GABP REGULATION OF THE MURINE GABPα/ATPSYNTHASE COUPLING FACTOR SIX AND HUMAN GLUTATHIONE REDUCTASE PROMOTERS

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Doctor of Philosophy

By
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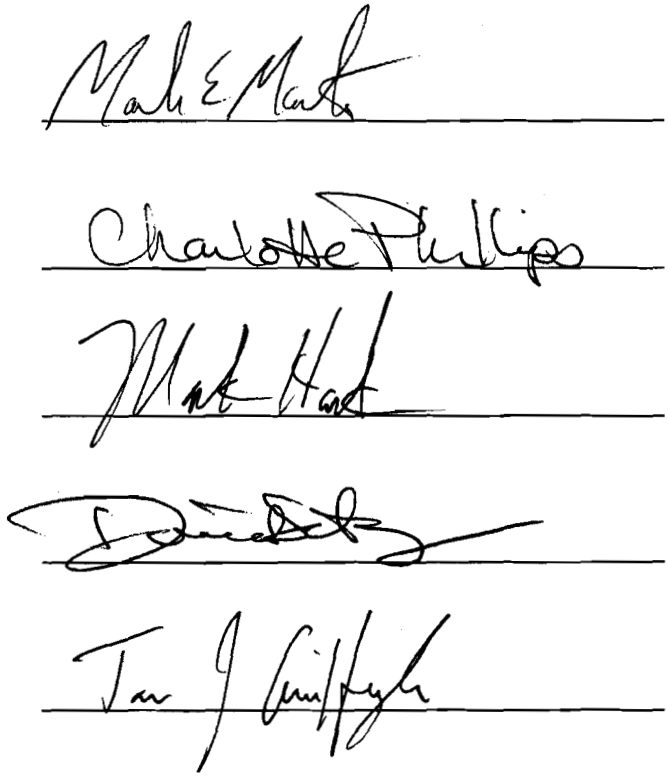
The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

GABP REGULATION OF THE MURINE GABPα/ATPSYNTHASE COUPLING FACTOR SIX AND HUMAN GLUTATHIONE REDUCTASE PROMOTERS

Presented by John D. Patton

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.



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John David Patton

Dr. Mark E. Martin, Dissertation Supervisor

ABSTRACT

The ubiquitously expressed ETS-family transcription factor GA-repeat binding protein (GABP) is involved in the transcriptional regulation of a variety of genes. GABP is a unique ETS-family protein being an obligate multimer, consisting of the GABPα subunit containing an ETS-family DNA binding domain and the unrelated GABPβ subunit containing a Notch/ankyrin repeat and the transcription activation domain. The *Gabpα* gene has previously been identified as being expressed from a bi-directional promoter expressing ATP synthase coupling factor six (CF6) (*Atp5j* gene) in the opposite direction. The core region of the promoter is a 400 base pair fragment that contains four GABP binding sites, and single Sp1/3 and YY1 binding sites. Site-directed mutagenesis of these transcription factor binding sites and subsequent analysis of these mutants demonstrate that while no single binding site is essential for expression from the promoter, the GA1 binding site functions cooperatively with the other three GABP binding sites and the Sp1/3 and YY1 binding sites to activate transcription in both directions from the promoter. The remaining transcription factor binding sites are functionally redundant for basal promoter activities. EMSA analysis reveals that DNA-protein complexes containing GABPα, GABPβ, Sp1, Sp3, and YY1 proteins form *in*

vitro. GABP binding to the promoter provides a potential mechanism of autoregulation of GABPα expression.

The ability of GABP to bind the promoter region also provides a potential mechanism for regulation of GABP under oxidative stress. Oxidative stress is primarily caused by dysfunction of the electron transport chain leading to incompletely reduced oxygen. The reactive oxygen species produced in this manner can cause severe cellular damage and must be quickly removed or reduced. One part of this process involves the glutathione (GSH) antioxidant system. GSH is an important reducing agent within the cellular antioxidant response but in the process is itself oxidized (GSSG). The GSSG is recycled back to GSH through the action of glutathione reductase (GSR). Two isoforms of GSR, a mitochondrial GSR (mtGSR) and a cytoplasmic GSR (cytGSR), are expressed from the same gene. The promoter region of GSR contains three GABP binding sites and three Sp1 binding sites, allowing for a potential regulation of the gene under oxidative stress. Site-directed mutagenesis of the transcription factor binding sites demonstrates that no single factor is essential for expression from the GSR promoter but the GC2 (Sp1/3) binding site functions cooperatively with the other binding sites to coordinate expression from the GSR promoter. EMSA analysis supports the formation of DNA-protein complexes on promoter containing GABPα, GABPβ, Sp1, and Sp3. The ability of these transcription factors to bind the DNA provides a potential mechanism for transcriptional regulation of the expression of mt- and cytGSR. The expression of mt- and cytGSR can be regulated at either the transcriptional or translational level, depending on the start site of the RNA message synthesized. Experimental evidence supports both of

these mechanisms. More experiments will be needed to determine the mechanism for regulation of expression of mt- and cytGSR.

INTRODUCTION AND BACKGROUND

ETS-domain transcription factors

Gene expression has long been thought of as a series of events that occur in succession to decode information from a gene to a protein. Recent evidence suggests the process of transcribing an mRNA and translation of a protein are linked and not a series of separate events (Mata et al., 2005; Erkmann and Kutay, 2004; Maniatis and Reed, 2002). The process of gene expression is regulated by mechanisms involving both transcription, RAN processing, and translation. On the transcriptional level, gene expression is regulated partially by the recruitment of transcription factors, which in turn recruit the remaining transcription machinery to the promoter sequence. The effect of these transcription factors can be one of repression or of activation of gene expression depending on the transcription factor, its mechanism of operation, and the gene that is being regulated. The ETS-domain family of transcription factors is a group of related factors (Sharrocks et al., 1998) that are distinguished by their conserved DNA binding domain (DBD). Within the DBD, the conserved winged helix-turn-helix motif of the ETS-domain contains four β-sheets and three α-helices (Donaldson et al., 1996; Werner et al., 1997; Kodandapani et al., 1996; Batchelor et al., 1998; Mo et al., 1998; Mo et al., 2000). This conserved DBD also is a potential site of regulation as a target of protein-protein interactions. A single amino acid mutation within the carboxy terminus of the helix responsible for DNA recognition can change the DNA-binding specificity and alter

the protein-protein interactions with other transcription factors (Kim, 1999; Goetz et al., 2000).

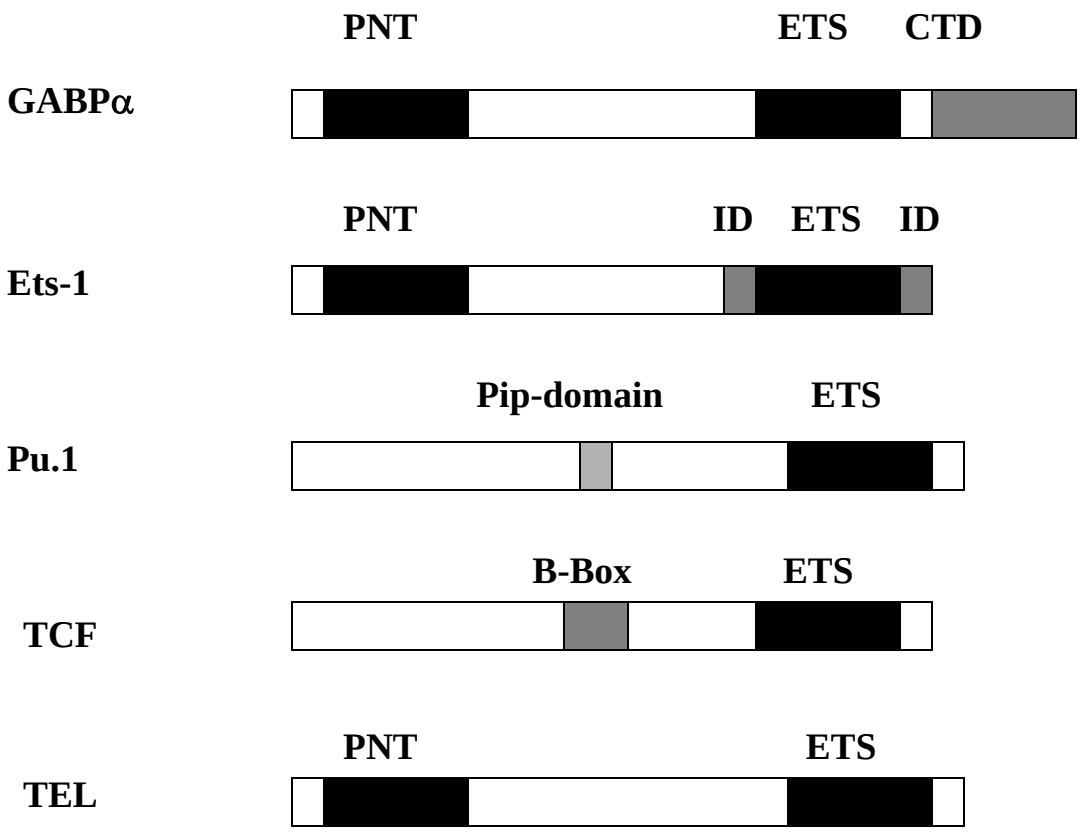
ETS-domain proteins are regulated by protein-protein interactions at several domains.

Evidence suggests that ETS-domain proteins can be regulated at several different locales through protein-protein interactions (Li et al., 2000). Interactions between the DBD of ETS-domain proteins and the DBD of other transcription factors can enhance the specificity and regulate the activity of the ETS-domain proteins. Ternary complexes, such as the complex formed by Ets-1 and AML-CBFα2, are formed through interactions between the DBD of the two transcription factors activating Ets-1 (Kim, 1999; Goetz et al., 2000). Ternary complexes form between ETS-domain proteins and other transcription factors in which the site of interaction is not the ETS-domain protein's DBD. These interactions are illustrated by the interaction of the SRF DBD with the B-box of the ternary complex factor (TCF) subfamily proteins causing activation of TCFs (Fig. 1) (Shore and Sharrocks, 1994; Hassler. and Richmond, 2001). Other ETS-domain family members require interactions with specific proteins, which make little or no contact with the DNA. In the GA-repeat binding protein (GABP) α-GABPβ complex, the ankyrin repeats of GABPβ contact GABPα as the tip of each loop within the ankyrin repeats is inserted into a depression between the first alpha helix in the ETS-domain and the two carboxy-terminal helices, but GABPβ has no contact with the DNA (Batchelor et al., 1998).

ETS-domain proteins bind specific DNA sequences.

The ETS-domain family of transcription factors binds to a conserved DNA sequence centered around GGA, although the surrounding sequence can be unique for individual ETS-proteins (Sharrocks et al., 1997; Graves and Peterson, 1998). The surrounding nucleotides can limit the ability of ETS-domain proteins to bind, but because of the conserved structures within the DBD of ETS-domain proteins, there is overlap of the specificity of ETS-domain proteins. Changes to the carboxy-terminal end of the DBD in the ETS domain can alter the specificity of its DNA-binding (Shore et al., 1996; Fitzsimmons et al., 1996). Co-regulatory protein partners can introduce specificity into the ETS-domain proteins. Many times a co-regulatory protein masks the activity of an ETS-domain protein until the appropriate trigger is present. In Ets-1, an inhibitory module, which blocks DNA binding, is formed by regions located at the amino- and carboxy-terminal regions of the ETS domain (Fig. 1) (Jonsen et al., 1996; Skalicky et al., 1996). A trigger, such as interactions with the transcription factors AML-1-CBFα2 and Pax-5, which causes the conformation of this region to change and one of the helices to unfold, removes the inhibition of DNA-binding (Jonsen et al., 1996; Peterson et al., 1995; Kim, 1999; Goetz et al., 2000). Alternately, the inhibitory structure of Ets-1 is stabilized by phosphorylation mediated by the calcium-calmodulin-dependent protein kinase II reinforcing the autoinhibition (Cowley and Graves, 2000).

Figure 1. The presence of several common and unique domains within ETS-domain proteins. Each of the ETS domain proteins maintains an ETS-DNA binding domain. Several have a PNT domain, which is a known site of protein-protein interactions. Also shown for GABPα is the carboxy-terminal domain (CTD) that interacts with GABPβ. The Ets-1 inhibitory domain (ID) is shown on either side of the ETS-DBD. The PU.1 has a domain that interacts with the co-regulatory protein Pip (labeled Pip-domain). The TCFs have a B-Box that is specifically bound by serum response factor.


PNT
ETS
CTD
GABPα
PNT
ID
ETS
ID
Ets-1
Pip-domain
ETS
Pu.1
B-Box
ETS
TCF
PNT
ETS
TEL

GABPα is an ETS-domain transcription factor.

GABP binds to a promoter region containing a characteristic (A/C)GGAA(G/A) ETS-domain binding site (EBS) (Sharrocks et al., 1997; Thompson et al., 1991; Yu, et al., 1997). The alpha subunit of GABP contains an ETS-domain DNA binding domain (DBD), while the beta subunit of GABP contains a series of repeated amino acid sequences similar to that observed in the Notch/ankyrin-repeat proteins (LaMarco et al., 1991; Virbasius et al., 1993; Sawada et al., 1994; Batchelor et al., 1998). The characteristic "winged-helix-turn-helix" structure of an ETS DBD contacts the DNA in the major groove at the GABP binding sequence. The DBD of GABPα most closely resembles ETS-domain family members Ets-1 and PU.1. (Donaldson et al., 1996; Kodandapani et al., 1995; Sharrocks, 2001). GABPα, like several ETS-domain proteins, has a pointed (PNT) domain within the protein (Fig. 1). The PNT domain represents a common site of protein-protein interaction in other ETS proteins, but at present, proteins that interact with this PNT domain of GABPα have not been identified (Golub et al., 1994; McLean et al., 1996; Jousset et al., 1997; Bush et al., 2001).

GABPβ is required for activation of transcription.

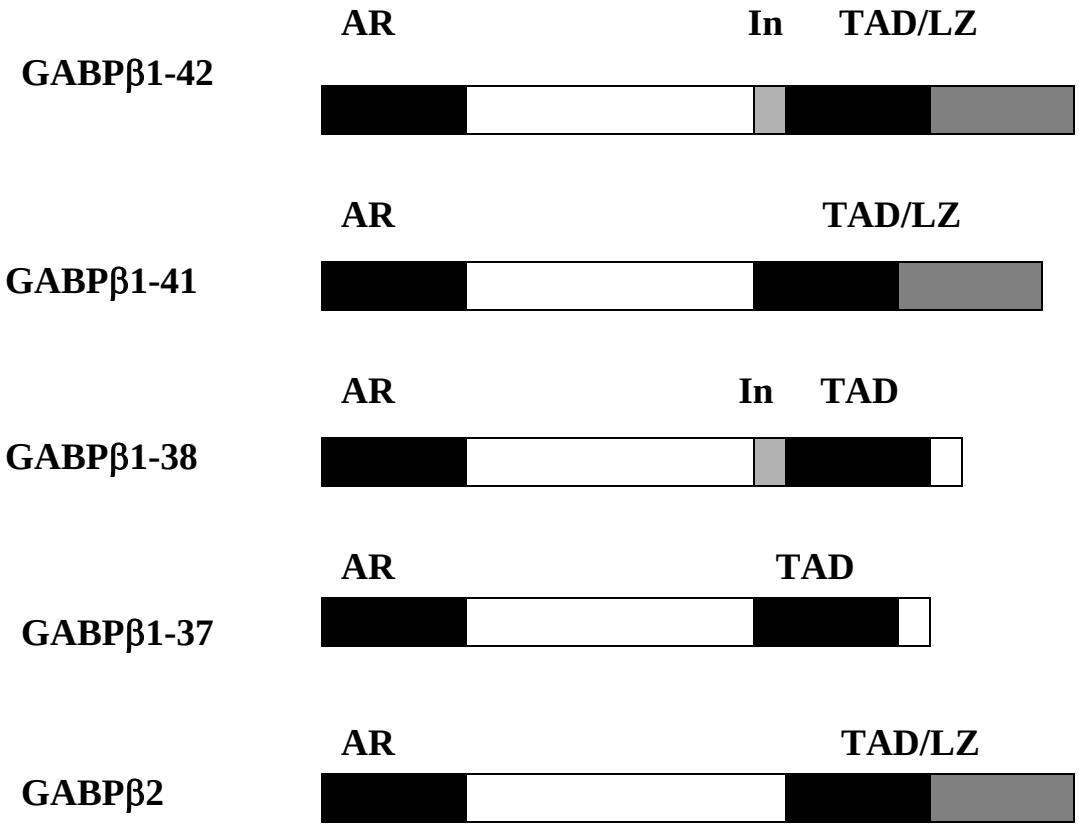
The presence of the β subunit of GABP is required for transcriptional activation. GABPβ expression is extremely complex as there are two different chromosomes which encode different forms of GABPβ: GABPβ1 and GABPβ2 (De la Brousse et al., 1994). There are also four different isoforms of GABPβ1 expressed as a result of alternative

mRNA splicing (Gugneja et al., 1995). All isoforms of GABPβ maintain a conserved region at the amino terminus that contains the four and one-half ankyrin repeats and is the site of interaction with GABPα and necessary for recruiting GABPβ to the DNA (Gugneja et al., 1995; Sawa et al., 1996; Thompson et al., 1991). Each of the ankyrin repeats is 33 amino acids in length and contains a pair of alpha helices that run antiparallel to each other. The four isoforms of GABPβ1 differ by the presence of a 12 amino acid region (Gugneja et al., 1996) and a leucine zipper region in the isoforms (Fig. 2) (De la Brousse et al., 1994). The carboxy-terminal region of GABPβ contains two important conserved features, a nuclear localization signal and a transcription activation domain (TAD). The exact location of the TAD has not been universally agreed upon but all evidence does support the TAD localization to the carboxy-terminal region of GABPβ (Gugneja et al., 1995; Sawa et al., 1996). GABP is a unique ETS-domain family as an obligate multimer with the TAD and nuclear localization signal for GABP are located on the beta subunit of GABP and the ETS-DBD located on GABPα (Sharrocks et al., 1997; Sharrocks 2001; Oikawa and Yamada, 2003).

GABP exists in solution as a heterodimer.

The crystal structure of the GABPα ETS-domain and the GABPβ ankyrin repeats shows the interactions between the two subunits of GABP. The alpha helices within the ankyrin repeats form hydrophobic interactions and water-mediated hydrogen bonds with GABPα. The ankyrin repeats of GABPβ contact GABPα within a depression between the helices in the 31 amino acid tail and the first helix of the ETS-DBD. Without the 31

Figure 2. The structure of the GABPβ isoforms. All isoforms of GABPβ contain an AR (activation region), which mediates interactions with GABPα. The differences exist in the inclusion or exclusion of other carboxy-terminal regions. There is a 12 amino-acid region (In) which has unknown function but is included in the GABPβ1-42 and GABPβ1-38 isoforms. There is also an LZ (leucine zipper) region that is shared by the GABPβ1-42 and GABPβ1-41 that is not present in the other GABPβ1 isoforms. The GABPβ2 isoform is encoded on a different chromosome than the other isoforms of GABPβ, which are encoded by the same gene. GABPβ2 maintains similarities to GABPβ1 in that it has an AR region, a similar TAD, and contains a LZ region.


AR
In
TAD/LZ
GABPβ1-42
AR
TAD/LZ
GABPβ1-41
AR
In
TAD
GABPβ1-38
AR
TAD
GABPβ1-37
AR
TAD/LZ
GABPβ2

amino acid tail, dimerization of GABPα and GABPβ does not occur, indicating the importance of the carboxy-terminal tail of GABPα. This brings GABPβ into close proximity to the DNA but there are no direct interactions between the ETS DNA binding site and GABPβ (Batchelor et al., 1998). GABP binds DNA as a heterodimer, when only one GABP binding site is present in the promoter region, and in solution, GABP also exists as a stable heterodimer (Chinenov et al., 2000a). GABP is a multimeric protein complex consisting of two alpha subunits and two beta subunits in its most active heteroterameric form. GABP activates transcription much more efficiently when two GABP binding sites are present on the same face of the DNA and are separated by as many as three helical turns on an artificial promoter as a heterotetramer complex ($GABP\alpha_2\beta_2$) can form (Yu et al., 1997).

GABP interacts with several other transcription factors.

GABP has several cooperative regulatory partners in gene expression. The physical interactions between other proteins and GABP have been documented. GABP is known to functionally interact with Sp1 in several contexts. Sp1 binds to many DNA promoters that lack a TATA motif and physically interacts with parts of the transcription machinery. GABP and Sp1 have been shown to functionally interact on many such promoters which encode a wide range of genes including: utrophin (Galvagni et al., 2002; Gyrd-Hansen et al., 2002), CD18 (Rosmarin et al., 1998), the folate receptor β (Sadasivan et al., 1994), the pem pd homeobox (Rao et al., 2002), and heparanase-1 (Jiang et al., 2002). The zinc finger domain of Sp1 was shown to interact physically with

an unidentified region of the GABPα subunit both *in vitro* and *in vivo* (Galvagni et al., 2002). In the adenovirus early 4 (E4) promoter, GABP is known to interact with ATF/CREB transcription factor family. The physical interaction occurs between ATF/CREB and GABPα as this subunit alone can synergize with ATF/CREB (Sawada et al., 1999). GABPα and another ETS-domain transcription factor, PU.1, functionally cooperate in the regulation of CD18. Although no physical interaction has been described between the two transcription factors, GABPα and PU.1 are both required to activate CD18, indicating functional cooperation (Rosmarin et al., 1995). One method for possible GABP and PU.1 interaction is through an intermediary such as p300 with which both transcription factors are known to interact, and the presence of p300 increases the responsiveness of CD18 (Bush et al., 2003; Bannert et al., 1999; Blobel 2001). Microphthalmia transcription factor (MITF) can interact with both subunits of GABP. Its role, though, is not one of transcriptional regulation but of nuclear localization. In the presence of mutant MITF, both subunits of GABP are not localized to the nucleus and remain in the cytoplasm, even though GABPβ has its own nuclear localization signal (Morii et al., 2002). GABP was first discovered as important in expression of herpes simplex virus intermediate early genes. One of the co-activators of these genes, HCF, interacts with GABPβ to stimulate low levels of transcription, even though HCF alone is functional as a repressor (Wilson et al., 1993, Vogel and Kristie 2002). Both YEAF1 and YAF-2 interact with GABPβ (Sawa et al., 2002). Both of GABPβ and YEAF1 are known to interact with YY1 as a ternary complex consisting of GABPβ-YEAF1-YY1 has been shown to form in a yeast three hybrid system (Du et al., 1998), providing evidence of the potential mechanism for YY1-GABP interactions. A known interaction that occurs

between GABPβ and E2F1, a transcription factor, which aids in regulating the G1/S cell cycle transition links GABP to cell cycle signaling (Hauck et al., 2002). These interactions provide potential mechanisms to show how GABP can be utilized to regulate the wide-ranging genes which it controls.

GABP is a target of intracellular signaling.

GABP is also involved in the regulation of several intracellular signaling pathways, serving as both a target of intracellular signaling pathways and as a component of the signaling pathways. GABP is modified by common mechanisms, like phosphorylation, that can cause GABP to be activated and induce activation of the targeted genes. GABP regulates signaling pathways through activation of transcription of several proteins. Interleukin-2 (IL-2) is under the transcriptional control of GABP, and is activated by GABP in response to T-cell receptors activation (Avots et al., 1997). GABP also regulates the transcription of several other cytokines, such as IL-16, that are expressed in lymphocytes and other cell types as well (Bannert et al., 1999; Kamara et al., 1997; Markiewicz et al., 1996). The γc chain is a component of several cytokine transmembrane receptors and its transcription is activated by GABP. GABP also regulates the expression of several hormone and hormone receptors that are not a part of the T-cell response. GABP regulates the expression of prolactin and thyrotropin receptor, which are responsible for the development of the mammary (Ouyang et al., 1996; Schweppe and Guitierrez-Hartmann, 2002) and thyroid glands, respectively (Yokomori et al., 1998). GABP is also the target of several signaling pathways. T-lymphocytes are

activated when IL-2 is induced. When the activation of T-lymphocytes is mimicked through chemical methods, the ERK pathways and MAP kinase pathways are activated. The end products of these pathways are able to phosphorylate GABP (Flory et al., 1996; Hoffmeyer et al., 1998). The phosphorylation of GABP is known to increase the expression of IL-2, suggesting the possible integration of the signaling pathways and activation of transcription of IL-2 (Avots et al., 1997; Hoffmeyer et al., 1998). The gene that expresses prolactin is activated by insulin. Insulin induces the MAPK signal cascade which results in increased phosphorylation of GABPα and may play a role in the induction of the prolactin gene (Ouyang et al., 1996).

GABP is required for mitochondrial biogenesis.

The enzymatic machinery of the electron transport chain (ETC) is encoded from both mitochondrial DNA and nuclear DNA. Many of the nuclear encoded components of the ETC, including cytochrome c oxidase subunits IV and Vb (Virbasius et al., 1993), VIA1 (Wong-Riley et al., 2000), VIIAL (Seelan et al., 1996), VIIC (Seelan and Grossman, 1997), XVII (Takahashi et al., 2002), and two of the components of succinate:ubiquinone oxidoreductase (Complex II) (Bibb et al., 1981), are regulated through the action of GABP. Thirteen of the remaining ETC proteins are encoded by the mitochondrial genome including components of NADH:ubiquinone oxidoreductase (Complex I), ubiquinone:cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV), and ATP Synthase (Complex V) (Brown, 1992). GABP also directs transcription of mitochondrial transcription factor A (MTFA), the major

transcription factor in the mitochondria (Virbasius and Scarpulla, 1994). The GABP regulation of MTFA provides a potential link between nuclear and mitochondrial transcription. GABP plays a major role in the regulation of much of the ETC, and may provide a link between ETC function and regulation of gene expression. GABP is indeed necessary for mitochondrial biogenesis due to its role in the expression of many proteins of the ETC (Villena et al., 1998).

GABPα is required for embryogenesis.

As has been discussed, GABP regulates a variety of genes *in vitro*. However, to better examine the *in vivo* functions of GABP, a mouse model was generated by cloning a single null mutation into the mouse *Gabpα* gene. The homozygous null mutant was lethal prior to implantation in embryogenesis (Ristevski et al., 2004). This is consistent with the elevated expression levels of the *Gabpα* gene in embryonic stem cells and throughout embryogenesis. The lethal homozygous null mutation of *Gabpα* is not entirely unexpected either, as similar results have been previously shown in knockout mouse models of ETS-domain proteins Fli-1 (Spyropoulos et al., 2000), Ets-2 (Yamamoto et al., 1998), and TEL (Wang et al., 1997). This illustrates the importance of the individual ETS-domain proteins and that despite the similarities between the protein families, they are not functionally redundant (Ristevski et al., 2004). In mice that are heterozygous for the *Gabpα* gene, the levels of GABPα are maintained at similar levels to the GABPα in wild-type mice in all tissue types examined. The heterozygous mouse also displayed no phenotypic changes with regard to gait, grip, growth rate, histopathology,

immunophenotype, and X-ray tomography (Ristevski et al., 2004). Conversely, when gene dosage with trisomy 21 is present in human Down's Syndrome the GABPα protein expression is maintained at wild-type levels (O'Leary et al., 2004). This is consistent with a compensatory mechanism being present which allows the expression of GABPα to be maintained at wild-type levels even with only a single gene copy present, and to prevent overexpression when an extra copy is present (Ristevski et al., 2004; O'Leary et al., 2004). The tight regulation over GABPα expression and the lethality of the homozygous null mutant indicates the requirement for GABPα. The characterization of the regulatory sequences directing GABPα is necessary to provide insight to role of GABP and mechanism of regulation of GABP expression during mitochondrial biogenesis and embryogenesis.

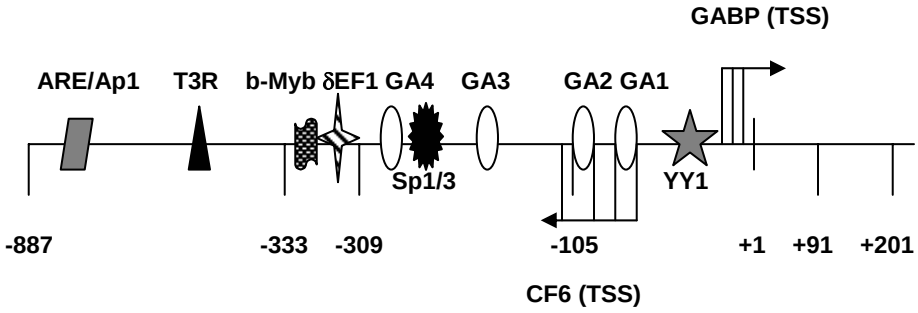
GABPα Promoter Region.

The promoter for the alpha subunit of GABP was identified through chromosomal walking and cloned into a pGL3-Basic (Promega, Madison, WI) vector expressing luciferase (Chinenov et al., 2000a). There are four consensus GABP binding sites; two of these sites (GA1 and GA2) are located approximately 100 base pairs (bp) upstream of the translation start site while the other two GABP binding sites (GA3 and GA4) are located approximately 170 bp and 257 bp upstream of the translation start site, respectively. The core promoter region also contains two additional transcriptional factor-binding sites, one Sp1 binding site, between the GA3 and GA4 binding sites, and one YY1 binding site, downstream of GA1 binding site (Fig. 3). YY1, Sp1, and GABP have been shown in

other promoters to be able to work cooperatively to regulate the transcription from those promoters. This cooperative regulation of gene expression can be either activation or repression as these transcription factors regulate transcription differently on different promoters. GABP alone is capable of initiating transcription in the absence of a TATA motif, although two binding sites allow GABP to be much more effective, as GABP$\alpha_2\beta_2$ complex can form (Yu et al., 1997). Because of the presence of GABP binding site in the GABPα promoter, GABP is likely autoregulated. The presence of the GABP binding sites along with the binding sites of transcription factors known to interact with GABP make it likely that GABP, Sp1, and YY1 coordinate the expression from the GABPα promoter region. This dissertation will focus on the regulation of expression from the GABPα promoter by GABP.

Further examination of the sequence surrounding the mGABPα promoter region revealed that GABPα is expressed from a bi-directional promoter, which expresses ATP synthase coupling factor six (CF6) in the opposite direction (Fig. 3) (Chinenov et al., 2000a). CF6 expression is regulated by GABP from its promoter as well. CF6 is an essential subunit of the ATP producing machinery in the mitochondria. The production of ATP in the mitochondria is closely linked to the ETC, a major producer of reactive oxygen species (ROS) and oxidative stress. The presence of GABP binding sites within the promoter region make CF6 another mitochondrially targeted protein that is regulated by GABP.

Figure 3. Map of the mGABPα/CF6 bi-directional promoter. The core promoter region with the flanking sequences of the mGABPα/CF6 bi-directional promoter is outlined with the transcriptional start sites labeled (TSS). The promoter was cloned into a pGL3 plasmid expressing luciferase.


GABP (TSS)
ARE/Ap1
T3R
b-Myb
δEF1
GA4
GA3
GA2
GA1
Sp1/3
YY1
-887
-333
-309
-105
+1
+91
+201
CF6 (TSS)

GABP directs transcription of a wide variety of genes.

GABP belongs to a group of transcription factors that, depending on the promoter, act on a gene as an initiator, activator, or repressor of transcription (Genuario et al., 1996; Sawanda et al., 1994; Yu et al., 1997). GABP was first identified in the herpes simplex virus as an initiator of transcription in the absence of a TATA motif (Carter et al., 1992; Carter and Avadhani, 1994; Virbasius et al., 1993). Because of its ability to activate and repress transcription, in promoters with more than one binding site is present GABP has the potential to act as both positive and negative transcription signals. The gene targets of GABP are wide ranging from lineage restricted genes to those associated with cellular "housekeeping" mechanisms, including: lineage-restricted genes, like CD18 (Rosmarin et al., 1995; Böttinger et al., 1994), nuclear genes that form proteins involved in the ETC (Virbasius et al., 1993; Carter and Avadhani, 1994; Sucharov et al., 1995; Martin et al., 1996; Villena et al., 1998), ribosomal proteins (Curcic et al., 1997; Genuerio et al., 1993), cell cycle control (Savoysky et al., 1994; Shiio et al., 1996), and some important genes in viral pathogens infecting humans, such as, HIV (Verhoef et al., 1999) and herpes viruses (Douville et al., 1995; Vogel and Kristie, 2002).

GABP regulation is sensitive to oxidizing agents.

The addition of an oxidizing agent to cell cultures decreased expression from a GABP regulated gene. It has been shown that the function and DNA binding ability of GABP are sensitive to oxidative stress (Martin et al., 1996). Treatment with diethyl

maleate (DEM), a glutathione (GSH) depleting agent, inhibited the ability of GABP to activate transcription and even to bind DNA, likely through glutathionylation of GABPα. The DNA binding ability of GABP could be recovered by treatment with n-acetyl cysteine (NAC), a glutathione precursor, indicating the effects of oxidative stress can be reversed (Martin et al., 1996). *In vitro* the glutathionylation of three cysteine residues in the carboxy-terminal domain of GABPα either prevent DNA binding or dimerization with GABPβ, thereby inhibiting gene expression, and illustrating the sensitivity of GABP to the reduction/oxidation (redox) state of the cell (Chinenov et al., 1998). The glutathionylation of GABP has not been shown to occur *in vivo*. Because GABP directs the transcription of several components of the ETC, dysfunction of which brings about oxidative stress, GABP is a potential link between oxidative stress, gene expression, and mitochondrial function. This link will be investigated by measuring the expression from the mGABPα/CF6 bi-directional promoter under oxidative stress induced conditions.

Oxidative Stress.

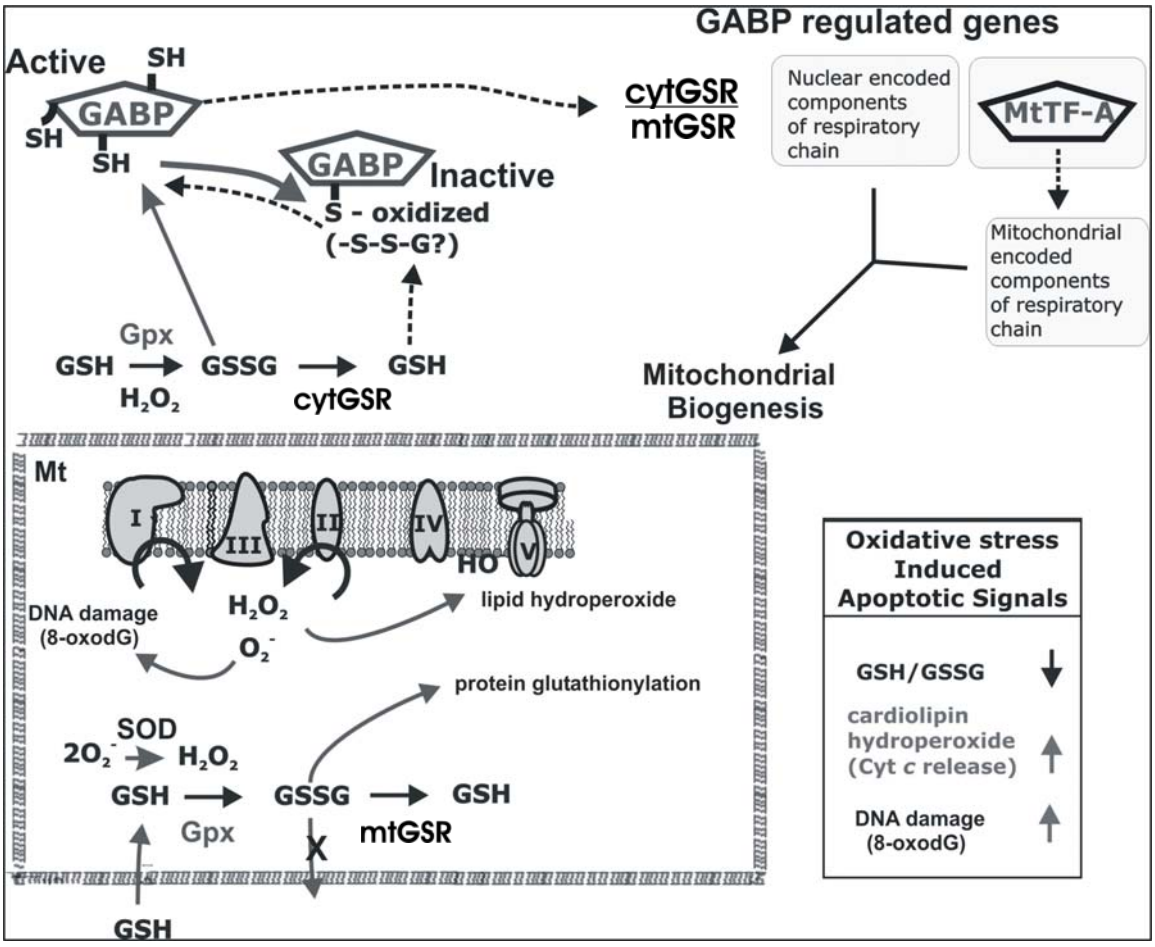
GABP is critical to the expression of ETC protein, and the ETC is the major consumer of oxygen in the cell. Oxygen is a critical component of the atmosphere and critical to life on earth. The paradox in this situation is that while oxygen is required for life it also is a source of oxidative stress. Oxygen is reduced in cellular metabolism to enable production of ATP. Dysfunction of the ETC leads to incomplete reduction of oxygen producing reactive oxygen species (ROS), which cause a perturbation to the redox level in the cell (Fig. 4). Cellular oxidative stress has been linked to several disease

states, including Parkinson's disease (Cohen et al., 1997), Alzheimer's disease (Aksenov et al., 1999), Huntington's disease (Butterfield and Kanski, 2001), and cancer (Frischer et al., 1993, Matsui et al., 2000, Peake et al., 1999), although it is uncertain whether the oxidative stress is causative or is the end result of the disease. Oxidative stress is also involved in the processes of aging (Atamna et al., 2001; Kokoszka et al., 2001) and apoptosis (Celli et al., 1998; Nomura et al., 2000; O'Donovan et al., 1999; Voehringer et al., 2000; Wang et al., 2001a; Wang et al., 2000). Cellular responses to oxidative stress play a critical role in maintaining homeostasis and redox balance of the system (Fig. 4).

ROS are produced by dysfunction of the ETC.

Cellular damage from oxidative stress has been well defined. Increased levels of oxidative stress can target proteins for degradation, oxidize lipids, and cause damage to DNA (Fig. 4) (Coffey et al., 2000; Esteve et al., 1999; James et al., 1996; Polyak et al., 1997). Oxidative stress is a result of perturbation of the redox level of the cell, either through the production of excess ROS or a decrease in cellular antioxidants. Dysfunction of the ETC involves incomplete reduction of oxygen, forming the superoxide radical ($O_2^{\cdot -}$) in up to five percent of the oxygen reduction events (Davidson et al., 2001; Klein and Ackerman, 2003; Kokoszka et al., 2001). The location at which the $O_2^{\cdot -}$ is produced can present a problem for cells. Mitochondrial DNA is much more susceptible to damage by oxidizing agents than the nuclear encoded DNA because mitochondrial DNA lacks introns meaning more of the mitochondrial DNA encodes proteins making the damage much more likely to cause damage that can affect protein formation. Without the quick

Figure 4. The GABP regulated redox cycle of the cell. GABP directs the transcription of genes involved with the production and removal of ROS. Many of the nuclear components of the ETC are expressed under the control of GABP. Mt-TFA expression is also regulated by GABP. The mitochondrial components of the ETC are regulated by Mt-TFA, expression of which is regulated by GABP. GABP is therefore required for mitochondrial biogenesis. The mitochondria are the major source of energy and ROS. The redox level of the cell is under tight regulation. GABP is part of the regulation process of GSR, which reduces GSSG back to GSH in both the mitochondria and cytoplasm. When excess GSSG is present, susceptible proteins, including GABP, are glutathionylated inhibiting the function of these oxidized proteins.


GABP regulated genes
Active
SH
GABP
SH
SH
SH
GABP
Inactive
S - oxidized
(-S-S-G?)
cytGSR
mtGSR
Nuclear encoded components of respiratory chain
MtTF-A
Mitochondrial encoded components of respiratory chain
Mitochondrial Biogenesis
Gpx
GSH
GSSG
GSH
H2O2
cytGSR
Mt
I
III
II
IV
V
HO
DNA damage (8-oxodG)
H2O2
O2-
lipid hydroperoxide
protein glutathionylation
SOD
2O2-
H2O2
GSH
GSSG
GSH
Gpx
mtGSR
X
GSH
Oxidative stress Induced Apoptotic Signals
GSH/GSSG
cardiolipin hydroperoxide (Cyt c release)
DNA damage (8-oxodG)

removal of $O_2^{\cdot -}$ from the mitochondria, it will quickly react either to oxidize guanine nucleotides of DNA or donate its free electron starting a dangerous free radical cascade (Fridovich, 1995). The $O_2^{\cdot -}$ also rapidly inactivates aconitase and similar dehydratases within the ETC, which potentially causes greater dysfunction of the ETC and more production of $O_2^{\cdot -}$ (Fridovich, 1998) (Fig. 4).

Oxidative stress is controlled through several defense mechanisms.

Cells have several mechanisms that defend against oxidative stress. Many cellular antioxidants, such as vitamin E and vitamin C, react with oxidizing agents in a terminal manner and are removed from the cell (Halliwell and Gutteridge, 1999; Fridovich, 1995; Özben, 1998). Several enzymes and proteins provide protection from oxidative stress by reactions that reduce the toxicity of ROS. Superoxide dismutases (SODs), the first line of defense, quickly scavenge superoxide radicals (Fridovich, 1995; Özben, 1998). SODs catalyze the removal of $O_2^{\cdot -}$ radicals by rapidly reducing the free radicals. Cytoplasmic and extracellular forms of SODs exist employing Cu and Zn as transition metals within their active sites. A mitochondrial targeted SOD employing Mn within the active site exists. The importance of Mn-SODs in lower organisms has been illustrated by the generation of a null mutation, which causes lethal lesions (Kokoszka et al., 2001). The $O_2^{\cdot -}$ radicals is converted to a less reactive peroxide by SODs (McCord, 1969; Davies, 2000). H_2O_2 is toxic as well and if the H_2O_2 is not reduced quickly, it can react through Fenton chemistry, an acid and iron (II) catalyzed reaction of H_2O_2 that produces iron (III), water and highly reactive hydroxyl radicals ($OH^{\cdot}$) (Cotgreave and Gerdes, 1998;

Davies, 2000). Catalases and glutathione peroxidases (Gpx) participate in the removal of ROS by reducing H_2O_2 to water and molecular oxygen (Fig. 4) (Aebi, 1984). Catalase reacts in a terminal manner and is removed from the cell. Gpx utilizes the reducing power of glutathione (GSH), a tripeptide consisting of L-γ-glutamyl-L-cysteinyl-glycine, to remove the hydrogen peroxide from the system (Esposito et al., 2000; Kussmaul et. al., 1999; Davies, 2000). In the process of reducing H_2O_2, two GSH tripeptides are linked through a disulfide bond to form the oxidized glutathione molecules (GSSG). The GSSG can be recycled back to GSH through the action of glutathione reductase (GSR) (Fig. 4).

Glutathione is an essential antioxidant.

In most cell types, GSH is present in concentrations ranging from five to ten millimolar. GSH is able to directly scavenge ROS and provides the reducing power by which Gpx reduces H_2O_2 to water and molecular oxygen, oxidizing GSH in the process. Mutations to the γ-glutamylcysteine synthetase gene, a gene required for GSH synthesis, result in a lethal phenotype (Shi et al., 2000) illustrating the absolute requirement for GSH. Depletion of the GSH pool, by oxidative stress, results in a rise in GSSG levels from the typical submillimolar concentrations to millimolar concentrations, results in the glutathionylation of susceptible proteins, peroxidation of lipids, alterations in the mitochondrial membrane, reduced ETC activity, enhanced ROS production, and contributes to apoptosis in some instances (Atamna et al., 2001; Celli et al., 1998; Coffey et al., 2000). GSSG in the cytoplasm can be dealt with by exporting GSSG from the cell or reduced by GSR (Akerboom et al, 1982; Eklöw et al., 1981; Adams et al., 1983;

Eklöw et al., 1984). In the mitochondria GSSG must be reduced, as it cannot be exported. Mitochondrially targeted GSR (mtGSR) is the only enzyme that reduces the GSSG within the mitochondria (Fig. 4) (Brodie and Reed, 1992; Chen et al, 2003; Liu and Kehrer, 1996; Olafsdottir and Reed, 1988; Spector et al., 2001).

Glutathione reductase is targeted to both the mitochondria and the cytoplasm.

The action of GSR becomes more important when the localization of oxidative stress is considered. The ETC, a major supplier of energy and consumer of oxygen, is located within the mitochondria, leading to the production of oxidizing agents in the mitochondria increasing the risk of oxidizing damage within the mitochondria. Mitochondrial DNA encodes many of the proteins and enzymes within the electron transport chain including: NADH:ubiquinone oxidoreductase (Complex I), ubiquinone:cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV), and ATP Synthase (Complex V) (Brown, 1992), so oxidative damage to mitochondrial DNA can ultimately affect the structure and function of proteins of the ETC (Davies, 2000). In addition the oxidizing agents can directly damage the proteins of the ETC, which brings about greater dysfunction of the oxygen reducing capabilities of the ETC leading to the production of even more ROS. Because one of the major producers of ROS is the mitochondria, it is important for the mitochondria to have protection from the oxidative stress (Davies, 2000). Mitochondrial targeted GSR provides this protection. It has been shown that GSR expressed with an artificial mitochondrial leader sequence (MLS) can protect cells from oxidative stress (O'Donovan et al., 1999).

Two isoforms of GSR are encoded within the human genome differing only by the presence of a MLS at the amino terminus of the mitochondrial specific form of GSR (mtGSR) (Fig. 5) (Kelner and Montoya, 2000, Tamura et al., 1997). This MLS allows GSR to be transported from the cytoplasm, where it is translated, into the mitochondria to enable GSSG to be reduced back to GSH utilizing NADPH as a reducing agent. This allows for GSH to be recycled within the mitochondria to provide better protection against oxidative stress within the mitochondria (Brodie and Reed, 1992; Chen et al., 2003; Liu and Kehrer, 1996; Olafsdottir and Reed, 1988; Spector et al., 2001).

NADPH is required for GSR activity.

The inability to export GSSG from the mitochondria requires that a mtGSR protein be expressed. For mtGSR to be functional, NADPH must be present in the mitochondria to maintain mtGSR activity and prevent the build-up of GSSG in the mitochondria. If the GSH:GSSG ratio in the mitochondria is out of balance to favor GSSG, glutathionylation of susceptible proteins can begin to take place. Glutathionylation of susceptible proteins includes proteins of the ETC, which could bring about greater dysfunction in the ETC and production of more ROS. NADPH is supplied to the mitochondria by several sources. The major source of NADPH in the mitochondria is from mitochondrial isocitrate dehydrogenase (mtICDH). Decreasing the expression of mtICDH causes a marked increase in ROS production and an accompanying increase in the damage due to ROS, illustrating the importance of mtICDH. Alternatively, when there is over-expression of mtICDH, cells are protected from damage caused by the presence of ROS (Jo et al., 2001). On a much more limited basis mitochondrial malic

enzyme (Vogel et al., 1998; Vogel et al., 1999; Loeber et al., 1991), proton-translocating transhydrogenase (Bykova, et al., 1999; Yamaguchi and Hatefi, 1991), and NADH kinase (Magni et al., 1999) increase the amount of NADPH present in the mitochondria.

Several systems are present to repair damage due to oxidative stress.

Because the removal of oxidants from cells is important but not 100% efficient, there are numerous mechanisms utilized by cells to repair damage caused by oxidants. One of these repair mechanisms involves reducing both intermolecular and intramolecular disulfide bonds. Glutathionylation has been shown to be either reversible or irreversible depending on the protein. Proteins that get irreversibly glutathionylated are marked for degradation or removal from the cell. Many times, oxidized proteins are degraded and replaced rather than removing the oxidants from the individual residues (Berlett and Stadtman, 1997; Grune et al., 1997; Pacifici and Davies, 1991; Sitte et al., 2000; Taylor et al., 1997; Ullrich et al., 1999). Generally, there are only two or three oxidized amino acids within an oxidized protein, so almost all of the amino acids can be recycled and used in the production of new proteins. During periods of high oxidative stress the degradation machinery of the cell can become overwhelmed. Proteasomes, the complexes responsible for protein degradation, may not be able to degrade all the proteins that are oxidized under highly oxidizing conditions. Under these conditions proteins begin to be crosslinked through disulfide bonds. These protein aggregates can cause further cellular dysfunction. The oxidation of other cellular components, such as DNA and lipids, can also be repaired or replaced following defined pathways for each of

these cellular components. DNA repair mechanisms have been shown to occur by both direct methods where the damage is directly repaired, and methods involving excision and replacement of the bases (Demple and Halbrook, 1983; Keyse, 2000; Wang et al., 1998; Halliwell and Aruoma, 1993).

The failure to remove oxidants can cause apoptosis or necrosis.

For some cells, the net result of a highly oxidative state, especially mitochondrial oxidative stress, is apoptosis (Sastre et al., 2000). In other instances, this highly oxidative state triggers necrosis. The energy requirements for multicellular organisms to repair all the damage due to oxidative stress can be quite great. At times, an organism will sacrifice a few cells through apoptosis rather than have an even greater energy drain on the organism if the immune response from necrosis is triggered. The apoptotic pathway involves a clearly defined pathway that is recognized by phagocytes, which then engulf the cells. The phagocytosis of the apoptotic cells prevents an immune response (Ankarcrona et al., 1995; Polyak et al., 1997; Farber et al., 1990), unlike necrosis, which as the cell disintegrates, attracts an immune response that can damage neighboring cells. For this reason, apoptosis can be a protective mechanism from a larger immune response. By undergoing apoptosis rather than necrosis the cells are, in effect, protecting their neighboring cells.

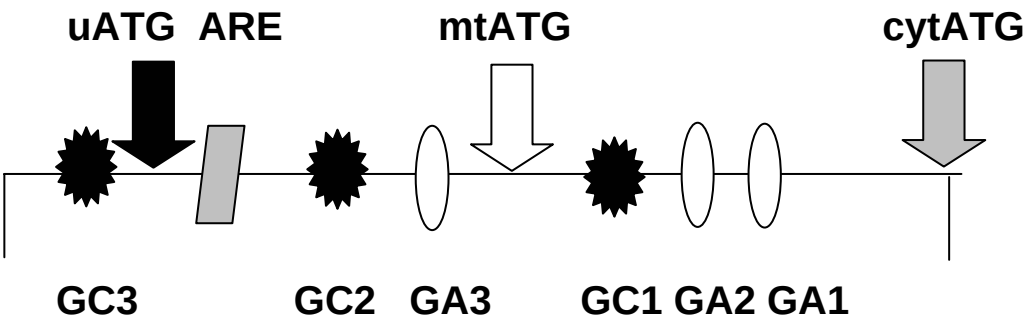
Reactive oxygen species function in the activation of several transcription factors.

ROS have also been shown to play an important role in gene expression. Several genes are expressed in a redox sensitive manner. Increased levels of hydrogen peroxide have been shown to increase the expression of AP-1-like factors in yeast cells. In addition to the expression of AP-1 factors being regulated in a redox sensitive manner, nuclear factor κB (NF-κB) expression is also under the control of the redox state of the cell, via the control of inhibitor factor-κB (IF-κB) (Haddad et al., 2000; Chandel et al., 1998; Sen and Packer, 1996). Although both Ap-1 and NF-κB require reducing conditions *in vitro* for DNA binding to take place, *in vivo* both Ap-1 and NF-κB are activated in pro-oxidant conditions (Sen and Packer, 1996). It has been suggested that oxidants, such as ROS, activate kinases that phosphorylate IF-κB, inactivating it and constitutively activating NF-κB (Sen and Packer, 1996). ROS also have been implicated in phosphorylation of c-jun and c-fos proteins, which activate Ap-1 (Sen and Packer, 1996; Laderoute and Webster, 1997; Knight and Buxton, 1996; Puri et al., 1995). In contrast to these two transcription factors, pro-oxidant conditions lead to the inhibition of GABP DNA binding activity and loss of ability to enhance transcription by modifying three cysteine residues in the DNA binding domain or the carboxy-terminal domain that interacts with GABPβ (Martin et al., 1996; Chinenov et al., 1998).

hGSR promoter.

The human GSR (hGSR) promoter was identified through genomic cloning of the human chromosome. The structure of the hGSR gene was found to be highly similar to the mouse gene, with an amino-terminal mitochondrial leader sequence (MLS), with homology to the mouse MLS sequence (Tamura et al., 1997; Iozef et al., 2000; Kelner and Montoya, 2000), present between two in-frame start codons. The hGSR promoter region has several potential start sites for transcription, both upstream and downstream of the translation start site of the MLS. The MLS is the only difference between the mt- and cytGSR and is removed once inside the mitochondria making the two isoforms of GSR indistinguishable (Taniguchi et al., 1986). The promoter region, similarly to the mGABPα/CF6 bi-directional promoter, does not have a TATA motif upstream of either of the translational start sites, however, there are several potential binding sites for both Sp1 and GABP within the promoter region of the hGSR gene (Fig. 5). As previously stated, GABP and Sp1 are capable of initiating transcription in the absence of a TATA motif. There is also an antioxidant response element present within the promoter region, indicating the possibility of redox sensitivity. GSR is intimately involved in the cellular protection from oxidative stress (Fig. 4). The transcription factor binding sites present in the promoter region of GSR indicate the expression of GSR is likely regulated in a redox sensitive manner. The presence of GABP and Sp1/3 binding sites and an ARE in the hGSR promoter potentially allows for the redox sensitive transcription of both mtGSR and cytGSR, possibly enhancing mtGSR expression under oxidative stress.

Figure 5. The promoter map for the hGSR gene. Each of the transcription factor binding sites is labeled as follows: GC (potential Sp1/3 sites), GA (potential GABP binding sites), and ARE/Ap1 (potential antioxidant response element/Ap1 binding site). The three translational start sites are labeled, as well. The uAUG is out of frame with the other two translation start codons. The mtAUG and cytAUG are in the same reading frame. The construct was cloned upstream of a luciferase gene to measure expression from the promoter. This construct was cloned into both the pCDNA1 construct and the pGL3 construct.


uATG
ARE
mtATG
cytATG
GC3
GC2
GA3
GC1
GA2
GA1

Potential methods of regulation of hGSR expression.

In addition to the transcription factor binding sites present within the hGSR promoter, there are also three potential AUG start codons for translation, which gives rise to two different isoforms of GSR. Different isoforms of proteins expressed from the same gene can be regulated by several mechanisms. One common means of regulating alternative isoforms of a protein is through alternative splicing of the mRNA (Agorio et al., 2003; Kozak, 1989; Kozak, 2001; Kozak, 2002). There is no evidence that alternative splicing occurs in transcripts initiated from the hGSR promoter, nor is there any evidence of splicing elements within the region near the mtAUG. Another mechanism for regulating the expression of different isoforms of a gene from a single promoter is the expression of transcripts with different 5'-ends. Several promoters, including the *S. cerevisiae* glutathione reductase gene, have been characterized by more than one transcript (Jitrapakdee et al., 1998; Outten and Culotta, 2004; Roberts et al., 1997). The available expressed sequence tags (ESTs) and cDNAs sequences are consistent with this type of regulation from the hGSR promoter. The expression could also be regulated by the alternative mechanisms of translation initiation. Initiation of translation is typically directed by the recruitment of the 43S ribosome complex to a capped mRNA by eukaryotic initiation factor 4F (eIF4F) (Gingras et al., 1999). The ribosome then scans the mRNA and selects the 5'-initiation codon. The efficiency by which translation is initiated by the scanning mechanism is dependent of the availability of eIF4F, the 5'-secondary structure of the mRNA, the presence of an upstream open reading frame, and the sequence context of the AUG (Kozak, 1989; Kozak, 2001; Kozak, 2002; Meijer and

Thomas, 2002). The mtGSR AUG has an adequate Kozak sequence context, with a single mismatch at the –3 position, while the cytGSR AUG has an ideal Kozak sequence (Byrd et al., 2002; Kozak, 1989; Kozak, 2001; Kozak, 2002). Any transcript containing both the AUGs for both mtGSR and cytGSR could exhibit leaky scanning at the mtGSR AUG allowing initiation to occur at the cytGSR AUG. The secondary structure of the 5'-untranslated region of mRNAs has been shown to be an inhibitor of translation initiation (Kozak, 1989; Kozak, 2001; Kozak, 2002; Gross, et al., 1990; Svitkin et al., 2001). The secondary structure of the 5'-untranslated region of hGSR mRNAs is predicted to be quite extensive by the mfold software (Mathews et al., 1999). The predicted secondary structures of the hGSR mRNAs have similar regions but do contain enough differences that could lead to alternative translational initiation. The presence of the upstream AUG (uAUG), which is in a poor Kozak sequence context, could inhibit expression from mtGSR AUG due to the presence of an open reading frame including the mtGSR AUG. It has been shown in other genes that when translation is initiated efficiently from the uAUG, the expression from the main AUG in the transcript is repressed while an AUG further downstream is affected to a much lesser extent, due to reinitiation from the downstream AUG (Hemmings-Mieszczak et al., 2000; Kochetov, 2005; Meijer and Thomas, 2002). The presence of an internal ribosomal entry site (IRES), which has been a subject of much debate, could also explain the selection of alternative AUGs from the hGSR transcript (Han and Zhang, 2002; Komar and Hatzoglou, 2005; Stoneley and Willis, 2004). Currently, no evidence of an IRES in the hGSR transcript exists but the predicted secondary structure includes features similar to the Y-type stem loop of the BiP IRES (Le and Maizel, 1997).

EXPERIMENTAL DESIGN

The transcription factor binding sites that affect expression from the mGABPα/CF6 bi-directional promoter will be determined.

GABP has been shown to be sufficient to initiation transcription in the absence of a TATA motif. The presence of four GABP binding sites in the core promoter region of the mGABPα/CF6 bi-directional promoter indicates a likelihood that the promoter is regulated by GABP. The GA1 and GA2 binding site are located in close proximity to each other and to the transcriptional start sites of both the GABPα and CF6 promoters. These two binding sites provide a platform for GABP$\alpha_2\beta_2$ complex to form and activate transcription. The remaining two binding sites can function as either positive or negative regulators of transcription. The YY1 and Sp1/3 binding sites provide binding sites for possible coregulatory proteins (Galvagni et al., 2002; Gyrd-Hansen et al., 2002; Rosmarin et al., 1998; Sadasivan et al., 1994; Rao et al., 2002; Jiang et al., 2002; Du et al., 1998). GABP regulates expression from the mGABPα/CF6 bi-directional promoter cooperatively with YY1 and Sp1. Because GABP is sensitive to oxidative stress (Martin et al., 1996; Chinienov et al., 1998) it is expected that the expression from the mGABPα/CF6 bi-directional promoter would be decreased under oxidative stress. Because the formation of GABP$\alpha_2\beta_2$ complex activates transcription, and oxidative stress inhibits GABP's ability to bind DNA, it is hypothersized that under oxidizing conditions the expression from the mGABPα/CF6 bi-directional promoter will be inhibited. The

regulation of the mGABPα/CF6 bi-directional promoter by GABP has been investigated by mutagenesis of the transcription factor binding sites and through EMSA analysis of the transcription factor binding sites. Oxidative stress was induced by treatment of cells cultures with H_2O_2 and the expression levels from the mGABPα/CF6 bi-directional promoter will be measured to determine the effect of oxidative stress on promoter activity.

The hGSR promoter will be characterized for the transcription factor binding sites that regulate expression from the promoter.

The presence of binding sites for GABP and Sp1/3 on the hGSR promoter and the absence of a TATA motif make it likely that GABP and Sp1 or Sp3 form complexes on the hGSR promoter and regulate expression from the hGSR promoter. GABP functions cooperatively with Sp1 to regulate transcription from other promoters (Galvagni et al., 2002; Gyrd-Hansen et al., 2002; Rosmarin et al., 1998; Sadasivan et al., 1994; Rao et al., 2002; Jiang et al., 2002). There are two GABP binding sites in close proximity to each other (GA1 and GA2), which would enhance the formation of GABP$\alpha_2\beta_2$ complex and activation of transcription. The remaining GABP binding site could function as either a positive or negative regulator of transcription. Sp1/3 functions cooperatively with GABP to regulate expression from the hGSR promoter. The transcription factor binding sites that regulate transcription will be determined by site-directed mutagenesis of the transcription factor binding sites. The factors that bind to the promoter will be determined through EMSA analysis.

Expression of the two isoforms of GSR has been investigated.

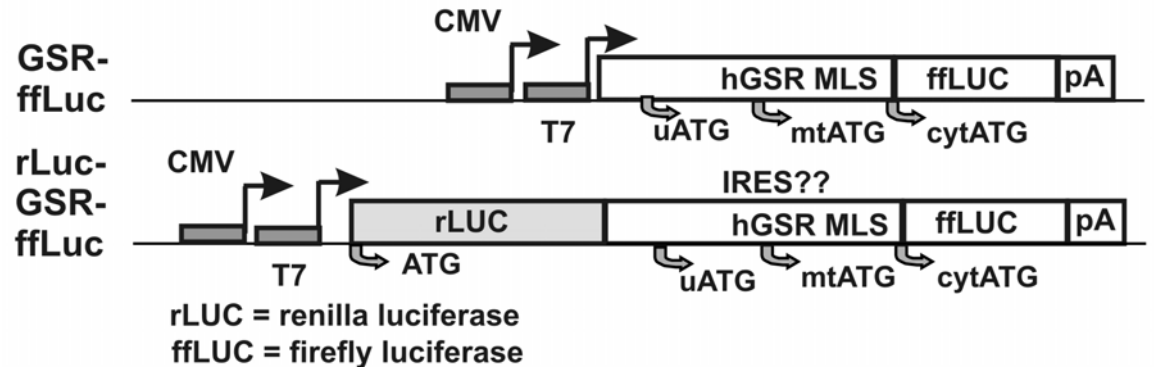
There are several possible mechanisms that have been outlined above for the possible regulation of mtGSR and cytGSR expression. The known ESTs and cDNAs initiating from sites both upstream and downstream of the mtGSR AUG would indicate the main source of regulation appears to be at the transcriptional level, controlling which type of transcript is formed. The expression of different transcripts will be confirmed by 5'-RACE analysis and measurement of the types of GSR transcripts present in a cDNA library of HeLa cells.

MATERIALS AND METHODS

Promoter constructs.

Promoter constructs for both the mGABPα/CF6 and hGSR promoters were generated for site-directed mutagenesis analysis of the transcription factor binding sites of the promoters. The generation of the mGABPα and CF6 promoters has been previously described (Chinenov et al., 2000a). The 400 base pair (-309/+91 relative to the ATG for GABPα translation) core promoter region as well as promoters containing the flanking sequences of the promoter region were cloned into pGL3-Basic (Promega, Madison, WI) expressing luciferase from the promoter. The hGSR promoter region (nucleotides –310/+1 relative to the cytGSR ATG) was likewise cloned into the pGL3-Basic (Promega) promoter allowing expression of luciferase from the hGSR promoter. To study the translational regulation of hGSR gene expression the promoter region was cloned into a pCDNA1-Amp expression vector (Promega). The strong T7 promoter region upstream of the cloned vector directs all transcription of the RNA to begin upstream the mtAUG. The entire GSR promoter region and luciferase expression vector was cloned into the pCDNA1-Amp expression vector by inserting a unique BamHI restriction site upstream of the GA3 binding site and an XhoI site downstream of the luciferase gene in the pGL3-hGSR-Basic expression vector, excising the DNA via enzymatic digestion, and cloning the hGSR-luciferase expression construct into the pCDNA1-Amp expression vector downstream of the T7 promoter. To examine the

Figure 6. The promoter construct to determine the possibility of internal ribosomal entry sites within the mitochondrial leader sequence of the hGSR promoter.


GSR-
ffLuc
CMV
T7
hGSR MLS
ffLUC
pA
uATG
mtATG
cytATG
rLuc-
GSR-
ffLuc
CMV
T7
rLUC
ATG
IRES??
hGSR MLS
ffLUC
pA
uATG
mtATG
cytATG
rLUC = renilla luciferase
ffLUC = firefly luciferase

possibility of an IRES in the hGSR promoter, the renilla luciferase gene was cloned into the pCDNA1-amp-hGSR-Luc expression vector (Promega). The renilla luciferase is expressed from the T7 and CMV promoter and is upstream of the hGSR promoter. The renilla luciferase gene was cloned into the pCDNA1-amp-hGSR-Luc expression vector between the firefly luciferase gene and the T7 promoter (Fig 6).

Mutagenesis.

Point mutations were inserted at each transcription factor binding site to disrupt the transcription factor's binding ability. Each site was mutated using the Stratagene Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). A 50 microliter (μl) reaction cocktail was prepared with mutagenesis reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton® X-100, 0.1 mg/ml nuclease-free bovine serum albumin (BSA)), 125 ng primer #1, 125 ng primer #2 (Table 1 & 2), one μl dNTP mix (25 mM dATP, dGTP, dCTP, and dTTP), 10 ng dsDNA template, and double deionized water was added to bring the reaction mixture to 50 μl. Two and one-half units of Pfu Turbo DNA polymerase (2.5 U/μl) was added to the reaction mixture. The reaction mixture was overlaid with 30 μl of mineral oil to prevent evaporation during the polymerase chain reaction (PCR). PCR was as follows: one cycle at 95ºC for 10 minutes, 18 cycles of one minute at 95ºC, one minute at 58ºC, and 10 minutes at 68ºC, and one cycle of 10 minutes at 68ºC.

Ten units of Dpn I was added to the PCR products, to digest the methylated and hemimethylated DNA, and the reaction digested for two hours at 37ºC. Two μl of the

Table 1. The mutagenesis primers and the restriction enzyme sites for the mGABPα/CF6 promoter are shown. The mutated nucleotides are underlined.

Mutation site	Primer sequence (5’➔ 3’)	Restriction sites
GA1	GTAAGTGCTTAAGGGTCCCTCA	AflII
GA2	AAACAGGAGGCTTAAGCGGAGGG	AflII
GA3	CGACGCTCACCGGACCTTAAGCGCCTCGAAGGG	AflII
GA4	CGTTCTACTTAAGGCCCTGGCT	AflII
YY1	GCTAAGACTCCGATATCTTTCCTACACTTTAAC	EcoRV
Sp1	GCCCTGGCTCAACCTACACACG	PstI

Table 2. The mutagenesis primers and the restriction enzyme sites are shown for the hGSR promoter. The mutated nucleotides are underlined.

Mutation site	Primer sequence (5’➔ 3’)	Restriction site
GA1	CGCGCCTTCCGAGGCTTCTTGCTGCTTCTGCCCGAG	BstBI
GA2	CGGGCGGCGCGCGCCTTCAGAGGCTTCCTGCTGCTT	BstBI
GA1/2	GCGCGCGCCTTCGAAGGCTTCGAGCTGCTTCTGCCC	BstBI
GA3	CCCACTTGGAGCGCCACTTAAGCGTGCATGGCCCTG	AflII
GC1	GCGGGACCGAGCTGGAGAAGAGCGGCGCGCGCCTTC	BpmI/MboI
GC2	GACCGACCCCGGGCCGGATCCCCCACTTGGAGCGCC	BamHI
GC3	TGGGTCTTGCCTAGCGGATCCCGCATGCTTAGTCAC	BamHI
ARE	GGCGGGCGCATGCTTAAGCACCGTGAGGCTGCGCTT	BamHI
mtAUGko	AGCGCCACTTCCGCGTCTTAAGCCCTGCTGCCCCGA	AflII
mtAUGopt	AGCGCCACTTCCGCGGCCATGGCCCTGCTGCCCCGA	NcoI
cytAUGko	CTCTCCCGTGCCATCGCCTGCAGGCAGGAG	NcoI KO
uAUGko	CTCGGATCCCGCATCGATAGTCACCGTGAGG	ClaI

digestion product were added to 45 μl of XL-1 Blue supercompetent cells. The mixture was incubated on ice for 20 minutes, transferred to a 42ºC water bath for 45 seconds, and then placed back on ice for 2 minutes. Five hundred μl of NZY^+ broth (10 g NZ amine, 5 g yeast extract, 5 g NaCl in one liter, pH=7.5; after autoclaving, 12.5 ml 1 M $MgCl_2$, 12.5 ml 1 M $MgSO_4$, and 20 ml 20% (w/v) glucose) were added to the mixture, which was incubated at 37ºC in an incubator shaker for one hour. Two hundred μl of the mixture was plated on a LB (10 g NaCl, 10 g tryptone, 5 g yeast extract in one liter, pH = 7.2) - agar (20 g agar per one liter LB) + ampicillin (200 ug/ml) plate and incubated overnight inverted at 37ºC.

Selecting and preparing mutant DNA.

Several colonies were picked from an overnight culture grown on an LB agar + ampicillin plate. Each colony was grown in a five-ml LB culture overnight at 37ºC with shaking, and harvested by centrifugation at 1000 x g for five minutes. The LB was then decanted off the cell pellet. The DNA was harvested from the cell pellet by Qiagen (Valencia, CA) Spin miniprep kit. The DNA was resuspended in 250 μl of resuspension buffer, lysed by adding 250 μl of lysis buffer, and then neutralized by adding 350 μl of neutralization buffer. The cell lysate was centrifuged at maximum rpm for 10 minutes in a microcentrifuge. The supernatant was then decanted into a spin miniprep column. The supernatant was centrifuged at maximum rpm for one minute to allow the plasmid DNA to bind to the column. The DNA was then washed with 500 μl of PB (Qiagen) buffer and with 750 μl of PE (Qiagen) buffer containing ethanol. After the ethanol wash, the column

was centrifuged one more time to remove any excess ethanol. The DNA was then eluted with 50-μl double deionized water after incubation for five minutes. The column was then centrifuged at maximum rpm for one minute and the DNA was eluted into a 1.5 ml tube.

The DNA was then screened for the proper mutants by enzymatic digestions. Each site that was mutated had an engineered restriction enzyme site (Tables 1 & 2). The digestions were run on a 0.7% agarose gel, the appropriate digestion pattern was selected, and that DNA was sequenced by the DNA core facility at the University of Missouri. Once the sequence was confirmed the DNA was transformed into XL-1 Blue supercompetent cells (Stratagene, La Jolla, CA) and 25 μl were spread on a LB-agar + ampicillin plate. The cells were incubated overnight at 37ºC. One colony was picked from the plate and transferred to a five-ml LB + amp culture and grown overnight with shaking at 37ºC. The DNA was then extracted from the XL-1 Blue cells by a Qiagen (Valencia, CA) Spinminiprep kit (see above) and stored in Tris-EDTA (10 mM Tris, 1 mM EDTA, pH = 7.6) at –20ºC.

Tissue Culture.

Several mammalian cell lines were maintained and utilized for the data collection in these experiments. Mouse fibroblasts, $A9_{2L}$, were graciously provided by Dr. David Pintel (Department of Molecular Microbiology and Immunology, University of Missouri-Columbia School of Medicine) and utilized for transient transfections and electrophoretic mobility shift assays (EMSA) for the mGABPα/CF6 promoter constructs. The EMSA

analysis of the mGABPα/CF6 promoter constructs was also done using 3T3-L1 cells kindly provided by Dr. Peter Wilden (Department of Pharmacology, University of Missouri-Columbia School of Medicine). For the transient transfection of the hGSR promoter constructs, human kidney cells, HEK293T cells, were graciously provided by Dr. Mike Henzl (Department of Biochemistry, University of Missouri-Columbia). The hGSR EMSA analysis was performed with nuclear extracts from HeLa cells kindly provided by Dr. David Pintel (Department of Molecular Microbiology and Immunology, University of Missouri-Columbia School of Medicine). All cell types were cultured by growth in Dulbecco's Modified Eagle Medium with high glucose (DMEM-HG) and 5% fetal bovine serum (FBS), penicillin and streptomycin, fungizone, and L-glutamine (DMEM-complete) added until confluent (Tullis et al., 1988; Young et al., 2005). The HEK293T and HeLa cells required media with 10% FBS to maintain the cell culture but the media other wise remained the same. The cells were split 1:6 and re-plated for use in other experiments. Frozen stocks of these cells were made by freezing the cells in DMEM-complete containing DMSO (HeLa, $A9_{2L}$, and 3T3-L1, 7.5% DMSO; HEK293T, 5% DMSO and 45% FBS).

Transfection.

The pGL3- or pCDNA1amp-DNAs were adjusted to the same concentration by comparison on 0.7% agarose gels for use in transfection. Once the DNA concentrations were confirmed to be the same, the DNAs were transfected into the appropriate cells lines. The DNA constructs of the mGABPα/CF6 promoter were transfected into $A9_{2L}$

cells. The DNA constructs containing the hGSR promoter were transfected into HEK293T cells. The cells were seeded into 60-mm plates (2 * 10^5 cells/plate) for transfection the following day. Each transfection was performed in quadruplicate. Each mutant DNA plasmid (0.4 μg) and an internal control plasmid (0.4 μg, pSV-βGal, Promega) were transfected with eight μl of PLUS reagent (Invitrogen, Carlsbad, CA), and 12 μl of Lipofectamine (Invitrogen) according to manufacturer's protocol. The transfections were harvested into 400 μl Reporter Lysis Buffer (Promega) after 42 hours. The cell lysate was harvested into 1.5 ml centrifuge tubes and centrifuged at 12300 rpm for 2 minutes to remove the cell debris. The supernatant was then collected to use to determine the relative expression from the mutant DNA promoters and the internal control. Luciferase and β-Gal assays were performed on each of the supernatants. Luciferase assays were performed by adding 10 μl of cell extract to a clear microcentrifuge tube containing 90 μl of luciferase Assay Buffer (100mM KPO_4, pH = 7.8, 10mM $MgCl_2$, 0.25mM ATP). One hundred μl of cold 1 mM Luciferin was added to each tube and then the luciferase expression was immediately measured in a Turner 20/20 Luminometer. For β-gal assays, thirty μl of cell supernatant were added to a microfuge tube containing 120 μl of ddH_2O and 150 μl of 2x β-galactosidase reaction assay buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml o-nitrophenyl-beta-D-galactoside). This reaction was incubated in a 37ºC water bath for one hour, after which the reaction was stopped with 500 μl of 1 M Na_2CO_3. After stopping the reaction, the absorbance of each reaction was measured at 420 nm (Chinenov et al., 2000a; Yu et al., 1997). The luciferase expression was normalized to the expression of β-Gal for each transfection and reported as an average of

luciferase expression divided by the β-Gal expression, with wild-type expression being reported as 100% activity for the promoter.

Oxidative stress induction.

Oxidative stress was induced on the cells by addition of hydrogen peroxide (H_2O_2) to transient transfections. The mGABPα wild type construct was transfected into several plates as described above. The cells were treated with 150 μM concentrations of H_2O_2 for 4 hours (Davies K.J.A., 1999; also experiments performed in our lab, See Fig. 21-23). The cells were then harvested as previously described into Reporter Lysis Buffer (Promega) and the luciferase and β-Gal assays were performed on the supernatants (Chinenov et al., 2000a; Yu et al., 1997). The luciferase expression in supernatants of cells that were treated with H_2O_2 was reported as a percentage of the same mutant that remained untreated in the experiment.

Electrophoretic mobility shift assay (EMSA).

The ability of GABP, Sp1, Sp3, and YY1 to bind to the mGABPα/CF6 promoter and the hGSR was determined by EMSA. HeLa, $A9_{2L}$ and 3T3-L1 cells were grown to near confluence (~10^8 total cells) in 150 mm cell culture plates in DMEM-complete as previously described. The cells were scraped off the plate, harvested in phosphate buffered solution (PBS), and centrifuged at 1200 rpm for 10 minutes at 4ºC. The supernatant was removed and the cell pellet was resuspended in 25 ml Buffer A (10 mM

HEPES (pH = 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), and 1 mM benzamidine). The cells were centrifuged again for 10 minutes at 1200 rpm and 4ºC. The supernatant was removed and the cell pellet was resuspended in two cell pellet volumes of Buffer A. The cells were incubated on ice for 30 minutes and lysed gently in a dounce homogenizer, being dounced 10 times with a loose-fitting pestle and then 20-30 times with a tight-fitting pestle. The nuclei were collected by centrifugation at 3000 rpm for 10 minutes at 4ºC. The nuclei were resuspended in two volumes of 0.42 M NaCl. The nuclei were incubated on ice for 30 minutes with occasional stirring. The nuclei were collected by centrifugation at 3000 rpm for 10 minutes at 4ºC, and the supernatant was collected as nuclear extract. The supernatant was dialyzed (12,000 MW cutoff) against 500 ml of Buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 1.0 mM benzamidine, 20% glycerol, 1 mM DTT) for at least four hours. After a minimum of four hours, the buffer was changed and dialysis was continued overnight, after which the nuclear extracts were collected from the dialyzed solution. Recombinant GABPα and GABPβ (rGABPα and rGABPβ) were prepared by expressing and purifiying the His_6-tagged proteins as has previously been described (Martin et al., 1996; Chinenov et al., 2000b)

The double stranded oligonucleotide probes (Fig. 7-8) were prepared for the gel by annealing the complementary oligonucleotides at 2.0 μg/μl in 10 mM Tris (pH = 8.0) and 50 mM NaCl. The mixture was then boiled for 10 minutes, and then transferred to a 68ºC water bath for 20 minutes. The mixture was incubated for twenty minutes at each of the following temperatures: 55ºC, 42ºC, 37ºC, and 30ºC. The mixture was then moved to the bench top for twenty minutes and placed on ice. The double stranded oligonucleotide probes were 5' end-labeled with [γ-^{32}P]ATP by incubation for 1 hour at 37ºC with T4

Figure 7. Probes used in the EMSA analysis of the mGABPα/CF6 bi-directional promoter. The first four probes correspond to sequences within the promoter region. The last three probes are control probes.

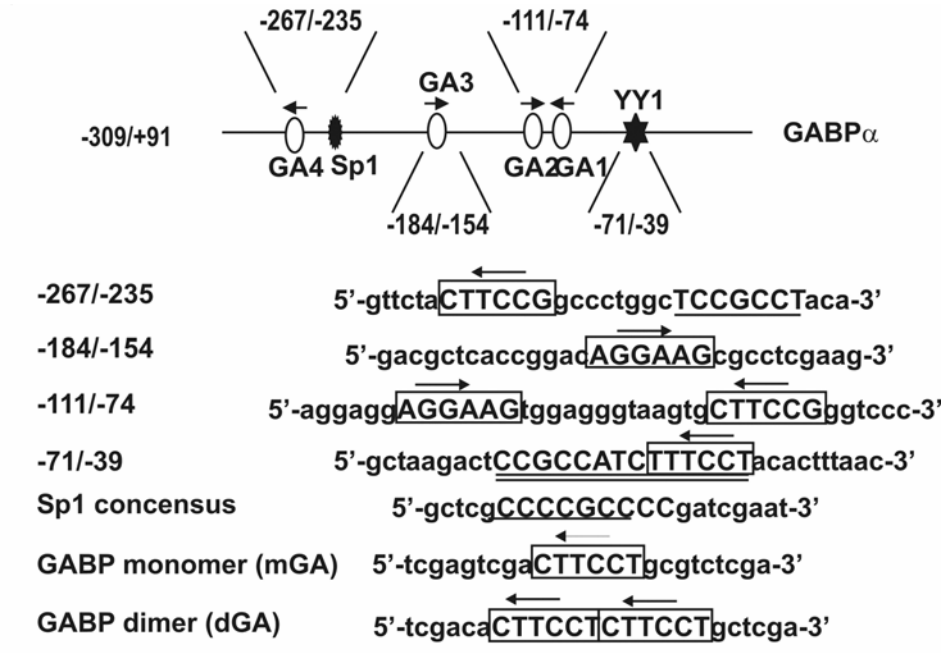
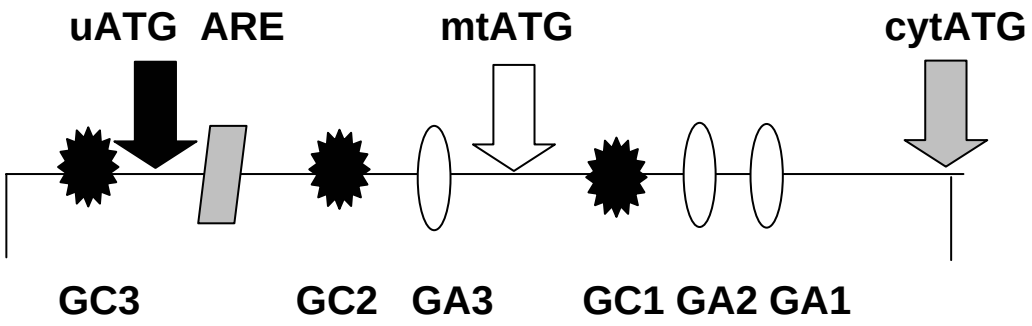

-267/-235
-111/-74
GA3
YY1
-309/+91
GABPα
GA4 Sp1
GA2 GA1
-184/-154
-71/-39
-267/-235 5'-gttctaCTTCCGgccctggcTCCGCCTaca-3'
-184/-154 5'-gacgctcaccggacAGGAAGcgcctcgaag-3'
-111/-74 5'-aggaggAGGAAGtggagggtaagtgCTTCCGggtccc-3'
-71/-39 5'-gctaagactCCGCCATCTTTCCTacactttaac-3'
Sp1 concensus 5'-gctcgCCCCGCCCCgatcgaat-3'
GABP monomer (mGA) 5'-tcgagtcgaCTTCCTgcgtctcga-3'
GABP dimer (dGA) 5'-tcgacaCTTCCTCTTCCTgctcga-3'

Figure 8. The EMSA probes for the hGSR promoter. The sequence of each probe is shown as well as the placement of the nucleotide sequences relative to the translational start site for cytGSR.



Binding site	Nucleotides	Sequence
GA1	-63/-40	ttccgaggcttcctgctgcttctg
GA2	-75/-52	gcggcgcgcgccttccgaggcttc
GA1/2	-72/-43	gcgcgcgccttccgaggcttcctgctgctt
GA3	-149/-126	ggagcgccacttccgcgtgcatgg
GC1	-91/-64	ccgagctggcggcgggcggcgcgcgcc
GC2	-174/-145	cgaccccgggccgcccgccccacttggagc
GC3	-253/-230	tcttgcctagcggcgggcgcatgc
ARE	-243/-214	cggcgggcgcatgcttagtcaccgtgaggc

polynucleotide kinase. The 5' end-labeled probes were purified with the Mermaid kit (BIO 101, Irvine, CA). The EMSA reactions were run with nuclear extracts or rGABPα and rGABPβ. Fifteen μg of the nuclear extract was incubated with poly(dI-dC)·pol (dI-dC) for 10 minutes at room temperature in 0.2 ml EMSA buffer (20 mM HEPES, pH 8.0, 50 mM KCl, 1 mM benzamidine, 5% glycerol, 1 mM DTT). The labeled double stranded oligonucleotide probes were added to the reactions and incubated for 15 minutes at room temperature. The reaction mixture was separated over a 5.0% or 6.3% non-denaturing polacrylamide gel in 0.25X TBE (35 mM Tris, 20 mM boric acid, 12.5 mM EDTA, pH 8.0). The gels were dried and subjected to autoradiography (Martin et al., 1996, Chinenov et al., 2000b). X-ray film was placed on top of the dried gels, exposed to a flash of light, and incubated at -80ºC. The three potential GABP DNA-protein complexes were observed by adding rGABPβ (~0.03 μg) to a slight excess of rGABPα in 20 μl of EMSA buffer containing 0.1 μg poly(dI-dC)·poly(dI-dC) and 0.5 mg/ml bovine serum albumin. The labeled probe containing two GABP binding sites (dGA) was added to the reaction, and the EMSA was run as described (Chinenov et al., 2000a, 2000b, 1998).

Competition assays and immuno-supershift assays were also performed with the nuclear extracts to confirm the binding of the transcription factors. The competition assays were performed by incubating the nuclear extract with a 500-fold excess of unlabeled probe with the nuclear extract for 30 minutes prior to addition of the labeled oligonucleotide probe. For the immuno-supershift assays, the nuclear extracts (15 μg) were incubated with 2 μl of antisera to a transcription factor for four hours prior to the addition of the labeled probe. The EMSA was then performed as described previously. The antibodies to rGABPα (sc-22810), rGABPβ (sc-13445), Ets-1/Ets-2 (sc-112X), Sp1

(sc-59), Sp3 (sc-13018) and YY1 (sc-1703) transcription factors were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

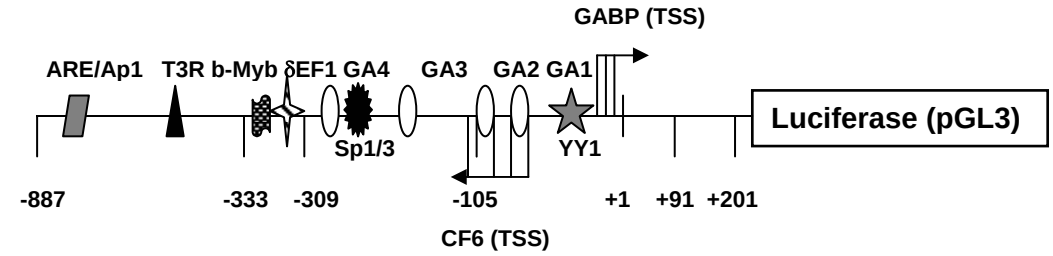
RESULTS

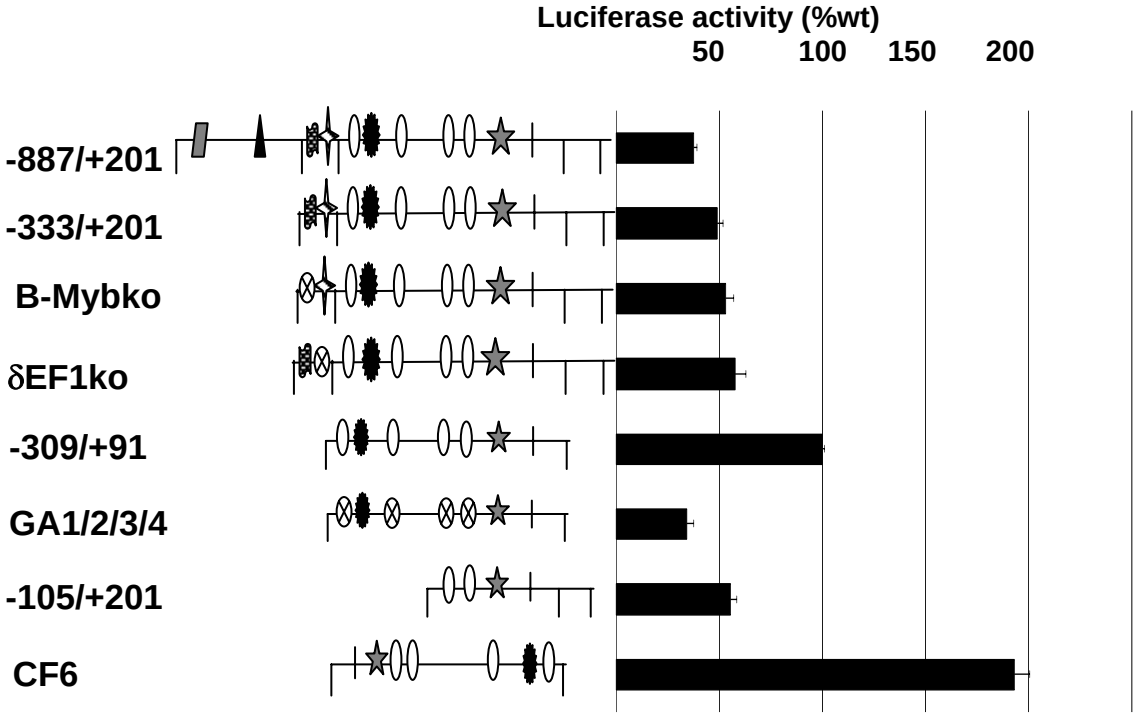
The mGABPα/CF6 Promoter Analysis

Identification of the functional core promoter region in the mGABPα/CF6 bi-directional promoter.

The mGABPα/CF6 promoter was previously isolated by long range PCR of the mouse genomic libraries (Chinenov, Y., et al., 2001a). The PCR products were cloned into the pGEM-T cloning vector (Promega) and sequenced by the University of Missouri DNA core facility. One truncated PCR product had a unique KpnI site at the junction of the cloning vector. A second KpnI site was introduced into the PCR product by site-directed mutagenesis at +91 relative to the 5'-teminus of the GABPα cDNA (Acc# M74515). The resulting fragment was cloned into the pGL3-basic (Promega) luciferase expressing vector in both the GABPα and CF6 orientations, and the promoter was found to direct transcription from both promoters (Chinenov et al., 2001a). Other workers subsequently identified three regions of the mGABPα/CF6 gene as potential regions for the core promoter (P1, P2, P3) by the NIX suite of software program (O'Leary et. al., 2005). The P2 region, which was predicted to be the dominant region of the promoter for GABPα expression, contained the –309/+91 core promoter region previously identified. The boundaries of the core mGABPα promoter were determined through deletion analysis and the examination of additional flanking sequences. Several 5'- and 3'-

Figure 9. The analysis of the effect on expression of the deletion mutants of the mGABPα promoter. The deletion flanking sequences to the core promoter reveals that the core promoter region is almost twice as active as any of the deletions. The knockout of the additional binding sites (in the –333/+201 construct) in the flanking sequences had no effect on the expression from the promoter. The functionality of the GABP site was tested as well and the expression from the promoter is reduced to 33% of the wild-type level in the mutant with all four GABP sites mutated.


GABP (TSS)
ARE/Ap1
T3R
b-Myb
δEF1
GA4
GA3
GA2
GA1
Luciferase (pGL3)
Sp1/3
YY1
-887
-333
-309
-105
+1
+91
+201
CF6 (TSS)


Luciferase activity (%wt)
50
100
150
200
-887/+201
-333/+201
B-Mybko
δEF1ko
-309/+91
GA1/2/3/4
-105/+201
CF6

deletion mutants were generated and cloned into the pGL3-basic expression vector (Promega). Analyses of the luciferase activity from the promoters of these deletion mutants reveal the –309/+91 promoter region to be the region of greatest interest, as the expression of luciferase from this region of the promoter is greater than the other regions of the promoter. The –105/+201 and –333/+201 promoters were shown to give expression levels slightly greater than the expression level of the –887/+201 promoter but have significantly (~40%) lower expression levels than the –309/+91 promoter (Fig. 9) demonstrating the dominance of the core promoter. The flanking sequences contain transcription factor binding sites that are most often utilized in tissue specific transcription. It has been demonstrated these transcription factor binding sites do not significantly affect the expression from the mGABPα/CF6 promoter. This was demonstrated by mutating the the δEF-1 and b-Myb binding sites are knocked out in the –333/+201 promoter (Fig. 9).

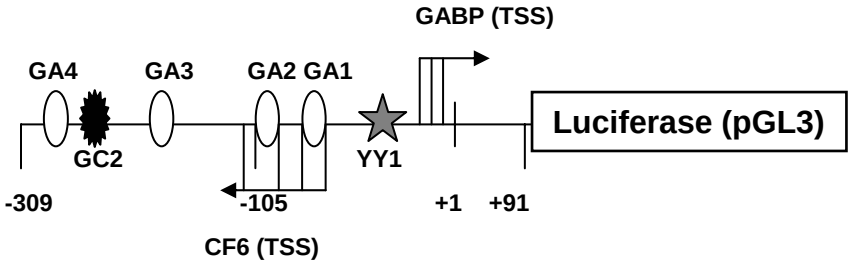
Identification of the functional GABP, Sp1/3, and YY1 binding sites in the mGABPα promoter.

Sequence analysis of the –309/+91 core promoter reveals six potential transcription factor-binding sites, one YY1, one Sp1/3, and four GABP binding sites (GA1, GA2, GA3, and GA4). Two of these GABP binding sites are located near the transcription start sites for both promoters (Chinenov et al., 2000b). The ability of GABP, Sp1, and YY1 to initiate, activate, and repress transcription is well documented (Galvin and Shi, 1997; Genuerio and Perry, 1996; Sucharov et al., 1995; Yu et al., 1997), so these

transcription factor binding sites are candidates for the regulation of the mGABPα promoter. These putative transcription factor-binding sites were then analyzed for their ability to regulate expression from the mGABPα promoter, by site-directed mutagenesis and subsequent analysis of gene expression. The mutant with all four GABP sites knocked out demonstrates that GABP is an important regulator of the transcription from the mGABPα promoter. When all four GABP binding sites are mutated the activity of the promoter is decreased to about 30% of the wild-type activity (Fig. 9), demonstrating that GABP or another ETS-related factor regulates transcription from the mGABPα promoter. Analysis of the single mutants of the six transcription factor-binding sites revealed varying levels of luciferase expression from the mGABPα promoter. The mutation of a single GABP site or the YY1 site reduced the expression of luciferase from the promoter to about 50% to 80% of the wild-type activity (Fig 10). The expression from the mutation of the Sp1 site was actually increased to 159% of the wild-type activity, suggesting a possible inhibitory role for this factor-binding site. The GA3 mutant showed the least change in activity as this mutant still maintained over 80% of the wild type activity. These data indicate a complex regulation system in which each of the transcription factors could play a role.

Since no single factor-binding site displayed dominant regulation over the mGABPα promoter activity and GABP requires two binding sites for the active heterotetramer complex to assemble on DNA (Sawada et al., 1994; Sucharov et al., 1995; Yu et al., 1997), the four GABP sites may be functionally redundant. YY1, Sp1, and GABP can all have redundant functions, so it is possible that these factor-binding sites are redundant or function in concert with each other. Multiple transcription factor-binding

Figure 10. The effect of single site mutations of putative factor binding sites on GABPα promoter activities. Each of the putative factor binding sites illustrated in the schematic of the GABPα core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core GABPα promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

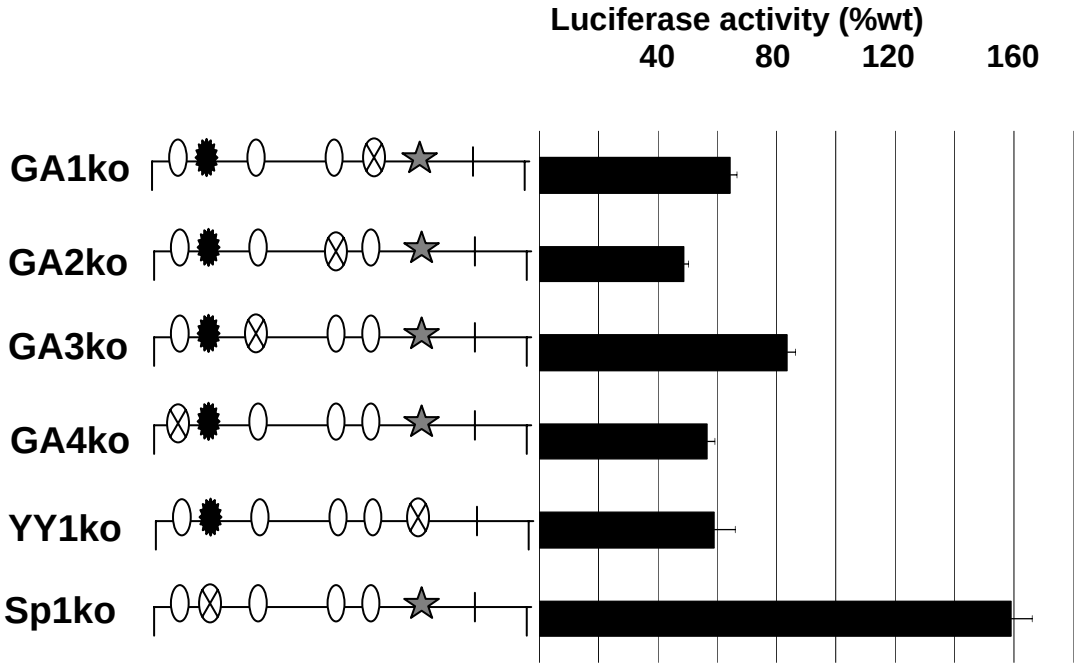
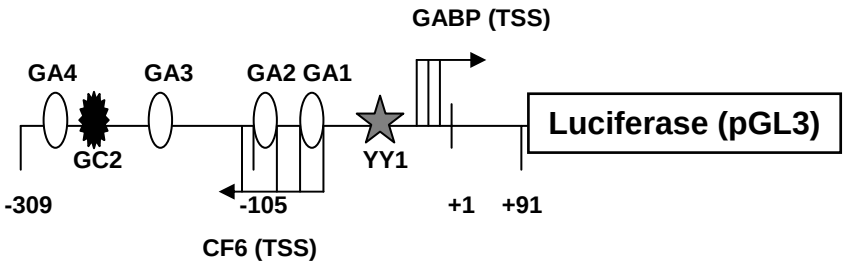

Luciferase activity (%wt)
40
80
120
160
GA1ko
GA2ko
GA3ko
GA4ko
YY1ko
Sp1ko

Figure 11. The effect of GA double site mutations of putative factor binding sites on GABPα promoter activities. Each of the putative factor binding sites illustrated in the schematic of the GABPα core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core GABPα promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

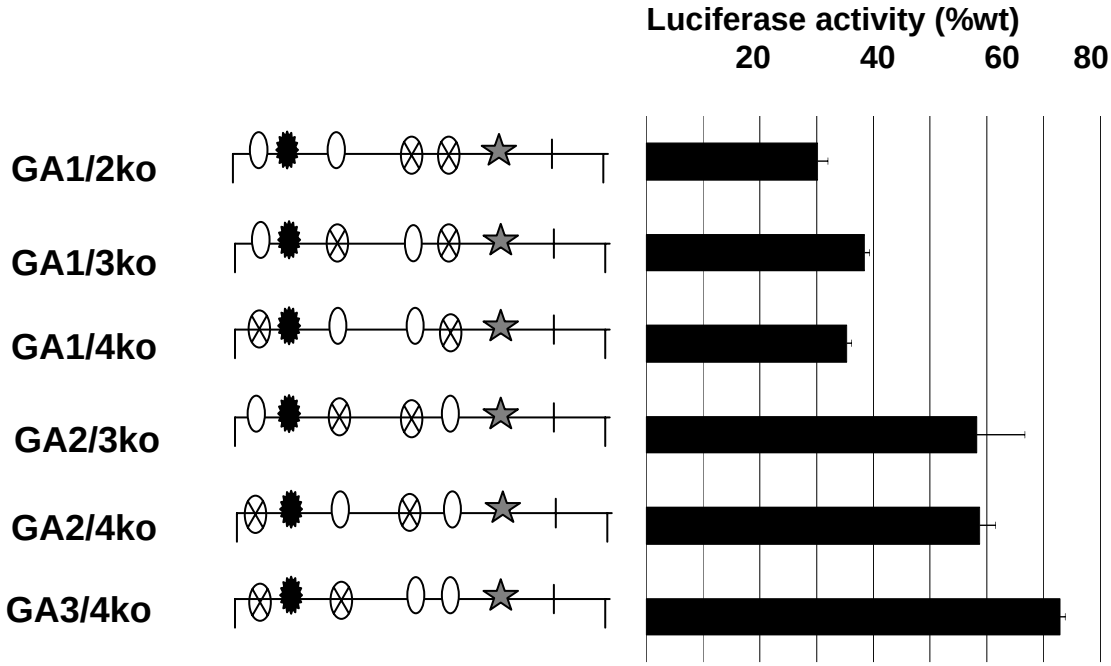
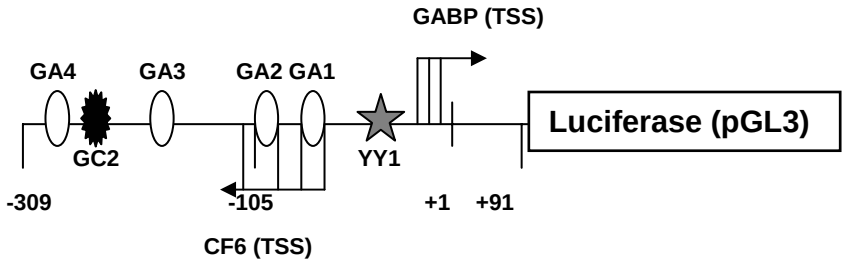

Luciferase activity (%wt)
20
40
60
80
GA1/2ko
GA1/3ko
GA1/4ko
GA2/3ko
GA2/4ko
GA3/4ko

Figure 12. The effect of YY1 and Sp1/3 double site mutations of putative factor binding sites on GABPα promoter activities. Each of the putative factor binding sites illustrated in the schematic of the GABPα core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core GABPα promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
GC2
YY1
Luciferase (pGL3)
-309
-105
+1
+91
CF6 (TSS)

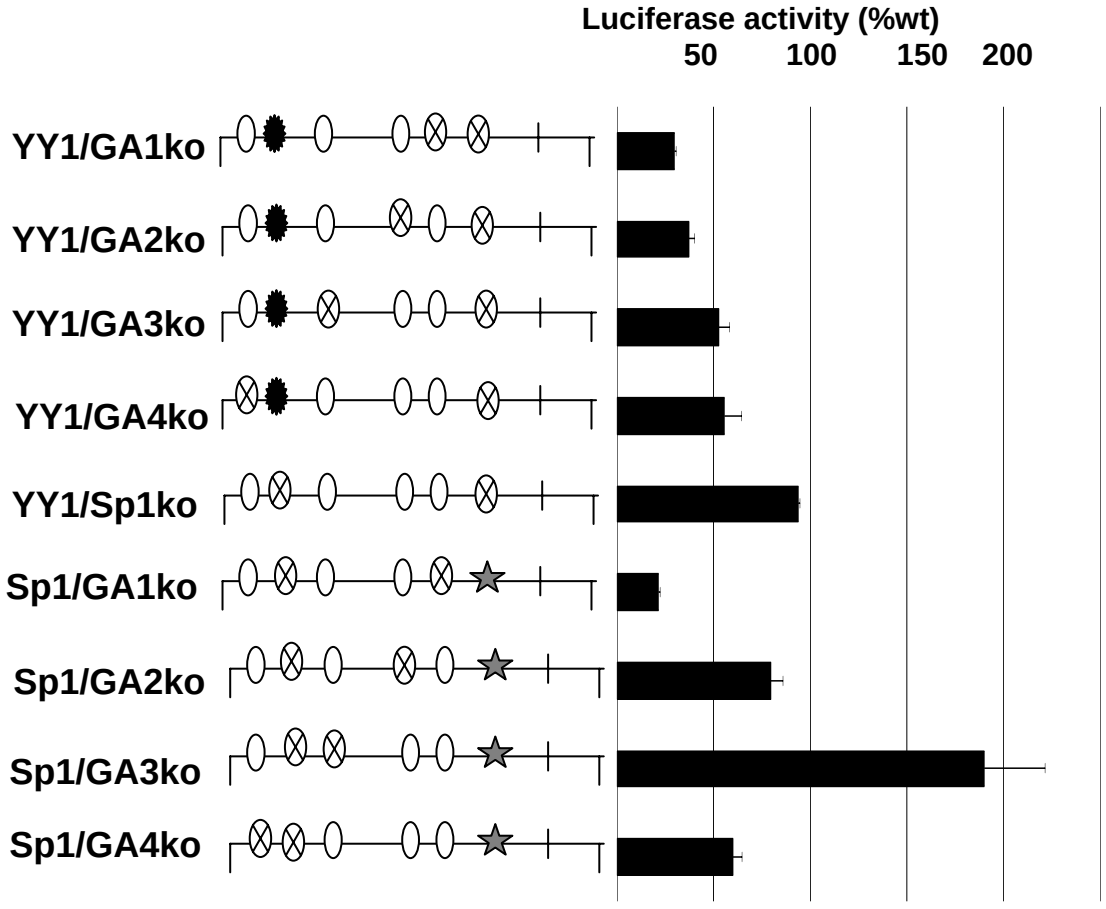
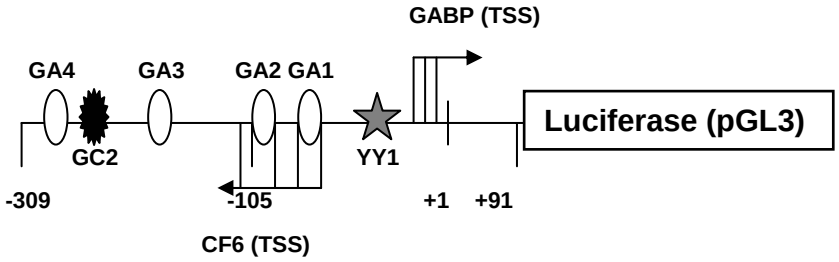
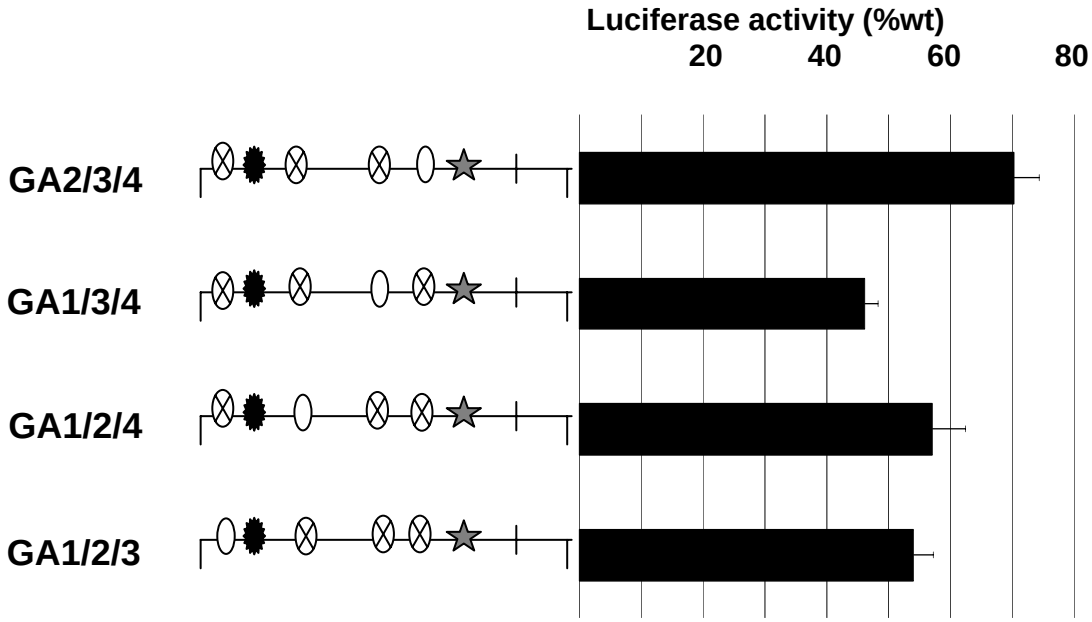

Luciferase activity (%wt)
50
100
150
200
YY1/GA1ko
YY1/GA2ko
YY1/GA3ko
YY1/GA4ko
YY1/Sp1ko
Sp1/GA1ko
Sp1/GA2ko
Sp1/GA3ko
Sp1/GA4ko

Figure 13. The effect of GABP triple site mutations of putative factor binding sites on GABPα promoter activities. Each of the putative factor binding sites illustrated in the schematic of the GABPα core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core GABPα promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)


Luciferase activity (%wt)
20
40
60
80
GA2/3/4
GA1/3/4
GA1/2/4
GA1/2/3

site mutants were constructed, by site-directed mutagenesis, to investigate this possibility, and the effect on luciferase expression levels was determined. Each of the multiple mutants revealed, as with the single mutants, varying levels of expression, ranging from about 20% to up to 110% of the wild type activity (Fig 11-13). Any mutant in which the GA1 site was mutated along with another GABP site the activity was reduced to approximately 20-40% of the wild-type expression levels compared to the mutant with the GA1 site intact (Fig 11). The double mutants with the GA1 site remaining intact gave expression levels that were affected to a lesser extent (40 to 80% of wild type activity). These data implicate the GA1 site as important for promoter activity and support the notion that the remaining GABP sites function redundantly and in concert with the GA1 site. The importance of the GA1 site is confirmed in the triple mutants of the GABP binding sites. When the GA1 site is the only intact GABP site, the expression from the mGABPα promoter is greater than for any of the other triple mutants (Fig 13).

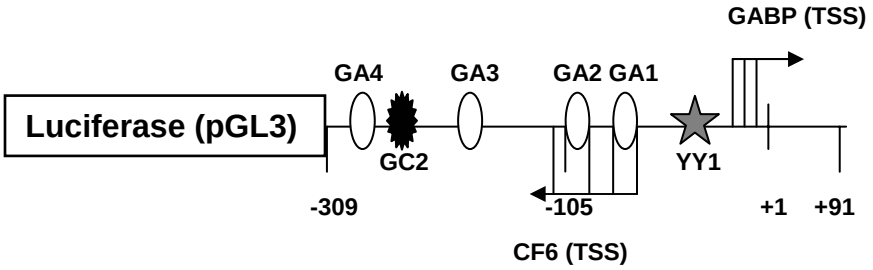
GABP is known to regulate transcription from several promoters in concert with YY1 and Sp1/3 (Du et al., 1998; Galvagni et al., 2001; Hempel et al., 2004) suggesting the possibility that YY1 and Sp1/3 functionally interact with GABP to regulate promoter transcription from the mGABPα promoter. YY1, Sp1, and GABP double knockouts were generated, via site-directed mutagenesis, and the resulting constructs analyzed for their ability to regulate expression from the promoter (Fig 12). The most severely affected double mutants were the YY1/GA1 (about 22% wild-type activity) and the Sp1/GA1 (about 30% wild-type activity) mutants. In the case of the YY1/GA double mutants, the closer the GABP site was to the YY1 site, the greater the effect the double mutant had on expression from the promoter, demonstrating the GABP interaction with YY1 is at least

somewhat dependent on distance between the two binding sites. The Sp1 binding site appears to have an important role in regulating the activity of the GABPα promoter as mutating that site along with the GA1 site decreased the activity of the promoter, indicating a likely interaction between GABP and Sp1 activates transcription and the GA1 binding site is not functionally redundant with the other GABP binding sites.

Identification of functional GABP, Sp1/3, and YY1 binding sites in the CF6 promoter.

The GABPα promoter includes a 165 bp region that shared remarkable sequence homology with rat CF6 gene oriented in the opposite direction on the DNA, suggesting the presence of a bi-directional promoter, which was subsequently confirmed (Chinenov et al., 2001b). Because this promoter is bi-directional, the transcription factor binding sites were examined for their role in regulating expression from the CF6 promoter, as well. Luciferase is expressed from the CF6 core promoter at about twice the level of expression from the mGABPα core promoter (Fig 9). The six transcription factor-binding sites were examined for their effectiveness in regulating transcription from the CF6 promoter. The only mutant that demonstrated a significant change in the expression from the promoter was the GA1 mutant, which expressed about 30% of the wild-type activity. The remaining single mutants expressed luciferase at between 70 and 111% of the wild-type levels (Fig. 14). These data once again implicate- the GA1 site as a significant binding site for transcription. The analyses of the double mutants in the CF6 promoter confirm this conclusion. When the GA1 site was mutated in concert with another GABP binding site, there was a reduction in the expression level from the CF6 promoter (about

Figure 14. The effect of single site mutations of putative factor binding sites on CF6 promoter activities. Each of the putative factor binding sites illustrated in the schematic of the CF6 core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core CF6 promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

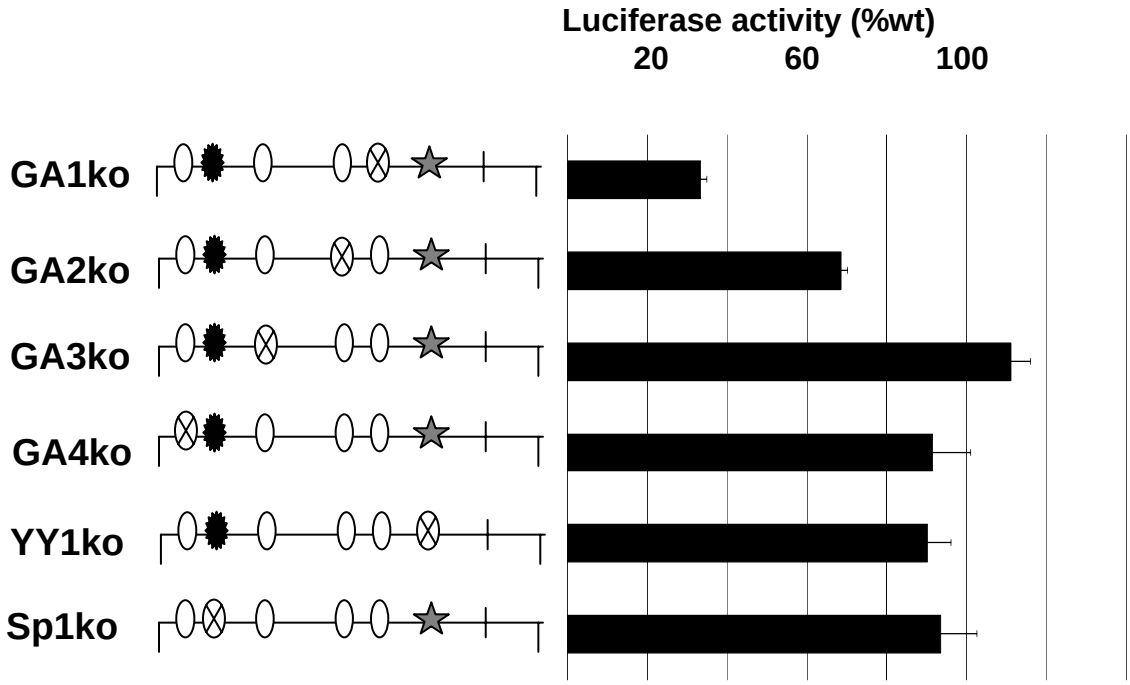
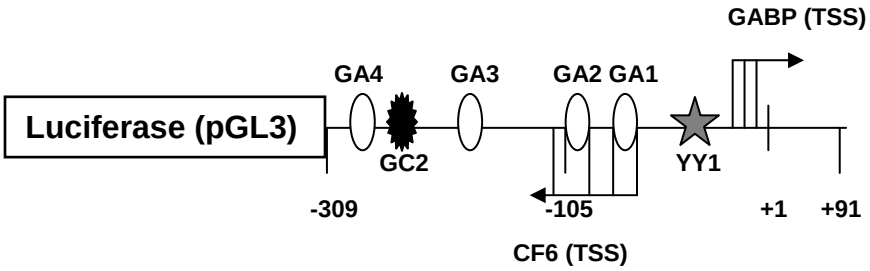

Luciferase activity (%wt)
20
60
100
GA1ko
GA2ko
GA3ko
GA4ko
YY1ko
Sp1ko

Figure 15. The effect of GA double site mutations of putative factor binding sites on CF6 promoter activities. Each of the putative factor binding sites illustrated in the schematic of the CF6 core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Boxes containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core CF6 promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

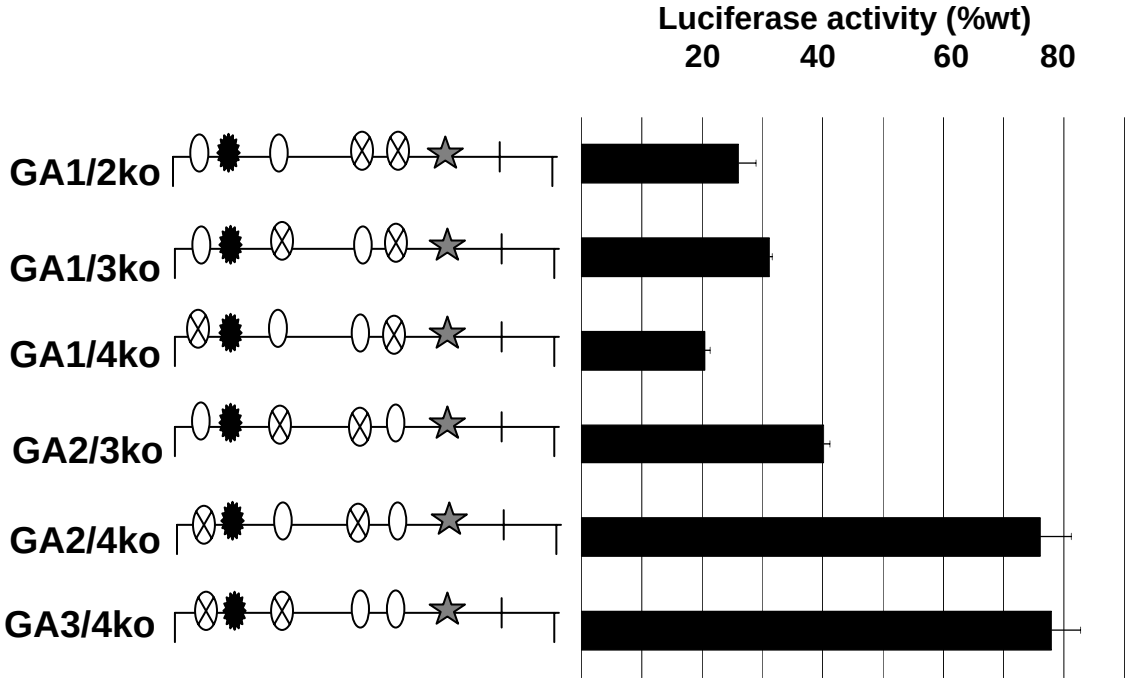
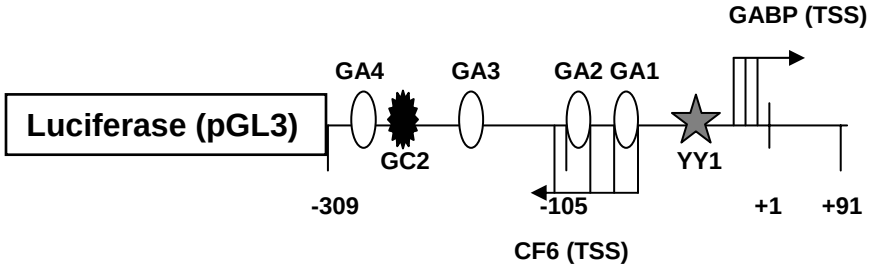
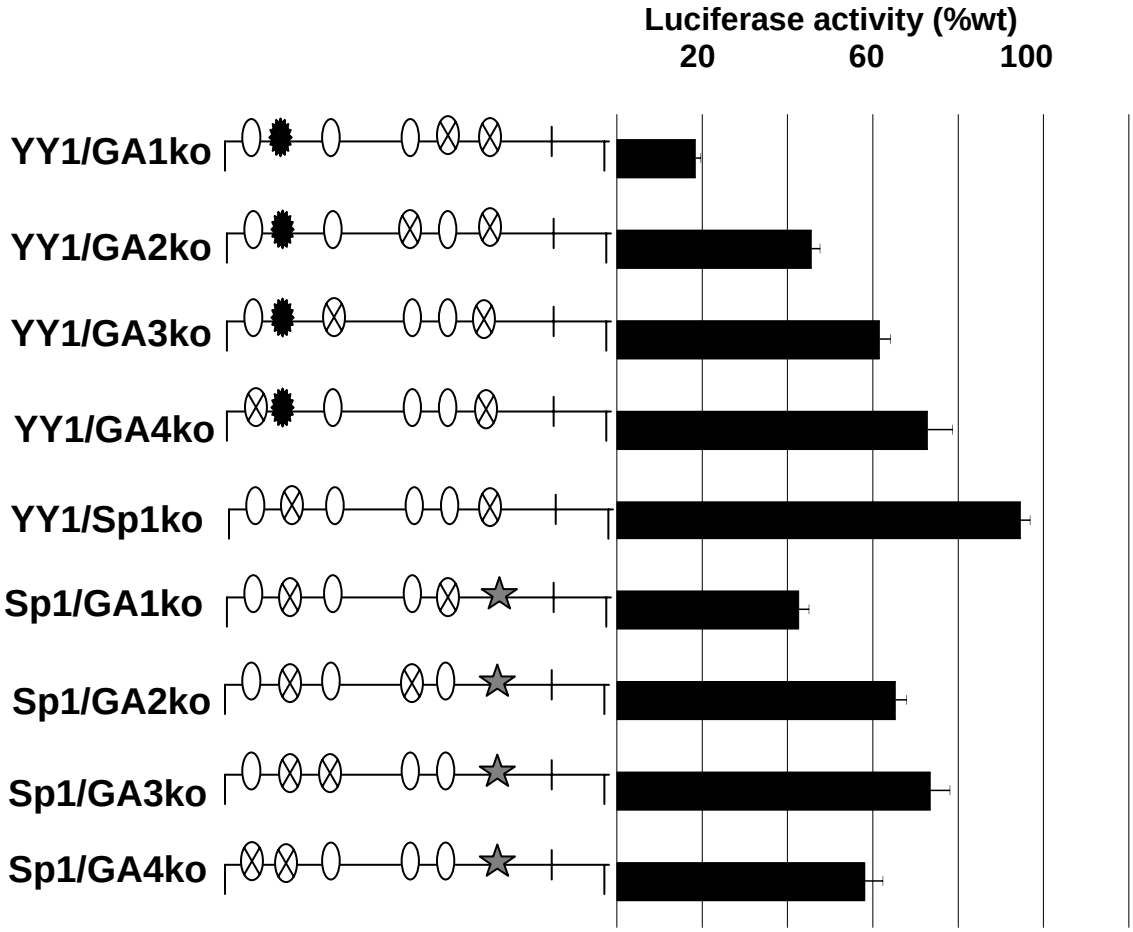

Luciferase activity (%wt)
20
40
60
80
GA1/2ko
GA1/3ko
GA1/4ko
GA2/3ko
GA2/4ko
GA3/4ko

Figure 16. The effect of YY1 and Sp1/3 double site mutations of putative factor binding sites on CF6 promoter activities. Each of the putative factor binding sites illustrated in the schematic of the CF6 core promoter was mutated and the effect on luciferase expression measured in $A9_{2L}$ fibroblasts. Boxes containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt core CF6 promoter.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

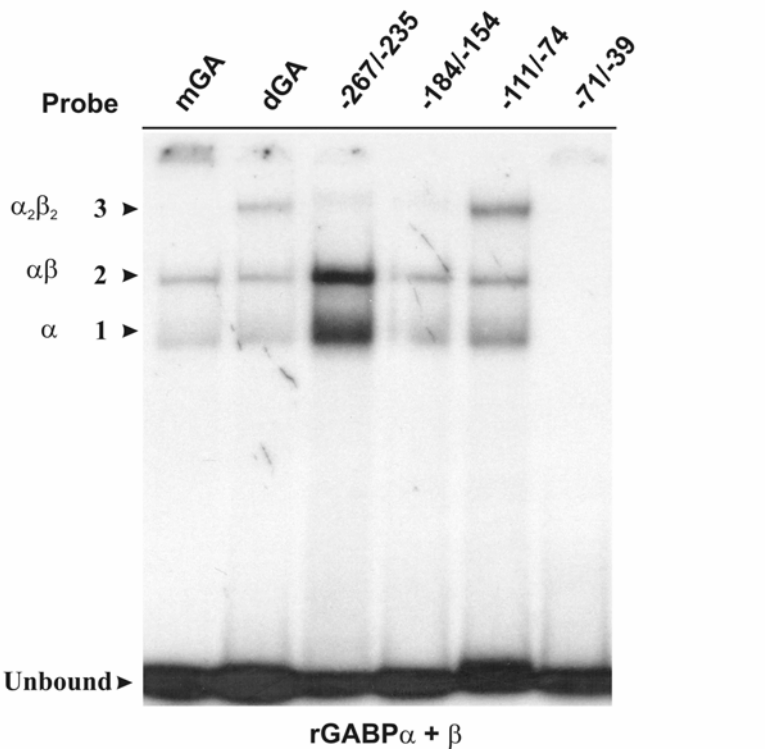

Luciferase activity (%wt)
20
60
100
YY1/GA1ko
YY1/GA2ko
YY1/GA3ko
YY1/GA4ko
YY1/Sp1ko
Sp1/GA1ko
Sp1/GA2ko
Sp1/GA3ko
Sp1/GA4ko

20% to 40% of wild-type activity) (Fig. 15). When the GA1 site is mutated in concert with the YY1 and Sp1/3 binding sites, there was a greater loss of promoter activity with only 18-42% of the wild type expression levels remaining (Fig 16). In the double mutants with intact GA1 sites, there was much less of an effect on the expression levels from the promoter (about 40% to 80% of the wild-type expression). In the double mutants lacking the YY1 site, the closer the GA mutation is to the YY1 site the greater the effect on expression (Fig. 16). These effects implicate an important role for YY1 and GA1 sites in transcription regulation of the CF6 promoter and illustrating as with the mGABPα promoter that the GABP binding sites are functionally redundant.

Identification of the factors binding to the mGABPα/CF6 bi-directional promoter.

The transcription factor binding sites that were identified as potential regulators of the mGABPα/CF6 bi-directional promoter were investigated by EMSA utilizing recombinant GABP (rGABP) and nuclear extracts from $A9_{2L}$ and 3T3-L1 fibroblasts. The six transcription factor-binding site were analyzed for binding using four synthetic probes (Fig 7). The –267/-235 probe contained a single GABP binding site (GA4) and the Sp1/3 binding site. The –184/-154 probe contained only a single GABP binding site (GA3). The –111/-74 probe contained the remaining two GABP binding sites (GA1 and GA2). The –71/-39 probe contained the binding site for YY1. The GA1 and GA2 binding sites are closely spaced within the promoter, inverted from each other, and could potentially promote the assembly of a heterotetramer complex on the promoter (Fig 7). Recombinant GABP had been previously shown to bind to probes containing both a

Figure 17. EMSA analysis of the ability of rGABP to bind the probes of the mGABPα/CF6 bi-directional promoter region and the control probes. The monomer GABP probe had been previously shown to be bound by GABPα and GABPαβ complexes. The dimmer GABP probe had previously been shown to be bound by GABPα, GABPαβ, and GABP$\alpha_2\beta_2$ complexes. Three complexes form on the –111/-74 probe as there are two GABP binding sites. The –267/-235 and –184/-154 probes are bound by two complexes. The –71/-39 probe is not bound by rGABP.

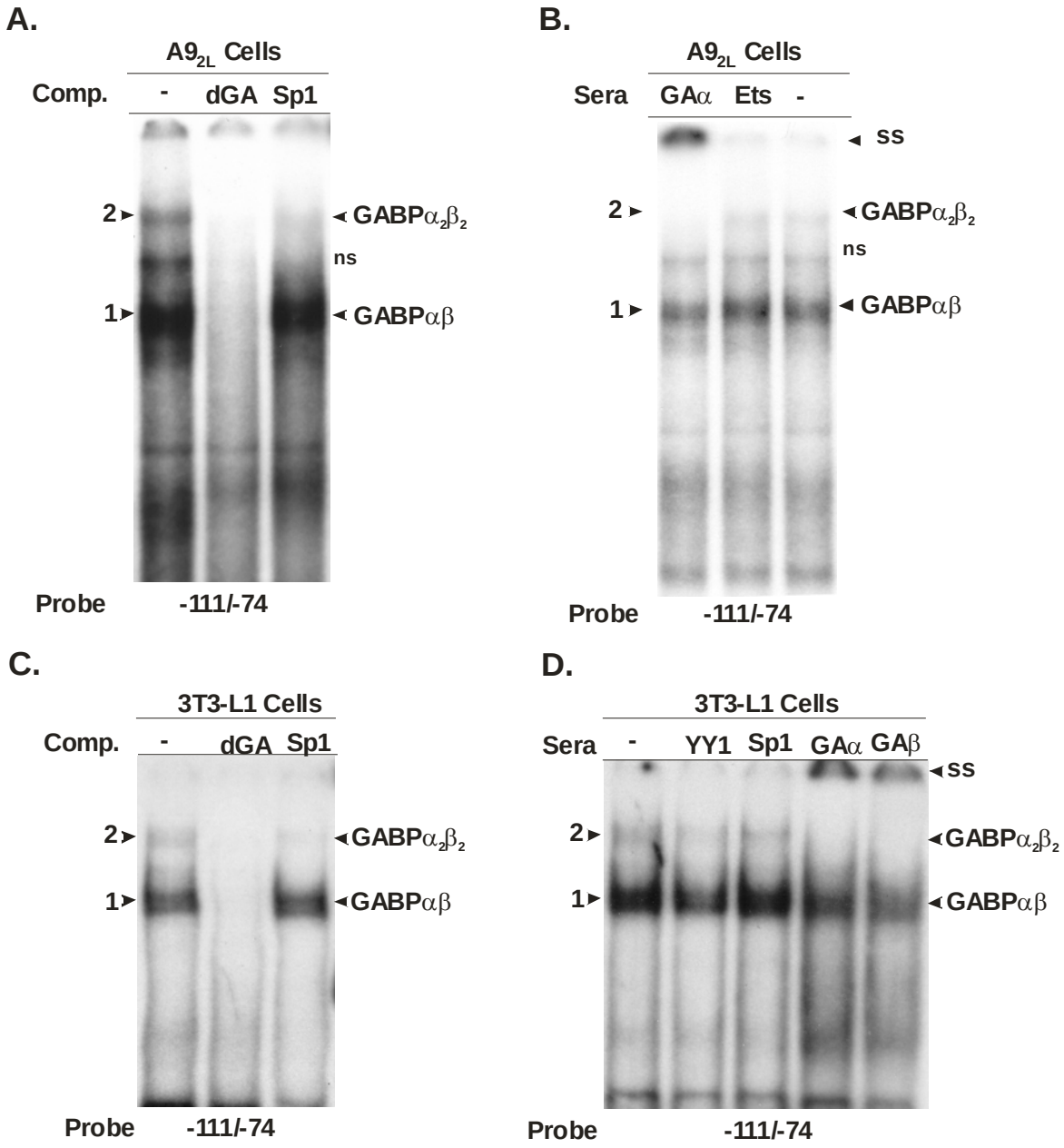

Probe
mGA
dGA
-267/-235
-184/-154
-111/-74
-71/-39
α2β2 3 ►
αβ 2 ►
α 1 ►
Unbound ►
rGABPα + β

single GABP binding site (mGA) as GABPα and GABPαβ heterodimer. When a probe contains two GABP binding sites (dGA), there are three complexes that may form on the probe: the α monomer, the αβ heterodimer, and the GABP$\alpha_2\beta_2$ heterotetramer (Chinenov et al., 2000b). Recombinant GABP was able to bind the three probes containing GABP binding sites. The results were similar to what had previously been reported (Chinenov et al., 2000b). The probes that contained only a single GABP binding site (the GA3 and GA4 probes) were bound by GABPα monomer and the GABPαβ heterodimer. The –111/-74 probe containing the two GABP binding sites was bound by GABPα monomer, the GABPαβ complex, and GABP$\alpha_2\beta_2$ complex, demonstrating the ability of the GABP$\alpha_2\beta_2$ complex to assemble on the probe containing the GA1/2 binding sites (Fig. 17). The –267/-235 probe was bound tightly by the rGABP as both the GABPα monomer and the GABPαβ complex. The –184/-154 probe was bound only weakly by the rGABPα and rGABPαβ complexes (Fig. 17) and did not form any complexes with nuclear extracts from either $A9_{2L}$ or 3T3-L1 cells (data not shown). In addition to the YY1 site, the –71/-39 probe contains a potential GABP binding site, but the –71/-39 probe was not bound by rGABP, indicating GABP does not bind this region of the promoter.

GABP in mouse cell nuclear extracts binds the –111/-74 probe corresponding to the mGABPα/CF6 bi-directional promoter.

All three GABP complexes bound the potential GABP binding sites in the –111/-74 probe when rGABP was examined. EMSA was performed to determine that GABP from $A9_{2L}$ and 3T3-L1 cell nuclear extracts does in fact bind the mGABPα/CF6 bi-

Figure 18. Electrophoretic mobility shift assays analyzing the –111/-74 region of the mGABPα/CF6 bi-directional promoter. A and C) Competition assay in which the binding activity of GABP to the promoter is examined in $A9_{2L}$ (A) and 3T3-L1 (C) cells utilizing excess probe containing two GABP binding sites (dGA) or a Sp1 binding site. B and D). Supershift assays in which antisera against transcriptions factors were added and the complexes that were supershifted were examined.


A.
A9$_{2L}$ Cells
Comp.
-
dGA
Sp1
2
1
GABPα$_2$β$_2$
ns
GABPαβ
Probe
-111/-74
B.
A9$_{2L}$ Cells
Sera
GAα
Ets
-
ss
2
1
GABPα$_2$β$_2$
ns
GABPαβ
Probe
-111/-74
C.
3T3-L1 Cells
Comp.
-
dGA
Sp1
2
1
GABPα$_2$β$_2$
GABPαβ
Probe
-111/-74
D.
3T3-L1 Cells
Sera
-
YY1
Sp1
GAα
GAβ
ss
2
1
GABPα$_2$β$_2$
GABPαβ
Probe
-111/-74

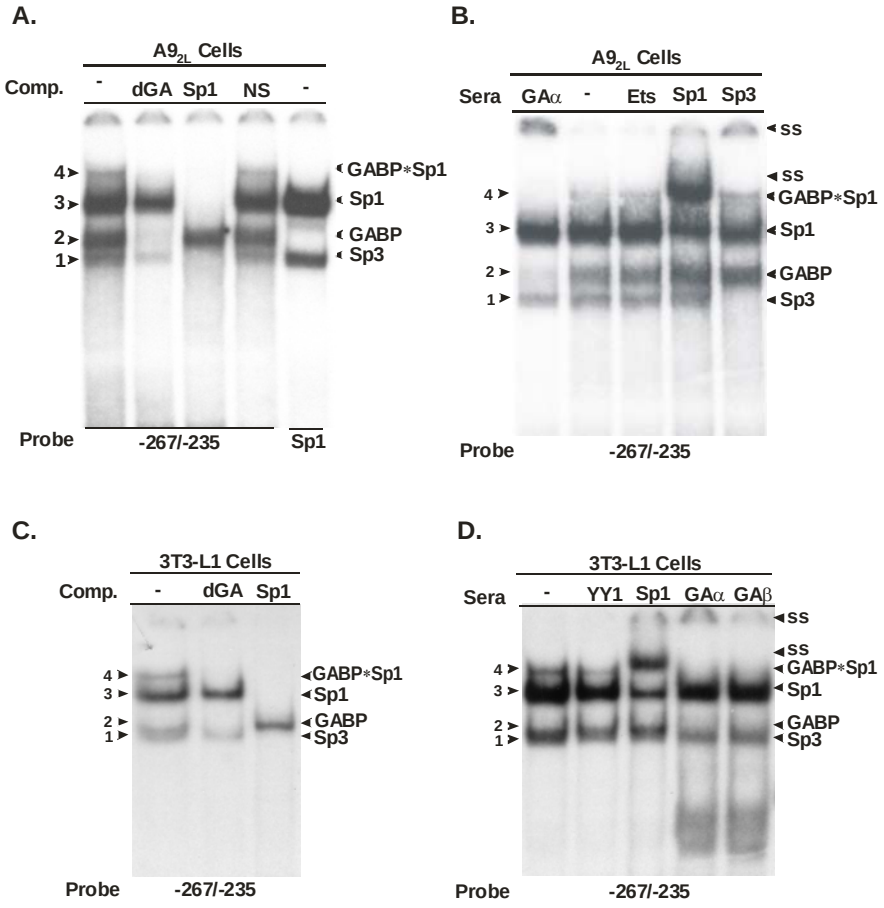
directional promoter (Fig 18). The –111/-74 probe bound both the heterodimer and heterotetramer complexes in nuclear extracts from both $A9_{2L}$ and 3T3-L1 cells, with no free GABPα complex apparent. Competition assays with a 500-fold molar excess of unlabeled dGA oligonucleotides prevented both GABP complexes from forming on the promoter with nuclear extracts from both $A9_{2L}$ and 3T3-L1 cells. Unlabeled oligonucleotides specific for Sp1 were unable to compete for the factor binding and prevent the GABP complexes from forming on the labeled probe (Fig. 18A & C). With both $A9_{2L}$ and 3T3-L1 cell nuclear extracts the heterodimer complex formation was reduced and resulted in a supershifted complex that remained in the well of the gel when either GABPα or GABPβ specific antiserum was used (Fig. 18B & D). The GABP heterotetramer complex was completely inhibited from binding the probe. The incomplete inhibition of the GABP heterodimer formation is likely due to insufficient antiserum in the experiment. There is also the possibility that another ETS-domain protein could be co-migrating with the GABP heterodimer but since Ets-1/Ets-2 specific antisera had no effect on the formation of the complex in $A9_{2L}$ nuclear extracts, it is likely that complex is GABP heterodimer. The second complex formed on the probe by $A9_{2L}$ and 3T3-L1 cell nuclear extracts is consistent with GABP heterotetramer as the complex is abolished with either GABPα or GABPβ specific antisera in 3T3-L1 cells.

Sp1, Sp3, and GABP proteins in mouse cell nuclear extracts bind the –267/-235 probe corresponding to the mGABPα/CF6 bi-directional promoter.

The factor binding to the GA4 and Sp1/3 sites was analyzed by EMSA, with the –267/-235 probe containing both sites. Four complexes bound the probe from nuclear extracts from both $A9_{2L}$ and 3T3-L1 cells. The smallest two of these complexes were not well resolved in the 3T3-L1 nuclear extracts, but competition assays support the presence of both complexes. In competition assays with excess unlabeled control dGA oligonucleotides, two of the complexes were competed away (complexes two and four) (Fig. 19A & C). Three of the complexes (complexes one, three, and four) could be competed away by an unlabeled probe containing a consensus Sp1 binding site. Only complex two was unaffected by the competition assay with the Sp1 probe in both $A9_{2L}$ and 3T3-L1 cells The formation of complex four was abolished by both the dGA and Sp1 concensus unlabeled nucleotides. These results are consistent with the complexes being identified as follows: complex one as Sp1 or Sp3, complex two as GABP, complex three as Sp1 or Sp3, and complex four as a ternary complex of Sp1 or Sp3 and GABP.

The difference between complex one and two can be seen in the 3T3-L1 cell nuclear extracts by comparing the lanes with dGA and Sp1 competitor DNAs (Fig. 19C). The DNA complexes that form on the –267/-235 probe were unaffected by preimmune serum and YY1 specific serum (Fig 19B & D), confirming that YY1 does not bind the promoter in this region. In both $A9_{2L}$ and 3T3-L1 cell nuclear extracts, complex three and complex four were inhibited or supershifted by the presence of Sp1-specific antisera, indicating that complex three is Sp1 and complex four contains Sp1. In $A9_{2L}$ cell

Figure 19. Analysis of the –267/-235 region of the mGABPα/CF6 bi-directional promoter. A and C) Competition assays in which the dGA and Sp1 unlabeled probes were added in excess and binding of transcription factors was examined in both $A9_{2L}$ and 3T3-L1 cells. B and D). Supershift assay with antisera against specific transcription factors added.

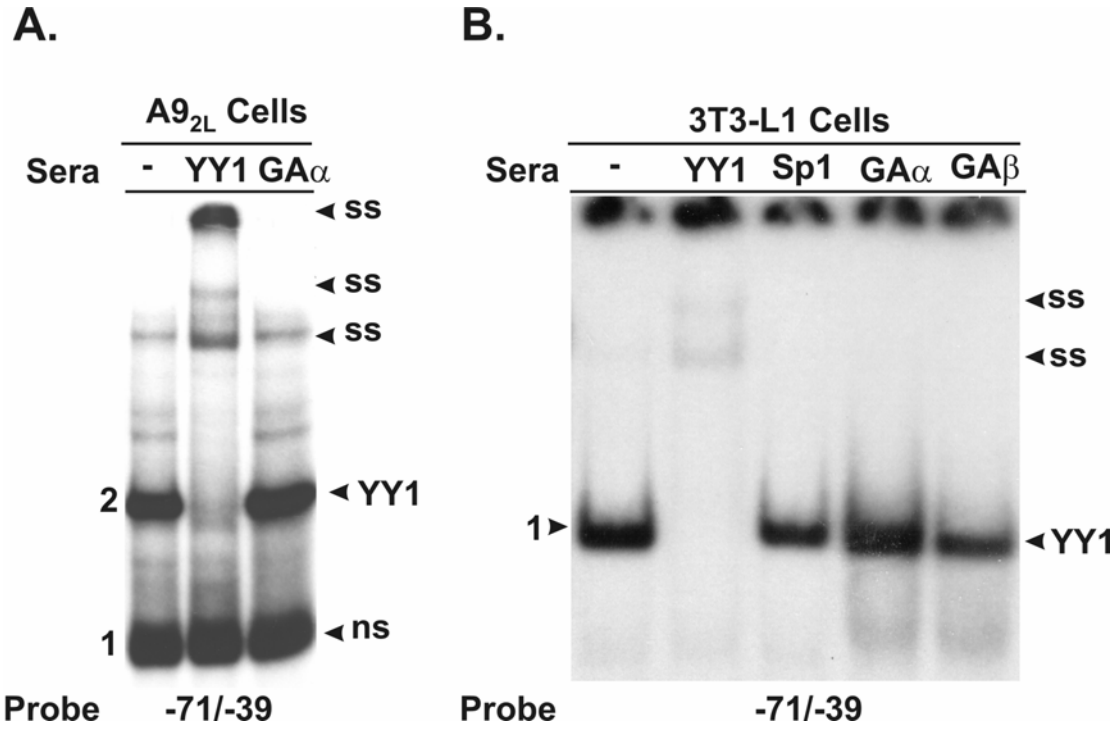

A.
A9$_{2L}$ Cells
Comp.
-
dGA
Sp1
NS
-
4
3
2
1
GABP*Sp1
Sp1
GABP
Sp3
Probe
-267/-235
Sp1
B.
A9$_{2L}$ Cells
Sera
GAα
-
Ets
Sp1
Sp3
ss
ss
GABP*Sp1
Sp1
GABP
Sp3
Probe
-267/-235
C.
3T3-L1 Cells
Comp.
-
dGA
Sp1
GABP*Sp1
Sp1
GABP
Sp3
Probe
-267/-235
D.
3T3-L1 Cells
Sera
-
YY1
Sp1
GAα
GAβ
ss
ss
GABP*Sp1
Sp1
GABP
Sp3
Probe
-267/-235

nuclear extracts, only complex one was inhibited or supershifted by antiserum specific to Sp3 (Fig. 19B), indicating the only complex one contains Sp3. In nuclear extracts from $A9_{2L}$ and 3T3-L1 cells, both complex two and four were able to be either inhibited or supershifted by the presence of antiserum specific for GABPα (Fig. 19B & D). Ets-1/Ets-2 antiserum had no effect on the any of the complexes formed from the $A9_{2L}$ cell nuclear extracts. These results confirm that complex two is GABP and complex four is a ternary complex of Sp1 and GABP.

YY1 in mouse cell nuclear extracts bind the –71/-39 probe corresponding to the mGABPα/CF6 bi-directional promoter.

The –71/-39 probe contains the consensus YY1 binding site and one potential binding site for GABP. Two complexes bind to the probe in the $A9_{2L}$ cell nuclear extracts, indicating the possibility that both GABP and YY1 bind to the probe. Antiserum specific for YY1 demonstrates that only complex two can be inhibited and supershifted in $A9_{2L}$ cells. In the nuclear extracts from the 3T3-L1 cells only one complex is present in the EMSA. The major complex is completely abolished by antiserum against YY1, and two supershifted complexes are produced (Fig. 20). Antiserum specific for Sp1, GABPα and GABPβ had no effect on the binding of the probe in either of the nuclear extracts. The second complex in the nuclear extracts from the $A9_{2L}$ cells is likely due to nonspecific binding. Similarly, antiserum specific for Sp1, GABPα, and GABPβ showed no effect on the binding of the probe in 3T3-L1 cells (Fig. 20). These results confirm YY1 from mouse cell nuclear extracts bind the mGABPα/CF6 bi-directional promoter.

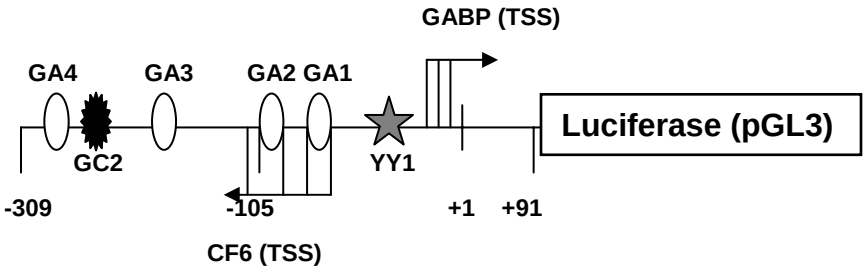
Figure 20. Analysis of the –71/-39 region of the mGABPα/CF6 bi-directional promoter. A) Supershift assays utilizing antisera against YY1 and GABPα in $A9_{2L}$ cells. B). Supershift assays utilizing antisera against YY1, Sp1, GABPα, and GABPβ in 3T3-L1 cells.


A.
A9$_{2L}$ Cells
Sera
-
YY1
GAα
ss
ss
ss
2
YY1
1
ns
Probe
-71/-39
B.
3T3-L1 Cells
Sera
-
YY1
Sp1
GAα
GAβ
ss
ss
1
YY1
Probe
-71/-39

Determination of the effect of oxidative stress on the expression from the mGABPα/CF6 bi-directional promoter.

The mGABPα/CF6 bi-directional promoter is regulated, at least in part, by GABP (as demonstrated by the mutagenesis data presented above), a redox sensitive transcription factor. Because GABP binding sites are present in many of the promoters of different components of the ETC and GABP is sensitive to the redox state of the cell (Martin et al., 1996; Chinenov et al., 1998), the expression levels of luciferase from the mGABPα/CF6 bi-directional promoter under oxidative stress conditions were examined as well. The oxidizing agent chosen was H_2O_2, due to its availability and its direct, whole-cell oxidizing effects. Based on published reports, the greatest effects in redox response, without starting the apoptotic pathways, are observed when cells are treated with concentrations of H_2O_2 ranging from 150 μM to 250 μM (Davies, 1999). The response time depended on whether the gene was involved with early response, middle response, or late response to addition of the oxidizing agent. $A9_{2L}$ cells were treated with H_2O_2, then harvested over a time course, and the greatest effect on the expression of luciferase was determined to be at the four hour time point (Fig 21). Several concentrations of H_2O_2 were analyzed for the effect on expression from the mGABPα/CF6 bi-directional promoter. The greatest effect on the promoter occurred when the cells were treated with 150 μM H_2O_2 (Fig 22). After analyzing several experiments in which the cells were treated with 150 μM H_2O_2, there was no real effect on expression from the mGABPα/CF6 bi-directional promoter when H_2O_2 was used to treat the cells. The mGABPα/CF6 bi-directional promoter showed no effect from

Figure 21. A time course experiment to determine at what time the greatest amount of luciferase will be expressed in response to treatment of cells with 150 μM H_2O_2. The wild-type core promoter region of the mGABPα promoter was used for these experiments. Based on this experiment, it was determined that four hours after treatment was the optimal time to measure the response to the treatment with H_2O_2.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

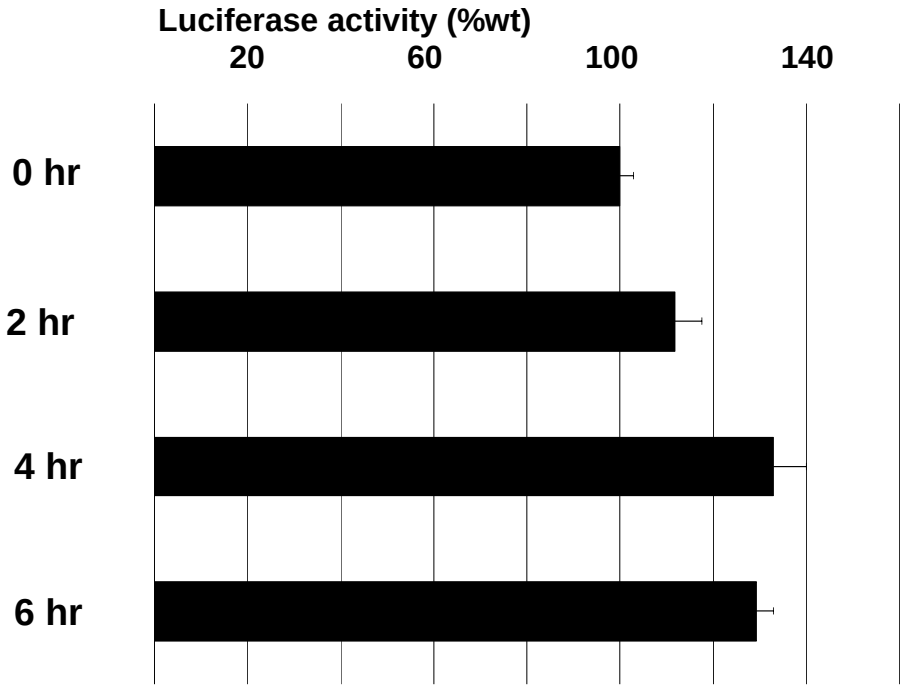
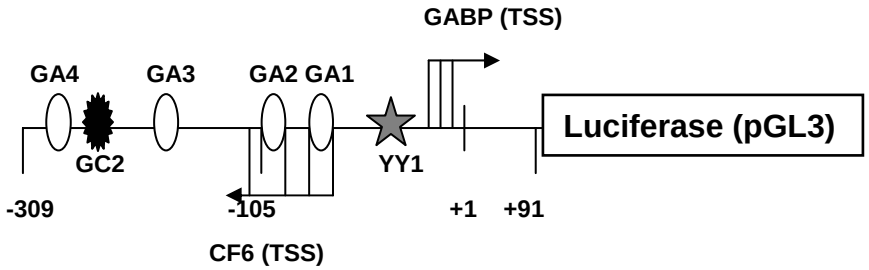

Luciferase activity (%wt)
20
60
100
140
0 hr
2 hr
4 hr
6 hr

Figure 22. The effect of varying concentrations of H_2O_2 on the mGABPα promoter was measured by determining the normalized luciferase expression. The cells were treated with H_2O_2 for four hours as had previously been determined as the appropriate treatment time. Cells were treated with different concentrations of H_2O_2 and the concentration that appears to have the greatest effect on the mGABPα promoter is 150 μM. The wild-type mGABPα promoter was utilized for these experiments.


GABP (TSS)
GA4
GA3
GA2 GA1
Luciferase (pGL3)
GC2
YY1
-309
-105
+1
+91
CF6 (TSS)

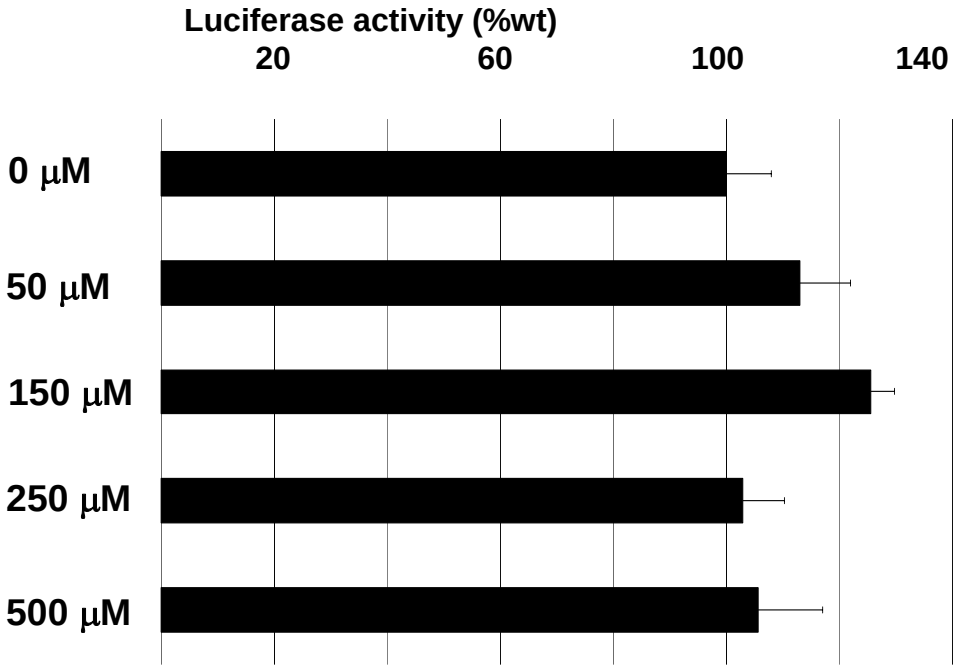
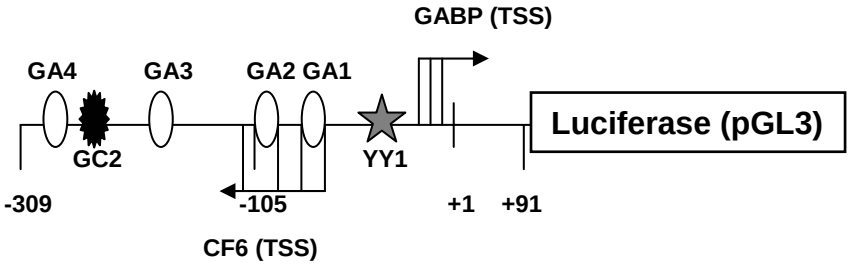
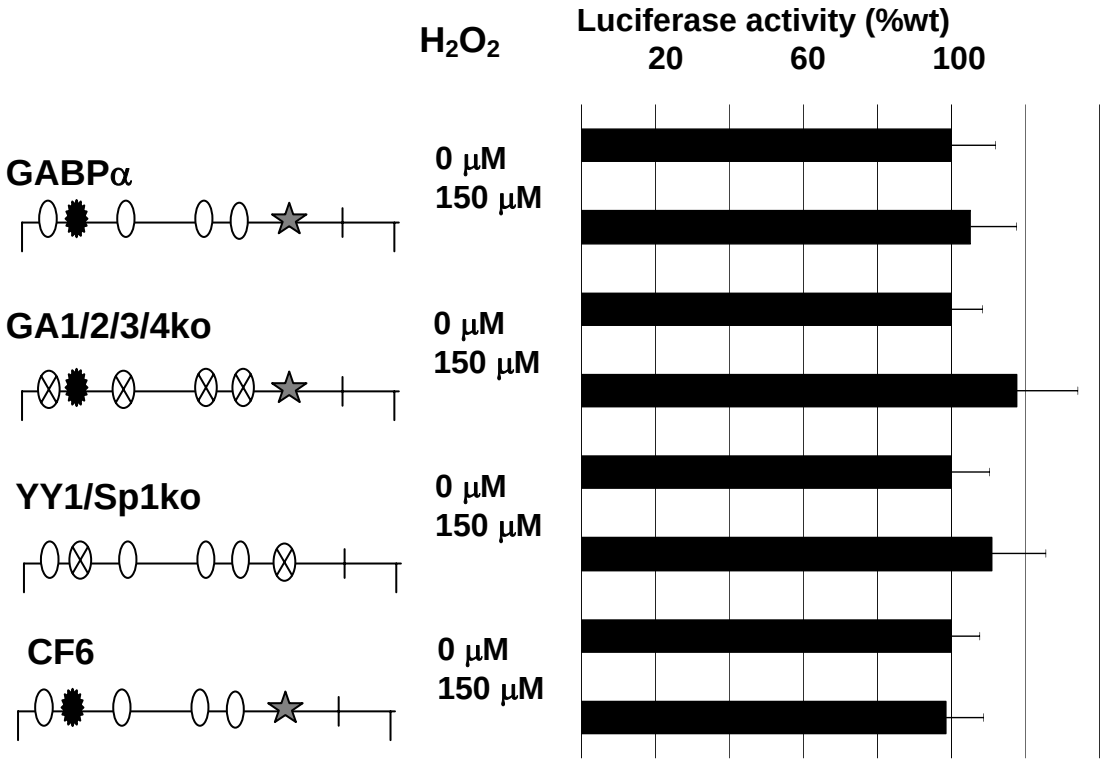

Luciferase activity (%wt)
20
60
100
140
0 μM
50 μM
150 μM
250 μM
500 μM

Figure 23. The effect of oxidative stress on the expression from the mGABPα/CF6 bi-directional promoter was examined by treatment with H_2O_2. The effect of the treatment with H_2O_2 was surprisingly nonexistent. Any difference in expression is within the error for the experiment, showing no significant difference in expression cause by treatment with H_2O_2.


GABP (TSS)
GA4
GA3
GA2 GA1
GC2
YY1
Luciferase (pGL3)
-309
-105
+1
+91
CF6 (TSS)


H2O2
Luciferase activity (%wt)
20
60
100
GABPα
0 μM
150 μM
GA1/2/3/4ko
0 μM
150 μM
YY1/Sp1ko
0 μM
150 μM
CF6
0 μM
150 μM

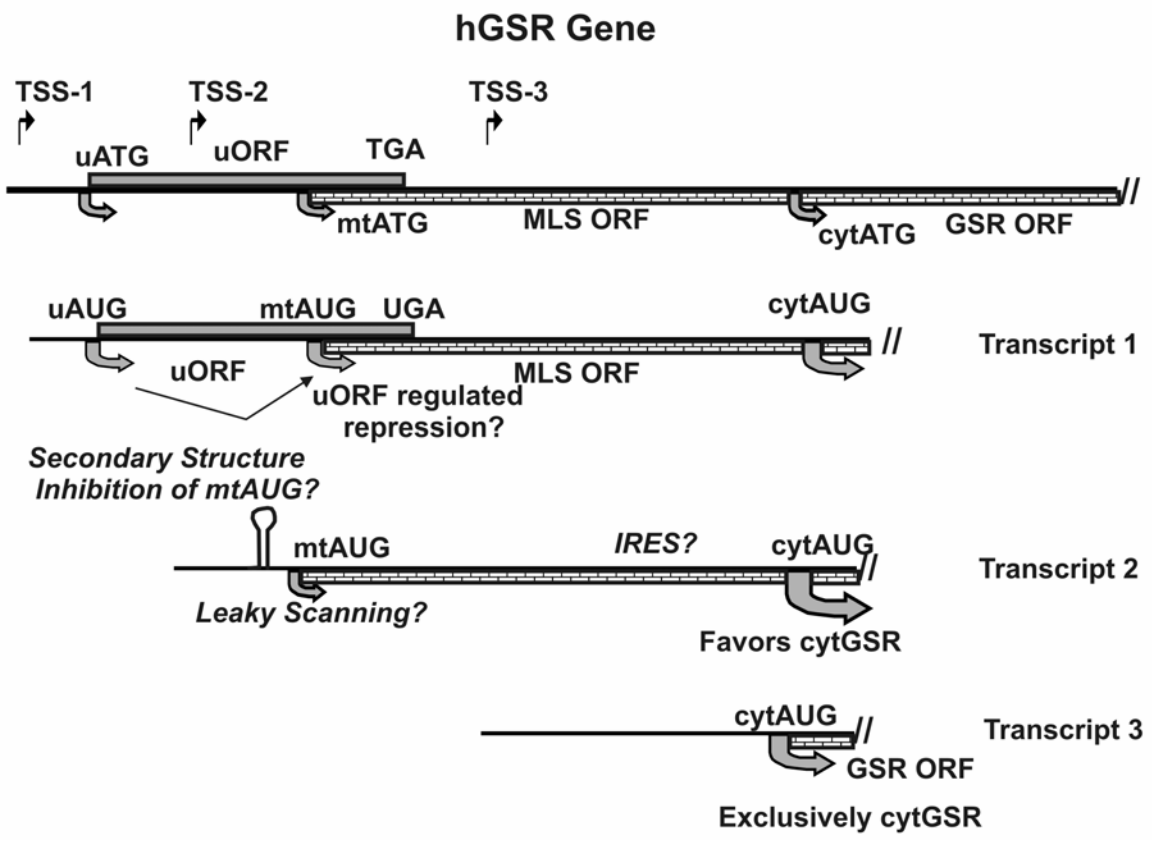
addition of the oxidizing agent in the CF6 direction either (Fig 23). Likewise, treatment of select multiple mutants with H_2O_2 resulted in expression levels that were unchanged in the GABPα direction. Both the YY1/Sp1 and GA1/2/3/4 mutants were examined under oxidative stress conditions. Neither of these mutants showed a significant change in the level of expression when placed under oxidative stress (Fig 23).

Analysis of hGSR promoter

The hGSR promoter expresses three potential classes of mRNAs.

Both isoforms of GSR are expressed from the same gene, from two in-frame AUGs, in both mice and humans, differing only by a MLS that is cleaved once mtGSR has been transported into the mitochondria, so the active form of GSR is identical in the mitochondria and cytoplasm (Iozef et al., 2000; Kelner and Montoya, 2000; Tamura et al., 1997). A third upstream AUG that is out of frame with the mt- and cytAUGs can potentially initiate translation, preventing translation from the mtAUG. The stop codon for the upstream open reading frame is within the MLS, potentially allowing the ribosome to stop translation and restart at the cytAUG (Hemmings-Mieszczak et al., 2000). Reported cDNAs and ESTs from GSR have 5'termini that are located both upstream and downstream of the mtAUG. Only one transcript has been reported initiating upstream of the uAUG, supporting the presence of three classes of hGSR transcripts (Fig 24). Transcripts one and two are capable of directing translation from either the mtAUG or the cytAUG, while transcript three can only direct translation from the cytAUG. Based on this model, expression of the two isoforms of GSR can be accomplished through regulation of the transcriptional start sites and translational efficiencies of the AUGs.

Figure 24. Three classes of transcripts have been detected from the hGSR promoter. The first transcript initiated upstream of the uAUG and potentially encode both mtGSR and cytGSR. Transcript two, which initiates upstream of the mtAUG but downstream of the uAUG, encodes both mtGSR and cytGSR. This transcript also lacks the uORF to potentially modulate translation from the mtAUG. Transcript three initiates between the mtAUG and the cytAUG and encodes only cytGSR. Secondary structure of the mRNA, the presence of a 5'-cap, and mRNA binding proteins are all potential modulators of AUG selection from the first two transcripts. The three potential translation start sites all are in different sequence contexts. The uAUG is in a poor context for translation initiation. The mtAUG is in an adequate context for translation initiation. The cytAUG is in the consensus context for translation initiation.


hGSR Gene
TSS-1
TSS-2
TSS-3
uATG
uORF
TGA
mtATG
MLS ORF
cytATG
GSR ORF
uAUG
mtAUG
UGA
cytAUG
Transcript 1
uORF
MLS ORF
uORF regulated repression?
Secondary Structure Inhibition of mtAUG?
mtAUG
IRES?
cytAUG
Transcript 2
Leaky Scanning?
Favors cytGSR
cytAUG
Transcript 3
GSR ORF
Exclusively cytGSR

Identification of transcriptional start sites of the hGSR promoter by 5'RACE.

The 5'-termini of the hGSR mRNAs were mapped by 5'-rapid amplification of cDNA ends (RACE). HeLa cell cDNA library from Invitrogen (Carlsbad, CA) was made RACE ready by the addition of a 5'-terminal GeneRacer RNA oligonucleotide that is ligated to the capped mRNA (Fig 25) (Frohman et al., 1998). The GeneRacer oligonucleotide was then used as the target of the 5'-primer, and a 3'-primer was targeted to the junction between the second and third exons of hGSR in PCR. The PCR product was then resolved on an agarose gel, revealing two PCR products of interest on the gel (Fig 25). There are other PCR products but there is not enough present to adequately purify and clone the product. The two PCR products were gel purified, cloned, and sequenced. The larger PCR product revealed four transcriptional start sites for the PCR product (-20, -49, -59, and –98, relative to the cytGSR ATG). All four of these PCR products revealed start sites downstream of the mtATG, corresponding to transcript three (Fig. 24). Only a single transcript was cloned from the smaller PCR product initiating from the -145 position, upstream of the mtATG, corresponding to transcript two (Fig. 24). These data would indicate at least a portion of the regulation to be at the transcriptional level. To confirm these results, the total GSR mRNA levels were measured by PCR. Three primers to the GSR mRNAs were constructed, one targeted to the mitochondrial leader sequence (P1), one targeted just downstream of the cytoplasmic start site (P2), and a third primer (P3) targeted to the junction between the second and third exons (Fig. 26). The PCR product from the reaction with P2 and P3 will determine the total GSR mRNA. The PCR product formed when using primers P1 and P3 will

Figure 25. 5’ RACE analysis of Hela cell cDNAs supports a two transcript model. Two populations of capped hGSR mRNAs are detected by 5’ RACE PCR. The more abundant population is represented by the transcripts that initiate downstream of the mtGSR AUG codon and encode only the cytGSR protein. The bottom panel shows the start sites of the different GSR transcripts identified by the 5’-RACE.

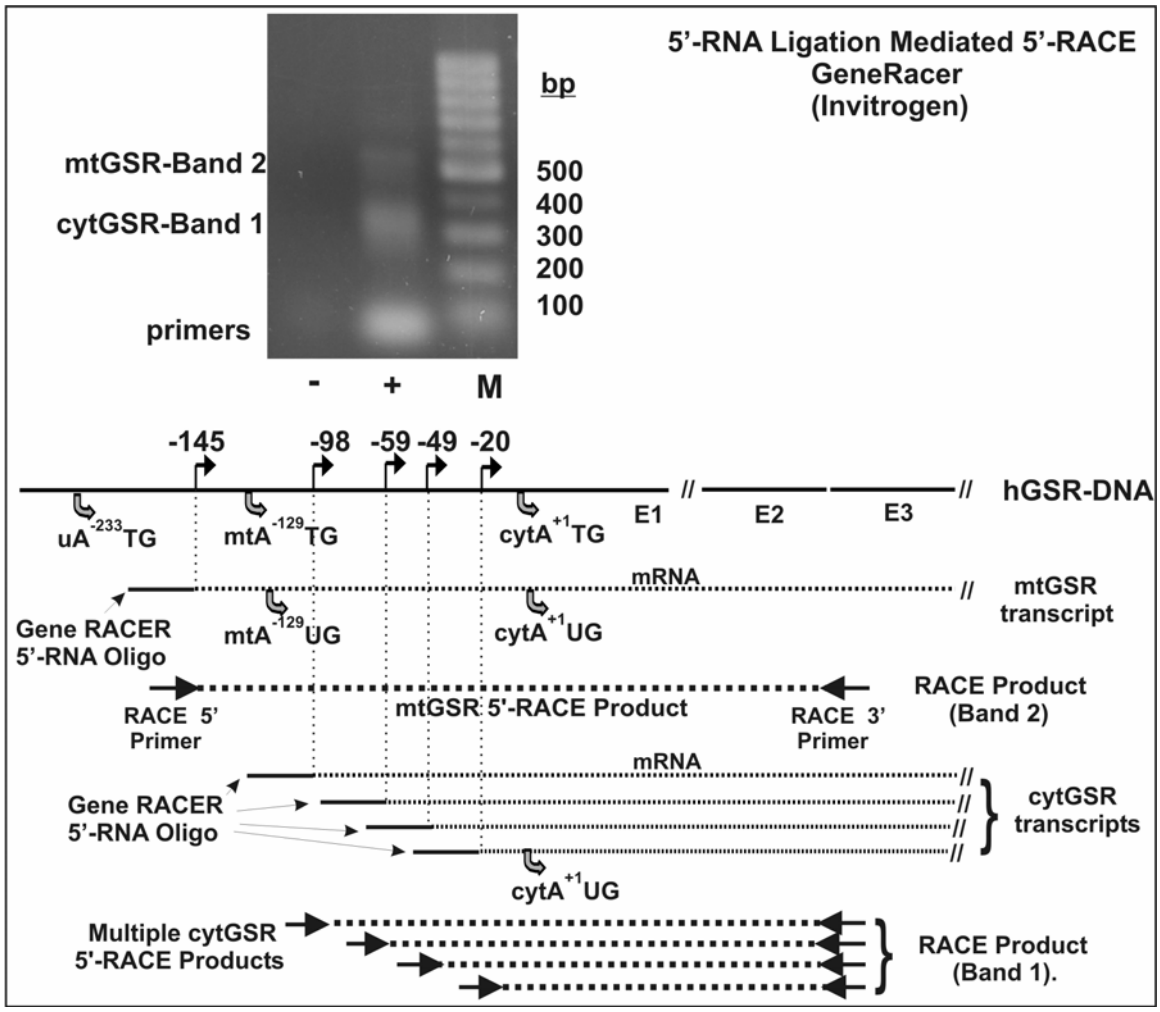
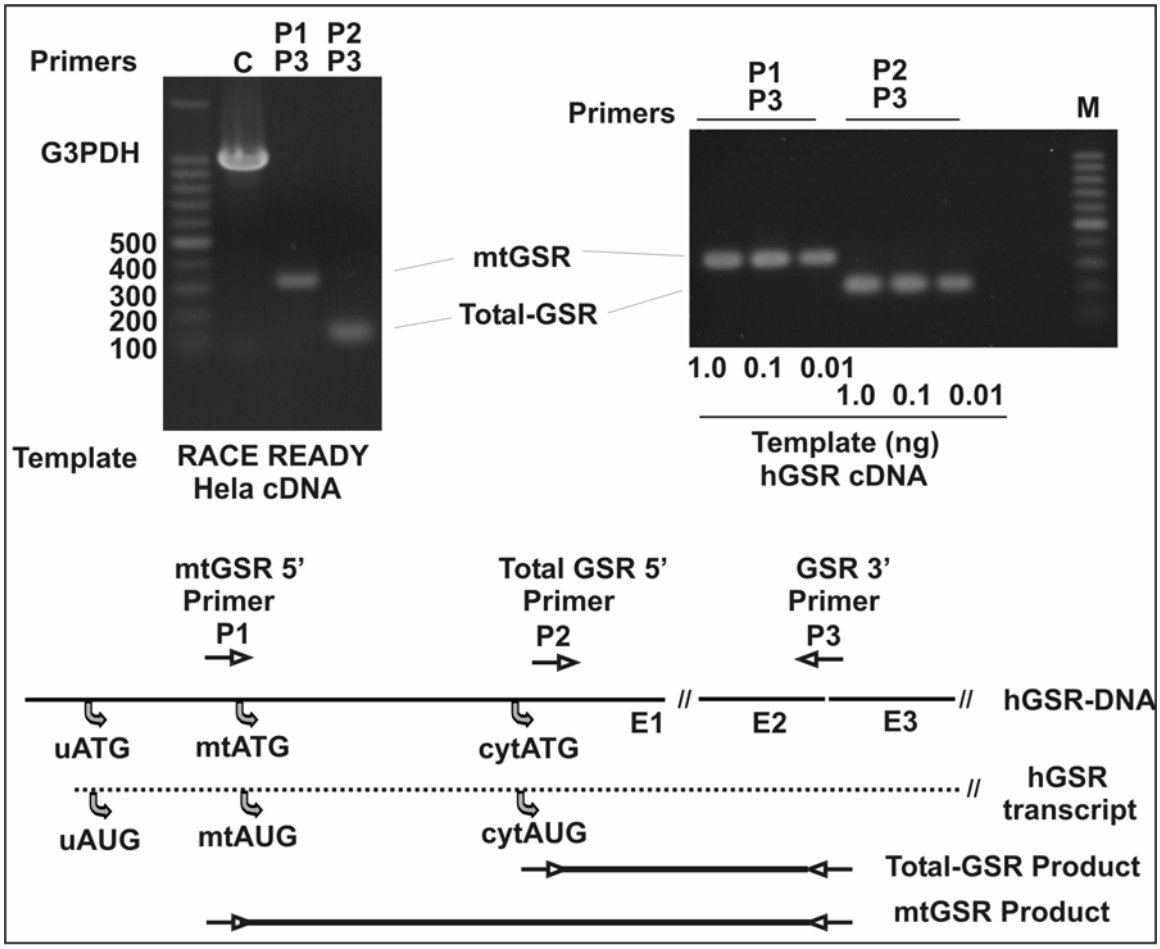

5'-RNA Ligation Mediated 5'-RACE
GeneRacer
(Invitrogen)
bp
mtGSR-Band 2
cytGSR-Band 1
primers
500
400
300
200
100
-
+
M
-145
-98
-59
-49
-20
hGSR-DNA
E1
E2
E3
uA-233TG
mtA-129TG
cytA+1TG
mRNA
mtGSR
transcript
Gene RACER
5'-RNA Oligo
mtA-129UG
cytA+1UG
RACE 5'
Primer
mtGSR 5'-RACE Product
RACE 3'
Primer
RACE Product
(Band 2)
mRNA
Gene RACER
5'-RNA Oligo
cytGSR
transcripts
cytA+1UG
Multiple cytGSR
5'-RACE Products
RACE Product
(Band 1).

Figure 26. PCR analysis of HeLa cell cDNAs detected identical amount of mtGSR and total GSR PCR products, suggesting the majority of the hGSR initiate upstream of the mtATG. PCR analysis of cloned hGSR cDNA produced identical amounts of PCR products indicating the PCR primers were equivalent in their amplification efficiency (right panel). The bottom panel indicate the design for the PCR.

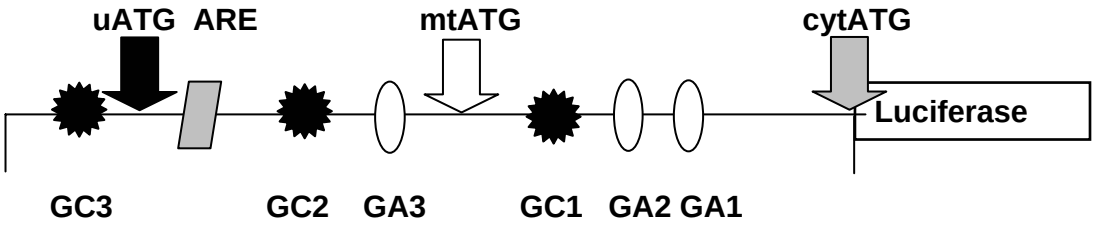

Primers
C
P1 P3
P2 P3
G3PDH
500
400
300
200
100
Template
RACE READY Hela cDNA
mtGSR
Total-GSR
Primers
P1 P3
P2 P3
M
1.0 0.1 0.01
1.0 0.1 0.01
Template (ng) hGSR cDNA
mtGSR 5' Primer P1
Total GSR 5' Primer P2
GSR 3' Primer P3
E1
E2
E3
hGSR-DNA
uATG
mtATG
cytATG
uAUG
mtAUG
cytAUG
hGSR transcript
Total-GSR Product
mtGSR Product

determine the GSR mRNAs that are initiated upstream of the mtATG. Based on the 5'RACE results, it was expected that the PCR product from the reaction with primers P2 and P3 would produce a much greater amount of product than the reaction with the P1 and P3 primers. However, it was seen that the two PCR reactions produced indistinguishable amounts of product (Fig 26). The result is not an artifact of having noncomparable PCR primer as the primers were shown to be identical (Fig 26). This result would seem to indicate that nearly all the transcript is initiated from upstream of the mtATG.

Identification of the functional transcription factor binding sites in the hGSR promoter.

The hGSR promoter presents a potentially difficult mechanism of regulation to identify. The promoter region examined contains several transcription factor-binding sites, including three GC rich regions that could serve as potential Sp1/3 binding sites, three potential GABP binding sites, and an ARE. The transcriptional regulation from the hGSR promoter has been studied by examining the expression levels from the promoter in which the transcription factor binding sites have been mutated (Fig 27). When the GA2 site is mutated, the expression level of luciferase from the promoter decreases to about 40% of the wild-type expression level. The GC1 site mutation shows a similar decrease in the expression level to about 40% of the wild type activity. The GC2 site mutation also shows a decrease in the expression level but only to about 60% of the wild-type expression levels. The remaining point mutations, GC3, GA1, GA3, and ARE, all express

Figure 27. The effect of single site mutations of putative factor binding sites on hGSR promoter activities. Each of the putative factor binding sites illustrated in the schematic of the hGSR promoter was mutated and the effect on luciferase expression measured in HEK293T kidney cells. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt hGSR promoter.


uATG
ARE
mtATG
cytATG
Luciferase
GC3
GC2
GA3
GC1
GA2
GA1

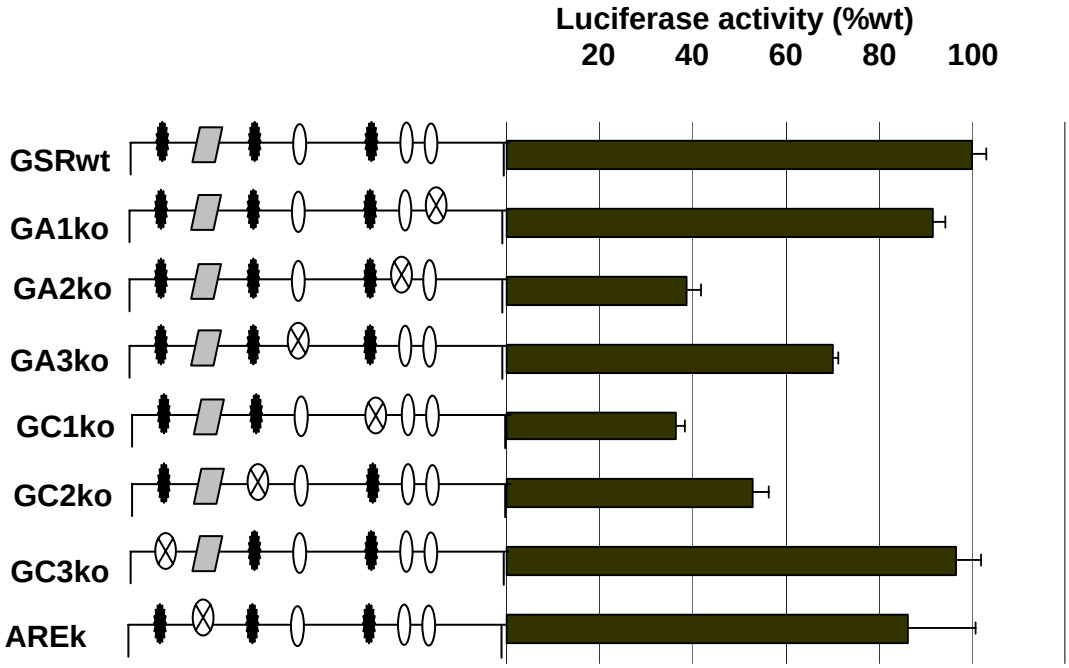
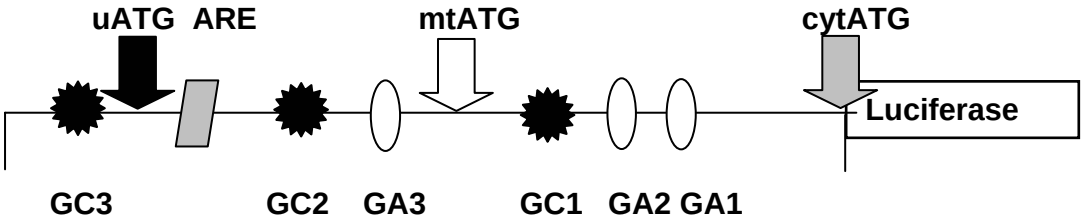
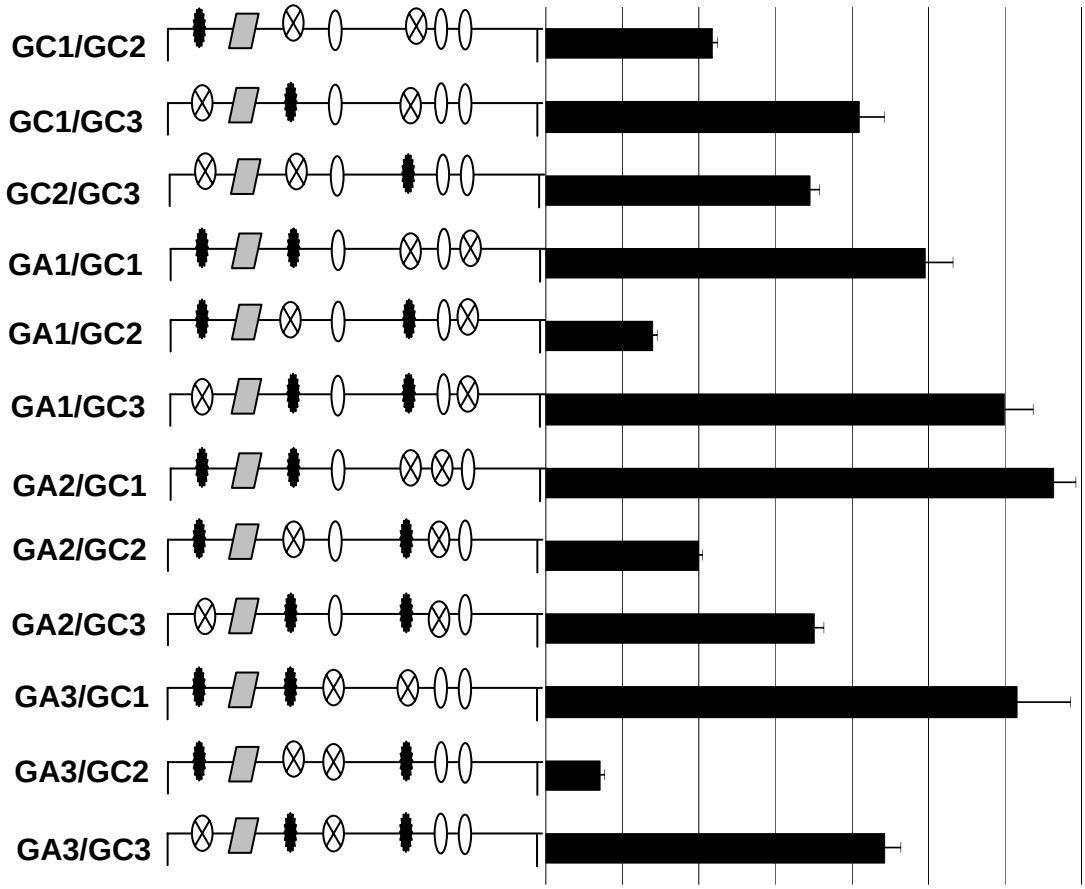

Luciferase activity (%wt)
20
40
60
80
100
GSRwt
GA1ko
GA2ko
GA3ko
GC1ko
GC2ko
GC3ko
AREk

Figure 28. The effect of double site mutations of putative factor binding sites on hGSR promoter activities. Each of the putative factor binding sites illustrated in the schematic of the hGSR promoter was mutated and the effect on luciferase expression measured in HEK293T kidney cells. Circles containing an X denote mutant factor binding sites. Luciferase activity was expressed relative to the wt hGSR promoter.


uATG
ARE
mtATG
cytATG
Luciferase
GC3
GC2
GA3
GC1
GA2
GA1


GC1/GC2
GC1/GC3
GC2/GC3
GA1/GC1
GA1/GC2
GA1/GC3
GA2/GC1
GA2/GC2
GA2/GC3
GA3/GC1
GA3/GC2
GA3/GC3

luciferase at levels varying between about 70% and 100% of the wild-type expression level (Fig. 27). Interestingly, when the GA2/GC1 double mutant is made in the same promoter, instead of having an inhibitory effect on transcription, the level of expression of luciferase increases to about 130% of the wild type activity (Fig 28). This provides evidence that a complex may be formed by transcription factors binding at these two transcription factor binding sites. In the absence of one of these binding sites the complex does not form but the other transcription factor still binds the promoter in the single mutant acting as an inhibitor of transcription. When both transcription factor-binding sites are mutated, this potential inhibition is removed by the removal of the second transcription factor-binding site, and the expression from the promoter increases above the wild type activity.

The other major transcription factor-binding site that has a role in regulation of expression from the hGSR promoter is the GC2 binding site. As a single mutant the GC2 site mutation has expression levels that are about 60% of the wild type expression from the promoter. As part of any double mutants the GC2 mutation in combination with any other mutant showed decreased levels of luciferase expression than that site mutated individually (Fig 28). The lone exception to this was in the GC2/GC1 double mutant, when the expression level was about 45% of wild-type activity, between the expression levels of the two single mutants. The most significant reduction of expression occurred in the GA3/GC2 double mutant, which expresses luciferase at only 15% the level of the wild type promoter. The remaining GC2 double mutants all reduced expression levels to between 30% and 40% of the wild type expression levels (Fig. 28). These data implicate

the GC2 site as an important transcription factor binding site for activation from the hGSR promoter.

Identification of the transcription factors binding to the hGSR promoter.

Mutation of the transcription factor binding sites of the hGSR promoter shows that the GC2 and GA3 sites are the most important in the regulation of the expression from the promoter. The presence of this regulation indicates that transcription factors can bind to these sites. The identity of the transcription factors that bind to these sites was analyzed through EMSA. The GABP binding sites were all analyzed for the ability of rGABP to bind the promoter regions. Control probes containing one (mGA) and two (dGA) GABP binding sites were also examined as reference compounds. Only the GABPαβ complex bound the mGA probe, but the dGA probe was bound by both the GABPαβ and GABP$\alpha_2\beta_2$ complexes. There were four probes from the hGSR promoter that were examined. A probe containing each of the GABP binding sites individually was tested. The GA1 probe only bound the αβ heterodimer weakly and interestingly, the GA2 probe was not bound by rGABP even though the mutation of this binding site has a great effect on expression from the hGSR promoter. The GA1/2 probe was bound by both the GABPαβ and GABP$\alpha_2\beta_2$ complexes although only weakly in each case. The GA3 site was bound by both the GABPαβ and GABP$\alpha_2\beta_2$ complexes of rGABP (Fig 29), likely due to nonspecific binding of a second heterodimer, due to a large excess of rGABP in the experiment. The same probes were analyzed by the ability of GABP to bind these sites utilizing nuclear extracts from HeLa cells. In HeLa cells both the GA1 and GA2

Figure 29. The ability of GABP to bind to the hGSR promoter binding factor sites was examined by EMSA. Recombinant GABP was shown to bind to the GA3 site strongly and either weakly or not at all to the other potential GABP binding sites. GABPα from nuclear extracts from Hela cells binds each of probes, but the only probe that had the heterodimer bound was the GA3 site.

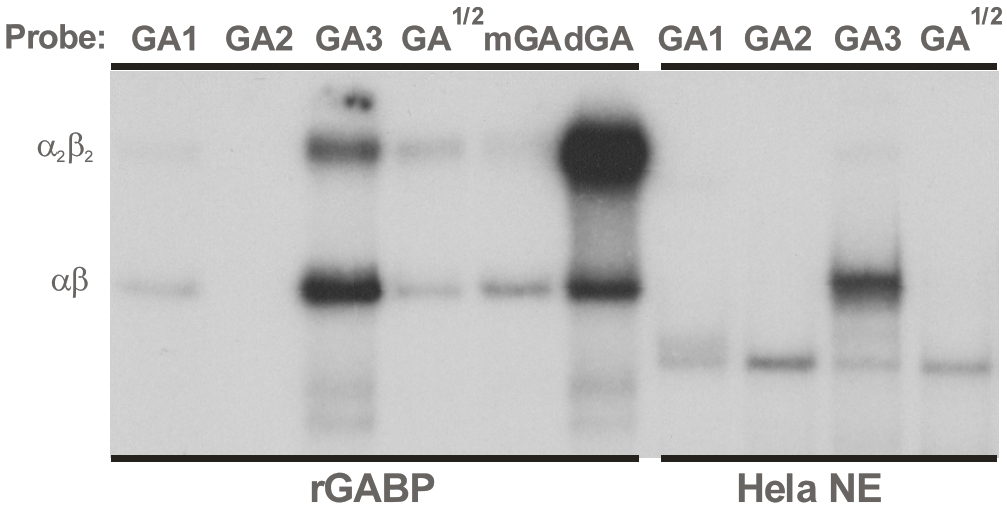
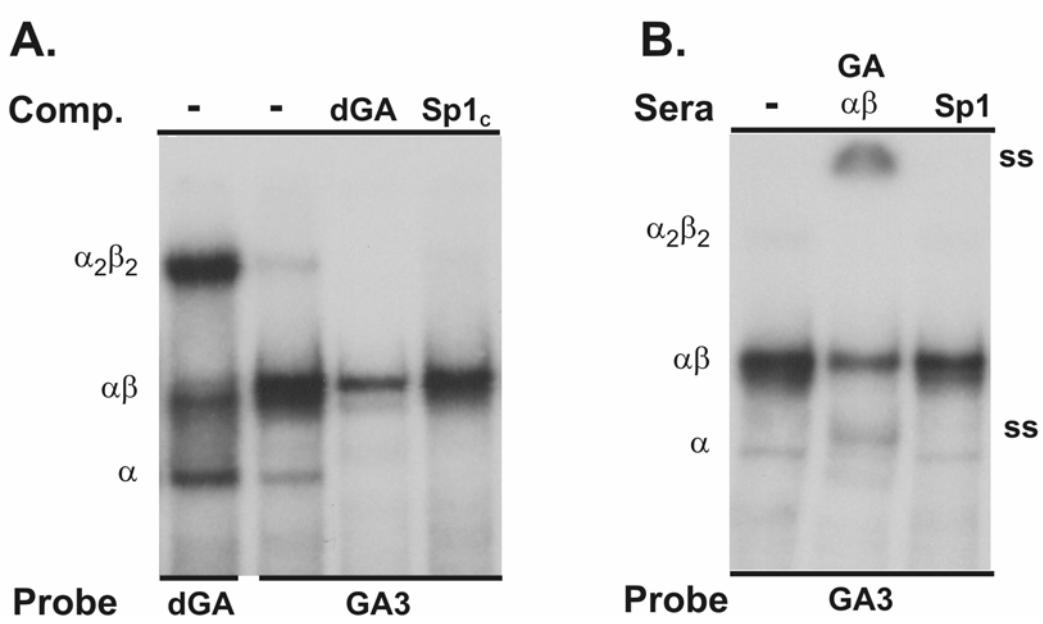

Probe: GA1 GA2 GA3 GA1/2 mGA dGA GA1 GA2 GA3 GA1/2
α2β2
αβ
rGABP
Hela NE

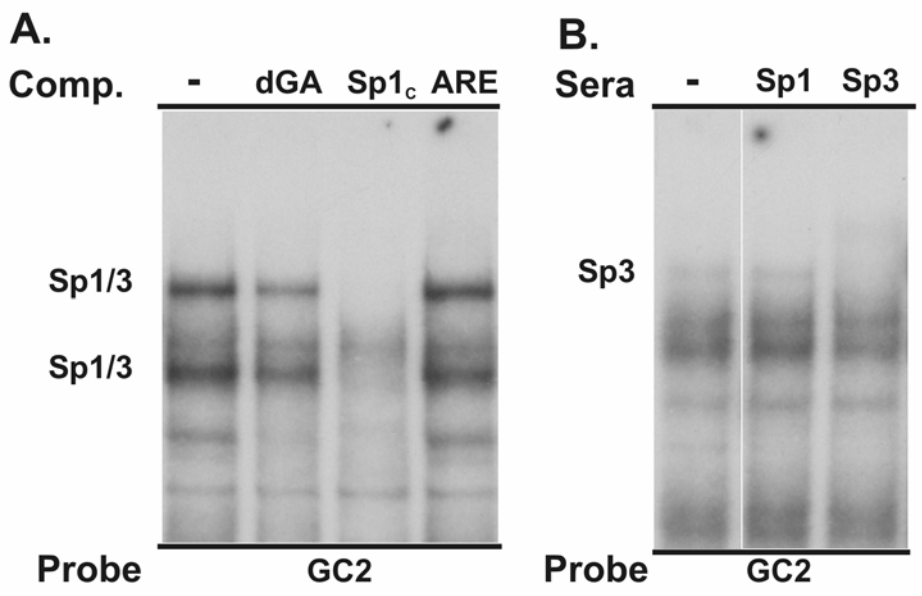
Figure 30. The GA3 probe was analyzed for the ability of transcription factors to bind to the promoter region. A). A competition assay was performed using the labeled GA3 probe and unlabeled dGA probe and Sp1 probe. B). Supershift assay were performed using antisera against both the α and β subunits of GABP (lane 2) and against Sp1 9 (lane 3).


A.
Comp.
-
-
dGA
Sp1c
α2β2
αβ
α
Probe
dGA
GA3
B.
GA
Sera
-
αβ
Sp1
ss
α2β2
αβ
α
ss
Probe
GA3

probes were bound by a complex smaller than the GABPαβ complex possibly the GABPα monomer complex. The same complex binds to the GA1/2 probe as well. The GA3 probe is bound by both the GABPα monomer complex and the GABPαβ complex (Fig 29). The GA3 site was examined more closely through competition and supershift assays because of the effect of the GA3/GC2 double mutant. In the competition assays binding of the GABPα and GABP$\alpha_2\beta_2$ complexes were completely eliminated. There was still some probe bound by a complex that had the same migration pattern as the GABPαβ complex, but the complex was greatly reduced compared the GA3 probe with no competing probes (Fig. 30). A probe containing an Sp1 consensus binding site also prevents binding of the weaker binding GABPα and GABP$\alpha_2\beta_2$ complexes but does not affect the binding of the GABPαβ complex to the GA3 probe (Fig 30), indicating a possible complex formed between Sp1 or Sp3 and GABP that migrates at the same place as GABP$\alpha_2\beta_2$ complex. In supershift assays, when antisera against GABPα and β were added to the assay, both the GABPα and GABPαβ complexes were supershifted. The GABPαβ complex was not completely shifted or inhibited, indicating that another factor possibly binds to the probe and co-migrates with GABPαβ complex. Antiserum against Sp1 had no affect on the migration pattern of the complexes bound to the GA3 probe (Fig 30), indicating that Sp1 is not a part of the complex formed on this probe.

The GC2 transcription factor-binding site also displays a regulatory role in expression from the hGSR promoter based on the data from the mutagenesis experiments. A probe containing the GC2 binding site was analyzed to determine which transcription factors bind to this region of the promoter (Fig. 8). The probe identifies several complexes that bind to the GC2 region of the promoter. Competition assays involving

Figure 31. Analysis of the ability of transcription factors to bind the GC2 site was examined by EMSA utilizing nuclear extracts from HeLa cells. A). Competition assays with unlabeled promoters reveal that several complexes can bind the GC2 site including a potential Sp1/Sp3 complex. B). Supershift assays with antisera against Sp1 and Sp3 reveal Sp3 as the transcription factor that binds the promoter.

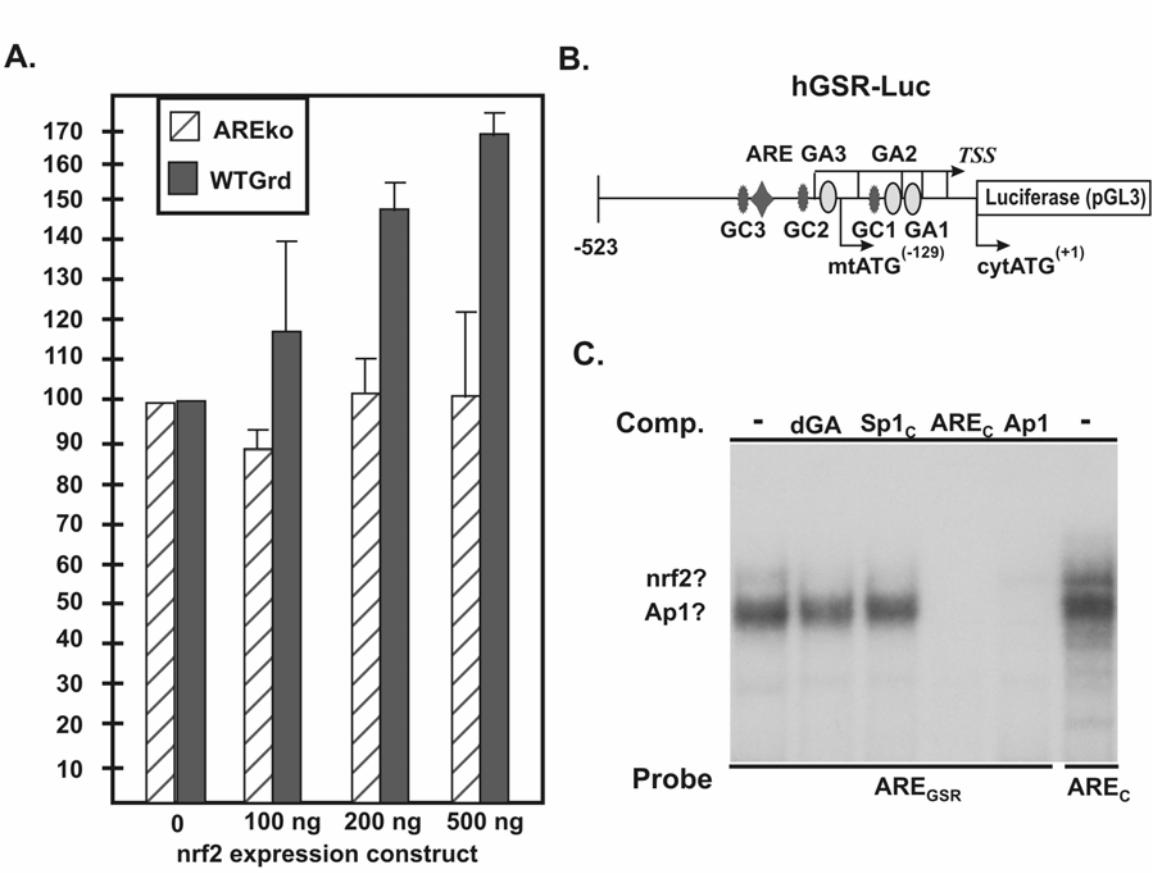

A.
Comp.
-
dGA
Sp1c
ARE
Sp1/3
Sp1/3
Probe
GC2
B.
Sera
-
Sp1
Sp3
Sp3
Probe
GC2

HeLa cell nuclear extracts and unlabeled dGA probe or unlabeled ARE probe have no effect on the binding of complexes to the GC2 probe, indicating that GABP does not bind in this region of the promoter. An Sp1 consensus site is also used as a competitor and is able to compete the binding of the two most prominent complexes to the probe (Fig 31), implicating Sp1 and Sp3 as possible factors within these complexes. When antiserum against Sp3 is added to the reaction and the EMSA analysis is run, there is a supershift in one of the complexes. The antiserum against Sp1 does not affect the migration pattern of any complexes that bind the GC2 probe (Fig 31B). Sp3 from HeLa cells is able to bind the GC2 transcription factor-binding site of the hGSR promoter. These data implicate GABP and Sp3 as the prominent regulatory transcription factors bound to the hGSR promoter.

Determination of the activity of the ARE in the hGSR promoter.

Within the promoter of the hGSR there is an antioxidant response element (ARE) upstream of the mtAUG and cytAUG but downstream of the GC3 site (Fig 32). The wild-type and ARE knock out promoters were analyzed for their ability to activate expression of luciferase from the hGSR promoter. The expression from the ARE mutant is the same as the expression from the wild-type promoter. The functionality of the ARE was examined by contransfecting the ARE mutant promoter and the wild-type promoter with a construct expressing nrf2 constitutively. The nrf2 (human homolog to GABP) expression vector was titrated into the transfection to determine if varying amount of nrf2 affected expression from the promoter. The expression level of luciferase from the

Figure 32. The ARE within the hGSR promoter was examined for activity. A). The hGSR promoter is activated by cotransfection with a nrf2 expression construct in a dose dependent manner. B). The ARE sequence is bound by Ap1, which can be competed from binding the hGSR ARE by unlabeled excess consensus ARE or an Ap1 binding site.

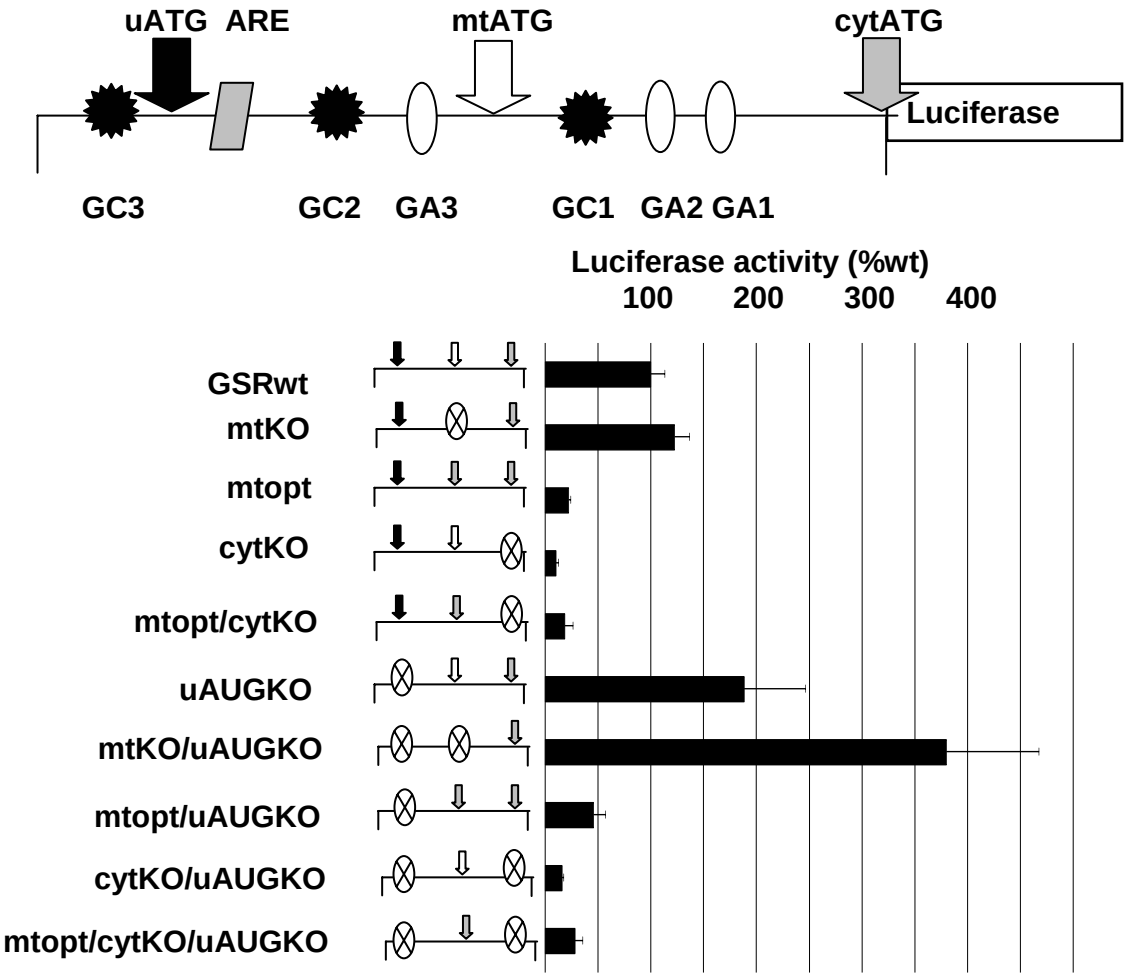

A.
AREko
WTGrd
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
100 ng
200 ng
500 ng
nrf2 expression construct
B.
hGSR-Luc
ARE GA3
GA2
TSS
Luciferase (pGL3)
GC3
GC2
GC1 GA1
-523
mtATG(-129)
cytATG(+1)
C.
Comp.
-
dGA
Sp1C
AREC
Ap1
-
nrf2?
Ap1?
Probe
AREGSR
AREC

wild-type promoter increased as the amount of nrf2 expression vector was increased. The ARE mutant promoter showed no change in expression related to the addition of the nrf2 expression vector. The ARE mutant promoter maintains the expression level of wild type promoter with no nrf2 cotransfected (Fig. 32A). The ARE region was also examined for the ability of transcription factors to bind to the region. In competition assays with unlabeled probes for consensus transcription factor binding sequences, the binding to the promoter could be out competed by an ARE consensus sequence and a binding site for Ap1. The binding to the probe could not be completely competed by the Sp1 consensus oligonucleotide or a dGA oligonucleotide. The dGA oligonucleotide did appear to inhibit the binding of a small complex that could potentially be nrf2 bound to the ARE probe (Fig. 32). These results indicate that the ARE is active, can be bound by Ap1, and can be activated by overexpression of nrf2 by cotransfection with the nrf2 expression vector.

Identification of the functional translational start sites in the hGSR promoter.

There are three potential translational start sites in the hGSR promoter, an upstream, out of frame AUG (uAUG), an AUG at the start of the mtGSR (mtAUG), and an AUG at the start of the cytoplasmic (cytAUG) form of the protein. The uAUG has a translational stop site within the mitochondrial leader sequence that is out of frame with the mtAUG open reading frame. The position of the stop codon within the uAUG open reading frame is such that translation cannot be initiated from the mtAUG as the mtAUG is located within the open reading frame, however, the stop codon is located far enough upstream of the cytAUG that translation could be stopped from the uAUG and possibly

Figure 33. The expression of luciferase from the pCDNA1-hGSR-luciferase vector with mutants to the AUGs. The mutated translational start sites are noted by the circle with an X through it. The white arrows represent the sites that have not been mutated. The gray arrow represents the optimization of the mtAUG context. The activity of each mutant increases when the uAUG is knocked out implicating the uAUG in repression.


uATG
ARE
mtATG
cytATG
Luciferase
GC3
GC2
GA3
GC1
GA2
GA1
Luciferase activity (%wt)
100
200
300
400
GSRwt
mtKO
mtopt
cytKO
mtopt/cytKO
uAUGKO
mtKO/uAUGKO
mtopt/uAUGKO
cytKO/uAUGKO
mtopt/cytKO/uAUGKO

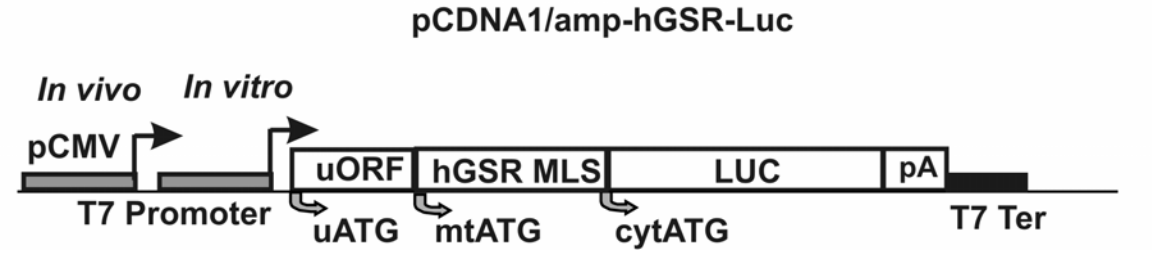
reinitiate at the cytAUG (Fig 33). Within the context of the pCDNA1-GSR-luciferase expression vector, each of the AUGs was mutated and the expression from each mutated promoter was analyzed. In single mutant with the cytAUG knocked out, the expression level was reduced to only about 10% of wild-type activity. With the uAUG still intact, the mtAUG was knocked out and the expression level from the pCDNA1-GSR-luciferase expression vector was increased slightly, to just over 120% of the wild-type expression. This slight increase in expression from the hGSR promoter when the mtAUG is knocked out could be due to the nonconsensus Kozak sequence near the mtAUG slightly inhibiting translation. If this were the case, it would be expected that the expression from the promoter would be increased if the mtAUG Kozak sequence was optimized by site-directed mutagenesis to be identical to the cytAUG Kozak sequence. When the mtAUG context is optimized, the mutant has only about 22% of the activity of the wild-type promoter. In the double mutant with the cytAUG knocked out and the mtAUG optimized, the analysis of the expression levels of the mutant revealed that expression levels of luciferase were only about 19% that of the wild-type promoter. The fact that this did not restore wild-type activity could be caused by translation initiating from the uAUG. Because of the strong CMV promoter upstream of the hGSR promoter, all the transcripts should have an mRNA 5'-terminus upstream of the uAUG. When the uAUG is mutated, overall expression is increased to about 140% of wild-type activity, indicating the uAUG can initiate translation, interfering with translation from the mtAUG. The uAUG mutant has been incorporated into the other pCDNA1-hGSR-luc as multiple mutants with the other translational start site mutants. The uAUG-mtAUG double knockout mutant gave the greatest change in expression from the promoter as this mutant had expression levels

of over 380% greater than the wild type activity, possibly by removing the inhibition of the two upstream AUGs. When the uAUG mutant is incorporated into the vector already containing the optimized mtAUG increases the expression from 22% to 46% of the wild-type activity when compared to the mtAUG optimized. In the uAUG-cytAUG double knockout mutant, the levels of expression increase from 10% to 16% for the cytAUG knockout. When the uAUG is mutated in the mutant promoter that has the mtAUG optimized and the cytAUG knocked out, the expression from the promoter increases from 18% to about 28% (Fig 33). These results indicate that there is some regulation of the expression from the promoter at the translational level. The uAUG has a role that does inhibit some translation from the GSR transcript as can be evidenced by the observation that all of the mutants of the uAUG increase the expression from the promoter. Also, the mtAUG has a role in the regulation as well. Mutation of the mtAUG to the optimized form has only about 20% of the activity of knocking the mtAUG out, indicating a role for the mtAUG in the expression from the hGSR promoter.

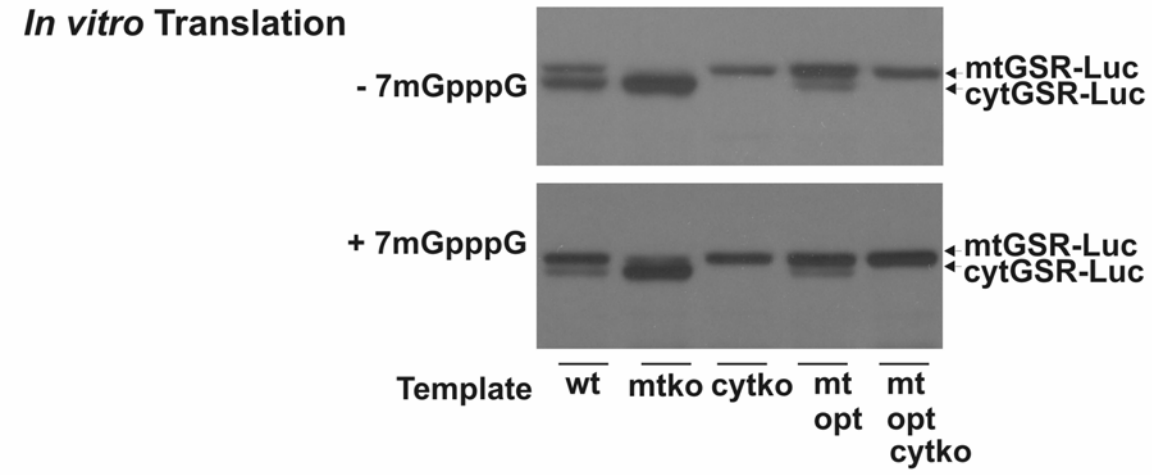
The results of the 5'RACE experiment seem to be contradicted by the results of the PCR analysis of the HeLa cells cDNA library. There is also some evidence based on the mutagenesis of the potential translation start sites that the expression of GSR is regulated translationally. In the 5'RACE the GeneRacer RNA-oligo is added only to the 5'-termini of capped mRNAs. In an *in vitro* translation assay the effect of the cap on translation from the hGSR promoter was investigated. The transcript was expressed from the previously described pCDNA1-hGSR-luciferase expression vector. The translation of the fusion protein in an *in vitro* assay was then determined. Translation from the uncapped transcript expressed more protein in which translation initiated from the

Figure 34. Analysis of the ability of the hGSR transcripts to be translated from both capped and uncapped messages. A). The design of the promoter construct to ensure that all the transcripts are initiated from a single promoter. B). The translation from uncapped and capped mRNA is examined. The major difference is in the wt and the mtopt hGSR promoters between the translation from capped and uncapped message. The translation from the uncapped mRNA favors the mtAUG when the sequence surrounding the AUG is made identical to the sequence surrounding the cytAUG, indicating a possible IRES. This in not the case in the expression from capped mRNA.

A.


pCDNA1/amp-hGSR-Luc
In vivo
In vitro
pCMV
uORF
hGSR MLS
LUC
pA
T7 Promoter
uATG
mtATG
cytATG
T7 Ter

B.

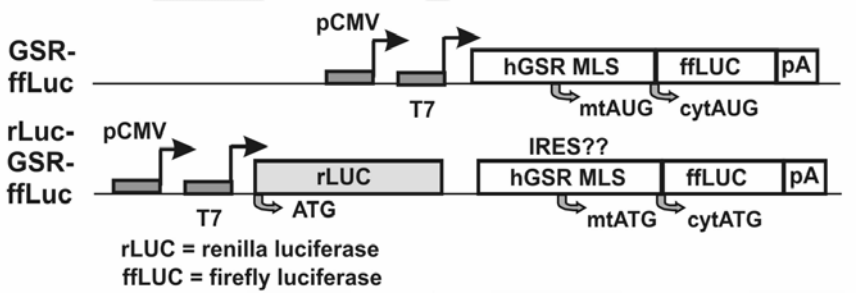

In vitro Translation
- 7mGpppG
+ 7mGpppG
mtGSR-Luc
cytGSR-Luc
mtGSR-Luc
cytGSR-Luc
Template
wt
mtko
cytko
mt opt
mt opt cytko

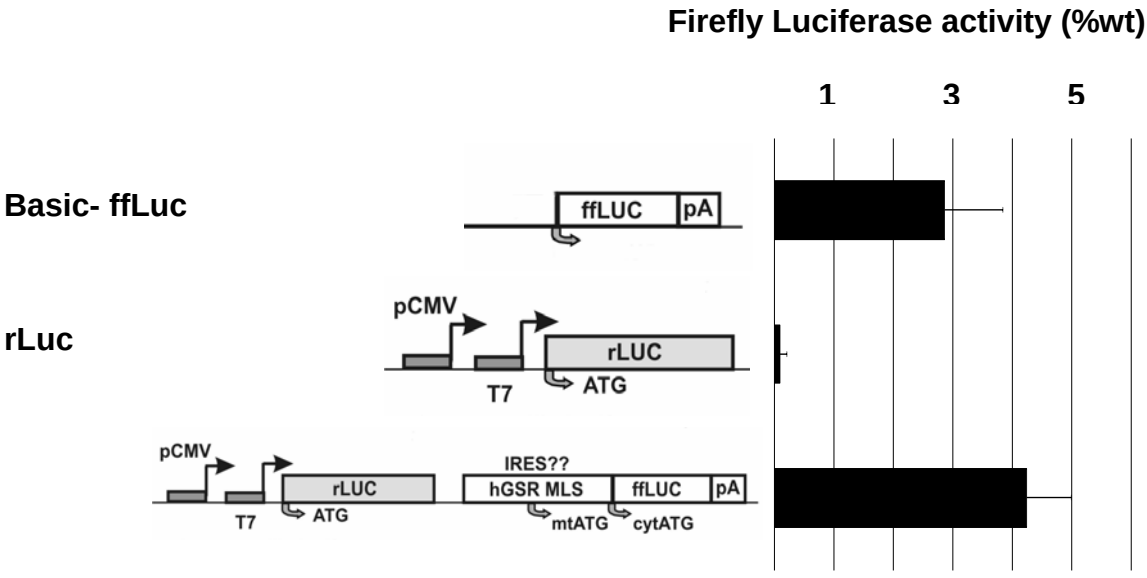
cytoplasmic start site. When the mtAUG was knocked out the transcription occurred exclusively from the cytAUG, and the expression appears to increase from the cytAUG. When the cytAUG is knocked out, the expression is exclusively from the mtAUG, but the amount of mtLuc does not appear to increase (Fig. 33). The expression from the mutant with the mtAUG context optimized shifted the expression from the cytAUG to a majority of the expression then coming from the mtAUG. The double mutant with the mtAUG optimized and the cytAUG knocked out gives exclusive expression from the mtAUG. The significant mutant is the mutant with the optimized mtAUG Kozak sequence, where the translation shifts to start at the mtAUG, indicating in uncapped mRNAs leaky scanning could regulate translation start site selection in the hGSR mRNA. In a capped mRNA the wild type AUGs the majority of the translation initiates from the mtAUG. When the mtAUG is optimized, almost all of the translation is initiated from the mtAUG (Fig 34). This result is even more significant when it is considered that the capping reaction is only about 50-80% effective, meaning that essentially all of the capped message initiates from the mtAUG. These data would support a model of translational regulation in uncapped mRNA but the leaky scanning model would not be consistent with translation from the capped mRNA.

An IRES does not appear to be present in the hGSR mRNA.

Another possible mechanism for the regulation of the expression of hGSR is through the presence of an IRES. There has not been any evidence presented to suggest that an IRES is present in the hGSR mRNA, but the predicted secondary structure of the

Figure 35. The expression of the dual luciferase from the promoter construct (Fig 6) shows that an internal ribosomal entry site is unlikely. If it does occur it is at a very small amount and likely would not affect expression in a significant manner. The expression of firefly luciferase from the construct testing for the IRES, gives the same amount of expression of firefly luciferase as a promoter-less construct. The expression of firefly luciferase from the GSR-ffLuc vector was set at 100 % and expression of the other expression vectors were compared to expression from the GSR-ffLuc expression vector.


GSR-
ffLuc
pCMV
T7
hGSR MLS
ffLUC
pA
mtAUG
cytAUG
rLuc-
GSR-
ffLuc
pCMV
T7
rLUC
ATG
IRES??
hGSR MLS
ffLUC
pA
mtATG
cytATG
rLUC = renilla luciferase
ffLUC = firefly luciferase


Firefly Luciferase activity (%wt)
1
3
5
Basic- ffLuc
ffLUC
pA
rLuc
pCMV
T7
rLUC
ATG
pCMV
T7
rLUC
ATG
IRES??
hGSR MLS
ffLUC
pA
mtATG
cytATG

5’-untranslated region of the hGSR mRNA contains a region with similar structure to the Y-type stem-loop of the BiP IRES (Le and Maizel, 1997). To investigate the possibility of an IRES, a promoter construct was made to test the expression of both renilla and firefly luciferase (Fig. 6). The expression of firefly luciferase from the rLuc-GSR-ffLuc promoter would provide evidence for an IRES. The expression of firefly luciferase from the promoter was measured and found to express luciferase at levels similar to the pGL3-BASIC promoterless vector (Fig. 35). These data indicate that an IRES is likely not present within the hGSR mRNA, indcating another method of regulating expression of hGSR.

DISCUSSION

Analysis of mGABPα/CF6 Bi-directional Promoter

The primary source of ROS in most cell types is the ETC (Boonstra and Post, 2004; Halliwell and Gutteridge, 1999). ROS are formed when molecular oxygen is incompletely reduced by the ETC about three to four percent of the time forming superoxide radicals. Superoxide radicals can be reduced to hydrogen peroxide and the hydroxyl radical. In order to minimize the formation of ROS the expression of the proteins ETC must be coordinated (Wallace, 1999). Genes from many of the proteins of the ETC have potential GABP binding sites within their promoter regions, implicating GABP as an important regulator of ETC protein expression (Gleyzer et al., 2005; Kelly and Scarpulla, 2004; Scarpulla 1997; Villena et al., 1998; Virbasius and Scarpulla, 1994). GABP is also critical for early embryogenesis (Ristevski et al., 2004). GABPα is critical in most cell types, and there are regulatory mechanisms for ensuring proper levels of expression. In mice heterozygous for the GABPα protein the expression levels of GABPα are similar to the expression levels in wild-type mice, even though they have only a single copy of the gene (Ristevski et al., 2004), and gene dosage does not cause an overexpression of GABPα (O'Leary et al., 2004). Understanding the regulatory mechanism of the expression of GABPα is essential for understanding the regulation of GABP and the role GABP has in the regulation of embryogenesis and ETC coordination. The presence of GABP binding sites within the promoter region of the mGABPα/CF6 bi-directional promoter provides a potential auto-regulatory mechanism for the expression

of GABPα. The redox sensitive cysteine residues present in the DNA binding and dimerization domains of GABPα provide a potential method for regulating the expression of genes under the control of GABP under oxidative stress conditions (Martin et al., 1996; Chinenov et al., 1998 and 2000b).

The mGABPα/CF6 bi-directional promoter has a YY1 and a Sp1/3 transcription factor binding site in addition to the four GABP binding sites. These binding sites contribute to the regulation of expression from the promoter. There are other binding sites in the flanking sequences of the core promoter, including binding sites for δEF1/SIP1, B-Myb, T3R, and an antioxidant response element (Chinenov et al., 2000a), which potentially participate in the regulation of the promoter under stress conditions or in a tissue specific manner. The mutation of these potential binding sites has no effect on the expression from the promoter when transfected into $A9_{2L}$ cells indicating that the other transcription factor binding sites likely do not effect transcription from the core promoter. Mutation of the GA1 binding site does have the most significant effect on expression from the promoter (Fig 10-13). For efficient transcription from the promoter construct, it is necessary for the GA1 site to remain intact with another GABP binding site present. The expression from the promoter is coordinated between GABP, Sp1/3, and YY1. The presence of YY1 or Sp1/3 is not required for transcription from the promoter in either direction but does increase efficiency of expression. The YY1 binding site has a role in transcription, especially from the CF6 promoter. The expression levels from the double mutants involving the YY1 binding site and one of the GA binding sites demonstrate this regulation. The closer the GA binding site was to the YY1 site the greater the decrease in expression became. The regulation of promoter activity from the GABP binding sites

depends predominantly on the binding of GABP to these sites but the possibility remains that another Ets-related factor is able to bind the GABP sites and contribute to promoter activity, although none have been shown to bind *in vitro*.

There is a growing list of promoters that share binding sites for GABP, Sp1, and YY1 but lack a discernable TATA motif. These promoters direct the transcription of a wide variety of genes. There are many genes encoded from this type of promoter. Nth11/Tsc2 is an endonuclease III homolog and is associated with tuberous sclerosis (Ikedi et al., 2000). Several other proteins including Surf-1/Surf-2, within the Surfeit locus in mammals (Vernon and Gaston, 2000), Hsp60/Hsp10, which encodes mitochondrial chaperones (Hansen et al., 2003), poly ADP ribose polymerase/RNaseP (Ame et al., 2001), VCLAD/PSD-95, which encodes a very long chain acyl COA dehydrogenase and a postsynaptic density protein (Zhang et al., 2003), and DHFR/MRP1 (Shinya and Shimada, 1994) are expressed from promoters the contain GABP, Sp1, and YY1 binding sites but lack a TATA motif. The regulation of these bidirectional promoters may be coordinated or independent. The promoters share the regulatory elements within the promoter regions including the importance of the GA1 binding site. The response of the genes to oxidative stress was tested to determine the effect of oxidative stress on expression from the promoters.

The mGABPα/CF6 bi-directional promoter expression is regulated by transcription factors that are known to be responsive to oxidative stress. There are three cysteine residues in the DNA binding domain and the dimerization domain of GABPα which prevent the formation of complex on DNA when the proteins are placed under oxidizing conditions (Chinenov et al., 1998). GABP binding sites are also present in

many of the proteins in the electron transport chain, the major intracellular source of oxidizing agents and ROS. The need for coordinated regulation of the proteins of the electron transport chain also point to the need of a method of coordinating the regulation under oxidative stress. The presence of the GABP binding sites within the promoters of many of these proteins makes GABP a likely regulator for the expression of these proteins. Also, since many of these proteins are involved with processes that cause oxidative stress and the ability of GABP to bind DNA is redox sensitive, regulation through GABP is logical. Understanding how GABP is regulated under oxidative stress would aid in understanding the cellular response to oxidative stress. It was expected that the mGABPα/CF6 bi-directional promoter would be regulated in response to oxidative stress. These experiments have shown that the expression from the mGABPα/CF6 bi-directional promoter is unaffected by the addition of hydrogen peroxide to the cell culture. This surprising result could be explained by a couple of possibilities. One possibility is that the promoter is not responsive to oxidative stress. The regulation by GABP of the other proteins could be at the protein level with GABP unable to initiate transcription from the promoters of the proteins of the electron transport chain. There is also the possibility that the cells are already adapted to oxidative stress so the addition of hydrogen peroxide does not affect expression from the mGABPα/CF6 bi-directional promoter. The growth of cells at 20% oxygen does cause cells to be under constitutive oxidative stress. Since the cells are under oxidative stress the cells do not further respond to the addition of an oxidizing agent. The alternative to examine whether the cells had already adapted to oxidative stress would be to grow the cells in a hypobaric chamber where the oxygen is regulated at about three percent, which is the normal level of oxygen

encounter *in vivo*. This would not allow the cells to preadapt to the oxidative stress. These conditions would likely elicit a different response to the addition of the H_2O_2.

Analysis of the hGSR promoter

The expression of GSR has the potential to be regulated at both the transcriptional and translational level. The transcription factor binding sites within the hGSR promoter regulate the expression of luciferase from the hGSR promoter. The mutation of these transcription factor-binding sites shows that the GC2 site displays the significant control of expression in the promoter. When the GC2 site is knocked out as a single mutant, the expression from the promoter is reduced to less than 60% of the wild-type expression levels. When this site is mutated as part of a double mutant with any of the other transcription factor binding sites, the expression is reduced, implicating the GC2 site as an important regulator. The most significant decrease in expression is observed in the GA3/GC2 double mutant, which reduces expression to 15% of the wild-type level (Fig. 28). The GA3 probe was bound more tightly by the rGABP than the other GABP binding sites. There was even some formation of the GABP tetramer complex bound to the GA3 probe, likely due to nonspecific binding of the GABP heterodimer complex to the DNA, as there was a large excess of GABP present (Fig. 29-30). In HeLa cell extracts, the GA3 probe was bound the strongest by GABP but no heterotetramer complex formed on the probe, while the other probes were bound only by GABPα monomer (Fig. 30). Interestingly, when the GA3 site was mutated the promoter still expressed luciferase at 90% of the wild-type level (Fig. 27), indicating that promoter binding does not necessarily relate to promoter activity. This implicates the GC2 site as the critical site for expression in this mutant. Sp3 binds the GC2 site (Fig. 31), so GABP and Sp3 appear to

form a critical complex on this promoter that forms efficiently on the GC2 and GA3 binding sites and inefficiently on any of the other binding sites. It appears from these data that the other GABP binding sites can at least somewhat effectively compensate for the lack of the GA3 site, but the GC1 and GC3 sites are not functionally redundant with the GC2 site. These data do provide a potential mechanism for transcriptional regulation of expression from the promoter.

Transcriptional regulation of expression from the promoter is also suggested by the data from the 5'-RACE, suggesting that the majority of transcripts initiate from the within the MLS (transcript 3, Fig. 24). The major product from 5'-RACE was purified, cloned, and sequenced to reveal four transcripts that originate within the MLS, and the minor PCR product reveals only a single transcript initiating upstream of the MLS. There are other minor PCR products present in the 5'-RACE experiment that would produce longer transcripts (Fig 25), however none of these PCR products was present in large enough concentration to separate the transcripts from the gel. This would suggest that the isoform of GSR expressed is regulated on the transcriptional level. However, when the amount of HeLa cell RACE ready cDNA was analyzed by PCR, it produced identical amounts of product for both the total GSR transcripts and mtGSR transcripts. This result indicates that a large majority of the transcripts initiate upstream of the mtAUG, implicating translational control as the major regulator of mt- and cytGSR expression. These results indicate a confliction between possible translational and transcriptional regulation of expression of GSR.

If the majority of transcripts initiate upstream of the mtAUG, there must be some form of translational regulation over the expression of GSR. There are other data that

support a theory that the major source of regulation is from translation. For the regulation to be at the translational level there would have to be a mechanism by which the AUG can be selected for expression. Eukaryotic mRNAs consist of the 5'-cap (m^7GpppN), the 5'-untranslated region (UTR), the initiator AUG, the open reading frame, the 3'-UTR, and the poly A tail (Kozak, 1989; Kozak, 2001; Kozak, 2002). The translation is initiated by the recruitment of the 43S subunit of the ribosome to the capped mRNA by eukaryotic initiation factor 4F (eIF4F). The ribosome will then scan the mRNA for the first available AUG to initiate translation from which to initiate transcription (Gingras et al., 1999). Many mRNAs with a highly structured 5'-UTRs are not translated efficiently unless there is excess eIF4E available. The requirement of a cap to recruit eIF4F, which recruits the 43S subunit of the ribosome, could have a role in the regulation of GSR expression. An *in vitro* translation assay was performed on the pCDNA1-GSR-luciferase construct in both uncapped and capped transcripts. In the uncapped transcript, the cytAUG was preferentially selected, but when the mtAUG context is optimized, the mtAUG is preferentially selected. Transcription is more efficient from the mtAUG in a capped message, which is the only type of mRNA expected in cells, unless the mRNA is targeted for degradation. The efficiency of the translation from an AUG can depend on several features of the mRNA including the sequence context of the AUG, the secondary structure of the mRNA, and the presence of an upstream open reading frame.

Nonideal sequence context of the AUG can lead to "leaky scanning" of the ribosome in which the ribosome does not initiate translation from the AUG furthest upstream. The sequence context of the cytAUG is an ideal Kozak sequence for translation initiation. The mtAUG and the uAUG are both in suboptimal contexts, as the mtAUG has

a mismatch at the –3 position, and the uAUG has mismatches at the –3 and +4 positions (Byrd et al., 2002; Kozak, 1989; Kozak, 2001; Kozak, 2002). The presence of these suboptimal initiation sequences alone cannot explain the differences in expression of mtGSR and cytGSR. The mtGSR has an adequate context for translation initiation so the levels of mtGSR expressed in most cells would not be expected to be as low as what is observed (~three to five percent), except in cardiac and skeletal muscle cells where up to 40% of the cellular volume is mitochondria and the mtGSR is expressed at levels in accordance with the percentage of mitochondrial cell volume. The data from the mutagenesis of the translational start sites does not support a "leaky scanning" hypothesis. If the sole method of regulating mtGSR and cytGSR was through leaky scanning then when the mtAUG is mutagenized into an optimal translation context then it would be expected that expression from the promoter would be maintained close to wild-type levels. Also, when the mtAUG is optimized and the cytAUG is knocked out we would expect expression to be maintained near wild-type levels. However, this is not what is observed. The reason for the low levels of expression from the GSR promoter when the mtAUG is optimized remains a mystery as more data will be required to determine why expression from the mtAUG is not maintained near wild-type levels, but "leaky scanning" does not appear to be a major method of regulating mtGSR and cytGSR expression.

Inhibition due to an upstream open reading frame (uORF) could help to explain some of the difference in expression of the two GSR proteins. The uORF initiates translation upstream and out of frame with the mtAUG and cytAUG. Translation could initiate from this uAUG, although the context is poor, and prevent translation from the

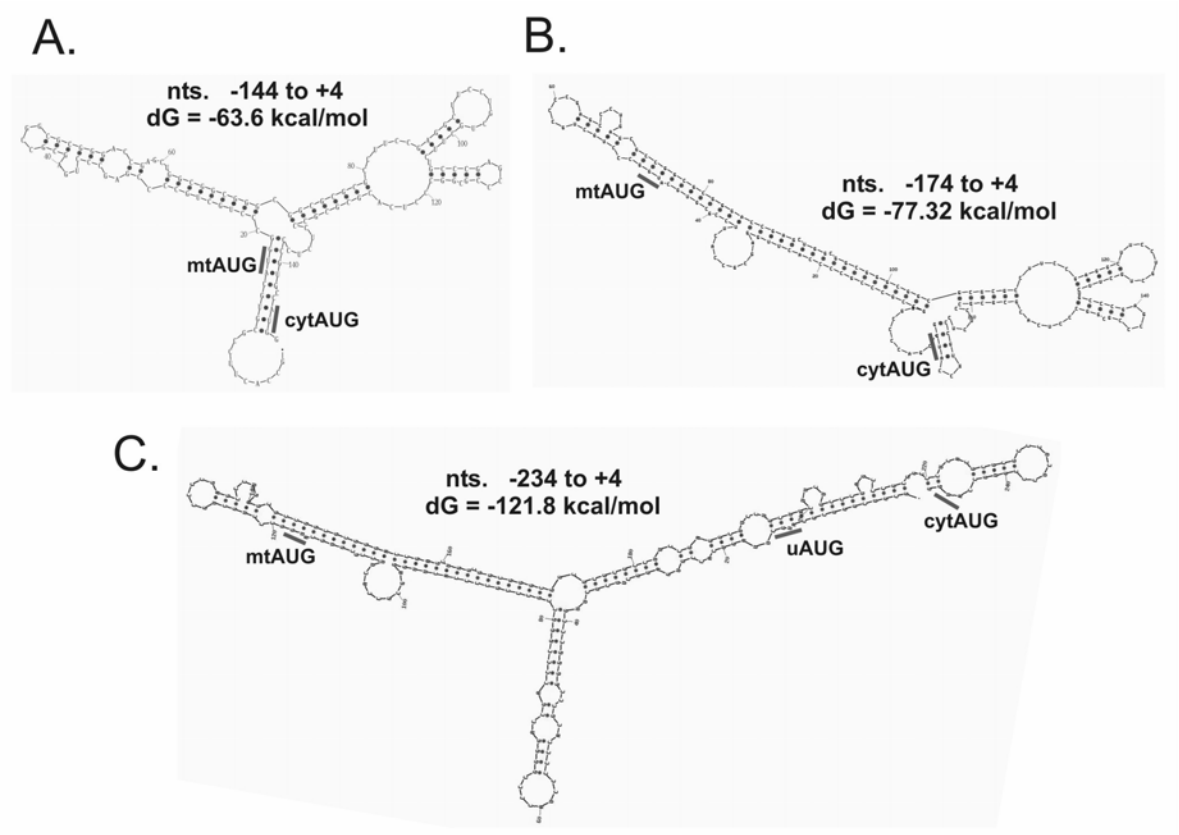
mtAUG. The translation of this uORF would not affect translation of the cytGSR though. The uORF has a stop codon within the mitochondrial leader sequence (hence, being out of frame with the mtAUG is important) that would cease translation but could allow the ribosome to reinitiate transcription downstream at the cytAUG (Huez et al., 1998; Kochetov, 2005; Meijer and Thomas, 2002). The uAUG is also in a poor context for the initiation of translation and likely would be subject to "leaky scanning" resulting in initiation from the mtAUG. The effect of the uAUG on the expression of luciferase was examined by mutating the uAUG in conjunction with mutations to the mt- and cytAUGs. The uAUG has a negative effect on expression from the hGSR promoter, as knocking out the uAUG increases expression in each of the mutants. The expression increases to almost double the single mutant activity for each of the mutants with the exception of the uAUG-mtAUG knockout double mutant, which has activity levels about triple the mtAUG knockout single mutant. The uORF likely has a regulatory role in which form of GSR is expressed provided that all transcripts initiate upstream of the uAUG. The expression from the single mutants of the pCDNA1-hGSR-luciferase construct does not present a clear translational regulation model. If the translational regulation was only through "leaky scanning," it would be expected that the optimization of the mtAUG and knockout of the cytAUG would be able to express luciferase at the same level as the wild-type vector, but this is not observed (Fig 34). The uAUG KO- mtAUG optimized double mutant has less than 20% the activity of wild-type promoter, implicating other methods of regulation as well.

A third potential regulator of the two translational start sites would be the secondary structure of the mRNA. All three possible types of transcripts of the GSR

mRNA are predicted to exhibit extensive secondary structure when using the mfold software (Mathews et al., 1999) (Fig 36). The secondary structure of the mRNAs from transcript one and two are able to exhibit secondary structure that is very stable in nature making translation initiation difficult (Gross et al., 1990; Kozak, 1989; Kozak, 2001; Kozak, 2002; Svitkin et al., 2001). While some regions of the three transcripts are common, other portions have significant enough differences that produce alternative start sites for translation initiation. The mRNA secondary structures could play a role in selection of the mt- or cytAUG.

There is also a fourth possibility that an internal ribosomal entry site (IRES) is present in the transcript. IRES have been described in viral systems, however their presence on cellular systems is not universally accepted (Han and Zhang, 2002; Komar, and Hatzoglou, 2005; Stoneley and Willis, 2004). Cellular IRES have been described in some mRNAs encoding a wide variety of proteins, including immunoglobulin heavy chain binding protein (BiP) (Le and Maizel, 1997), vascular epidermal growth factor (Huez et al., 1998), Apaf-1 (Mitchell et al., 2001), eIF4GI isoforms (Nevins et al., 2003), and c-myc (Stoneley et al., 2000). While the presence of an IRES has not been described to date in the hGSR mRNA, the secondary structure predictions for the GSR mRNA include structures similar to those seen in the Y-type stem-loop of the BiP IRES (Le and Maizel, 1997). Further investigation will need to be done to determine which of these mechanisms contribute to the regulation of hGSR promoter as well as whether there is an affect of oxidative stress on promoter function.

Figure 36. Predicted secondary structures for three possible transcripts of hGSR, depending on the transcriptional initiation site.


A.
nts. -144 to +4
dG = -63.6 kcal/mol
mtAUG
cytAUG
B.
mtAUG
nts. -174 to +4
dG = -77.32 kcal/mol
cytAUG
C.
nts. -234 to +4
dG = -121.8 kcal/mol
mtAUG
uAUG
cytAUG

The GSR promoter has been shown to be sensitive to the redox state of the cell. The presence of the ARE in the promoter region could be part of the regulation of expression of GSR under oxidative stress. When the ARE site is knocked out there is no response to the expression of GSR, however, when the ARE knockout and wild-type promoters are cotransfected with nrf2, the wild-type promoter displayed increased expression from the promoter while the expression from the ARE knockout remained unchanged. The binding to the ARE could be competed away by an excess of unlabeled oligonucleotides that contained a consensus site for either an ARE or the Ap1 transcription factor. These results indicate the ARE is functional and implicate the ARE site as necessary for stimulation by nrf2, likely through a complex of Ap1/nrf2.

The results observed in these experiments implicate a complex method of regulation of the hGSR promoter. The ability to increase the expression of mtGSR in response to oxidative stress is critical for cellular function and survival. The understanding of the regulation of the hGSR promoter will provide a great deal of insight to the mechanism by which the cell responds to oxidative stress. The role of translation regulation will also provide additional understanding to the field of translational regulation. The transcriptional and translational aspects to regulation from the hGSR promoter provide an interesting platform for further investigations to take place. The lab will continue experiments to examine the possible methods of regulation of GSR expression. The factors which bind to the secondary structure will be determined. Also, it will be necessary to better define the 5'-termini of the hGSR mRNA. This will be accomplished through high resolution 5'-RACE or primer extension.

CONCLUSIONS

The mGABPα/CF6 bi-directional promoter is regulated by GABP but does not respond to treatment with H_2O_2.

The mGABPα/CF6 bi-directional promoter is regulated by GABP in cooperation with YY1 and Sp1/3. The role of Sp1/3 appears to be one of inhibition, while GABP and YY1 function to activate expression from the promoter. Complexes form on different regions of the promoter to coordinate the regulation of transcription. These studies have shown a complex including GABPαβ/Sp1/3 binds to the probe containing the region of the promoter containing the GA4 binding site and the Sp1/3 binding site. Functional interactions have been demonstrated between GABP and YY1, as the mutation of these two binding sites has an inhibitory effect on transcription. It is reasonable to assume that YY1 and GABP interact physically on this promoter although the interaction may be through an intermediary such as YEAF-1, which has been shown to interact with both proteins and even form a ternary complex (Du et al., 1998).

Since GABP is sensitive to oxidative stress, the binding of GABP to the mGABPα/CF6 bi-directional promoter allows for the possibility of expression from the mGABPα/CF6 bi-directional promoter being sensitive to oxidative stress. The expression of luciferase from the mGABPα/CF6 bi-directional promoter was unaffected by the addition of H_2O_2. These results do not necessarily mean the mGABPα/CF6 bi-directional promoter would not respond to another type of oxidant. Confirmation of the response to

oxidative stress of the mGABPα/CF6 bi-directional promoter should be attempted. To ensure that the cells were not preadapted to oxidative stress due to exposure to atmospheric levels of oxygen, the oxidative stress tests should be repeated in cells that are grown at oxygen levels similar to physiological levels. Other oxidants should be used as well. GABP was sensitive to oxidative stress when treated with DEM. The response of the mGABPα/CF6 bi-directional promoter to other forms of oxidants including DEM should be tested. DEM is a GSH depleting agent and GABP is inactivated by treatment with DEM. The concentration of H_2O_2 used in this experiment may not be enough to cause depletion of GSH and therefore be unable to inactivate GABP. The response of the mGABPα/CF6 bi-directional promoter to other oxidants may cause a response to oxidative stress.

The GSR gene appears to be regulated at both the transcriptional and translational level.

The hGSR promoter is regulated primarily through complexes that form at the GC2 and GA3 site on the promoter. The GA3 complex is bound more tightly by GABP than the other GABP binding sites. A Sp3 complex binds the promoter at the GC2 binding site. The binding of these two transcription factor binding sites by transcription factors indicate that the hGSR promoter is regulated by the binding of GABP and Sp3 to the promoter complex.

The regulation of translational start site selection has not been clearly illustrated at this time. The results of the 5'RACE experiment displays a transcriptional control over

AUG selection as transcripts are present with only the cytGSR AUG present in some of the transcripts. These results though are contradicted by the measurement of GSR cDNAs present in a HeLa cell cDNA library. PCR measurements of the GSR present in the cDNA library from HeLa cells indicates that the vast majority if not all of the GSR transcripts are initiated upstream of the mtGSR AUG. The contradiction between these two results needs to be resolved. The 5'RACE experiment should be repeated with a thermal stable reverse transcriptase. The secondary structure of the RNA upstream of the cytGSR AUG is rather extensive. The thermal stable reverse transcriptase would allow the secondary structure to be denatured at the higher temperatures that the experiment will be repeated.

The presence of three AUGs within the mRNA does display some regulation over the selection of the start site. The uAUG regulates expression from the mRNA containing all three AUGs, decreasing the GSR expression. The mtGSR AUG has a regulatory role in translation as well as optimizing the context of the AUG decreases expression rather than maintaining wild-type expression.

The regulation of the translational start site selection of the hGSR promoter has not been clearly illustrated at this time. The results presented in this dissertation provide evidence for both transcriptional and translational control over start site selection. Further experimentation will be needed to determine the regulation of expression of mt- and cytGSR from the hGSR promoter.

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