-2194luc; 2. EcoR1, gadd45b-1118luc; 3. SbfI, gadd45b-1018luc; 4. PvuII, gadd45b-638luc; 5. Rsrl, gadd45b-387luc; 6. BstEI, gadd45b-227luc; 7. BsgI, gadd45b-133luc; 8. EcoRI, gadd45b-81luc.

2.9 Generating gadd45B Promoter Linker-Scanning Mutant Luciferase Constructs

To identify narrow regions of the gadd45b promoter required for stress-mediated activity, a series of linker-scanning mutant constructs were produced on the gadd45b promoter in the pGL3-basic luciferase backbone. A 15-bp DNA linker
(5'-ggtaccgagctctta-3’) was designed to replace wild-type DNA sequence in the Gadd45b-1656LUC promoter construct to pinpoint the specific required sequence for MMS-mediated induction. The sequence was designed with two important considerations in mind: 1. Lacking of stress-mediated sequence itself, and 2. Containing a SalI restriction site for screening of the cloned products.

This was accomplished in two parts using the Stratagene Quikchange II XL mutagenesis system. First, a specific 15-bp wild-type sequence was deleted from the Gadd45b-1656LUC construct. Then, the 15-bp linker cassette was inserted into the deleted sequence location. Linker-scanning mutant constructs were screened for successful insertion by restriction digest analysis, and then confirmed by DNA sequence analysis using primers to the pGL3-basic backbone, GL3 and RV2.

2.10 Generating gadd45B Promoter Site-Specific Point Mutation Luciferase Constructs

To identify specific transcription factor binding sites in the gadd45b promoter required for stress-mediated activity, site-directed mutagenesis was carried out using the Stratagene Quikchange II XL system to mutate individual cis
elements. The specific base changes created are shown in Chapter 5 (Figure 27). The base change mutations were made in order to eliminate the binding sites for Egr1/Sp1/MZF1 or NFY. The specific sequence changes required to eliminate binding was determined from the literature and then tested by EMSA. All mutant constructs were sequence verified for accuracy.

2.11 DNA Sequencing and Analysis

All constructs generated and cloned were screened and/or sequence verified using the DNA sequencing facility at the University of Pennsylvania, B1 Richards Building, 3700 Hamilton Walk Philadelphia, Pennsylvania.

2.12 Luciferase Promoter-Reporter Assay

To test the activity of the gadd45b promoter-luciferase constructs generated, the Dual Luciferase Reporter Assay (Promega) was performed using the single tube Berthold Luminometer. 1.0x10^5 RKO cells were seeded into 10-3.5cm Falcon tissue culture treated dishes for each promoter-luciferase construct to be tested. 24 hours after seeding, each construct was transfected (0.5ug/plate) and co-
transfected with pRL-null (Renilla Luciferase, 50ng/plate) using lipofectin (5ul/plate, Invitrogen), according to the product insert procedure. Following the protocol, 1mL of lipofectin/DNA mix was added in the absence of FBS at 1mL per plate and incubated overnight (16 hours) before media was replaced with 2mL DMEM + 10% FBS per plate. 33-35 hours after the initial transfection, plates were treated or left untreated and harvested at the desired time-point (usually 8-10 hours). To harvest, the protocol from the Dual Luciferase Assay kit was followed. 500ul of 1X Passive Lysis buffer was used per plate to harvest according to protocol. Lysate was diluted 1:10 in 1X Passive Lysis buffer and stored at -80°C until ready to assay.

To assay, lysate was thawed at RT until samples were RT. Samples were randomly assayed at RT by using 10ul lysate with 100ul Luciferase Assay Reagent II (LARII) and 100ul Stop and Glo Reagent.

Relative Luciferase Activity for each promoter construct, for a given treatment, was determined as follows: The Absolute activity value for any given sample was attained by averaging the ratio of the Firefly luciferase light units to the
Renilla luciferase light units. The Relative light unit values were then attained by taking the ratio of treated to untreated light units calculated above. Values were normalized to the control construct, \textit{gadd45b-1656luc}, to which a value of 100 was assigned. All luciferase experiments were repeated at least three times.

\textbf{2.13 Electrophoretic Mobility Shift Assay (EMSA)}

To identify and/or confirm the binding of specific proteins or protein complexes to the \textit{gadd45b} promoter \textit{in vitro}, the Electrophoretic Mobility Shift Assay (EMSA) was carried out. $2 \times 10^6$ RKO cells were seeded in 10cm tissue culture plates and treated after 24 hours. At the appropriate time, the nuclear extracts were isolated using the NE-PER reagent (Pierce). Protein concentration was determined prior to freezing and storing the nuclear extracts at $-80^\circ\text{C}$. 3 pool PAGE purified duplex oligonucleotides (IDTDNA) were radiolabeled with gamma-P32 (3000 Ci/mmol) with T4 polynucleotide kinase (NEB), purified with oligo spin column (Roche), and 70fmol used per binding reaction. 20ul binding reactions included 10mM Tris, 50mM KCl, 1mM DTT pH 7.5, 1ug Poly(dl-dC), 70fmol radiolabeled probe and 5ug nuclear protein extract. Reactions were incubated at room
temperature for 20 minutes before adding 1X loading buffer and loading onto precast 6% DNA retardation gels (Invitrogen) in 0.5X TBE at 100V for 1 hour. Gel was dried at 80°C for 25 min. and exposed to film.

2.14 Chromatin Immunoprecipitation Assay (ChIP)

To identify and/or confirm the binding of specific proteins or protein complexes to the gadd45b promoter in vivo, the Chromatin Immunoprecipitation Assay was carried out. 2.5x10^6 RKO cells were seeded into 10cm plates. 24 hours after seeding, cells were either treated or left untreated for 8 hours prior to in vivo crosslinking and lysis using the EZ ChIP system (Upstate). Sonication was carried out on 8.2x10^6 cells in 410 SDS lysis buffer on a setting of 3 for 30 pulses per tube. 2x10^6 cell equivalents in 100ul SDS lysis buffer was used for each Immunoprecipitation in the protocol. All steps were followed verbatim for the crosslinking, DNA shearing, DNA isolation, Immunoprecipitation, and PCR according to the EZ ChIP protocol (Upstate). The one exception was in the pre-clearing step prior to the immunoprecipitation, in which an additional overnight incubation with sepharose beads was carried out. For the immunoprecipitation,
5μg of the following antibodies were used: Egr1 (Santa Cruz SC189X, C-19),
NFY-A (Santa Cruz SC-10779X, H-20), MZF1 (Santa Cruz SC-764X, N-262),
Sp1 (Santa Cruz SC-420X, 1C6). PCR primers to gadd45b were designed as
suggested in the protocol. The primers used were, for upstream, 5’-
ggcattcgccgtcacctacc-3’; and downstream, 5’-attgggctggccctcagtg-3’. For the
PCR reaction, Roche Faststart PCR master-mix was used in 20ul total reaction
volume, with the following cycling conditions: 95°C 3min; 95°C 20 sec.; 68°C 30
sec; 72°C 30 sec.; 72°C 2min, for a total of 32 cycles.
5'- gggaggggat tcacggcccc ccgaaagctc cgacgccttgc aatccgccg
cgcccccga acoczgggctc gcgtgcggag aatcagggg aaaaaaactt
ctgctttttt tttcttttct
ggc
attcgcggtc acctacccgg ccccccccgcg ccctcctcc cggttctcg cccccacgtg
c gggcccccggcg ccctcctcc cggttctcg cccccacgtg gggggtgggg gcacgccgct
cctcccccccgcg cctcccccccgc gccaacggca ggcagcgtg cactgcggt cgttttccta ccaattaggag
gggcgaatga ctccactgag gcccagccccca atgtcaagt ctataaagt cggtgccgga
ggctcccaacg tcaagatggc ggaagccggcg actacggttg gttccctggag ctattcctggc gcccttcccttct
gagacgttg caataatttt tccgctttt tctggaagga tttgctcgtg cccccaaagg ctggtgagag
tctctagct ctgtgggaag gttttgggct ctctggctcg gattttgcaa tttctccctg gggactgccg
tgagccgca tccactgtgg attataattg caacATG -3'-LUCIFERASE

Figure 8. The gadd45b promoter – the ChIP amplified sequence is in red. The primer pair is indicated by the underlined/bold sequence. The amplicon is 183 bp.

2.15 Small Interfering RNA (siRNA) Knockdown of Egr1

To determine the effect of Egr1 on stress-mediated gadd45b induction, endogenous Egr1 mRNA levels were reduced in RKO cells by RNA interference using siRNA RNA oligonucleotides. 1.0x10^6 RKO cells were seeded per 10cm plate. After 24 hours SiRNA oligonucleotides (50nM Egr1, Ambion Silencer pre-designed SiRNA AM16704, ID: 146223; 20uM scrambled oligo) were transfected using lipofectin (Invitrogen) at 30ul per plate according to product insert. 6mL of the siRNA/lipofectin mixes were transferred to each plate for an overnight incubation in 10% CO2 incubator. Media was removed and replaced with
10%FBS/DMEM for 8hrs before treating the plates with MMS (100ug/mL). After 8 hours, cells were harvested and total RNA was isolated with Trizol (Invitrogen). To determine the levels of gadd45b and Egr1 mRNA, Northern blot analysis was carried out as described above (section 2.2).

2.16 DNA Isolation, Quantization, and Quality Analysis
Sequence-verified gadd45b promoter-luciferase constructs were produced and isolated by High Speed Maxi-prep (Qiagen). All luciferase constructs used in the luciferase assay were stored at -20deg. C in a frost-less freezer, subjected to only one freeze-thaw. DNA quantity and quality were assessed spectrophotometrically and by gel electrophoresis.

2.17 Tissue Culture
A human colorectal carcinoma cell line (RKO) was maintained in Dulbeco’s Modified Eagle Media (DMEM) containing 10% Fetal Bovine Serum (FBS) (without antibiotics) at 37° C and 10% CO2. Cells were maintained and split every three days to a density of 30-40%.
2.18 \textit{gadd45b} Promoter Z-DNA Sequence Analysis

The \textit{gadd45b} sequence was analyzed by Graham Wang for Z-DNA regulatory sequence.

2.19 Analysis of Transcription Factor Consensus Elements in the \textit{gadd45b} Promoter

To identify candidate stress-mediated cis-element in the \textit{gadd45b} promoter, the gadd45B promoter sequence was computationally analyzed and compared with two independent transcription factor analysis programs. First, the sequence was analyzed against the TRANSFAC transcription factor database in the program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). This data was confirmed by analysis of the same sequence with the Transcription Factor Element Search System from the University of Pennsylvania (http://www.cbil.upenn.edu/cgi-bin/tess/tess).
CHAPTER 3
THE INCREASE IN GADD45B MRNA LEVELS IN MAMMALIAN CELLS BY VARIOUS STRESS STIMULI IS MECHANISTICALLY DISTINCT
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3.1 INTRODUCTION

induction by these agents is not known. An understanding of this stress-mediated mechanism would be beneficial for the development of targeted gene therapy. For example, \textit{gadd45b} mRNA levels are de-regulated in a wide-ranging number of diseases and conditions (Table 2). To illustrate, it has been well established that \textit{gadd45b} under-expression is associated with Hepatocellular Carcinoma (Jiang C, 2007; Qiu W, 2007; Qui W, 2004; Qui W, 2003). Conversely, \textit{gadd45b} is highly up-regulated in malignant pancreatic neuroendocrine tumors (Duerr EM, 2008). More generally, \textit{gadd45b} over expression is associated with chemotherapeutic stress-resistant tumors (Engelmann A, 2008). In practical terms, since most, if not all, chemotherapeutic drugs induce \textit{gadd45b}, mechanistic knowledge could facilitate chemotherapeutic-resistant intervention through pathway-specific adjustment using customized gene therapy strategies.

An increase in the levels of cytoplasmic mRNA, following a specific stimulation event, can be controlled either by transcriptional activation, through post-transcriptional mRNA stabilization, or epigenetically through site-specific DNA

Much more is known about the stress-mediated regulation of \textit{gadd45a}, an isotype of \textit{gadd45b}. Over the past decade, numerous factors affecting \textit{gadd45a} stress-mediated induction have been identified: activating transcription factors (p53, WT1, Oct-1, NF-Y, FoxO3a, Egr1, CEBP\textsubscript{\alpha}), suppressing transcription factors (Myc, ZBRK1), stabilizing mRNA factors (Nucleolin), destabilizing mRNA factors (AUF1), translational repressing factors (TIAR), and epigenetic modifiers, (Table 4). In spite of this vast information however, there is limited data on
induction regulation of \textit{gadd45a} involving the specific panel of stress stimuli used in this study. Nonetheless, these prior studies of gadd45a, may prove useful, given the significant number of shared cis elements between the two promoters.

In order to begin to elucidate the stress-mediated pathways controlling \textit{gadd45b} regulation, we determined the degree of transcriptional activity of the \textit{gadd45b} promoter following various types of stress treatment. This was carried out after first confirming the extent of \textit{gadd45b} induction in a human colorectal carcinoma cell line (RKO). Next, we confirmed the results obtained in the promoter activity assay by determining the rate of transcription initiation of \textit{gadd45b} with the nuclear run-on assay.

We hypothesized that we would observe an increase in \textit{gadd45b} mRNA levels in RKO cells following treatment with different types of stress stimuli, given the induction observed by wide ranging stress stimuli in most tissues (\textit{Table 6}). Based on the past studies of \textit{gadd45a} stress-mediated regulation, we would
propose that differences in stress-mediated gadd45b induction would be due to transcriptional activation by combinations of unidentified transcription factors.
<table>
<thead>
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<th>Inducing agent</th>
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<tr>
<td>Histone Deacetylase inhibitor LABH589</td>
<td>Scuto A, 2008</td>
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<td>5-azacytidine depsipeptide</td>
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<tr>
<td>Gamma irradiation</td>
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Table 6. Stimuli that induce *gadd45b*
3.2 RESULTS

3.2.1 *gadd45b* mRNA levels increase in a human colorectal carcinoma cell line (RKO) following treatment with various types of stress stimuli.

In order to determine the levels of *gadd45b* mRNA in RKO cells following various types of stress treatment, a northern blot analysis was carried out on total mRNA isolated from stress-treated RKO cells over an eight hour time course (Figure 9). As a point of reference, *gadd45a* mRNA levels were determined as well, by re-probing the same blot. To verify equal sample loading, the 28S and 18S ribosomal RNA bands are included. Basal levels of *gadd45b* and *gadd45a* mRNA in RKO cells is low, but detectable (Figure 9, lane 1). We observed similar kinetic profiles for *gadd45b* mRNA over eight hours in RKO cells exposed to MMS (100ug/ml) or UVC (500J/M²) -- two base-altering DNA damaging agents. In both cases, a steady increase in *gadd45b* mRNA is observed beginning at < 1 hour post treatment that plateaus by eight hours (Figure 9, lane 4 and data not shown). We observed maximum increases of 17-fold and 15-fold (Figure 10) for *gadd45b* mRNA at eight hours post-MMS and -UVC treatment respectively. In contrast, *gadd45a* MMS- or UVC-mediated induction is different. For *gadd45a*, we observe a marginal increase following UVC treatment, that is
maintained over the entire eight hour time course, while for MMS treatment, we observe a more abrupt increase of \textit{gadd45a} mRNA at four hours, that is maintained through eight hours (Figure 9, lanes 2-4).

For sorbitol and anisomycin treatments, we observe similar kinetic profiles for \textit{gadd45b} induction, in which an abrupt and robust increase in \textit{gadd45b} mRNA is seen at four hours, and which is then sustained through eight hours (Figure 9, lanes 8-10, 14-16). The maximum levels of induction for \textit{gadd45b} mRNA is 27-fold for sorbitol and 27-fold for anisomycin (Figure 10). In contrast, we see less robust induction for sorbitol-mediated \textit{gadd45a} induction, with a gradual increase of mRNA over eight hours (Figure 9, lanes 8-10). For anisomycin treatment, however, \textit{gadd45a} mRNA induction is similar by kinetics and level to that of \textit{gadd45b} mRNA (Figure 9, lanes 12-16). Finally, hydrogen peroxide (500uM) treatment results in a maximum increase of 5-fold for \textit{gadd45b} at 1 hour and slowly declines through the eight hour time course. \textit{Gadd45a} mRNA peaks at four hours to 5-fold and then declines.
Figure 9. Gadd45b mRNA levels increase in RKO cells following treatment with various stress stimuli. Northern blot analysis using a human 1.3kb gadd45b- or 0.7kb gadd45a-cDNA probe on 10ug total RNA extracts from human RKO cells following treatment with different stress agents. Total RNA was extracted with Trizol (Invitrogen) at 1, 4, and 8 hours following treatment of RKO cells. Treatments were applied 24 hours after seeding 2.5x10^6 RKO cells per 10-cm tissue culture dish.
Figure 9 is a representative example of four northern blots carried out using similar conditions and that included time courses of up to 12 hours post-treatment. Relative *gadd45B* mRNA levels from the northern blot in Figure 9 were determined by band densitometry using the MacBas program (Figure 10). The values of the –fold induction in Figure 10 were calculated by normalizing each band value to the 28S ribosomal RNA band and calculating fold-increase relative to the respective untreated sample (lane 1).
Figure 10. Graph of northern blot from Figure 9 by quantification of *gadd45b* mRNA levels by densitometry using the MacBas program. Fold-induction values were determined by normalizing the densitometry values to the 28S ribosomal band and then calculating fold-increase relative to the untreated band (lane 1, Figure 9).
3.2.2 The Gadd45b promoter is activated in RKO cells upon treatment with MMS, but not with sorbitol.

A ~4kb gadd45b promoter-luciferase reporter construct is activated by treatment with MMS, but not with sorbitol, in RKO cells. The luciferase activity of treated nuclear extracts was determined at ten hours post-treatment using the dual luciferase assay (Promega). As seen in Figure 11, compared to the highest level of promoter activity by MMS treatment, UVC and H$_2$O$_2$ treatment yielded 45% and 30% activity relative to MMS treatment, respectively. In contrast, we observe promoter activity at background levels (<10%) upon treatment with either sorbitol or Anisomycin. Relative luciferase activity was determined as described previously. The red arrows over MMS and sorbitol indicate those treatment for which further study was carried out. Our interest in these two particular agents is described in the discussion section of chapter 3.
Figure 11. A *gadd45b* promoter-luciferase reporter construct is activated by MMS (100ug/ml), but not sorbitol (0.3M), in RKO cells. The relative luciferase activity value is the ratio of the average **treated** Relative Light Unit (RLU) ratio to **untreated** relative light unit ratio. RLU ratios are obtained by dividing the firefly luciferase RLU by the Renilla luciferase RLU. Relative luciferase activity values were normalized to a “calibrator” construct, which in all experiments was the “*gadd45b-1656luc*” construct, set to “100” Relative Luciferase activity. N=5 per sample and this experiment was repeated four times.
3.2.3 The rate of transcription of *gadd45b* is increased following treatment with methylmethane sulfonate (MMS), but not with sorbitol.

Treatment of RKO cells with MMS, but not with sorbitol, results in an increase in transcription of the *gadd45b* gene, as determined with the nuclear run-on assay. At six hours post-MMS treatment, *gadd45B* mRNA transcript levels significantly increase compared to untreated RKO cells (Figure 12). Interestingly, at the same time-point (and all time points tested up to eight hours), there was no increase in *gadd45B* mRNA transcript level following treatment with sorbitol.

This data is consistent with that observed in promoter-luciferase experiments (Figure 10, 11). As a loading control, we measure the degree of transcription of β-actin acting at the same time points. From Figure 12, it is clear that even as *gadd45b* levels increase with MMS treatment, actin levels decrease, indicating an even greater rate of than is indicated by *gadd45b* alone. Likewise, the negative control consisted of including the vector alone. The negative control, as expected, remains at near background levels, verifying the validity of the results. The result from Figure 12 was observed in three independent nuclear run-on experiments.
Figure 12. *gadd45b* gene transcription initiation occurs following MMS treatment, but not sorbitol treatment, in RKO cells. The nuclear run-on isolation, labeling, and hybridization protocols were carried out using combined and modified versions of protocols by Greenberg, et al. and Schubeler, et al (see Materials and Methods). 5μg full length human *Gadd45B* cDNA targets were spotted by vacuum slot blot to Gene Screen Plus (NEN) nylon membranes according to the NEN product insert.
3.3 DISCUSSION

Prior to investigating any mechanistic aspect of gadd45b regulation, we first sought to determine if gadd45b was indeed induced by a variety of stress agents in a human colorectal carcinoma cell line (RKO). Previously, we had observed robust gadd45b induction by MMS in this cell line over a time course consistent with that seen in other tissues and cell lines. We therefore decided to use this cell line to assess a number of different types of physiologically relevant stressors. In Figure 9 we carried out a northern blot analysis of gadd45b mRNA from RKO cells treated with either MMS, UVC, sorbitol, H$_2$O$_2$, or Anisomycin. As a pseudo-control, we also probed for gadd45a mRNA on the same blot. From Figure 9, gadd45b mRNA levels increase to varying degrees with all stressors tested. For gadd45b, we observe similar kinetic profiles for both MMS and UVC treatment – a gradual increase in mRNA levels over an eight hour time course that plateaus by eight hours (data not shown). This kinetic profile is starkly different from sorbitol or anisomycin treatment, in which a sharp and sudden increase occurs at 4 hours and remains at that high level through eight hours.
Hydrogen peroxide is unique, in that induction peaks early by one hour and then steadily drops off over eight hours. These data and kinetics are consistent with the types and nature of the stress agents used. MMS and UVC both damage DNA at the base level, by introducing bulky adducts that disrupts transcription and DNA replication. Sorbitol generates osmotic stress and anisomycin is a protein synthesis inhibitor. Both elicit cytotoxic effects, that include protein unfolding, cell shrinkage, and indirect DNA damage. Hydrogen peroxide causes different types of DNA damage through reactive oxygen species (ROS).

Interestingly, the degree of induction and the kinetics are largely different for gadd45a. The overall levels of induction for gadd45a are much lower for all stresses, compared to gadd45b. Other than anisomycin, the kinetics are entirely different as well. These differences are presumably the result of differences in regulation – different transcription factors, or combinations of transcription factors, or different modes of regulation entirely.

We observed on testing the promoter activity of gadd45b by luciferase assay, that each stress activated the gadd45b promoter to varying degrees, and that
these levels do not necessarily correlate to the levels of mRNA seen by Northern.

For example, with UVC treatment, we observed roughly half the induction of MMS and likewise, half the promoter activity in the luciferase assay. Hydrogen peroxide treatment, interestingly, had a disproportionately high promoter activity relative to the mRNA levels seen by northern. On the other hand, the two cytotoxic treatments, sorbitol and anisomycin, which both had robust induction, had essentially no promoter activity by luciferase assay (Figure 10). This seeming contradictory observation can be explained in a couple of different ways. One possibility is that the sorbitol/anisomycin-responsive stress elements are outside of the tested promoter-luciferase construct. The sorbitol/anisomycin-responsive element may be upstream of -3897bp tested or contained within an intron of the gene. Alternatively, the increased mRNA levels may be via increased \textit{gadd45b} mRNA stabilization and/or decreased mRNA degradation.

The nuclear run-on assay (Figure 12) was run to confirm the results of the luciferase assay data for two of the different types of stress agents—that MMS-mediated \textit{gadd45b} induction involved increased transcription and that sorbitol
lacked transcription regulation. We see that at six hours post-treatment, MMS treated samples have increased levels of the *gadd45b* primary transcript by approximately two-fold compared to untreated control. Additionally, as expected, sorbitol-treated samples lacked any increase in transcription. For this particular assay we chose to measure the overall transcription rate using a full-length cDNA probe to *gadd45b*. Regulation of the rate of transcription of a gene can occur at two points in the process: 1.) Initiation of transcription, or the rate of formation of the RNA Polymerase II complex at the transcription start site, or 2.) transcript elongation, or the rate at which the RNA polymerase II moves along the gene to generate an RNA transcript. It is possible to experimentally determine the rate each of these two types of processes for a given transcriptionally active gene or to measure the overall net effect of both processes combined. To measure the rate of transcription initiation for a given gene, the radiolabeled transcripts from the run-on reaction must be hybridized to a cDNA probe consisting of the 5’ portion of a cDNA. This is because any newly generated transcript produced through a transcription initiation mechanism would incorporate radiolabeled nucleotide in the 5’-portion of the transcript in the
nuclear run-on reaction. Alternatively, to measure the rate of transcription elongation, a 3’cDNA probe must be used as the hybridizing target for the nuclear run-on transcripts. As noted, we included both types of regulation in our analysis by using a full-length cDNA probe.

Our use of a full-length cDNA probe may explain the relatively modest increase in transcriptional activity observed in the nuclear run-on assay (Figure 12). As mentioned, we observe an approximately 2-fold increase in transcription for gadd45b from treatment with MMS. The small increase in transcription observed here is much less than expected based on our MMS-mediated promoter-luciferase activity and robust mRNA induction by northern. This seeming inconsistency can be explained however, if transcription is being regulated at the level of elongation and not initiation. This would make sense, given that we observe a rather high level of constitutive transcriptional activity in the untreated sample (Figure 12). Thus, it is possible that transcription initiation occurs at a high rate constitutively in the untreated state, but that the elongation of this initiated transcript does not occur or only partially occurs. Upon MMS treatment,
transcript elongation may occur and/or is carried out more faithfully to generate full length pre-mRNA transcripts. Using a full length cDNA target probe, as we have done, would not show this. It would be possible in future experiments to differentiate between these two modes of regulation using the appropriate cDNA target probe. Nonetheless, our nuclear run-on data convincingly confirms the promoter-luciferase data – MMS mediates increased \textit{gadd45b} mRNA levels through a transcriptional mechanism, while sorbitol acts through a post-transcriptional mechanism.

We chose to investigate the two contrasting mechanisms mediated by MMS and sorbitol because of the stark difference in promoter activities, the robust increases in their mRNAs and because of their physiological relevance. An alkylating agent, MMS, essentially mimics a number of chemotherapeutic agents that utilize alkylating DNA damage to activate apoptosis and treat cancers such as leukemia, lymphoma, multiple myeloma, sarcoma, lung cancer, breast cancer, ovarian cancer, and other slow growing tumors (www.cancer.org) As such, knowledge of MMS-mediated induction would facilitate the development of
targeted pathway-specific therapy for such cases where tumors have become chemo-resistant to alkylating agents. For example, a recent study identified gadd45b up-regulation as being associated with chemotherapeutic stress-resistant tumors (Engelmann A, et al. 2008) On the other hand, sorbitol is an agent which, when applied to cells, creates a hyperosmotic environment. This type of osmotic stress is commonly encountered in the kidney, but also has been proposed to exist in proliferating cells and other cells with high metabolic activity (see chapter 7 for more information). Hence, the study of sorbitol-mediated mRNA stability facilitates a mechanistic understanding of the osmotic stress response and the signaling within proliferating cancer cells. Again, such knowledge would be key to targeted cancer therapy in the future.

As stated above, our initial observations (Figure 10, 11) indicate that gadd45b may be regulated either transcriptionally or post-transcriptionally (or both), depending on the inducing stress agent. In the next two chapters we will investigate the mechanism of MMS-mediated transcriptional activation. Finally, in chapter 6, we will address sorbitol-mediated gadd45b induction.
3.4 CONCLUSIONS

1. *gadd45b* mRNA levels increase in RKO cells to varying amounts and with different kinetics, depending on the type of stress stimuli.

2. MMS, which damages DNA by adding alkyl moieties to bases, robustly induces *gadd45b* mRNA over 8 hours, and induces *gadd45b* promoter activity by 17-FOLD.

3. sorbitol, which creates an osmotic imbalance in the cell, induces *gadd45b* mRNA over 8 hours by 27-FOLD and does not induce *gadd45b* promoter activity.
CHAPTER 4

TWO CIS ELEMENTS WITHIN THE PROXIMAL PROMOTER OF GADD45B ARE REQUIRED FOR MMS-MEDIATED TRANSCRIPTIONAL ACTIVATION
CHAPTER 4

TWO CIS ELEMENTS WITHIN THE PROXIMAL PROMOTER OF GADD45B ARE REQUIRED FOR MMS-MEDIATED TRANSCRIPTIONAL ACTIVATION

4.1 INTRODUCTION

In the previous chapter, it was shown that the robust MMS-mediated increase in gadd45b mRNA observed in RKO cells involves a transcriptional regulatory component, as determined by the promoter-luciferase and nuclear run-on assays. The MMS-mediated cis-acting DNA elements that activate gadd45b transcription have not been identified.

The identification of the -cis acting MMS-responsive elements in gadd45b is important for several reasons. First, the elucidation of the MMS-mediated transcriptional pathway is facilitated by knowledge of the cis elements. The identification of a p53 site within Intron 3 of gadd45a is a good example of this. In this case, gadd45a was found to be an important target of the p53 tumor suppressor protein in response to ionizing radiation (Kastan MD, et al. 1992).
Second, an understanding of the trans-activating machinery is not complete until the full complement of cis-acting DNA elements has been determined. Third, the possibility of novel protein-DNA interaction exists, in which non-consensus transcription factor binding occurs. Fourth, mapping the important cis-elements is a first step in defining how specific nucleotide polymorphisms, both wild-type and mutationally generated, may affect transcriptional induction. \textit{gadd45b} SNP analysis could have significant clinical relevance in determining specific disease propensity (Table 2), for which gene therapy pathway modulation may prove beneficial.

A number of studies carried out over the past decade have identified a variety of important cis elements within the \textit{gadd45b} promoter. These studies have predominantly focused on either non-alkylating sources of DNA damage or have investigated apoptotic mediated-mediated \textit{gadd45b} induction. Only one study has investigated the mechanism by which a DNA damaging agent induces \textit{gadd45b} mRNA. Thyss and colleagues demonstrate the dependence of Egr1 on UVB/C-mediated \textit{gadd45b} induction (Thyss R, et al. 2005). In this study
however, the specific binding site for Egr1 is not identified, which, given that at least four Egr1 sites may exist, is an important consideration.

At least eight other studies have identified important cis elements involved in different signaling cascades resulting in *gadd45b* induction. Some of the important elements identified were SMAD3, SMAD4, and NF-kB utilized in TGF-β and NF-kB signaling respectively (Major MB, et al. 2004; Jin R, et al. 2002).

Many more stress-mediated cis elements have been identified within the *gadd45a* promoter. These include the GC-rich region, p53 site in intron 3, WT1/Egr1 site, CCAAT box, Oct-1 element, and the FoxO3a site (Table 4). While much valuable information has been gleaned from these studies, one can argue that a number of them are biased and/or incomplete in the identification and analysis of important cis elements. In no study has an unbiased and rigorous approach been taken to identify important cis elements.

In order to identify the DNA elements that are required for MMS-mediated *gadd45b* transcriptional activation, a systematic dissection of the *gadd45b* promoter was carried out using the firefly luciferase reporter system. This
systematic analysis was accomplished in two stages. First, a gross analysis was undertaken, using 5′-deletion luciferase constructs of the gadd45b promoter, to identify those general regions that were required for induction. Next, specific MMS-responsive elements were identified using a series of mutant constructs, in which overlapping "inert" DNA replaces wild-type sequence within the gadd45b promoter. Because we used linker scanning mutagenesis across a continuous portion of the promoter, our analysis was not biased towards known consensus regions. With several MMS mediated activity-deficient linker scanning mutants identified, we carried out a sequence analysis to identify candidate activating transcription factors for subsequent verification (chapter 5).

We hypothesized that we would identify several specific elements within the gadd45b promoter, which are necessary for MMS-mediated induction. This we expected based on what has been discovered about gadd45a regulation – that several different elements in the promoter mediate stress-responsive induction.
4.2 RESULTS

4.2.1 Two regions within the \textit{gadd45b} promoter mediate full MMS-responsive transcriptional activity.

Two regions within the \textit{gadd45b} proximal promoter, located within the first 227 base pairs upstream from the transcription start site, are required for full MMS-mediated \textit{gadd45b} promoter activity. In Figure 13, ten hours after treatment of RKO cells with MMS (100ug/ml), we observe \textasciitilde40\% loss in activity with the \textit{gadd45b}-133luc construct and \textasciitilde80\% loss in activity with the \textit{gadd45b}-81luc construct, compared to \textit{gadd45b}-1656luc. A loss of activity in a given construct compared to a fuller length version, identifies a region of the DNA that is necessary for transcriptional activity to some extent. The loss of activity we observe in two constructs, establishes the importance of the following two regions, for MMS-mediated transcriptional activity: 1) Between -227bp and -133bp upstream from the transcription start site, a 94bp stretch of promoter, and 2) Between -133bp and -81bp upstream from the transcription start site, a 52bp stretch of promoter. In the other seven 5’- gross deletion promoter constructs, which collectively span -3877bp to -227bp, we detect no significant differences in
MMS-mediated activity compared to full length, \textit{gadd45b-3877luc}. The identical experiment using sorbitol to treat RKO cells, yielded \textit{gadd45b}-promoter activities at levels near background (Figure 13), as expected based on the lack of sorbitol-mediated activity from chapter 3.

\textbf{Figure 13.} Two regions within the first 227bp of the transcription start site of the \textit{gadd45b} promoter are important for MMS-mediated promoter activity. The Dual Luciferase Assay (Promega) was carried out on treated or untreated RKO cell extracts transfected with a series of 5' gross deletion \textit{gadd45b}-promoter constructs. Extracts were harvested ten hours after treatment with either MMS (100ug/ml), sorbitol (0.3M) or untreated control. \(N=5\) for each sample and the experiment was repeated five times.
4.2.2 Multiple cis elements within the MMS-responsive regions contribute to \textit{gadd45b} transcriptional activity.

Within the two regions of the \textit{gadd45b} promoter that contribute to MMS-mediated activity identified in section 4.2.1, -227bp/-133bp (94bp) and -133bp/-81bp (52bp), the stretches between -180bp/-127bp (53bp) and -115bp/-88bp (27bp), contain the MMS-responsive elements. To identify these more confined MMS-responsive regions within the two large broad regions, the Dual Luciferase Assay was carried out on a series of \textit{gadd45b} linker-scanning mutant promoter luciferase constructs. To this end, a series of overlapping linker-scanning mutants were generated on the \textit{gadd45b-1656luc} construct backbone, by replacing 15-bp wild-type sequence with 15-bp “inert” linker DNA sequence spanning the two MMS-responsive regions. Linker-scanning mutants were generated, as opposed to deletion constructs, in order to alter the wild-type sequence while preserving the spatial and topological landscape of the DNA. In so doing, specific transcription factor binding is disrupted while the overall tertiary structure of the promoter-transcription apparatus interface is maintained. From four independent experiments, we observed a loss of MMS-mediated \textit{gadd45b} promoter activity in six of ten linker scanning mutants shown in Figure 14 below:
1). Loss of ~25% activity in LS17 (-180bp/-166bp), LS18 (-167bp/-153bp), and LS22 (-115bp/-101bp); 2). Loss of ~35% activity in LS19 (-154bp/-140bp), and 3.) Loss of ~40% activity in LS20 (-141bp/-127bp) and in LS23 (-102bp/-88bp).

The losses of activity indicated above are in reference to the wild-type, \textit{gadd45b-1656luc}. In three Linker-scanning mutant constructs, LS15 (-206/-192bp), LS16 (-193/-179), and LS21 (-128/-114), we observe slight, but insignificant, increases in activity relative to wild-type control.
Figure 14. Multiple 15-bp regions between -180bp and -127bp and between -115bp and -88bp in the gadd45b promoter may contribute to MMS-mediated gadd45b induction. Dual Luciferase Assay was carried out as previously described using 10 15bp linker scanning mutants of the gadd45b promoter-luciferase construct. RKO cells were harvested 10hrs after treatment with MMS (100ug/mL). N=5 and 4 independent experiments were carried out with similar results.
4.2.3 Three 15-base pair cis-elements mediate full MMS-responsive *gadd45b* transcriptional activity.

Based on the linker-scanning luciferase data from section 4.2.2, in which we observed modest losses of MMS-mediated activity in several regions, we hypothesized that multiple cis elements may play a role in mediating *gadd45b* transcriptional activation. To test the idea, we generated several linker-scanning combination mutants in *gadd45b*-1656luc. To this end, we decided to mutate in combination, those three regions which when mutated, resulted in the greatest loss of activity following MMS treatment (Figure 14): (1). LS19 (-154bp/-140bp) - 35%; (2). LS20 (-141bp/-127bp) -40%; (3). LS23 (-102/-88).

The MMS-mediated activity of the four linker-scanning combination mutants was determined by dual luciferase assay. In the triple linker-scanning mutant, we observed an almost complete loss (>80%) of MMS-mediated promoter activity, compared to wild-type (Figure 15). Three other combination mutants consisted of three double mutants: (1). -154/-140 (LS19)/-102/-88 (LS23); (2). -154/-140 (LS19)/-141/-127 (LS20); and (3). -141/-127 (LS20)/-102/-88 (LS23). We
observed a reduction in luciferase activity between 75% and 50% between the three double linker-scanning mutants compared to the wild type control.

Figure 15. MMS-mediated gadd45b induction is reduced by 80% to near-background levels in a triple linker-scanning mutant gadd45b-promoter luciferase construct. Dual luciferase assay was carried out as previously described on RKO cell extracts. N=5 per sample and four independent experiments were carried out with similar results.
4.2.4 Sequence of the MMS-response cis-elements

The DNA sequence used as a reference for the “wild-type” human *gadd45b* promoter/gene was obtained from Pubmed. There are several versions that have been submitted for this gene. We chose the sequence derived from accession number NM_015675, since it was the most complete. Figure 16 below highlights the regions identified by the triple linker-scanning mutant luciferase assay as being important in conglomeration for MMS-mediated *gadd45b* induction. The wild-type sequence, which was replaced with a 15-bp “inert” linker DNA sequence, is colored for the three mutant constructs.
Figure 16. DNA sequence of the MMS-responsive regions in the human gadd45b promoter, identified by luciferase assay/combination linker-scanning mutagenesis. Wild-type sequence and promoter orientation map.
4.2.5 Several transcription factor binding sites are present within the three MMS-response regions.

Based on the observation that MMS-mediated activity is essentially abolished when three regions (-154bp/-140bp; -141bp/-127bp; -102/-88), of the gadd45b promoter are replaced with inert linker cassettes (Figure 15), we decided to identify candidate consensus cis elements within these regions. A significant number of transcription factor consensus binding sites were identified within the important MMS-responsive sequence identified above. Using two independent sequence analysis programs, TFSEARCH and TESS (University of Pennsylvania), a complete list of putative transcription factor binding sites for each region was generated. Below in Table 7 is a list of the top five candidate transcription factors for each region with the specific binding site color-coded. Details of the search parameters and search engine programs are described in the materials and methods.
<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Consensus sequence</th>
<th>Score*</th>
<th>% consensus</th>
<th>orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS MZF-1 19</td>
<td>tcctccccctccct</td>
<td>88.7</td>
<td>88%</td>
<td>antisense</td>
</tr>
<tr>
<td>SP1</td>
<td>tcctccccctccct</td>
<td>80.8</td>
<td>80%</td>
<td>antisense</td>
</tr>
<tr>
<td>GATA-1</td>
<td>tcctccccctccct</td>
<td>76.3</td>
<td>90%</td>
<td>antisense</td>
</tr>
<tr>
<td>GATA-2</td>
<td>tcctccccctccct</td>
<td>72.3</td>
<td></td>
<td>antisense</td>
</tr>
<tr>
<td>GATA-3</td>
<td>tcctccccctccct</td>
<td>69.4</td>
<td></td>
<td>antisense</td>
</tr>
<tr>
<td>LS V-myb 20</td>
<td>tccgtcgccaacccg</td>
<td>79.1</td>
<td>78%</td>
<td>antisense</td>
</tr>
<tr>
<td>C/EBP</td>
<td>tccgtcgccaacccg</td>
<td>68.8</td>
<td>86%</td>
<td>antisense</td>
</tr>
<tr>
<td>Lyf-1</td>
<td>tccgtcgccaacccg</td>
<td>66.2</td>
<td>67%</td>
<td>antisense</td>
</tr>
<tr>
<td>E2F</td>
<td>tccgtcgccaacccg</td>
<td>64.4</td>
<td>63%</td>
<td>antisense</td>
</tr>
<tr>
<td>SREBP</td>
<td>tccgtcgccaacccg</td>
<td>63.4</td>
<td></td>
<td>sense</td>
</tr>
<tr>
<td>LS Sox-5 23</td>
<td>ttccaccaatagga</td>
<td>80.4</td>
<td>90%</td>
<td>sense</td>
</tr>
<tr>
<td>STATx</td>
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<td>77.9</td>
<td>67%</td>
<td>sense</td>
</tr>
<tr>
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<td>ttccaccaatagga</td>
<td>76.9</td>
<td>71%</td>
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</tr>
<tr>
<td>SRY</td>
<td>ttccaccaatagga</td>
<td>75.2</td>
<td>75%</td>
<td>sense</td>
</tr>
<tr>
<td>CDP-cR</td>
<td>ttccaccaatagga</td>
<td>72.9</td>
<td>80%</td>
<td>antisense</td>
</tr>
</tbody>
</table>

Table 7. Transcription factor consensus sequences top five. Scoring scheme is based on the following formula: score = 100.0 * (‘weighted sum’ - min) / (max - min). (http://www.cbrc.jp/research/db/TFSEARCH.html).
4.3 DISCUSSION

In this chapter, we identified two regions of the *gadd45b* promoter -- -154bp/-127bp and -102bp/-88bp that contribute to the MMS-mediated induction of *gadd45b*. From these data, we determined the most likely transcription factors, based on DNA consensus sequence homology, which may act through these cis elements to mediate induction.

The process and strategy employed for the identification of these two MMS-responsive regions was a “blind” strategy, in which we initially made no assumptions about potential transcription factor candidates or MMS-responsive regions based on previous studies of other stress responsive genes, including *gadd45a*. The process of identifying the important cis-elements began with Figure 12, in which we first identified general regions of the *gadd45b* promoter that could be activated by MMS treatment. This involved observing activity in *gadd45b*-promoter luciferase constructs with the largest being -3877bp upstream from the transcription start site, and the smallest, -81bp upstream from the transcription start site. In carrying out promoter studies, there is always the
question as to how large an initial promoter piece to begin working with. In other words, where are the responsive elements most likely to be located in reference to the transcription start site? Traditionally, textbooks generally define the promoter of a gene as the 500-bp immediately upstream of the transcription start site (Alberts B, et al. 2002). While this is an arbitrary value defining the size of the promoter, most responsive elements are located within the first 500bp, and indeed most of these are within the first 200bp upstream of the transcription start site. Responsive elements in this region may mediate activation or suppression of a given gene.

Responsive elements which can affect the transcription of a given gene, and that are located anywhere other than the immediate promoter region (500bp) are termed enhancers or suppressors. There are examples of enhancer elements that are located as much as 50,000bp upstream of a transcription start site (Alberts B, et al. 2002). In addition to long-range transcription regulation, upstream of a given gene, enhancers and suppressors also exist downstream and/or within the introns of genes. Gadd45a is a prime example of this, in which
the p53 site in intron 3 induces transcription in response to ionizing radiation.


Thus, given the numerous possible locations of cis elements that may contribute to transcriptional regulation, it is nearly impossible to exclude the possibility that one or more contributing element has been missed in a given analysis. Nonetheless, for a given treatment, in this case MMS, it is possible that all contributing elements exist within the element-rich region of the promoter (-500bp). Our studies therefore included the promoter and an additional 3kb of upstream sequence to rule out any contributions from proximal enhancer elements.

In eight of ten gadd45b promoter 5’-deletion luciferase constructs, corresponding to the region between -227bp and -3877bp, we observed essentially full MMS-mediated activity (Figure 13). As described previously, we observed a loss of activity with the two smallest luciferase constructs, corresponding to the gadd45b promoter region between -227bp and -81bp. This 146bp stretch of promoter,
required for MMS-mediated promoter activity, is located within the proximal region of the promoter, which is in accord with the most common location of responsive elements in general. Based on our initial in-vitro experiments with the promoter-luciferase system (Figure 13), in which we observed robust activity with a series of construct, that was essentially eliminated in the absence of -146bp, we felt confident that any possible MMS-responsive elements between the transcription start site and -3877bp, are present in this small region. This data still does not rule out the possibility that other MMS-mediated cis-acting elements exist as enhancers, either upstream or downstream of the promoter.

Having determined the general MMS-responsive region of the gadd45b promoter, we next wanted to narrow in on and pinpoint the important regions, with higher resolution. To this end, we chose to employ the strategy of using 15bp linker-scanning mutants to meet this objective. The use of a series of consecutive linker-scanning mutants permits the identification of important elements without introducing the additional variables of 1). Disruption of native DNA topology, and 2). Affecting the spacio-variance of protein-protein
interactions along the DNA. Additionally, a strategy of this sort, in which a series of linker-scanning mutants overlap along an important region, ensures an unbiased analysis of that region. The 15-bp linker permits cis element identification to within 15bp resolution. This size was chosen based on the fact that the average cis element is about 10bp. The sequence of the linker cassette used in the mutants was chosen with two important factors in mind: 1). Of foremost importance, a linker cassette must be “inert” in that it lacks cis elements itself that might affect transcriptional activity 2). The linker cassette should include a restriction site, which can be used to identify positive mutant clones.

We designed our mutant linker cassette empirically by testing different combinations of linker cassettes containing the SacI restriction enzyme (for restriction enzyme screening) for the presence of cis elements against the TRANSFAC database. As described previously, we found that the sequence, $5'$-GGTACCGAGCTCTTA$-3'$, met all of these criteria.
In ten linker-scanning mutant constructs tested (Figure 14), we observed a significant reduction in MMS-mediated activity in 3 constructs and a modest reduction in 3 constructs, as described previously. Four of the six mutant constructs with reduced activity spanned a GC-rich region of the *gadd45b* promoter, from -180bp/-127bp (LS17-LS20). It has been well established that GC-rich regions are often regulatory in nature both transcriptionally and epigenetically (CpG methylation). The prevalence of transcriptional regulation via these GC-rich regions may be explained by the compatibility resulting from the DNA topology secondary structure with the typical protein DNA binding motifs (zinc-fingers, others). We focused on the two proximal linker-scanning mutants of this group together with LS23, since they consistently generated the greatest drop in activity (Figure 15). Since a combination of the three linker-scanning mutants tested resulted in a loss of activity to essentially basal levels, we focused our attention on these regions for the remainder of the study. It is not beyond possibility however that other combinations of Linker-scanning mutants, involving LS17 and LS18, could also result in a loss of activity, however, such an analysis is beyond the scope of this project.
Upon inspection of the actual DNA sequence that mediates MMS-responsive induction (Figure 16), two things immediately come to our attention: 1). In LS19, between -154bp/140bp we find an interesting stretch of cytosine residues punctuated with thymidines in a pseudo regular pattern (5’-TCCTCCCTCCCCCTC-3’). Indeed, this site is essentially a duplicate stretch of CTCCCCCTC, except that in the first half of the “repeat”, there is an additional cytosine in the stretch, CTCCCCCTC. 2). In LS23, between -102bp/-88bp sits the CCAAT box element (TTCCACCCAATAGGA). It is well established that the CCAAT box plays a role in both basal transcription and stress-mediated transcriptional activation.

Interestingly, during a fortuitous encounter with a scientist studying the effect of alternative forms of DNA on transcriptional activity, at a conference in California, we discovered a possible connection between the DNA sequence between -154/-140bp (LS19), the inherent tertiary structure, and transcriptional activity. “Standard” DNA sequence that consists of any given random sequence of nucleotides takes on the B-DNA form in-situ, in which a right-handed DNA helix
repeats every 10bp, and consists of a major and minor groove to which proteins access and bind DNA. It has been discovered in recent years however, that specific sequence stretches of DNA can generate alternative tertiary forms. Indeed, up to 3-forms have been identified. One form, Z-DNA, has been shown to have an effect on transcription (Wang G, et al. 2007). Astoundingly, a blind analysis of the gadd45b promoter by this group determined that the same stretch of DNA that mediates gadd45b transcription by MMS, -154/-140bp (LS19), forms Z-DNA (Figure 40, 41). It is unclear whether this Z-DNA structure simply generates the tertiary structure required for the binding of specific transcription factors, or whether it inherently can affect transcription in and of itself. Whatever the case, it is an interesting point to consider in our analysis of regulation.

We were quite interested to determine the specific required DNA sequence within these three LS mutants, to which activating transcription factors bind. To this end, we carried out an analysis of these three regions to identify candidate transcription factors that bind based on binding consensus sequence. For a given sequence, a list of potential binding proteins was determined from
TRANSFAC database, and scored according to potential for binding. Listed in Table 7 are the factors identified ranked according to binding score and % homology of the DNA with the exact consensus sequence. In some cases, a protein may have a higher binding score than might be expected from the % consensus sequence. This is because certain nucleotides within a stretch may be more important for binding than others.

A cursory review of the list of top binding candidates does not immediately generate an ah-ha moment. MZF1 (Myeloid Zinc Finger 1), the most likely binding protein to -154/-140bp region is a protein involved in the differentiation of myeloid progenitor cells. Sp1 (Specificity Protein 1), is a ubiquitous protein that seems to be involved in every type of transcriptional activation. Furthermore, a review of the literature reveals that, with the exception of SREBP (Sterol-response binding protein), which has a relatively low binding score of 63.4, binding to LS20 (-141/-127), none of the proteins appears to have precedent for activating transcription in response to MMS or any stress for that matter!
A close look at the top candidates for binding (Table 7) reveals that the majority of proteins bind to the antisense sequence of the promoter. This is interesting, but not unexpected, since DNA is a dynamic molecule, which is known to function at least in part at the level of tertiary structure to control transcription.
4.4 CONCLUSIONS

1. Two MMS-responsive regions are located at -154bp/-120bp and -102/-88bp in the \textit{gadd45b} promoter.

2. Multiple putative transcription factor binding sites are located within the two MMS-responsive regions of the \textit{gadd45b} promoter.
CHAPTER 5

SEVERAL TRANSCRIPTION FACTORS BIND AND ACTIVATE MMS-MEDIATED TRANSCRIPTION OF $GADD45B$ THROUGH THE TWO MMS-RESPONSIVE ELEMENTS
CHAPTER 5
SEVERAL TRANSCRIPTION FACTORS BIND AND ACTIVATE MMS-MEDIATED TRANSCRIPTION OF GADD45B THROUGH THE TWO MMS-RESPONSIVE ELEMENTS

5.1 INTRODUCTION

In the previous chapter, we identified two MMS-responsive regions in the gadd45b promoter, which, when mutated together, results in a loss of transcriptional activity to nearly basal levels. One of these elements is a 28-basepair element located at -154 bp to -127 bp upstream of the transcription start site; the other is a 15-basepair element located at -102 bp to -88 bp upstream of the transcription start site. While several potential transcription factor binding sites were identified within these regions by sequence analysis, it is not known experimentally which factors bind to and activate gadd45b through these elements.

The identification of the transcription factors, which bind to and modulate gadd45b activity, is an essential aspect of dissecting the mechanism of induction.
To fully construct a molecular pathway, typically this initial step of protein identification is followed by the retrograde re-construction of upstream protein-protein interactions up to an initiating “sensor” protein. For example, the previous study of UV-mediated $gadd45b$ induction, identified the NF$\kappa$B-Egr1-$gadd45b$ pathway in this manner (Thyss R, et al. 2005) The ATM-p53-gadd45a-p38 pathway was likewise elucidated in similar fashion.

Knowledge of the pathway for MMS-mediated $gadd45b$ induction is important for a number of reasons. First, $gadd45b$ induction is de-regulated (over- or under-expressed) in a number of cancers and conditions (Table 2). Knowledge of this pathway could facilitate disease treatment through $gadd45b$ induction-modulation using gene therapy at different points. Second, this work would add to the knowledge base of the stress response at the molecular level. Indeed, the true complexity and sheer number of genes involved in response to genotoxic and non-genotoxic assaults has recently become evident in large scale gene array studies. As such, there is much work to be done to fill in the gaps of this vast and complex response network. Third, knowledge of the MMS-specific $gadd45b$
induction pathway may allow for the development of new strategies in the

To identify the transcription factors which bind to and activate $gadd45b$ through
the two MMS-responsive cis elements (-154/-127, -102/-88), we used the
Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation
(ChIP). Next, we determined the contribution of each identified protein to MMS-
mediated $gadd45b$ induction, in two ways: 1). We evaluated $gadd45b$ promoter
activity using EMSA/ChiP-identified-protein-binding-deficient promoter-luciferase
constructs, and 2). We determined the induction of $gadd45b$ in RKO cells in
which the EMSA/ChIP-identified-proteins were knocked down by RNA
interference (RNAi).

Based on the loss of $gadd45b$ promoter activity with specific promoter mutants,
we expected to identify multiple transcription factors that activate $gadd45b$
transcription in RKO cells following MMS treatment.
5.2 RESULTS

5.2.1 Several transcription factors bind to the *gadd45b* promoter

5.2.1.1 *At least four proteins bind within the upstream MMS-responsive regions of the gadd45b promoter.*

In order to identify the proteins that bind to and activate the *gadd45b* promoter through the MMS-response elements, we carried out an electrophoretic mobility shift assay (EMSA). We first sought to determine which proteins bind to the most distal of the two primary response regions, located at -154/-127bp upstream of the transcriptional start site. To this end, a radiolabeled double stranded oligonucleotide, “EMSAoligo-174/-124”, which spans this MMS-responsive region, was used in EMSA binding reactions with untreated- or MMS-treated nuclear extracts from RKO cells (Figure 17). From this assay, we observed at least three distinct bands in binding reactions using either untreated or MMS-treated extracts. Interestingly, a fourth band is observed in the EMSA when MMS-treated extracts are used; this band is not present using the untreated extracts. The presence of a band indicates the presence of a bound protein or protein complex from the nuclear extracts, which has retarded the migration of the radiolabeled oligonucleotide in the gel. Thus, we can conclude that at least
three proteins or protein complexes bind constitutively, and one protein binds specifically with MMS, to the distal-most MMS-response region.
**Figure 17.** Electrophoretic Mobility Shift Assay (EMSA) using either MMS-treated (100ug/ml, 8hrs) or untreated nuclear extracts. There is increased binding of a protein to the *gadd45b* promoter MMS-responsive region following MMS treatment of RKO cells. In addition, several proteins bind the *gadd45b* promoter MMS-responsive region constitutively, before and after MMS treatment.
5.2.1.2 Sp1 and MZF1 transcription factors constitutively bind to the upstream MMS-responsive region of the gadd45b promoter.

In order to identify the proteins binding to the gadd45b promoter seen in section 5.2.1 above, we carried out a number of EMSA competition and/or supershift experiments, in which specific bound proteins could be identified in binding competition assays using either cold oligonucleotide competitors or antibodies to a putative bound protein. As a starting point in these experiments, we focused on the proteins most likely to bind to the MMS-response regions based on homology to the consensus binding sequence (Table 7, section 4.2.5).

An EMSA was carried out with MMS-treated nuclear extracts using the radiolabeled EMSAoligo-174/-124 probe in the presence a number of competitors (Figure 18). In lanes 1 and 2, are untreated and MMS-treated nuclear extracts respectively, in which no binding competitors were included. These results show the band pattern observed in Figure 17 – three constitutively bound bands and one additional band in the MMS-treated lane 2. In lane 3, the top band is significantly diminished when 1ug of Sp1 antibody is included in the binding
reaction. The immunodepletion of the top band by Sp1 is a positive indicator of the presence of Sp1 either directly binding to or indirectly binding to the probe as part of a complex. The antibody immunodepletion by Sp1 is specific since a non-specific IgG antibody control fails to immunodeplete or shift the top band (lane 4).

**Figure 18.** EMSA supershift or cold competition identified constitutive binding of transcription factor Sp1 to the MMS-responsive regions of the *gadd45b* promoter. Sp1 immunodepletion with Sp1 antibody and elimination with cold competitor oligonucleotide.

When a cold competitor oligonucleotide containing the Sp1 consensus sequence is incubated in 50-fold excess with nuclear extracts (lane 7), we see a similar
result, in which the top band is eliminated, or competed out of solution by
sequestering the Sp1 that is present. As a control, we see that a cold competitor
containing a mutated Sp1 site fails to eliminate the top band. All bands are
competed off using the cold probe EMSAoligo-174/-124 as expected (lane 5).
Most of the bands are competed off using the cold probe EMSAoligo-163/-138 as
expected (lane 6).

In another experiment, using the same radiolabeled probe and MMS-treated
nuclear extracts, we observe the immunodepletion of a second band when the
MZF1 antibody is included in the binding reaction (Figure 19). The band
eliminated by MZF1 antibody is the second largest protein or protein complex, as
it is the second band from the top on the gel (labeled #2). Interestingly, in
addition to elimination of this band, the Sp1 band (labeled #1) and a third lower
band (labeled #4) are less distinct following MZF1 immunodepletion, suggesting
binding cooperativity between Sp1 (#1), MZF1 (#2), and a third unidentified
protein (#4).
Figure 19. EMSA using -174/-124 radiolabeled probe with 5ug nuclear extracts. MZF1 immunodepletion with MZF1 antibody (1ug).
5.2.1.3 The MMS-inducible protein binding to the gadd45b promoter in vitro is not one of a number of factors identified by transcription factor sequence consensus analysis.

Using EMSA in vitro binding assays, we have discovered that Sp1, MZF1 and other proteins constitutively bind gadd45b promoter in the vicinity of the upstream MMS-response region (-154/-127bp). In addition to determining the constitutively-bound proteins, we sought to identify the MMS-specific protein that robustly binds the gadd45b promoter. We were surprised to find that antibodies to the most likely proteins, according to Table 3, failed to shift or immunodeplete the strongly induced MMS-specific band by EMSA (Figure 20). Lanes 1 and 2 of Figure 20 use untreated and MMS-treated nuclear extracts in binding reactions to the gadd45b promoter probe, -163/-138. Lane 3 includes the Sp1 antibody as a control, which immunodepletes the top band, as shown previously. In lanes 4-9, different antibodies fail to shift the inducible band. A number of other antibodies were used in other EMSA experiments, but none shifted the inducible band. Similar results were obtained in EMSAs using cold probes containing consensus sequences as competitors.
**Figure 20.** In an EMSA, the MMS-inducible band fails to supershift using antibodies to proteins that bind to sequence with high homology to the oligonucleotide probe.
5.2.1.4 The MMS-inducible protein binds to the sequence CTCCCCCTC in the gadd45b promoter.

Since numerous competition binding assays (EMSA) using antibodies and/or cold competitor oligonucleotides failed to shift the MMS-specific band, we decided to determine the specific required sequence to which this protein binds. A protein which binds to a specific sequence on the radiolabeled probe (and be seen on the gel), will be competed out of solution when excess cold probe of the same sequence is first included, and thus not be seen on the gel. In contrast, when a mutant cold competitor is included, which disrupts the normal binding sequence of a given protein, a band will be seen on the gel. Thus, in this way we can identify the specific required sequence for binding of any given protein to a specific sequence.

Using this type of strategy, we carried out an EMSA with nine cold competitor 3-basepair linker-scanning mutants in 50-fold excess to compete for binding with the MMS-specific protein (Figure 21). In this EMSA, lane 1 and 2 show untreated and MMS-treated nuclear extracts, respectively, binding to wild-type probe; the MMS-specific band is clearly observed. In lane 3, all protein binding is eliminated.
as expected when wild-type cold probe is present in 50-fold excess. Similarly,
we observed a complete loss of protein binding with mutants 1, 2, 3, 7, 8, and 9.
Since these cold competitor oligonucleotides are capable of binding the inducible
protein, we can conclude that the specific mutated sequence is not necessary for
the binding. In cold competitor mutants 4 and 5 in which the sequences CTC
and CCC are replaced with TTT, we observe no loss of binding of the inducible
band. In cold competitor mutant 6, in which CTC is replaced with TTT, we
observe a partial loss of binding. These data indicate that the sequence
CTCCCCCTC is required for binding of the MMS-inducible protein, and that the
first six nucleotides, CTCCCC may be more important for the binding.
Interestingly, the central three cytosine residues of the above nine base pair
sequence that are essential for the binding of the MMS-specific band, are also
essential for the binding of the three constitutively bound proteins (Figure 21,
lane 8).
Figure 21. EMSA competition to identify binding sequence. Cold competitor mutant oligonucleotides in 50-fold excess were incubated with radiolabeled EMSA oligo-163/-138 probe and MMS-treated nuclear extracts (100ug/ml, 8hrs). As a control, 50-fold excess wild-type -163/-138 cold probe was used (lane 3).
5.2.1.5 The MMS-inducible, which binds to the gadd45b promoter, is Egr1.

It was realized that the sequence to which the MMS-specific protein binds in EMSA is in anti-sense orientation and at 65% consensus to the binding sites for Wilm’s Tumor protein factor 1, WT1, and Early growth response gene 1, Egr1. Because of the 2-base mismatches and the reverse orientation, our previous analysis for transcription factor binding sites did not identify these candidates. Therefore, to determine if either Egr1 or WT1 binds the sequence inducibly, an EMSA was carried out using a cold competitor oligonucleotide that contains the Egr1 consensus binding sequence (Figure 22b). In lane 2 of the Figure, a wild-type cold oligonucleotide containing the consensus Egr1 binding site is able to compete away the MMS-specific band. More convincingly, when a similar binding reaction is carried out in the presence of the Egr1 antibody with MMS treated nuclear extracts, we observe a supershift of the inducible band (Figure 22a). This supershift is specific to Egr1 since antibodies to neither WT1, which has the same consensus binding sequence as Egr1, nor the non-specific antibody IgG, are able to shift the band.
Figure 22. Egr1 is the MMS-inducible protein that binds the gadd45b promoter. EMSA was carried out with either Egr1 antibody (A), or cold competitor to Egr1 (B).
5.2.1.6 One prominent protein constitutively binds the gadd45b promoter in the vicinity of the -141/-120bp MMS-responsive region

We previously demonstrated in chapter 4, by linker-scanning mutagenesis, the importance of the region at -141bp to -127bp, which encompasses the more proximal half of the overall upstream MMS-responsive region, -154bp to -127bp.

While the binding studies up to this point have utilized a probe (-174/-124), which spans the entire upstream response region (-154/-127), we failed to identify any proteins which bind to this sequence of DNA, located at -141bp to -127bp.

Because of the importance of this region to MMS-responsiveness, as determined by linker-scanning mutagenesis, we decided to carry out an EMSA using a smaller EMSA probe that would more isolate this region. To this end, we carried out an EMSA with untreated or MMS-treated nuclear extracts with a probe spanning -149/-124bp (Figure 23). We observed two prominent bands from the EMSA, which had the same intensity using either untreated or MMS-treated extracts.
Figure 23. EMSA. Untreated or MMS-treated (100μg/ml, 8hrs) extracts used with -149/-127bp radiolabeled probe.
5.2.1.7 The gadd45b sequence located at -141/-120bp is important for MMS-mediated transactivation, but a bound protein or protein complex is not detectable.

We observed constitutive binding (with and without MMS treatment) of a protein or protein complex (Figure 23) in an EMSA using a gadd45b probe, which spans an important MMS-responsive region (LS20, -141/-120). We were unable to identify this protein by supershift or competition oligo EMSA in experiments similar to those of Figure 22. Therefore, we attempted to identify the sequence of this region that is required for binding by competition mutant oligo EMSA as carried out previously to identify the binding sequence of Egr1 in Figure 21. To this end, we used six mutant oligos that span the MMS-response region (Figure 24), and observed a loss of protein binding in 4 of 6 binding reactions (M3-M6), a partial loss in one (M1) and a slight loss in one (M2). As a control, the wild-type cold oligo was included (lane 2) and competed out the binding of protein to probe, showing no band, as expected. These data indicate that the protein or protein complex requires the sequence substituted for in Mutant 2 and less so in mutant 1. These sequences are CTCCC for Mutant 1 and CCTCC for Mutant 2. Interestingly, this sequence overlaps with the sequence of Sp1, Egr1, and MZF1.
and is present in the proximal half of the upstream response region (-154/-140), suggesting an unidentified fourth protein bound to this 10bp GC-rich sequence. Furthermore, it has not escaped our notice that this constitutively bound band may be the unidentified lowest-running band seen when using the larger EMSA probe (-174/-124), since supershift and cold-competitor EMSA using either Sp1, MZF1, or Egr1 fail to shift or eliminate this band.

![Image of EMSA experiment](image)

**Figure 24.** An unidentified protein constitutively binds the *gadd45b* promoter sequence CTCCCCCTCC as determined by EMSA.
5.2.1.8 The NFY complex constitutively binds to the CCAAT box, located within the most proximal MMS-responsive region, of the gadd45b promoter

Up to this point, our focus has been on identifying proteins that bind to the distal-most MMS-response region (-154/-127bp). Next, we sought to identify the proteins that bind to the proximal response element, located at -102/-88bp. To this end, we carried out an EMSA using a probe that spanned this region of the gadd45b promoter, EMSAoligo-124/80bp. We observe identical protein binding to this probe and with equal intensity using nuclear extracts from either untreated or MMS-treated nuclear extracts (Figure 25). When a wild-type cold competitor oligonucleotide is included in excess in the binding reaction, we observe the loss of the band, as expected (lanes 2 and 6). When an oligonucleotide containing a mutated CCAAT box is included in excess, the band remains (lanes 3 and 7). Based on this mutant binding competition, the proteins binding to the probe bind specifically to the CCAAT box. This is fortuitous, since the CCAAT box is the primary cis element present in the proximal MMS-response region.

We next carried out the EMSA binding reactions in the presence of the NFY-A antibody, a protein that, as a complex with NFY-B and NFY-C, binds the CCAAT
box. When NFY-A antibody is included, we observe a supershift of the band, indicating that the NFY complex is binding at this location on the *gadd45b* promoter.

**Figure 25.** NFY complex binds the CCAAT box of the *gadd45b* promoter constitutively, before and after MMS treatment.
5.2.1.9 Egr1, Sp1, NFY, and MZF-1 bind to the endogenous gadd45b promoter in-vivo

Using the electrophoreotic mobility shift assay (EMSA), we have shown that Sp1, NFY, and MZF1 bind constitutively and that Egr1 binds inducibly to the MMS-response regions of the gadd45b promoter. The EMSA is useful for identifying specifically interacting proteins in vitro. However, due to the artificial nature of in vitro binding assays, we sought to verify that these proteins bind to the gadd45b promoter in-vivo. To accomplish this, we carried out the Chromatin Immunoprecipitation assay (ChIP) to show in-vivo binding of Egr1, Sp1, NFY, and MZF1 to the gadd45b promoter (Figure 26).

Figure 26. Egr1, Sp1, MZF1 and NFY-A bind to the endogenous gadd45b promoter as determined by Chromatin Immunoprecipitation (ChIP)
5.2.2 Transcription factor binding contributes to MMS-mediated activity

5.2.2.1 The Egr1/Sp1/MZF1 site and the CCAAT box in the gadd45b promoter were mutated to eliminate binding.

In order to determine the effect that Egr1, Sp1, MZF1 and NFY have on MMS-mediated gadd45b induction, we sought to determine the promoter activity from a luciferase construct in which each site is mutated to prevent binding. Because the Egr1, Sp1 and MZF1 binding sites overlap at the same location, we were unable to specifically mutate one site without affecting binding of the others. Therefore, we decided to generate a mutation that eliminated binding of all three. To this end, the Egr1/Sp1/MZF1 sites in gadd45b promoter-luciferase construct were eliminated by point mutation using a site-directed mutagenesis technique. Figure 27 shows the wild-type sequence of the gadd45b promoter in the vicinity of the Egr1/Sp1/MZF1 site and the mutation generated to eliminate transcription factor binding at this locus. The cytosine stretch that makes up this site was changed to thymidine residues. This mutation proved to eliminate binding of each of these proteins in the EMSA cold competition experiments carried out previously.
The CCAAT box of the \textit{gadd45b} promoter within the \textit{gadd45b1656luc} construct was mutated by site directed mutagenesis as described in the materials and methods. Figure 27a,b graphically shows the mutations made to eliminate the binding of the NFY complex to this site. Again, the specific mutations made, proved to eliminate binding in EMSA experiments carried out previously.
Figure 27. Mutated \textit{gadd45b} promoter sequence to eliminate protein binding. (A). Egr1/Sp1/MZF1 site mutation. (B). CCAAT box mutation. The mutation was generated in the \textit{gadd45b.1656luc} construct and sequence verified.
5.2.2.2 Mutation of the Egr1/Sp1/MZF1 site and the CCAAT box in the gadd45b promoter reduces MMS-mediated transcriptional activity.

Mutation of Egr1/Sp1/MZF1 site in the gadd45b promoter reduces MMS-mediated activity to that comparable to the linker-scanning mutant (LS19) of the same location. The relative luciferase activity of the mutant Egr1/Sp1/MZF1 gadd45b construct was found to be ~75% compared to the comparable wild-type construct (Figure 28).

The CCAAT box element of the gadd45b promoter contributes to MMS-mediated induction (Figure 28). In a dual luciferase assay using the gadd45b -1656luc – CCAAT mutant construct described in Figure 42, we observe a ~30% loss of MMS-mediated promoter activity, compared to the wild-type. The loss of activity seen with this mutant is comparable to that seen in the Linker-scanning mutant counterpart, LS23 (Figure 28). Interestingly, however, in a double point mutant, in which both the CCAAT box and the Egr1/Sp1/MZF1 sites are mutant in a luciferase construct we observe no additional loss of luciferase activity, as might be expected. Instead we see a drop of ~30% in the double mutant, which is comparable to that seen with either the Egr1/Sp1/MZF1 mutant, or the CCAAT
box mutants by themselves. A comparison of the activity of this double mutant to that of the Linker-scanning double mutant which covers the same sequence regions, LS19/LS23, shows a difference of ~35% activity.

Figure 28. A CCAAT box mutant of the gadd45b promoter luciferase construct has a decrease in MMS-mediated activity compared to wild-type.
**5.2.2.3 siRNA knockdown of Egr1 reduces gadd45b MMS-mediated transcriptional activity**

When the DNA sequence to which Egr1, Sp1, NFY and MZF1 bind is mutated to eliminate binding, we observed a reduction in MMS-mediated luciferase activity. We decided to follow up on this data by siRNA knockdown of Egr1, to determine the effect on MMS-mediated *gadd45b* induction. We observed that Egr1 knockdown by siRNA reduces the MMS-mediated induction of *gadd45b* significantly (Figure 29).

![RNAi knockdown of Egr1 by siRNA in RKO cells](image)

*Figure 29.* RNAi knockdown of Egr1 by siRNA in RKO cells. Egr1 knockdown reduces MMS-mediated *gadd45b* induction.
5.3 DISCUSSION

In this chapter, we investigated the trans-acting factors that act on the \textit{gadd45b} promoter in two ways: 1). In sections 5.2.1.1 through 5.2.1.9, we identified those transcription factors that bind to the two cis-acting elements of \textit{gadd45b in vitro} by EMSA and then confirmed these interactions \textit{in vivo} by ChIP. 2). In sections 5.2.2.1 through 5.2.2.3, we investigated the effect that the inducible protein, Egr1, has on MMS-mediated \textit{gadd45b} transcription by promoter-luciferase activity and by RNAi knockdown.

We initially identified those transcription factors that bind to the MMS-responsive regions \textit{in vitro} by Electrophoretic Mobility Shift Assay (EMSA). This assay is based on the concept that an oligonucleotide will migrate through a gel slower when proteins are bound to it. Each band represents either a single bound protein or a protein complex. Additionally, the intensity of the band may reflect either the degree of binding affinity of a given protein or protein complex to a particular site, or the abundance of a particular protein or protein complex in the nuclear extract tested.
In this way, DNA-protein interactions can be determined. Despite the numerous drawbacks to using the EMSA to identify DNA-protein interactions (artificial binding conditions, naked DNA, other), this assay is the best tool available to show protein binding to a specific sequence under different conditions and to positively identify those proteins by competition or supershift assays. Furthermore, it provides an excellent first indication of those binding proteins, which can then be verified with the in-vivo Chromatin Immunoprecipitation binding assay.

In our initial experiments, we used different sets of radiolabeled double-stranded oligonucleotide probes that contained the MMS-response elements identified in chapter 3, -154/-127bp and -102/-88bp. Our oligonucleotide probes included:

In an EMSA using either the EMSAoligo-174/-124 probe or the EMSAoligo-163/-138 probe we observed an identical pattern of bands: 3 constitutive and 1 MMS-specific (Figure 16, 18). The larger of the two probes, EMSAoligo-174/-124 spans the entire distal MMS-response regions (LS19-20, -154/-127). The alternative probe, EMSAoligo-163/-138 spans only the upstream half of the distal MMS-response region (LS19, -154/-140). This initial data indicated that the upstream half (-154/-140) of the distal MMS response region was bound by both constitutive and inducible factors, whereas the downstream half (-140/-127), while important for induction, itself did not bind proteins and was additionally not required for the binding of those proteins which bind upstream (Figure 30).

**Figure 30.** initial EMSA binding summary
In our binding studies, we employed EMSA binding studies that included either cold competitor oligonucleotides or antibodies to identify the proteins interacting with the promoter. In this manner, we showed that Sp1 and MZF1 are constitutively bound between -154/-140bp, the NFY complex is constitutively bound to the CCAAT box between -102/-88, and Egr1 is inducibly bound (MMS) between -154/-140bp (Figure 31).

**Figure 31.** Summary of protein binding data.
Sp1 and MZF1 were readily identified, as these two proteins are the two top candidates for binding, based on the analysis for transcription factor binding sites with the TRANSFAC database (Table 7). In contrast, we had difficulty in the identification of the inducible band, Egr1. While the region -154/-140bp is a consensus site for numerous proteins, none of those tested by either cold competitor EMSA or antibody supershift was able to shift or deplete the band (Figure 20).

By identifying the specific sequence required for binding by this inducible band, using the EMSA-linker scanning competitor strategy (Figure 21), we were able to narrow the possible protein binding possibilities. In this manner we identified Egr1 as a possible binding protein and subsequently showed this by supershift and cold competition EMSA. Interestingly, the Egr1 bindings site in the gadd45b promoter generates a low binding score (64.2) for Egr1 when analyzed against the TRANSFAC database.
In contrast to Egr1, we identified NFY binding to the proximal MMS-response element (CCAAT box) in a fortuitous manner. While the NFY complex is a prime candidate for CCAAT binding, it is not among the top candidates for binding to the *gadd45b* CCAAT box (Table 7). We nonetheless identified the single band as NFY with supershift analysis in our first experiment with this probe. Therefore, the difficulties with Egr1 were made up for with NFY; and both have similar binding scores (~64).

The numerous in-vitro EMSA binding assays carried out to identify the binding of Egr1, Sp1, MZF1, and NFY-A to the *gadd45b* promoter were followed up with ChIP analysis to assess endogenous binding. Our data confirmed the binding of each of these factors; however, there were some notable differences in binding intensity between the ChIP and EMSA. Most notably was the robust increase in Sp1 binding with MMS treatment compared to untreated by ChIP (Figure 26). This was consistently observed by ChIP, whereas, there was no increase in binding intensity for Sp1 by EMSA. Another difference in binding pattern is with Egr1. Where there is an increase in binding of Egr1 to the endogenous *gadd45b*
promoter with MMS treatment as assessed by ChIP, this increase is significantly less robust than that observed by EMSA. Differences in protein-DNA binding intensity for a given protein by EMSA versus ChIP can be explained by the status of the DNA itself. The endogenous DNA, in the form of chromatin, is a much more complex molecule compared to a transfected plasmid DNA, in terms of organizational tertiary structure, and in DNA-associated histones and other proteins.

From our binding studies, we are left with several questions that may be worth pursuing in future studies. First, how do three proteins (Sp1, NFY, MZF1) that are constitutively bound to two different MMS-responsive cis elements, mediate MMS-induced transcriptional activation? It is possible that these proteins are bound and become “activated” by a protein modification upon stimulation with MMS. Another likely possibility is that these proteins act as a docking station for other protein complexes, which bind to and activate transcription. Second, what additional proteins are binding to the GC-rich region of LS19 (-154/-140)? From the EMSA, it is clear that at least one other protein binds here. Could others also
be binding that cannot be detected by EMSA? Third, and most intriguingly, what role does the MMS-responsive region -140/-127 play in activating transcriptional activity? We have shown the importance of this region by linker-scanning mutagenesis on MMS-mediated activity (Figure 15); however, we are consistently unable to detect protein binding to this region. We have further determined that this sequence is not required for the binding of Egr1, Sp1, or MZF1, since an EMSA probe lacking -140/-127 binds Egr1, Sp1 and MZF1 equally well.

**Figure 32.** LS20 does not bind protein but is requires for MMS-mediated transcription
With respect to the role of this stretch of DNA, two possibilities come to mind: 1).

The sequence forms a secondary/tertiary structure that is required to bridge
upstream transcription factors to downstream transcriptional machinery, or 2).

This stretch of DNA does bind an important protein that is not detectable under
the binding conditions we used for the EMSA. It would be interesting to
investigate this issue in the future.

Ultimately, we wanted to determine what affect both the constitutively-bound
Sp1, NFY, and MZF1 proteins and the inducibly-bound Egr1 protein have on
MMS-mediated transcriptional activity. We approached this in two ways: 1).
Measure the MMS-mediated promoter activity when the Egr1, Sp1, NFY, and
MZF1 binding sites are eliminated by point mutation, compared to non-mutated
sites, and 2). Determine the levels of gadd45b mRNA in MMS-treated cells in
which the inducible Egr1 is knocked down.
Since the binding sites for Egr1, MZF1 and Sp1 overlap at the same locus, it was
not possible to eliminate binding of one by point mutation without affecting the
binding of the other two. Therefore, from our luciferase-mutagenesis experiment,
we can only conclude that either one of these proteins, a combination of two
proteins or all three contributes to MMS-mediated activity. Furthermore, since
eliminating the binding of all four proteins doesn’t completely eliminate
transcriptional activity, it is evident that additional, unidentified factors contribute
as well. It is possible that these factors bind to the cis-acting elements, but are
not detected by EMSA with the binding conditions used.
5.4 CONCLUSIONS

1. Sp1 and MZF1 bind the *gadd45b* promoter at -154/-140 before and after MMS-treatment.

2. The NFY complex binds the *gadd45b* promoter at the CCAAT box, between -102/-80 before and after MMS-treatment.

3. Egr1 binds the *gadd45b* promoter inducibly at -154/0140 after MMS-treatment.

4. The binding of NFY complex and Egr1, Sp1, and MZF1 contributes to MMS-mediated transcriptional activity.

5. Additional components (factors or DNA sequence) contributes to *gadd45b* MMS-mediated induction.
CHAPTER 6

SORBITOL INCREASES GADD45B MRNA LEVELS BY INCREASING THE
GADD45B MRNA HALF-LIFE
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SORBITOL INCREASES GADD45B MRNA LEVELS BY INCREASING THE GADD45B MRNA HALF-LIFE

6.1 INTRODUCTION

In chapter 3, it was shown that sorbitol treatment of RKO cells results in a robust induction of gadd45b mRNA in spite of the observation that there is no sorbitol-mediated promoter activity (luciferase assay), or transcription initiation (nuclear run-on assay). These observations suggest a post-transcriptional mechanism in the form of mRNA stability, however, it is also possible that a sorbitol-response element lies outside of the promoter sequence tested. An important enhancer element located outside of the proximal promoter of a gene is not without precedent; an active p53 site, for example, is present in the third intron of gadd45a.

Confirming that gadd45b mRNA is stabilized by sorbitol treatment is significant because it will confirm previous findings, using NaCl, that hypertonic stress stabilizes the gadd45b mRNA transcript (Chakravarty D, 2002). These findings
will additionally serve as a platform for subsequent studies to identify the components involved in this stabilization. \textit{gadd45b} is unique in this respect, since it does not possess a consensus mRNA stabilizing element in the 3'UTR (Esclatine A, et al. 2004). Thus, a novel mechanism of mRNA stabilization would be at play.

The hypertonic environment generated by sorbitol may exist in proliferating cancer cells. As such, it is not difficult to envision a strategy of customized pathway-specific therapeutic intervention to modulate \textit{gadd45b} levels to improve cancer treatment therapy.

In order to determine if the sorbitol-mediated increase in \textit{gadd45b} mRNA is the result of increased mRNA half-life, we used two different strategies to determine the rate of mRNA degradation following treatment. This was carried out by two different methods using the RNA transcription inhibitor, Actinomycin D: \textit{gadd45b} mRNA levels were determined over a time course in which either 1) 2.5uM Actinomycin D was added to four-hour post-treatment (MMS or sorbitol) RKO
cells; or 2). RKO cells were pre-treated with 2.5uM actinomycin D prior to MMS or sorbitol treatment. The *gadd45b* mRNA half-life was determined from these experiments by measuring the rate of mRNA degradation over time following the addition of Actinomycin D.

We expected to observe an increase in *gadd45b* mRNA stability in RKO cells following treatment with sorbitol, compared to MMS-treated and untreated cells.
6.2 RESULTS

6.2.1 Gadd45b mRNA is stabilized by treatment with sorbitol relative to treatment with MMS.

Sorbitol treatment stabilizes gadd45B mRNA relative to MMS treatment.

Treatment of RKO cells with either MMS or sorbitol increases gadd45b mRNA significantly by six hours (Figure 33, lanes 2, 8). When these treated cells are then subjected transcriptional inhibition with Actinomycin D, we observe a decrease in gadd45b mRNA levels over time for both MMS (Figure 33, lanes 3-7) and sorbitol (Figure 33, lanes 9-13). The rate of gadd45b mRNA decay is greater for MMS-treated cells compared to sorbitol treated cells.
Figure 33. Sorbitol treatment of RKO cells stabilized gadd45B mRNA relative to treatment with MMS. Northern blot analysis was carried out on total RNA extracted over 1.5 hours from RKO cells that were either treated with MMS (100μg/ml) or sorbitol (0.3M) for six hours, followed by Actinomycin D treatment (2.5μM). A human gadd45b cDNA probe was used to probe the northern blot and equal loading was determined by 18S, 28S bands of the northern gel (shown below).
Figure 34. Quantization of Northern analysis by densitometry using MacBas program.
6.2.2 Gadd45b mRNA half-life is stabilized by treatment of RKO cells with sorbitol, relative to MMS treatment or no treatment.

We observe similar results when RKO cells are pre-treated with Actinomycin D, immediately followed by either MMS, sorbitol, or DMEM treatment (Figure 35).

Here we clearly observed a rapid decay in gadd45b mRNA in both untreated and MMS-treated RKO cells, while sorbitol treated cells maintained higher levels of gadd45b mRNA over time. Analysis of these data by band densitometry indicates by two hours following inhibition of transcription with Actinomycin D, gadd45b mRNA levels in untreated or MMS treated cells are 30-40% that of Sorbitol treated cells (Figure 36).

**Figure 35.** Northern blot analysis was carried out on total RNA extracted over 4 hours from RKO cells that were pretreated with Actinomycin D (2.5uM) and then immediately treated with either MMS (100ug/ml), sorbitol (0.3M) or DMEM (control).
Figure 36. Quantization of northern blot by densitometry using MacBas program
6.2.3 sorbitol treatment of RKO cells fails to increase binding of Egr1 to the \textit{gadd45b} promoter.

Sorbitol treatment fails to increase Egr1 binding to gadd45B promoter. The inducible band observed in EMSA with MMS treatment, determined to be Egr1, is not observed with sorbitol treatment.

\textbf{Figure 37.} sorbitol treatment fails to increase the binding of Egr1 to the \textit{gadd45b} promoter. EMSA using two different probes spanning the MMS-responsive region with nuclear extracts from RKO cells treated with either DMEM (control), MMS (100ug/ml), or sorbitol (0.3M) and harvested after 8 hours.
6.3 DISCUSSION

We determined in chapters 3, 4 and 5 that MMS treatment mediates \textit{gadd45b} mRNA increase primarily through a transcriptional mechanism, involving a cohort of transcription factors acting in concert (Sp1, Egr1, NFY, others). The large increases in \textit{gadd45b} mRNA following sorbitol treatment involved some type of post-transcriptional mechanism.

As stated earlier, it is becoming more evident that post-transcriptional mechanism plays a larger role than previously appreciated. Recently, post-transcriptional mechanisms have been described for gadd45 gene members, including \textit{gadd45a} and \textit{gadd45b} [Lal A. 2006 (45A), Sakaue M. 1999 (45B), Abcouwer SF. 1999 (45A), Yoshida T. 2005 (45A), Zhang Y. 2006 (45A)]. It was shown that \textit{gadd45b} was regulated at the level of mRNA stability in human lung carcinoma cells following treatment with a novel synthetic retinoid, 6-[[3[(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN). The Gadd45b half-life was increased greater than 9-fold following treatment (Sakaue M., 2006). Additional genes have been shown to be regulated post-
transcriptionally, including cell cycle regulators, cdk4, E2F-1, p21, p27, cyclins A, B1, D1, tumor suppressors p53, BRCA1, regulators of apoptosis, Bcl-2, Bcl-x, Bax, DNA repair factors ERCC1, and mitogenic factors, c-fos, c-jun, c-myc (Lal, et al 2006).

While there are a number of potential points of regulation between pre-mRNA transcript and fully functional protein, only two points in the process would alter the levels of cytoplasmic mRNA detectable by Northern or by RT-PCR. Either 1.) increased transcript numbers (transcriptional activation or transcriptional de-repression) or 2.) increased steady state cytoplasmic mRNA numbers (increased mRNA stability or decreased mRNA degradation) would account for an increase in detectable cytoplasmic mRNA by Northern or by RT-PCR. As such, we chose to focus on the mRNA stability aspect of the gadd45b mRNA to determine how the treatments affected the half life.

mRNA stabilization has been reported for gadd45a mRNA with glutamine deprivation, arsenic treatment, and with MMS treatment. In the first set of
experiments, we monitored the levels of \textit{gadd45b} mRNA over time in cells in which transcription was inhibited (Actinomycin D, 2.5uM) after treatment (MMS or sorbitol) had been applied for four hours. In this experiment we determined that sorbitol treatment increased the \textit{gadd45b} mRNA half-life relative to MMS-treated cells by approximately 4-5 fold (Figure 33, 34). When RKO cells are first pre-treated with Actinomycin D prior to treatment with either sorbitol or MMS, we observed a marked increase in sorbitol-treated \textit{gadd45b} mRNA half-life relative to untreated or MMS-treated (Figure 35,36). In either type of experiment, sorbitol-treated samples increase the \textit{gadd45b} mRNA half-life significantly over either MMS-treated or untreated samples. The differences in these results between experiments could be for a number of different reasons. It’s possible that inhibiting transcription after MMS or sorbitol treatment masks the degree of sorbitol-mediated \textit{gadd45b} mRNA stabilization relative to the MMS-treated samples, since MMS-mediated transcriptional activity may continue to occur following addition of actinomycin D. It’s also possible that other factors affecting mRNA stability are altered in different ways between the two types of
experiments. Regardless of the why there is a difference, in both types of experiment, the gadd45b mRNA is stabilized by sorbitol treatment.

The fact that there is different mechanism of regulation depending on the type of stress, raises the interesting question as to why such a difference would exist. Is there a fundamental reason why sorbitol would stabilize mRNA, while MMS would increase transcription, with the end result being similar levels of gadd45b mRNA and with similar induction kinetics? Or, is this difference in mechanism simply an evolutionary off shoot? For instance, it’s not beyond the realm of possibility that different types of stresses played a more prominent role at different points in time stress response by mammalian cells. In this type of situation, the end result might be the same, while the route of getting there is different. Or, the different types of mechanisms may have been selected for other reasons, such as energetics.

Future work to uncover the specific post-transcriptional mechanism of mRNA stabilization involving gadd45b would help to resolve these questions.
6.4 CONCLUSIONS

1. Sorbitol treatment increases the *gadd45b* mRNA half-life by blocking mRNA degradation.

2. Egr1 does not play a role in the sorbitol-mediated induction of *gadd45b*, as it does with MMS treatment.
Here we have shown that the stress-responsive gene, \textit{gadd45b}, which is robustly induced by a wide variety of cellular stress agents, is regulated by distinct mechanisms, depending on the stress stimuli present. When human colon carcinoma cells (RKO) are subjected to alkylating DNA damage with MMS, we find that \textit{gadd45b} induction occurs through promoter activation. This MMS-mediated activation in part involves the transcription factors Egr1, Sp1, MZF1 and NFY acting through both the GC box and CCAAT box DNA elements, respectively. In contrast, in the presence of hypertonic stress generated with sorbitol, a high level of \textit{gadd45b} induction occurs in the absence of promoter activation. Instead, the robust increase in \textit{gadd45b} mRNA levels is the result of an increased mRNA half-life following sorbitol treatment. In the section that follows, I will discuss various aspects of both types of control mechanisms, the therapeutic implications, and hypothesize a general mechanism that integrates both. Finally, I will suggest important follow-up studies that would be useful to pursue in the future.
7.1 MECHANISM I – TRANSCRIPTIONAL ACTIVATION FROM ALKYL-BASE DNA DAMAGE

In Chapters 3 and 4, we demonstrated that transcriptional activation of \( gadd45b \) occurs in RKO cells following treatment with the DNA-alkylating agent, MMS. This was demonstrated in three ways: 1.) Using a \( gadd45b \)-promoter luciferase reporter construct, we observe a significant increase in promoter activity in MMS-treated cells compared to untreated cells, 2.) We observe an increase in transcription initiation events by nuclear run-on assay in cells treated with MMS compared to untreated cells, and 3.) When RKO cells are pretreated with the general transcription inhibitor, actinomycin D, there is a block in \( gadd45b \) induction in cells treated with MMS. Taken together, these independent sources of data are very convincing in establishing that MMS-treatment induces \( gadd45b \) primarily through transcriptional activation.

7.1.1 MMS and Chemotherapy

Methylo methane sulfonate (MMS) is a chemical that is effective at damaging DNA by chemically modifying DNA bases through the addition of alkyl moieties. The addition of alkyl moieties to DNA bases results in several deleterious effects to
the cell: 1.) abnormal base pairing 2.) bulky adducts, 3.) Increased DNA strand breakage, and 4.) Increased cross-linking between DNA strands. The cellular response to such DNA damage by alkylation agents is generally dose dependent such that low doses, elicits the growth arrest and DNA repair response, and high doses will result in programmed cell death. It is the latter cellular outcome that has made alkylation agent use a potent and effective chemotherapeutic treatment against a number of cancers.

The first “modern day” chemotherapeutic agent was an alkylation agent, which was accidentally discovered as such during World War II. Nitrogen mustard gas is an alkylation agent compound that has been used prevalently as a biological weapon since World War I. As a gas, this compound is a powerful irritant that affects the skin, eyes, respiratory tract, and quickly compromises the immune system (www.bt.cdc.gov). It was discovered in 1942, that soldiers exposed to this compound exhibited very low white blood cell counts (Hirsch J, 2006). From this observation, it was hypothesized that this compound could be an effective treatment for blood cancers. Indeed, this was demonstrated by Goodman in
1946, in which he demonstrating the effectiveness of intravenously injected liquid Nitrogen mustard for treatment of advanced lymphomas (Goodman LS, et al. 1946).

Today Alkylation agents continue to be used as effective chemotherapeutic agents against blood cancers and slow growing solid tumors. Some specific cancers treated in this manner include acute/chronic leukemia, lymphomas, Hodgkin’s disease, multiple myeloma, sarcoma, lung cancer, breast cancer, and ovarian cancer (www.cancer.org) While other alkylating agents have been developed (mechlorethamine, cyclophosphamide, chlorambucil, cisplatin, carboplatin, oxaliplatin) that have less side effects than nitrogen mustard, all alkylating agents act through the same mechanism of DNA base alteration, leading to apoptosis at all phases of the cell cycle. Methylmethane Sulfonate is no different, and is used as an affordable alternative to study the cellular response to alkylation-based DNA damage.
Hence, in part because of the robustness of \textit{gadd45b} induction (compared to other treatments) and additionally because of the chemotherapeutic implications of the widely used alkylation-based cancer treatment, we chose to investigate the mechanism of MMS-mediated \textit{gadd45b} induction. As mentioned previously, knowledge of this mechanism could provide a means to carry out pathway-specific modification to improve the efficacy of alkylation-based chemotherapy.

7.1.2 MMS-mediated transcriptional activation mechanism

That a genotoxic stress such as MMS mediates a transcriptional activation of a stress responsive gene is not surprising. Transcriptional up-regulation appears to be the most important and common mechanism in genes encoding stress response proteins (Guhaniyogi J, et al 2001). In the section that follows, I will briefly describe the components found to be required for MMS-mediated \textit{gadd45b} transcriptional activation: cis-elements (GC box, CCAAT box) and trans-factors (Egr1, Sp1, MZF1 and NFY), and describe these components in the context of transcriptional activation and the stress-response.
As a follow up to our initial observations with a promoter-luciferase construct and nuclear run-on assay, demonstrating MMS-mediated transcriptional activity, we sought to identify the specific cis-elements in gadd45b that are required for this activity. As mentioned previously, this was accomplished using a series of linker-scanning-luciferase mutants, which allows the unbiased identification of important regions. We identified two MMS-responsive regions located upstream from the transcription start site at 1.) -154bp to -127bp, and 2.) -102bp to -88bp. Inspection of the DNA sequence makeup of these regions reveals the presence of two well studied cis-acting elements: 1.) GC-box located at -154bp to -141bp, and 2.) CCAAT box located at -100bp to -88bp. These two elements are well known regulatory regions to which a number of different factors bind and modulate transcriptional activity.

7.1.3 CCAAT box

The CCAAT box is one of the most common elements in eukaryotic promoters, found in the forward or reverse orientation (Mantovani R, 1998). There has yet to be a study in which a CCAAT-box in a canonical position was not shown to play
a role in transcriptional activation (Mantovani R, 1999). In TATA-containing
promoters, including that of *gadd45b*, the CCAAT box is preferentially located in
the -80/-100bp regions with a mean position of -89. The *gadd45b* CCAAT box,
located at -90/-98, is situated as expected. The consensus CCAAT box
sequence as determined by comparing 96 unrelated promoters, extends
upstream and downstream of the CCAAT-box proper for a 13bp element: C-A/G-
transcription factors recognize and bind to this elements (C/EBP, CTF/NF-I,
CDP, other) NFY/CBP is by far the major CCAAT box binding factor. NFY
binding to the CCAAT box is the only transcription factor that absolutely requires
for binding, a 100% consensus of the internal pentanucleotide, CCAAT. In the
*gadd45b* promoter, the CCAAT box sequence is: \textcolor{red}{CA\textcolor{green}{ACCAAT}}\underline{A\textcolor{red}{GGAG}}. The
highlighted nucleotides perfectly match the consensus sequence, while the bold
underlined pentanucleotide is the core sequence. Thus, the cis element located
between -102/-88bp is a bona fide CCAAT box based on consensus sequence
and base on location within the *gadd45b* promoter.
7.1.4 Early growth response gene 1 -- Egr1

Early growth response gene product (Egr1) is a well known stress-responsive zinc finger transcription factor (59 kDa) that binds to the GC/GT boxes of a number of genes (including p53, TGF-β) to modulate the cell cycle and/or apoptosis depending on the cell type and stimulus (Yu J, et al. 2006). Egr1 induction is rapidly and transiently up-regulated by a wide variety of stress agents, similarly to gadd45b. Egr1 is unique among transcription factors in that it activates transcription as a monomer.

In our study, we have identified Egr1 as the inducible factor that binds to the gadd45b promoter at the GC box (-154/-141). In both In-vitro supershift mobility shift assay and in-vivo Chromatin Immunoprecipitation, we observe increased binding of Egr1 to the gadd45b promoter at the GC box. Furthermore, the importance of this binding on MMS-mediated gadd45b induction was demonstrated in Egr1 siRNA knockdown experiments. Here, we observe a reduction in gadd45b MMS-mediated induction in cells in which Egr1 expression is knocked down. Our results are consistent with a previous study in which Egr1
is shown to bind to and mediate UVB-mediated \textit{gadd45b} induction in mouse cells (Thyss R, et al 2005).

### 7.1.5 Specificity Protein 1 -- Sp1

Specificity protein 1 (Sp1) was the first transcription factor identified, cloned and shown to be a sequence specific binding protein that activates a broad and diverse spectrum of mammalian and viral genes (Dynan WS, et al 1983; Dynan WS, et al 1983; Dynan WS, et al 1985; Briggs MR, et al. 1986; Kadonaga JT, et al. 1987). Like Egr1, Sp1 recognizes GC/GT boxes and interacts with the DNA through three C2H2-type Zinc fingers (Safe S, et al. 2005). This is consistent with the binding that we observe by mobility shift assay, in which Sp1 binds to the GC box at the same location as Egr1. The primary difference between Sp1 and Egr1 binding however, is that Sp1 binds constitutively at the same level in both untreated cells and MMS-treated cells. It is not uncommon for Sp1 and transcription factors in general that activate transcription to remain bound and become activated by a modification such as phosphorylation or acetylation. Indeed, there are a number of examples of constitutively bound Sp1-mediated

Interestingly, like the GADD45B protein itself, Sp1 is thought to function primarily by interaction with other bound transcription factors to activate transcription (Chu S, et al 2005). For example, Sp1 has been shown to activate a number of genes through interactions with E2F, AhR, SMADs, NFkB, GATA proteins, jun and others (Lania L, et al, 1997). In addition to these transcription factors, Sp1 has also been shown to directly interact with the general transcriptional machinery components, including TATA-binding protein associated factors (TAFs) and other nuclear cofactors comprising the general transcriptional apparatus (Safe, S, et al 2005). Significantly, Sp1 strongly interacts with NFY to modulate transcription (Safe S, et al 2005). In-vitro studies suggest that Sp1 stabilizes binding of NFY to an adjacent CCAAT box and NFY stabilizes binding of Sp1 to a GC-rich promoter (Tanese N, et al 1991). Similarly, other studies demonstrate interesting
NFY-Sp1 interactions on a generic promoter: Sp1 does not affect the off-rate of NFY dissociation from a DNA complex, but Sp1 significantly increases the on-rate of NFY-DNA binding (Wang, W, et al 1999). Thus, it is clear that Sp1-NFY interaction is common and important in transcriptional activation.

Our results are consistent with a constitutively bound Sp1-containing complex that, upon MMS-treatment, becomes modified (perhaps phosphorylation) to become activated. This activated Sp1 complex then interacts with other transcription factors (possibly Egr1, NFY, and MZF1), and with the general transcriptional machinery to activate transcription.

### 7.1.6 Nuclear Factor Y complex -- NFY

Nuclear Factor Y (NFY), also known as CCAAT-binding factor (CBF) is a complex of three proteins (NFY-A/CBF-B, NFY-B/CBF-A, NFY-C/CBF-C) all of which are required to bind the CCAAT box DNA sequence C-A/G-A/G-C-C-A-A-T-C/G-A/G-G-A/C-G. This complex associates with the CCAAT box through a histone-fold motif (Mantovani R, 1999). Like Sp1, NFY is a ubiquitous
transcription factor that is involved in the transcriptional activation of a number of
types of genes, including stress-inducible genes (Roy, et al, 1995; Marziali, et al
1997). NFY interacts with other transcription factors, including Sp1, and
cofactors to modulate transcriptional activity.

As mentioned previously, the human gadd45b promoter contains one canonical
NFY binding site (CCAAT box), to which the complex was shown to bind with
equal intensity before and after MMS treatment. Mutation of this CCAAT box
abolishes binding and reduces the MMS-mediated transcriptional activity. So,
like Sp1, it appears as if the constitutively bound NFY is required to some extent
for transcriptional activity. It may be the Sp1-NFY interaction or an unidentified
interaction that is required for this activity. Indeed, a similar study showed that
NFY binding to the CCAAT box is required for similar MMS-mediated
7.1.7 Myeloid Zinc Finger 1 -- MZF1

Myeloid Zinc Finger 1 (MZF1) is a protein that binds to GC-rich regions and activates transcription. One study demonstrates cooperativity between Sp1 and MZF1 in the transcriptional activation of a gene involved in metabolism (Dong S, et al 2007). In our studies we observe constitutive binding of MZF1 both in-vitro and in-vivo.

7.1.8 Model Summary

We propose that a number of transcription factors binding to the \textit{gadd45b} promoter at the GC-box and CCAAT box contribute to the MMS-mediated transcriptional activation (Figure 38). A number of these factors (NFY, Sp1, and MZF1) remain constitutively bound and become activated following MMS-mediated DNA damage either by a protein modification (phosphorylation, acetylation) or when Egr1 inducibly binds. In this manner, with constitutively bound factors, \textit{gadd45b} is poised and ready for induction when DNA damage is present. This scenario indeed has precedent with another gadd45 family member, \textit{gadd45a}. gadd45a gene induction by genotoxic stress agents (Ionizing
radiation) occurs transcriptionally with no changes in the binding pattern of proteins after treatment (Graunke DM, et al. 1999). In this study, it is shown that proteins are constitutively bound to several cis elements (Oct-1, CCAAT box, p53 site, and AP-1 site) before and after IR treatment. Graunke, et al explains that the chromatin structure and transcription factor binding is preset for rapid up-regulation in response to stress (Graunke DM, et al 1999).

From our study, it is clear that additional transcription factors, co-factors, or the like, which we have not identified, contribute to MMS-mediated induction. This we know, since complete loss of gadd45b promoter activity is not observed when the binding of Egr1, Sp1, MZF1, and NFY are altogether mutated to prevent protein binding. Hence, it is likely that there are other factors (enhancers, or transcription factors) that are contributing to this induction. On the other hand we observe an essentially complete loss of MMS-mediated activity in a triple linker-scanning mutant that entirely eliminates the regions of the CCAAT box, the GC box and the immediate region downstream of the GC box. Thus, it appears
that despite our lack of complete characterization of the required transcription
factor complement, we have thoroughly defined the required cis-acting regions.
Figure 38. Model for MMS-mediated transcriptional activation of \textit{gadd45b}. Sp1, MZF1, and NFY transcription factors are bound to the GC box and CCAAT box respectively and poised for activation by protein modification (phosphorylation?). Upon MMS damage, a signaling cascade is initiated which involves activation and binding of Egr1 in conjunction with NFY, Sp1, and/or MZF1 activation to initiate transcriptional activity.
7.2 MECHANISM II – HYPERTONIC STRESS INCREASES \textit{GADD45B} mRNA HALF-LIFE

In chapters 3 and 6 we observed that upon treatment of RKO cells with sorbitol, an agent which generates a hyperosmotic imbalance across the cellular membrane (resulting in cell shrinkage and increased ionic concentration), \textit{gadd45b} mRNA half-life is increased approximately 10-fold relative to untreated or MMS-treated cells. Three pieces of evidence led us to this conclusion: 1.) A \textit{gadd45b}-promoter-luciferase construct was inactive following treatment with sorbitol, 2.) Initiation of transcription initiation after sorbitol treatment was equal to that of untreated cells, as determined by nuclear run-on assay, and 3.) pre-treatment of RKO cells with the universal transcription inhibitor, Actinomycin D, failed to inhibit the accumulation of \textit{gadd45b} mRNA. Taken together, we established that unlike the transcriptional activation that occurs with MMS treatment, sorbitol induces \textit{gadd45b} mRNA levels by a fundamentally different mechanism – by increasing mRNA half-life.
7.2.1 Cellular Hypertonic Stress

Tonicity is a measure of the relative osmotic pressure experienced by the membrane of a cell. In a hypertonic environment, the concentration of solutes is lower within the cell relative to the external environment. In this situation, an efflux of water occurs in an attempt to generate an osmotic balance (isotonicity), which has several detrimental cellular effects. Most noticeably, cell shrinkage occurs from the resulting loss of internal water content. This loss of water has the effect of increasing the overall ionic content of the cell, which directly affects the conditions required for optimal biochemical reactions, and in maintaining the tertiary structures of macromolecules like DNA, proteins, etc. Protein unfolding and malfunction occurs (Timasheff SN, et al 1993) as well as the generation of double-stranded DNA breaks and chromosomal aberrations (Kultz D, et al 1999, and Galloway SM, et al 1987).

As occurs with direct DNA damage, a complex network of intracellular signaling pathways are initiated by a cell when a hypertonic environment is encountered. There is much overlap with the pathways elicited with DNA damages, such as

Previously, hypertonic stress was thought to be a factor only in the Inner Medullary cells of the kidney, which are adapted to an environment of extreme changes in osmotic pressure, as cells that function to concentrate fluids. Indeed these cells can be said to have optimized the hypertonic stress response, and in cases of hypertonicity, initiate cell cycle arrest until isotonicity is re-established (Ho SN, et al 2006). However, the recent discovery of the ubiquitous expression of the osmo-sensing transcription factor NFAT5 has prompted a closer look at the organism-wide presence of hypertonic stress (Ho SN, et al 2006). To this end, it has been discovered that cellular hypertonicity is indeed a common type of cellular stress that correlates directly with metabolic activity rate and proliferation rate.
While it is not entirely clear why cellular hypertonicity increases with an increase in either metabolic rate or proliferation rate, it has been hypothesized that high metabolic activity generates a large number of macromolecules, which decrease the internal concentration of intracellular osmolytes (amino acids, inositol) (Ho SN, et al 2006). This situation is functionally identical to exposing a cell to extracellular hypertonicity, in that an efflux of water occurs from the osmolyte imbalance. Actively dividing cells in this context, as in rapidly dividing cancerous cells, are likewise subjected to this form of hypertonic stress. Indeed, the primary marker for hypertonicity, NFAT5 expression, is readily detectable in essentially all constitutively proliferating cell lines maintained in tissue culture (Lopez-Rodriguez, et al. 1999; Miyakawa, et al 1999b; Ko et al, 2000; Trama, et al. 2000).

Thus, in part because of the robustness of \textit{gadd45b} induction, which is comparable, if not greater than, MMS-treatment, and additionally because of the, implications in proliferation potential and cancer progression, we chose to investigate the mechanism of MMS-mediated \textit{gadd45b} induction. Pathway-
specific knowledge could allow us increase the efficacy of chemotherapeutic treatments.

7.2.2 Hypertonicity-mediated increase in gadd45b mRNA half-life

Our finding that hypertonic stress of RKO cells by sorbitol treatment results in the increased gadd45b mRNA half-life is interesting, given that most hypertonically regulated genes are transcriptionally induced (Burg MB, et al 1997). However, our finding in part corroborates a similar finding by Chakravarty, et al, in which they observed an increase in the half-life of gadd45a, gadd45b and gadd45g mRNAs in mouse Inner Medullary cells following treatment with NaCl, used to induce hypertonicity (Chakravarty D, et al 2002).

Like transcriptional activation, mRNA stability is modulated through cis-elements in the mRNA, usually within the 5’- or 3’-UTR, and with transcription factors that act on these elements to either increase or decrease mRNA stability. The most well characterized cis element is the AU-rich element (ARE), the consensus sequence being 5’-UUUUAUUAAU-3’ and usually found within the 3’-UTR. An
mRNA that contains one copy of this sequence in the 3’UTR is weakly destabilizing, while two copies result in a pronounced destabilization. When the AUF1 protein factor binds these sequences, mRNA degradation occurs. Some other known mRNA stability factors that act on the mRNA include HuR (stabilizes), Nucleolin (stabilizes) and YB-1 (stabilizes).

While a variety of stress-responsive genes are known to be regulated at the level of mRNA stability, including p21 and VEGF (Wang W, et al 2000; White, et al 1997), interestingly, a number by studies demonstrate mRNA stability control of the gadd45 family of genes. For example, gadd45a mRNA is shown to be stabilized by various trans-factors in at least five different independent studies by various types of stress, including Arsenic, MMS, glutamine deprivation, and UV (Zheng X, et al 2005; Zhang Y, et al. 2006; Abcouwer SF, et al 1999; Jackman J, et al 1994). In yet another study, the gadd45a mRNA stability is achieved following MMS treatment, through the degradation of the mRNA-destabilizing protein AUF1.
There is precedent for *gadd45b* mRNA stabilization as a control mechanism as well. As mentioned previously, NaCl-induced hypertonic stress stabilized *gadd45b* mRNA (Chakravarty D, et al 2002). The synthetic retinoid AHPN also stabilized Gadd45b mRNA (Sakaue M, et al 1999). Finally, the Herpes Simplex Virus protein U₄₁₄₁ stabilizes *gadd45b* mRNA (Esclatine A, et al 2004).

While precedent exists for both mRNA stabilization for gadd45a and *gadd45b* mRNA, it is interesting to note that while gadd45a possesses two ARE elements in the 3′-UTR (1060bp, 1278bp), *gadd45b* is lacking of any known mRNA stabilization cis elements. It is hypothesized that it is a stem-loop secondary structure that forms in the UTR of mRNA that is recognized by different stabilizing or destabilizing transcription factors. To date, these have not been located or identified in the *gadd45b* mRNA sequence. In spite of this, there is some evidence that components of the p38 MAPK pathway are involved in *gadd45b* mRNA stabilization, since a specific p38 inhibitor represses NaCl-induced *gadd45b* mRNA stabilization to a some degree (Chakravarty D, et al 2002).
7.2.3 Model Summary

Accordingly, we propose a mechanism by which \textit{gadd45b} mRNA half-life is increased through mRNA stabilization in the presence of hypertonic stress (Figure 39). Hypertonic stress initiates a signaling cascade which activates an unidentified mRNA stabilization protein which binds to \textit{gadd45b} mRNA to prevent degradation. This scenario is more likely over the alternative situation in which a mRNA de-stabilizing, which is normally bound to \textit{gadd45b} mRNA, is removed following hypertonic stress, thus increasing the normally short half-life.

We propose activation of a stabilizing protein over de-activation of a de-stabilizing protein because the \textit{gadd45b} mRNA UTR lacks any ARE sequences, which destabilize the mRNA through AUF1 protein.

There are a number of potential candidates that stabilize the \textit{gadd45b} mRNA in the presence of hypertonic stress. Two prominent ones, which have been shown to play a role in gadd45a mRNA stabilization include HuR and Nucleolin.
Figure 39. Model for hypertonic stress-mediated *gadd45b* induction. Hypertonic stress in RKO cells, generated with sorbitol treatment, increases the ½-life of *gadd45b* mRNA by approximately 10-fold relative to untreated or MMS-treated cells. We propose that *gadd45b* mRNA is stabilized by an unidentified protein in the presence of hypertonic stress.
We have demonstrated that two different types of cellular stress agents, MMS and sorbitol, increase the levels of $gadd45b$ mRNA with similar kinetics by entirely different mechanisms. Alkylation damage to DNA bases (MMS) increases $gadd45b$ mRNA by activating transcription through a complement of transcription factors (Egr1, Sp1, MZF1, and NFY) that are either activated or bind to the GC box and CCATT box. Hypertonic stress mediated damage (sorbitol) increases $gadd45b$ mRNA through mRNA stabilization, most likely by an unidentified protein which binds to and prevents $gadd45b$ mRNA degradation.

A fundamental and intriguing question is why such different mechanisms exist to achieve the same result? Do these different mechanisms exist out of necessity or is there a cellular advantage to stabilizing $gadd45b$ mRNA with one type of stress vs. another? I propose one possible rationale to explain these differences as follows.
A primary difference between hypertonic stress and direct DNA damage-type stress is in the overall cellular effect each has. Hypertonic stress has a global effect on biochemical reactions by changing ionic conditions and generally destabilizes protein structure. DNA damage on the other hand has a more specific effect of directly damaging the bases of DNA. Biochemical reactions and protein structure generally are not altered from the normal state by DNA damage, as they are with hypertonic stress. Thus, it is possible that the altered state of proteins and non-optimal conditions for biochemical reactions prevents efficient transcriptional activation of important stress-response genes. In this scenario, the mRNA stabilizing proteins would have evolved to be more robust than average in this hypertonic environment. Thus, mRNA stabilization would be favored over transcriptional activation in a hypertonic environment.
7.4 REGULATION OF GENE EXPRESSION – A CONTEMPORARY VIEW

We have unequivocally demonstrated that the alkylating agent, MMS, activates gadd45b transcriptionally, which is mediated through two cis-regions and involves at least several transcription factors. We have also positively determined that a hypertonic-inducing agent, sorbitol, induces gadd45b post-transcriptionally, by increasing mRNA stability.

While transcriptional activation and mRNA stability are two modes of regulation that have been identified as major mechanisms of control, in recent years it is becoming clear that multiple modes of regulation exist. During the course of our research, for example, we discovered that the MMS-responsive cis-acting GC-rich region forms Z-DNA in solution (Figure 40, 41). This is interesting given that Z-DNA has been shown to be involved in transcriptional activation (Wang G, et al 2005). Other modes of regulation that have recently been discovered to modulate mRNA include, methylation, histone modification, nuclear export control, mRNA processing, mRNA localization control, micro-RNA control, and transcriptional control through insulators, enhancers, and repressors. Thus, it is
clear that gene expression control is a dynamic process with many possible points of regulation (Figure 42). Lal, et al describes these multiple regulatory layers well, in the context of gadd45a expression:

Once the gadd45a gene is transcribed, the gadd45a pre-mRNA must first be spliced, and the resulting mRNA capped at the 5’ end, polyadenylated, transported through the nucleoplasm, and exported out of the nucleus. After it reaches the cytoplasm, the gadd45a mRNA must elude ribonucleolytic degradation, bear possible episodes of storage in subcytoplasmic domains, and eventually engage with the translational machinery to serve as a template for the synthesis of Gadd45a protein. Like transcription, posttranscriptional processes can be regulated negatively through repressors and positively via inducers (Lal, et al., 2006).

While we have learned a great deal about the precise control of gene expression regulation, it is clear that many more intricate details remain to be investigated. Indeed, it may be the case that processes like transcriptional and post-transcriptional control mRNA stability control are the primary gross means to control overall mRNA levels, while other mechanisms more finely tune these levels.
Figure 40. Secondary forms of DNA in solution. (Mogel, 2006). B-DNA is the “standard” and predominant form of DNA that exists in solution. Z-DNA is an alternative form that exists along specific stretches of nucleotide sequence. Z-DNA has been proposed to be involved in transcriptional regulation.
Figure 41. sequence of the gadd45b promoter. Highlighted region was determined to form Z-DNA in solution in an Analysis by Guliang (Graham) Wang from the Department of Carcinogenesis at UT MD Anderson Cancer Center.
**Figure 42.** Contemporary view of gene expression (Orphanides G, et al 2002). There are a multitude of points of regulation from the initial transcription initiation event to the production of a functional protein that is in the right place at the right time.
7.5 FUTURE STUDIES

In this study, we have undertaken the initial steps to elucidate the mechanism by which two different types of stress agents, MMS and sorbitol, mediate induction of gadd45b. Our primary findings were that 1.) MMS treatment activates gadd45b transcriptionally through the GC-box and CCAAT box elements, 2.) Several factors bind to and act through these cis elements to contribute to induction, including Egr1, Sp1, MZF1, and NFY, and 3.) sorbitol treatment induces gadd45b through an increase in mRNA stability.

Many questions still remain however about both the transcriptional and post-transcriptional regulation by these stress agents. Future studies should address the observation that in spite of the identification of the full complement of MMS-contributing cis-acting elements, full activity is not lost when the specific TF binding sites are mutated. There possibility the specific non-protein-binding sequence (Z-DNA?), or additional unidentified binding TF’s should be determined.
While we focused on two very different types of stress agents, MMS and sorbitol, it would be interesting to investigate other similar and different stress agents.

From the one previous study with stress-mediated gadd45b regulation (Thyss R, et al 2005), Egr1 appears to be the sole regulating factor with UVB treatment, which is different from our observation with MMS treatment. It would be worth defining more specifically, which type of stress agent elicits what type of regulatory cascade, and with what components. For example, how does ROS-mediated DNA damage compare to IR-mediated DNA damage in terms of gadd45b induction mechanism? Is there a specific type of DNA damage (double strand breaks, single strand breaks, nicks, base alteration) that mediates gadd45b induction by different pathways, using different components?

While our studies have focused on specifically regulation, it would worth also following up this work with functional studies in the same cell line and with the same stress agents. Determining the cellular outcomes, kinetics and contribution of gadd45b to the outcomes would be important in defining the role of gadd45b in this system. This could be accomplished by determining percent apoptosis
and/or growth arrest with and without \textit{gadd45b} in the presence of each stress agent.

We used one cell line for the bulk of our experiments. Initially we did investigate a number of cell lines however to determine overall \textit{gadd45b} induction levels. These included 293T, COS7, HCT116, and MCF-7. In all cells lines \textit{gadd45b} was induced by a variety of stress agents (UVC, UVB, MMS, sorbitol, IR). It would be worth carrying out binding studies and/or knockdown studies with the components identified in our study (Egr1, Sp1, NFY, MZF1) to determine if they contribute to \textit{gadd45b} induction in other cell lines as well.

On a more global level, it would be interesting to further define the role of \textit{gadd45b} in tumorigenesis and/or chemo-resistance. Which type of tumors and at what stage is \textit{gadd45b} de-regulated? What role is \textit{gadd45b} playing when it is over/under-expressed in specific types of tumors? These types of questions can be answered by measuring levels of \textit{gadd45b} and the other \textit{gadd45} isoforms in a variety of primary tumors and/or surveying literature for microarray studies. A
better understanding of \textit{gadd45b} function in these contexts would permit specific therapeutic intervention through the \textit{gadd45b} induction pathway.

Our other primary finding, that sorbitol increases \textit{gadd45b} mRNA not by transcriptional activation, but by mRNA stabilization, is a rich area for future study. Compared to transcriptional regulation, much less is known about the components and mechanism of mRNA stability. While several cis elements and trans factors have been identified that mediate stability, \textit{gadd45b} itself lacks any recognizable mRNA stability elements. Thus, it is possible that a novel pathway is playing a role in hypertonic stress-mediated \textit{gadd45b} mRNA stability.

To elucidate this mechanism, one could identify novel stability elements in the 3’ and/or 5’ UTR of \textit{gadd45b} mRNA. This could be accomplished using expression vectors with serial mutations in the 5’ and/or 3’ UTR of \textit{gadd45b}. An RNA-protein binding pull-down assay could identify candidate factors which interact with \textit{gadd45b} mRNA to modulate stability. Past unpublished data from
one lab (Chakravarty, et al 2002) has also demonstrated the possibility that the p38 MAPK is involved in stress-mediated mRNA stability.
REFERENCES
REFERENCES


Schrag, J.D., Jiralerspong, S., Banville, M., Jaramillo, M.L., and O'conner-McCourt, M.D. (2008). The crystal structure and dimerization interface of GADD45gamma. Proceedings from the National Academy of Sciences USA 105: 6566-6571.


Trama, J., Lu, Q., Hawley, R.G., Ho, S.N. (2000). The NFAT-related protein NFATL1 (TonEBP/NFAT5) is induced upon T cell activation in a calcineurin-dependent manner. Journal of Immunology 165(9):4884-94.


Yang, J., Zhu, H., Murphy, T.L., Ouyang, W., and Murphy, K.M. (2001). IL-18-stimulated Gadd45beta required in cytokine-induced, but not TCR-


APPENDIX
APPENDIX

EFFECT OF FREEZE-THAW OF DNA CONSTRUCTS ON LUCIFERASE-PROMOTER ACTIVITY

A significant portion of our overall study to identify important stress-responsive cis-elements involved the use of a number of gadd45b promoter-luciferase reporter constructs. Because of the highly sensitive nature of the luciferase assay, which often results in high variability from experiment to experiment, we sought to identify, early on in our studies, those variables that may contribute to experiment to experiment variability. We identified several factors over the course of two years, which affected this variability. These factors include: 1.) The use of fresh reagents, specifically the Promega Luciferase Assay Reagent II (LARII) and the Stop and Glo buffer. Promega explains that these reagents may be frozen and used again; however, this leads to variability. 2.) Measuring luciferase activity in samples thawed to room temperature. Temperature is a critical factor in variability and samples must be completely thawed and measured within 1 hour of being thawed to ensure consistent measurements. 3.) Cellular lysate should ideally be measured within 1 hour after lysis without a
freeze thaw. 4.) static electricity affects luciferase measurement and gloves should not be used to handle the polystyrene tubes. 5.) The luciferin substrate is light sensitive and the measurements should be carried out in the dark.

We additionally wanted to determine what effect DNA quality has on luciferase activity variability. This question was addressed by Jasmine Hill, a summer student from North Carolina, who worked in our lab as a summer intern in the summer of 2006. The experimental strategy used by Jasmine to assess DNA quality on luciferase activity was carried out by asking two specific questions: 1.) How do multiple freeze-thaws of a DNA construct affect DNA quality and luciferase activity? and 2.) How does long-term storage of DNA at various temperatures affect DNA quality and luciferase activity?

To answer question #1, a 6kb promoter-luciferase construct was subjected to up to 50 freeze-thaw cycles and assayed for quality by gel electrophoresis (Figure 43) and luciferase activity (Figure 44). Pure DNA that has been freshly isolated runs as a single band on an agarose gel. When plasmid DNA has been
subjected to DNA damage that generates nicks and/or strand breakage, multiple bands and/or smearing of bands is observed. As shown in Figure 43 below, there is no degradation of the plasmid DNA even up to 50 freeze thaws.
Likewise, we detected no significant decrease in promoter activity by luciferase assay from DNA subjected to up to 50 freeze thaw cycles (Figure 44).

**Figure 43.** Effect of freeze-thawing of a DNA plasmid (~6kb) on DNA quality. Plasmids were frozen at -80°C and thawed for the indicated number of times followed by quality assessment by agarose gel electrophoresis. Degraded or nicked DNA would yield multiple bands and/or smearing. Data produced by Jasmine Hill.
Figure 44. A 6kb promoter-luciferase plasmid DNA subjected to multiple freeze-thaw cycles was assays for promoter activity. Data produced by Jasmine Hill.
To answer question #2, we tested the quality of DNA and luciferase activity of the same 6kb luciferase construct when stored at different temperatures. Stability studies were carried out to simulate long term storage at a -20°C temperature, by storing at elevated temperatures for shorter periods of time. We applied the crude estimate that every degree C increase in temperature corresponds to 20 days at -20°C. For example, we stored constructs at room +4°C for 35 days, which is roughly equivalent to 515 days at -20°C. This stability-temperature equation was used previously and determined to be accurate in 2001 while I was member of a Research and Development group at a Biotechnology company in Maryland. We measured constructs stored at various temperatures for 35 days and measured luciferase activity (Figure 45). Our results indicate that plasmids can be stored at -20°C and maintain full luciferase activity up to 2.5 years.
Figure 45. A 6kb promoter-luciferase construct was stored at various temperatures over time and tested for luciferase activity. Data produced by Jasmine Hill.
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Key Events Timeline in Graduate School at The Fels Institute

2003

Join Liebermann/Hoffman lab – June 3, 2003
Rotation Review meeting – June 12, 2003
Committee meeting #1 – October 11, 2003

2004

Committee meeting #2 – May, 28, 2004
Outside Research Proposal seminar -- September 30, 2004
Committee meeting #3 – November 24, 2004

2005

Elevation to Candidacy – January 12, 2005
Classes Complete – May, 2005
Committee meeting #4 – May 17, 2005
Birth of Son, Ethan Warren Zumbrun – Sunday, August 14, 2005
Committee meeting #5 – December 5, 2005

2006

Committee meeting #6 – May 9, 2006
Committee meeting #7 – December 5, 2006
2007

Committee meeting #8 – May 3, 2007
Birth of Daughter, Valerie Dianne Zumbrun – Tuesday, July 24, 2007
Marks Research Day poster presentation – December 11, 2007
Committee meeting #9 – December 14, 2007

2008

Gordon Research Conference, “DNA Damage and Repair”. Ventura, CA – March 9-14 2008
Committee meeting #10 – May 22, 2008
Fels Research Day poster presentation – October 24, 2008
Dissertation Defense – November 14, 2008