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The Murine p8 Gene Promoter Is Activated by Activating Transcription Factor 4 (ATF4) in the Gonadotrope-Derived LβT2 Cell Line

Christina M. Million Passe, Garry Cooper, and Christine C. Quirk

Medical Sciences Program, Indiana University, Bloomington, Indiana 47405-4401

The factor p8 is a high mobility group (HMG) A family member that is upregulated during the cellular stress response in numerous tissues. Because expression of this protein encourages cellular transformation, our goal is to characterize the mechanism by which the p8 gene is regulated. Using LβT2 cells as a model of a transformed cell in which p8 plays a role in tumor formation, we dissected the p8 promoter into its minimal functional units and found that activating transcription factor 4 (ATF4), a factor also upregulated during cellular stress responses, enhances p8 promoter activity in a dose-dependent manner. In addition, ATF4 binds in the highly conserved major activation domain of the p8 proximal promoter between –130 and –100 bp. Furthermore, we show that six of the nine base pairs that encompass the putative element are essential for ATF4 binding. These findings increase our knowledge of the mechanisms regulating the p8 gene in a genetically defined tumor model.

Key Words: p8; ATF4; gonadotrope; LβT2.

Introduction

While investigating the factors involved in terminal differentiation of gonadotropes, we found that expression of the protein p8 is necessary for normal temporal initiation of luteinizing hormone β (LHβ) gene expression during embryonic development *(1)*. In the gonadotrope tumor–derived LβT2 and GH3 somatolactotrope tumor–derived cell lines we have also shown that p8 is a mediator of neoplastic transformation and tumorigenesis *(2)*.

p8, which is also known as candidate of metastasis 1 (com1) and nuclear protein 1 (*nupr1* or *np1*), is a nuclear phosphoprotein with a basic helix–turn–helix motif that shares a great deal of biochemical homology with high mobility group (HMG) A proteins *(3,4)*. HMG proteins function as architectural transcription factors that play a general role in regulating gene expression and cellular proliferation during development and the stress response *(5)*. Like its family members, p8 exhibits maximal levels of expression during embryonic development and is downregulated in normal adult post-mitotic tissue. However, several lines of research have shown that expression of p8, like HMG proteins, reemerges in response to cellular stress. A simple change of media to cells in culture transiently induces the expression of several stress-activated genes, including p8 *(6)*. In addition, p8 is markedly upregulated in acinar cells upon acute or chronic pancreatitis *(3,7)* and in the pancreas in response to lipopolysaccharide endotoxin injection in rats *(8)*. This stress response is also seen in liver and kidney, indicating that p8 expression is a consistent response to stress in a number of tissues.

Aberrant expression of HMG proteins has been associated with abnormal cellular proliferation, neoplastic transformation, and cancer. Some members of this family are known to be direct targets of oncoproteins and may themselves represent a new family of oncogenes *(9–11)*. As such, it is not surprising that the reemergence of p8 expression during stress has been linked to pathology. In diabetes, for example, p8 expression is induced by endothelin-1 and is associated with renal mesangial cell hypertrophy, a major cause of renal failure *(12)*. Expression of p8 is also associated with demyelination of axons in multiple sclerosis *(13)*. Moreover, p8 is a cancer marker in the breast *(14,15)*, pancreas *(16,17)*, thyroid *(18)*, prostate *(19)*, and pituitary gland *(2)* and its expression has been linked to enhanced metastatic potential *(14)* and the tumorigenic properties of cell lines *(2,20,21)*. p8 appears to be not only a marker, but also a facilitator, of tumorigenic events, as transformed mouse embryonic fibroblasts, LβT2 cells, and GH3 cells form tumors in nude mice only in the presence of p8 *(2,20,22)*.

Although it is clear p8 plays an oncogenic role, the mechanism by which it promotes cellular change is largely unknown. As p8 is HMG-like, it likely functions as a transcriptional regulator during development and the cellular stress response. However, p8 may also interact directly with

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Author to whom all correspondence and reprint requests should be addressed: Christine C. Quirk, Medical Sciences Program, Indiana University, 1001 East 3rd Street, JH104, Bloomington, IN 47405-4401. E-mail: cquirk@indiana.edu

other factors to target different pathways within the cell. For instance, a recent study found that p8 interacts with Jun-activating binding protein 1 (Jab1) to increase export of the cell cycle regulator p27 to the cytoplasm for ubiquitin-mediated degradation *(23)*. Another recent study found that in HeLa cells p8 interacts with the anti-apoptotic factor prothymosin α (ProTα) to block the apoptotic response induced by treatment with staurosporine *(24)*. These two studies provide novel mechanisms through which p8 may act to increase cell proliferation and allow cells to avoid apoptosis during the cellular stress response and pathology.

p8 expression has been shown to correlate with, and in some cases facilitate, tumorigenesis and other pathological events. Thus, understanding the mechanism by which p8 influences cellular homeostasis, including regulation of the p8 gene itself, is vital to the future use of p8 as a therapy to regulate tumor growth or block tumor formation. However, the elements and factors that control expression of the p8 gene have yet to be fully elucidated. Herein, we demonstrate that activating transcription factor 4 (ATF4), a factor that, like p8, plays a role both in development *(25–30)* and the cellular stress response *(31–36)*, is able to bind the major activation domain of the p8 gene to enhance promoter activity in the LβT2 cell line.

Results

The Major Activation Domain of the p8 Promoter Lies Between –130 and –100 bp

In the gonadotrope tumor–derived LβT2 cell line, we have found that p8 is a mediator of neoplastic transformation and tumorigenesis *(2)*. As such, we chose LβT2 cells as a model system to begin studying the mechanisms governing p8 gene regulation in a genetically defined tumor model in which p8 encourages the pathological state. To begin, we created a construct in which approx 1500 bp of the proximal promoter of the murine p8 gene drives expression of a luciferase reporter gene, p8(–1471/+36)-pGL3. Upon transient transfection of LβT2 cells, this construct is approx 50-fold more active than the promoterless control vector, pGL3 Basic (Fig. 1A). To further characterize the regulatory regions, 5' deletion constructs were made at –1230, –209, –130, and –72 bp. The –1230/+36, –209/+36, and –130/+36 constructs yielded no difference in promoter activity compared with –1471/+36. However, upon deletion to –72/+36, promoter activity was substantially decreased to a level not different from the promoterless control, indicating that a major activation domain resides between –130 and –72 of the p8 proximal promoter.

To better delimit the region required for p8 gene activation in the gonadotrope, two additional deletion constructs, –100/+36 and –90/+36, were created (Fig. 1B). Both promoter constructs displayed a greater than sixfold decrease in luciferase activity compared with the –130/+36 construct, indicating that a major activation domain of the p8 promoter lies between –130 and –100 bp. In addition, there appears to be an element(s) residing between –90 and –72 bp, as deletion of this region further diminishes activity of the p8 promoter to levels not different from the promoterless control.

To determine potential p8 regulatory elements, Genomatix MatInspector© software *(37)* was used to identify consensus sequences for known transcription factors within the p8 promoter. This analysis showed that the activation domain between –130 and –72 bp contains several putative elements that may regulate p8 promoter activation, including a region between –130 and –100 bp containing overlapping elements for three basic leucine zipper (bZip) transcription factors: ATF4, CCAAT enhancer binding protein (C/EBP), and albumin D-box binding protein (DBP). The region containing these elements is completely conserved in the human and rat p8 promoters (Fig. 1C), indicating it may be vital to p8 gene expression in a number of species.

ATF4 mRNA Is Highly Expressed in LβT2 Cells

The major activation domain of the p8 promoter contains highly conserved putative elements for the bZip factors ATF4, C/EBP, and DBP. These factors belong to three different bZip subfamilies: ATF4 to the activating transcription factor/cAMP response element binding protein (ATF/CREB) subfamily, C/EBP to the C/EBP subfamily, and DBP to the proline and acidic amino acid rich (PAR) subfamily of bZip factors *(38)*. Members of each bZip subfamily recognize the same palindromic or pseudopalindromic sequence; however, overlap exists between the elements these factors recognize *(39)*. Consequently, it is possible that any ATF/CREB, C/EBP, or PAR factor could recognize the overlapping bZip element within the major activation domain of the p8 promoter.

To identify potential candidate factors involved in p8 gene regulation, we utilized the data obtained from a set of Affymetrix GeneChips® we have recently performed on LβT2 cells that have been stably transfected with empty pcDNA3 (C-LβT2). This cell line was created to serve as a control to compare the effects of altering p8 expression in LβT2 cells and expresses factors characteristic of gonadotropes, including LHβ, gonadotropin-releasing hormone receptor, and p8, at levels similar to wild-type LβT2 cells *(1)*. When the expression levels of a variety of ATF/CREB, C/EBP, and PAR factors were compared, we found that both ATF4 and DBP mRNA are highly expressed (Fig. 2A), indicating that these factors may be potentially important in p8 gene regulation in LβT2 cells. The roles of DBP and its subfamily members, thyrotroph embryonic factor (TEF) and hepatic leukemia factor (HLF), appear to be predominantly linked to maintaining circadian control of transcription within the liver, brain, and other organs *(40)*. As such, they are constitutively expressed in a cyclic fashion in many tissues. To our knowledge, no link between p8 expression and

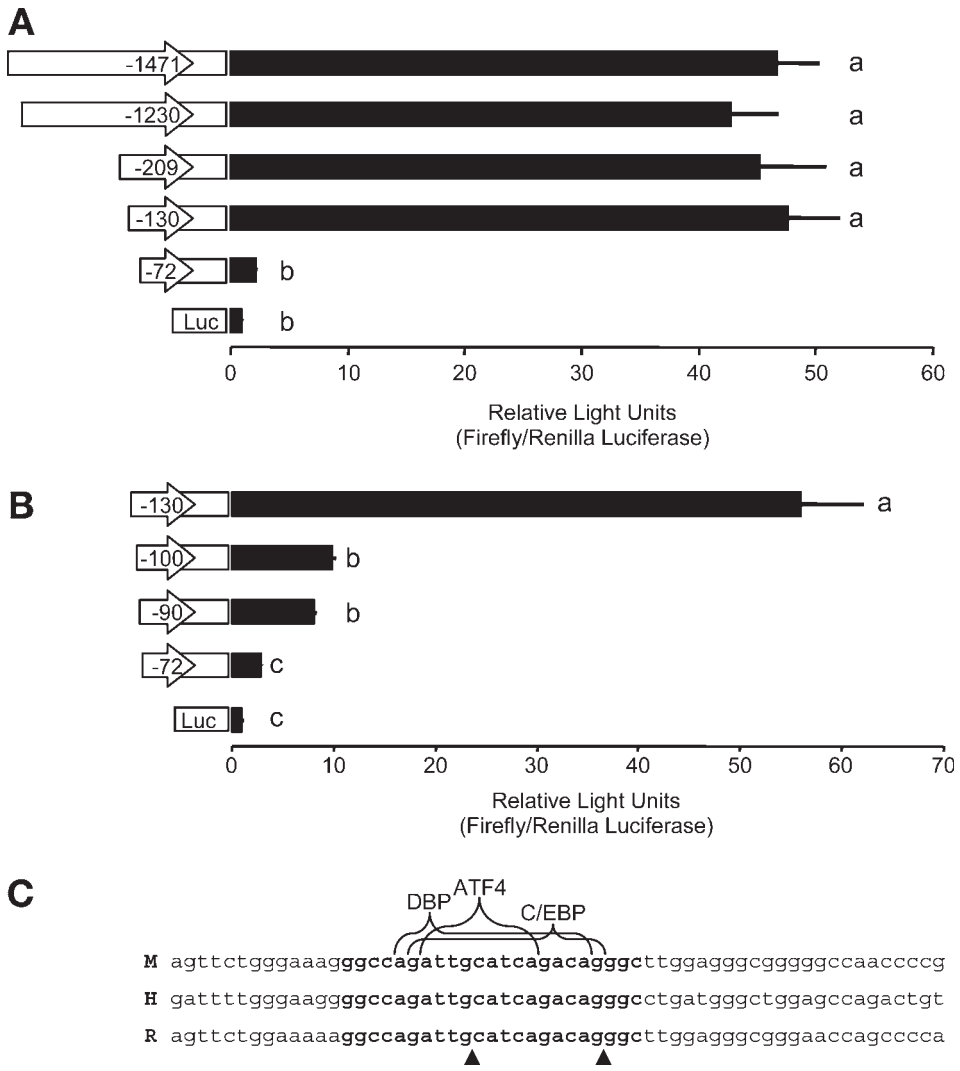

Fig. 1. The p8 promoter contains a major activation domain between –130 and –100 bp, a region that is highly conserved across species. (**A**) Transient transfection analysis utilizing p8 promoter-driven and promoterless luciferase constructs. Deletion analysis indicates that a regulatory element(s) necessary for activation of the p8 promoter in LβT2 cells resides between –130 and –72 bp. (**B**) Creation of additional promoter deletion constructs (–100/+36 or –90/+36) indicates that a major activation domain exists between –130 and –100 bp in the p8 promoter. Values are means ± SD of firefly luciferase activity normalized with renilla luciferase activity. Bars bearing different letters indicate luciferase values that are significantly different ($p < 0.01$). (**C**) The p8 promoter sequences from the mouse (–130 to –72 bp), human (–76 to –18 bp), and rat (–107 to –49 bp) are aligned to indicate a 23-bp region that is fully conserved in all three species (**bold**). Within this region, putative *cis*-acting elements within the p8 promoter are indicated. Black triangles mark the location of promoter deletions constructs created in the mouse sequence at –100 and –90 bp, respectively.

circadian rhythms has been established. Consequently, it seemed unlikely that DBP would be the factor regulating p8 gene expression in LβT2 cells. Conversely, like p8, ATF4 is expressed in both development *(25–30)* and the cellular stress response in a number of tissues *(31–36)* making it a suitable candidate for further investigation as a factor involved in p8 gene regulation in LβT2 cells.

To verify that ATF4 mRNA is expressed at similar levels in LβT2 and C-LβT2 cells, Northern blot analysis was performed (Fig. 2B). When the average amount of ATF4 in each cell line is normalized to 18S, the levels of ATF4 are not significantly different. The relative ATF4 signal intensity is 50.5 ± 16.8 in LβT2 cells 40.7 ± 16.8 in C-LβT2 cells.

ATF4 Activates the p8 Promoter in LβT2 Cells in a Dose-Dependent Manner

A putative ATF4 element lies within the major activation domain of the p8 promoter and ATF4 mRNA is present endogenously in LβT2 cells. To determine whether ATF4 is able to activate the p8 promoter when overexpressed in LβT2 cells, the –1471/+36 bp p8 promoter construct was

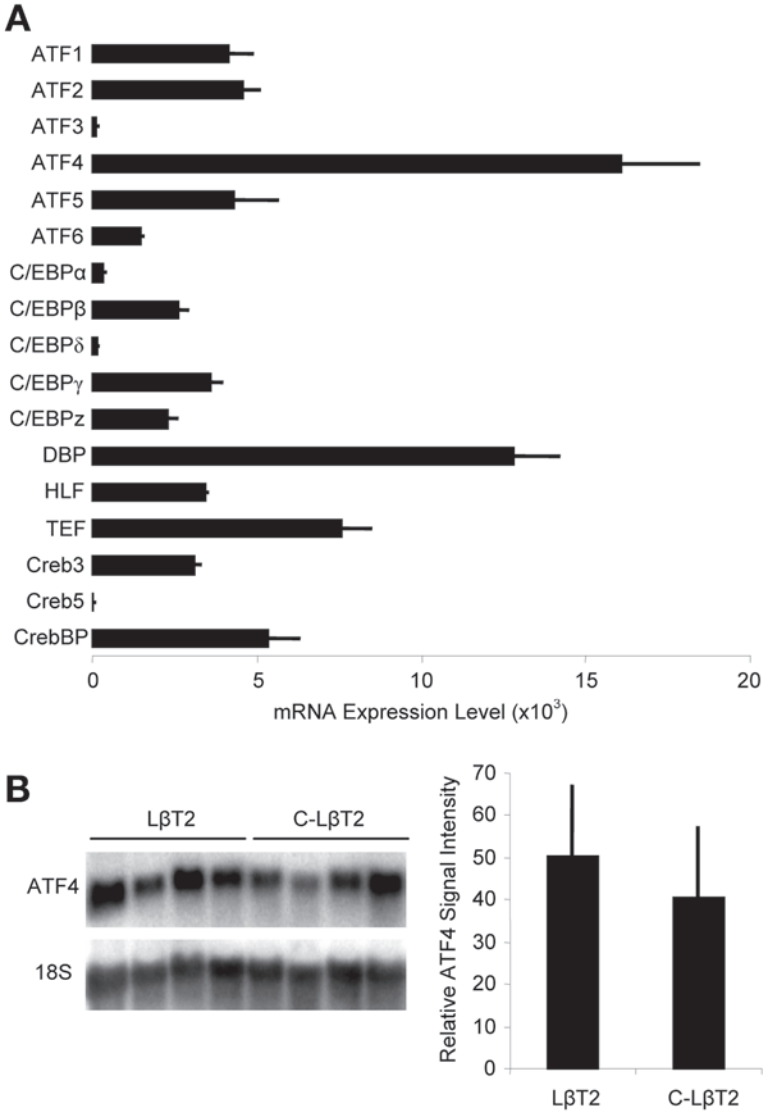

Fig. 2. ATF4 mRNA is highly expressed in LβT2 cells. (**A**) Affymetrix GeneChip® analysis was performed on mRNA from C-LβT2 cells. For each ATF/CREB, C/EBP, and PAR factor shown, values corresponding to the average of the probe set with the highest expression level. Error bars indicate SD across four GeneChips®. (**B**) Northern blot analysis of four biologically distinct pools of LβT2 and C-LβT2 mRNA. When normalized to 18S, the expression of ATF4 in the two cell lines is not significantly different; the relative ATF4 signal intensity is 50.5 ± 16.8 in LβT2 cells and 40.7 ± 16.8 in C-LβT2 cells.

transiently cotransfected with a vector encoding ATF4 or its corresponding empty vector, pCMV-Sport6. Cotransfection of ATF4 increases p8 promoter activity approx twofold over basal levels, which corresponds to approx 100-fold activation in promoter activity over the promoter-less control. The increase in promoter activity driven by ATF4 is dose-dependent; increasing the amount of transfected ATF4-containing vector from 2 to 200 ng per 10^6 LβT2 cells results in a corresponding increase in p8 promoter activity (Fig. 3A). To verify the level of ATF4 overexpression in these studies, Western blots were performed on protein harvested from transfected cells (Fig. 3B), showing that when overexpressed, ATF4 levels increase in a dose-dependent manner. When normalized to GAPDH expression, ATF4 expression peaks at 1.8-fold increase from basal levels upon transfection with 200 ng ATF4 vector per 10^6 cells.

A 13-bp Region Within the Major Activation Domain Is Crucial for ATF4 Activation

To verify that the region containing the highly conserved ATF4 element is critical for p8 promoter activation by ATF4 in LβT2 cells, a series of site-directed mutants in the –1471/+36 bp promoter were constructed and tested. First, a 13-bp mutation was created that abolishes the entire bZip region containing the ATF4 element (Fig. 4A). The second two mutations, 4 bp each, abolish sequences within the ATF4 ele-

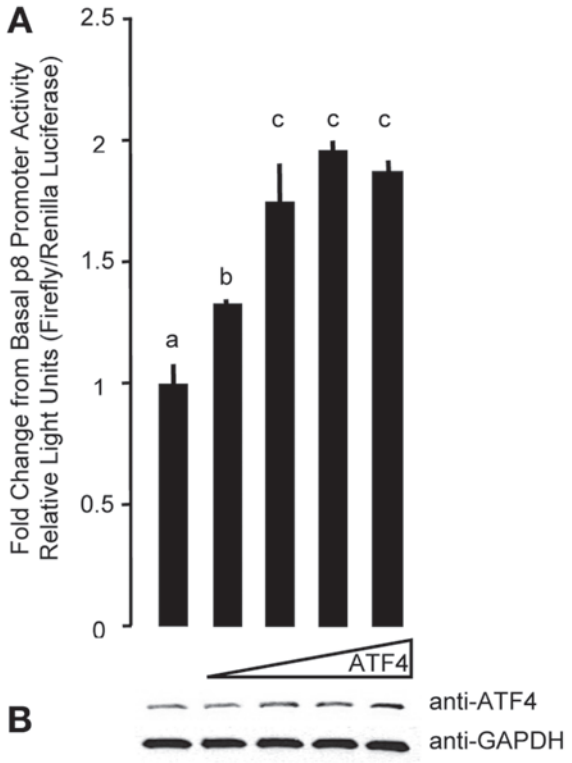

Fig. 3. ATF4 is able to activate the p8 promoter upon transient cotransfection in LβT2 cells in a dose-responsive manner. Cells were transiently cotransfected with –1471/+36 p8 promoter construct and 0, 2, 20, 100, or 200 ng per 10^6 cells of a vector constitutively encoding ATF4. (**A**) Upon cotransfection, ATF4 causes an increase in p8 promoter activity in a dose-responsive manner. Values are means ± SD of firefly luciferase activity normalized with renilla luciferase activity. Bars bearing different letters indicate luciferase values that are significantly different ($p < 0.01$). (**B**) Total protein was harvested from transfected cells and the dose-dependent increase in ATF4 expression was verified via Western blot, normalized to GAPDH as a loading control. Upon transfection with 200 ng ATF4 vector per 10^6 cells, ATF4 expression is 1.8-fold more than basal expression.

ment. Upon transient transfection in LβT2 cells, all mutants show a significant loss of p8 promoter activity to a level similar to that of the –100/+36 construct, indicating that the highly conserved region containing the ATF4 element is critical for p8 promoter activation in LβT2 cells. In addition, the –1471/+36 bp promoter construct in which the 13-bp region containing the ATF4 element has been mutated is not activated by ATF4 in cotransfection (Fig. 4B). Likewise, the –100/+36 bp promoter construct, which lies downstream of the ATF4 element, is not activated by cotransfection of ATF4, indicating that the region containing the ATF4 element is necessary for p8 promoter activation by ATF4 in LβT2 cells.

ATF4 Binds the Major Activation Domain of the p8 Promoter

To determine if endogenous ATF4 is capable of binding the major activation domain of the p8 promoter in LβT2 cells, electrophoretic gel mobility super-shift analysis was performed. Double-stranded radiolabeled probe containing the ATF4 element within the p8 promoter, γ-^{32}P-p8 (–119/–92), was incubated with LβT2 cell nuclear extract. When these reactions were separated by polyacrylamide gel electrophoresis, distinct bands representing factors able to bind the probe were observed (Fig. 5A). Binding of the factor indicated by arrow 1 represents a high-affinity, specific interaction as it is effectively competed away with a fivefold molar excess of nonradiolabeled homologous competitor, and is not competed away with 500-fold heterologous competitor. In addition, this band is super-shifted by addition of ATF4 antibody (1ss), but not by antibodies to the bZip factors C/EBPα and TEF, whose putative elements also lie within the major activation domain of the p8 promoter. Thus, it is likely that this band represents endogenous ATF4 binding its element within the major activation domain of the p8 promoter.

To determine which bases within the ATF4 element are important for ATF4 binding the p8 promoter, EMSA competition assays were performed. Radiolabeled probe encompassing the major activation domain of the p8 promoter, γ-^{32}P-p8(–119/–92), was incubated with LβT2 cell nuclear extract with 500-fold molar excess of non-radiolabeled competitor. In these reactions, each competitor utilized harbored a single transversion mutation in one of the 13 bp found critical for p8 activation by ATF4 (μ1 through μ13). In each set of oligos, the following nucleotide substitutions were made: A to C, C to A, T to G, or G to T. The EMSA competition strategy was used because competitors in which the mutated base is critical for ATF4 binding act as heterologous competitor; they do not compete for ATF4 binding and do not interfere with the intensity of its band on the gel. Conversely, the competitors whose mutated basepair is not necessary for ATF4 binding act as homologous competitors and compete for ATF4 binding, decreasing the intensity of the ATF4 band on the gel. The EMSA competition analysis revealed that six of the nine basepairs within the ATF4 element and none of the basepairs surrounding the element are necessary for ATF4 binding (Fig. 5B). Competitors in lanes 1, 4, 8, and 10–13 compete for ATF4 binding the wild-type probe; thus, they are not critical for ATF4 binding the sequence. Competitors in lanes 2, 3, 5, 6, 7, and 9 do not compete for binding of the probe to ATF4, indicating that these bases are necessary for ATF4 binding to the p8 promoter.

Discussion

LβT2 cells were derived using targeted oncogenesis in transgenic mice and readily form tumors upon allograft in athymic nude mice *(41)*. However, this tumorigenic ability appears to be dependent upon expression of p8 *(2)*, indicating that these cells are an appropriate model for the study of p8 gene regulation in tumorigenesis. Herein, we have

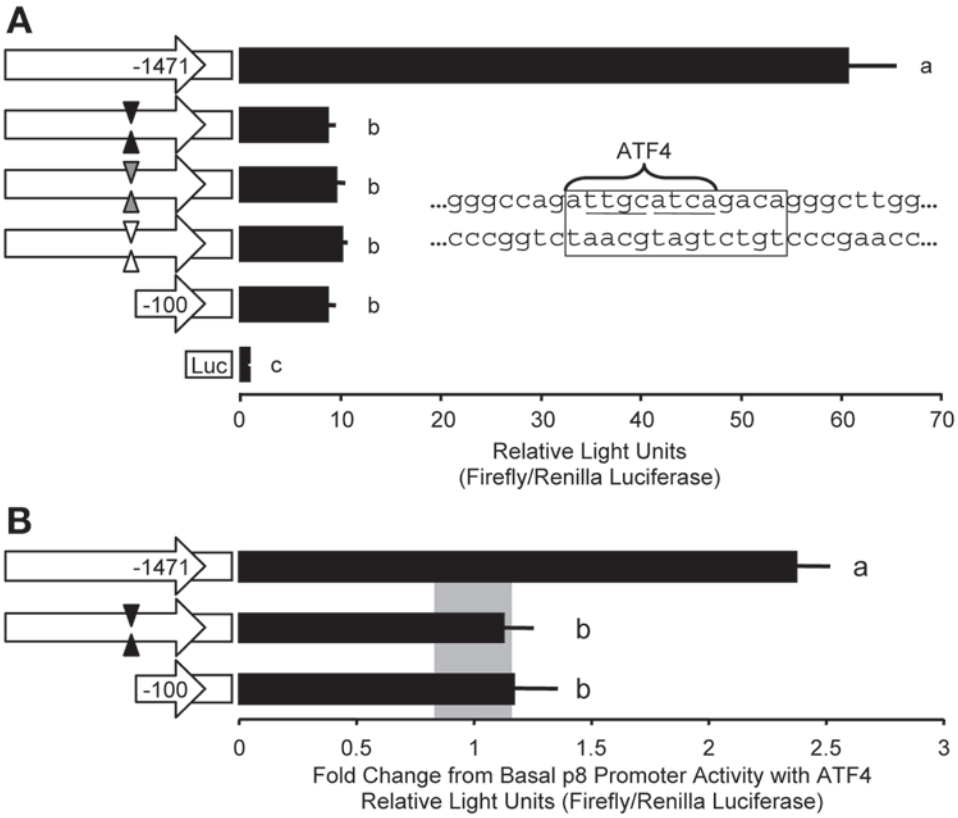

Fig. 4. The major activation domain of the p8 promoter contains a 13-bp region containing an ATF4 element that is crucial for promoter activation by ATF4 in LβT2 cells. (**A**) Cells were transiently transfected with promoter-driven luciferase constructs consisting of either the wild-type –1471/+36 or –100/+36 p8 promoter, one of three mutation constructs made in the –1471/+36 p8 promoter, or a promoterless luciferase plasmid. The mutations, which are indicated with triangles, represent a 13 bp mutation (black) abolishing the entire putative ATF4 element (boxed region in the inset sequence) or constructs in which 4 bp mutations were introduced within the putative ATF4 element (underlined sequences in inset, TTGC [gray] and ATCA [white]). All mutations of the ATF4 element decrease p8 promoter activity approximately sixfold. (**B**). Cells were transiently transfected as in **A** and cotransfected with 200 ng per 10^6 cells of ATF4. The vertical gray bar represents basal p8 promoter activity ± SD upon cotransfection of empty vector. Overexpression of ATF4 causes a significant increase in p8 promoter activity only in the wild-type –1471/+36 construct. Values are means ± SD of firefly luciferase activity normalized with renilla luciferase activity. Bars bearing different letters indicate luciferase values that are significantly different ($p < 0.01$).

mapped an ATF4 *cis*-acting element within the highly conserved overlapping bZip region of the major activation domain of the p8 promoter that is necessary for full promoter activation in LβT2 cells. ATF4 is present endogenously in LβT2 cells, binds its element within the major activation domain of the p8 proximal promoter, and enhances promoter activity in a dose-dependent manner upon transient overexpression.

Like p8, ATF4 is important in the development and differentiation of tissues and in cellular stress responses. During the stress response, multiple pathways lead to phosphorylation of eukaryotic translation initiation factor 2α, which causes a general inhibition of protein synthesis with a concomitant induction in the translation activation of ATF4, which is thought to play a critical role in the generalized response to stress *(31,33,42,43)*. Given the similar expression of ATF4 and p8 during the stress response, the coinciding presence of ATF4 and p8 in LβT2 cells, and the ability of endogenous ATF4 to bind and activate the p8 promoter in these cells, it is likely that ATF4 is a key factor that upregulates p8 gene expression in these cells and perhaps during the stress response in general.

We have shown that overexpression of ATF4 increases p8 promoter activity in a dose-dependent manner, peaking at approx twofold basal levels. Although this increase in activity may seem small, it is important to note that a twofold change in p8 gene expression has been proven to have significant physiological consequences. In the GH3 somatolactotrope tumor cell line, which endogenously expresses p8, decreasing expression of p8 mRNA leads to a corresponding decrease in the cells' ability to form tumors *(2)*. While GH3 cells expressing basal levels of p8 form tumors by 27 d postinjection, cells expressing approx half the basal level of p8 mRNA do not form tumors even after 42 d and cells with intermediate levels of p8 expression (up to 67% of basal mRNA levels) form tumors with rates and final volumes proportional to their p8 expression. In addition, a recent study has found that in cortical astrocytes, serum deprivation for 2 d increases p8 mRNA expression approx 2.8-fold *(44)*. This increase in p8 expression results in a cor-

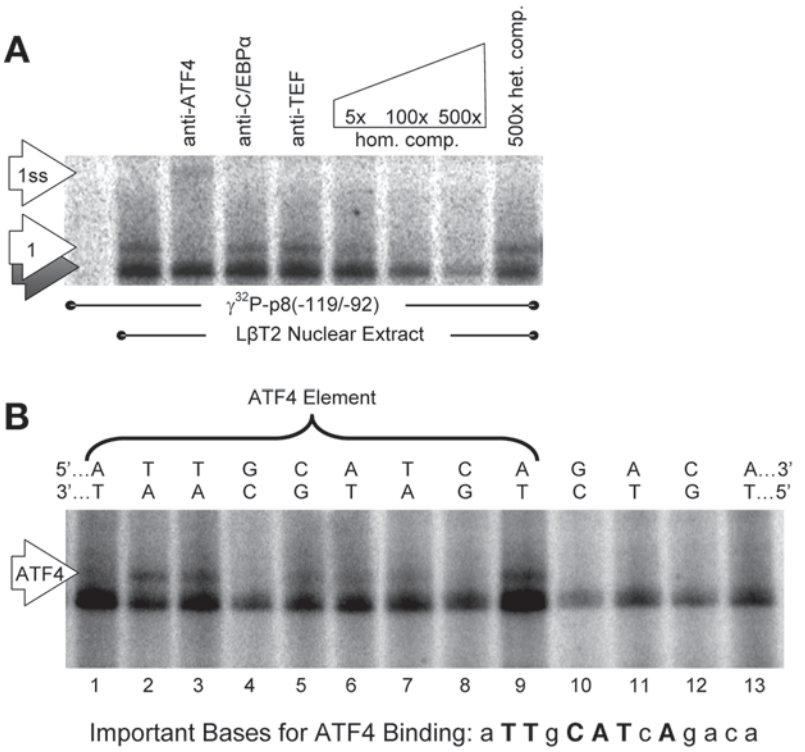

Fig. 5. ATF4 binds the major activation domain of the p8 promoter. (**A**) EMSAs were performed with LβT2 nuclear extract and wild-type p8 probe, γ-^{32}P-p8(–119/–92). Unlabeled p8(–119/–92) was added as homologous competitor as indicated. For heterologous competition, an unlabeled downstream portion of the p8 promoter, p8(–91/–64), was used. Arrow 1 indicates a factor that binds probe in a specific manner as its binding is competed away with homologous, but not heterologous, competitor. This band is super-shifted by the addition of ATF4 antibody (Arrow 1ss), but not by addition of antibodies to the bZip factors C/EBPα or TEF. (**B**) EMSAs were performed with LβT2 nuclear extract, wild-type p8 probe, γ-^{32}P-p8(–119/–92), and 500-fold molar excess of a double-stranded oligo similar to wild-type but which, in each lane, harbored a point mutation in one of the 13 bp within the overlapping bZip region [listed as p8(–119/–92)μ1 through μ13 in Material and Methods], corresponding to lanes one through 13. Below, bases found to be important for ATF4 binding this region of the p8 promoter are listed in capital letters.

responding decrease in the expression of heme-oxygenase 1, an enzyme involved in protecting cells against oxidative damage, indicating that a relatively small increase in p8 expression has the ability to sensitize cells to oxidative stress. Finally, another recent study has found that a 2.1-fold increase in p8 mediates cannabinoid-induced apoptosis in the A375 human melanoma cell line *(45)*. Intriguingly, this study found that a primary mechanism by which p8 mediates cannabinoid-induced apoptosis is by upregulating factors involved in the cellular stress response, including ATF4. Future studies will be necessary to discern whether p8-mediated ATF4 upregulation is a cell-specific phenomenon or whether it is possible that the two factors are able to form a positive feedback loop during the cellular response to stress.

We have found that in LβT2 cells, a 13-bp region of the p8 promoter is critical for activation by ATF4. However, it appears that ATF4 is not the sole factor able to bind the major activation domain of the p8 promoter in LβT2 cells (see Fig. 5A, gray arrow). It is not surprising that other factors can recognize this sequence. The 13-bp region crucial for p8 promoter activation contains overlapping putative elements for bZip factors from the ATF/CREB, C/EBP, and PAR subfamilies and it is possible that any of these factors, if present, could act at this site. We hypothesize that the 13-bp overlapping bZip domain is critical for p8 promoter activation in a number of cell types, where it is likely acted upon by a variety of bZip factors in a cell-specific manner. Moreover, the overlapping bZip region is conserved in a number of species (see Fig. 1C), indicating that it may be important not only in cell-specific regulation of the murine p8 gene, but also in p8 gene regulation in a number of species. This hypothesis is strengthened by the finding that the overlapping bZip region is required for promoter activation by C/EBPα and β in NIH 3T3 fibroblasts *(46)*. In addition, a recent study found that the p8 gene is activated by the ATF/CREB factor ATF3 in an in vitro model of angiogenesis *(47)*. It appears that regardless of the cell type, a bZip factor or multiple bZip factors are involved in regulation of p8 gene expression, likely at the highly conserved

overlapping bZip site on the p8 promoter. As a relatively small change in p8 expression appears to have major physiological consequences within the cell, these findings provide novel insight into a mechanism by which p8 expression could be targeted in the future to control abnormal cell proliferation, neoplastic transformation, and cancer.

Materials and Methods

Plasmid Vectors

All plasmid DNAs were prepared from overnight bacterial cultures using Qiagen DNA plasmid columns according to the protocol of the supplier (Qiagen, Chatsworth, CA) and were verified by sequencing and restriction enzyme digest prior to use in transfection. Luciferase reporter constructs were created in pGL3-Basic (Promega, Madison, WI). p8(−1471/+36)-pGL3 was created by digesting p8(−5000/+36)-pCAT *(46)* with *Hind*III and *Xba*I, blunt-ending with T4 DNA polymerase, and inserting the fragment into the *Sma*I site in pGL3-Basic. The −1230/+36, −209/+36, −130/+36, and −72/+36 constructs were created by digesting p8(−1471/+36)-pGL3 with *Sac*I and either *Swa*I, *Pvu*II, *Apa*I, or *Bstx*I, respectively. Constructs were blunt-ended with T4 DNA polymerase and ligated using Quick Ligase (New England Biolabs, Ipswich, MA). The −100 and −90/+36 constructs were created using a PCR strategy. Primers were created that annealed to the specific regions of the p8 promoter and included a flanking *Sac*I restriction site: 5'-GGTTTATTGAGCTCAGGGCTTGGAGGGCG GGGGC-3' and 5'-GGTTTATTGAGCTCAGGGCGGGG GCCAACCCC-GT-3'. These primers, along with a primer that anneals to a sequence within the polylinker of pGL3, GLprimer2 (Promega), were used to amplify specific products using p8(−1471/+36)-pGL3 as template for the PCR reaction using *PfuUltra*™ Hotstart High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA). The PCR products were digested with *Sac*I and *Xho*I and sub-cloned into the same sites in the pGL3 polylinker. Promoter mutation constructs were created using the standard QuikChange® II Site-Directed Mutagenesis Kit protocol (Stratagene) using mp8(−1471/+36)-pGL3 as template for the PCR reaction. In each construct, a transversion mutation was made to the wild-type promoter sequence: A to C, C to A, T to G, and G to T. Primers used were as follows (underlined bases indicate those that are mutated compared to the wild-type sequence): 13-bp mutation: 5'-GAAAGGGCCAGCGGTAC GACTCACGGGCTTGGAGGGC-3' and 5'-GCCCTCCA AGCCCGTGAGTCGTACCGCTGGCCCTTTC-3'.

First 4-bp mutation: 5'-GAAAGGGCCAGAGGTAAT CAGACAGGGC-3' and 5'-GCCCTGTCTGATTACCTC TGGCCCTTTC-3'.

Second 4-bp mutation: 5'-AGGGCCAGATTGCCGAC GACAGGGCTTG-3' and 5'-CAAGCCCTGTCGTCGGC AATCTGGCCCT-3'. The expression vector pCMV-Sport6-ATF4 (cat. no. EMM1002-708) was purchased from Open Biosystems (Huntsville, AL). p8(−5000/+36)-pCAT was a gift from Juan Iovanna, Centre de Recherche INSERM, Marseille, France.

Affymetrix GeneChip® Analysis

C-LβT2 cells were created by stable transfection of wild-type LβT2 cells with empty pcDNA3 (Invitrogen, Carlsbad, CA) as described *(1)*. C-LβT2 cells were maintained in high glucose Dulbecco's modified Eagle's medium containing 2 m*M* L-glutamine and supplemented with 10% fetal bovine serum and antibiotics (complete medium). RNA from four biologically independent replicate cultures of C-LβT2 cells was harvested using TRIzol reagent® (Invitrogen) and the integrity was verified by Northern blot analysis for p8. High-quality C-LβT2 RNA was then sent to the Center for Medical Genomics at the Indiana University School of Medicine (Indianapolis, IN). Quality was further assessed by spectrophotometry and electrophoresis using an Agilent Bioanalyzer. The standard Affymetrix protocols were used to synthesize biotinylated cRNA, starting with 5 μg total RNA from each, using the Affymetrix kits for cDNA synthesis, in vitro transcription, and sample cleanup. Each independent sample was hybridized to a separate Affymetrix Mouse Genome HG 430 2.0 GeneChip®, washed and labeled following standard protocols. GeneChips® were scanned using an Affymetrix Model 3000 scanner controlled by GCOS software. Data were extracted using the MicroArray Suite 5 (MAS5) algorithm and exported into MicroArray Data Portal for analysis.

Northern Blot Analysis

C-LβT2 cells were maintained in complete medium and RNA from four biologically independent replicate cultures of LβT2 and C-LβT2 cells was harvested using TRIzol reagent®. Ten micrograms of total RNA were separated by electrophoresis in a 1% denaturing agarose gel and transferred to nylon membrane (Hybond N+; Amersham Biosciences, Pittsburgh, PA) by gravity and capillary action. All probes were made by radiolabeling of cDNAs with α-^{32}P-dCTP (3000 Ci/mmol, MP Biochemicals, Solon, OH) using the standard DECA-prime II kit protocol (Ambion, Austin, TX). The random-primed labeled ATF4 probe used in the Northern blot analysis was created by digesting pCMV-Sport6-ATF4 with *Mlu*I, which yielded a 1400-bp fragment containing the full murine ATF4 cDNA, and the 18S probe consisted of a cDNA encompassing the murine 18S rRNA contained within primers included in the Quantum RNA 18S Internal Standards (Ambion). Northern blot analysis was performed as described previously *(1)*. Briefly, the membrane was prehybridized in hybridization solution for approx 3 h and hybridized with the appropriate radiolabeled probe overnight at 45°C in a roller-bottle hybridization oven. The final washes were in 0.5X SSC, 0.5% SDS at 65°C. The membrane was exposed for 1–24 h on a phosphor screen (Amersham Biosciences) followed by densito-

metric scanning and analysis using a Typhoon 9200 Variable Mode Imager with the ImageQuant TL v2003.01 software package (Amersham Biosciences).

Cell Transfection and Luciferase Assays

LβT2 cells were maintained in complete medium as described previously *(1)*. The day prior to transient transfection, cells were plated at a density of approx 1×10^6 cells/well in 15 mm wells in 500 μL complete media. Transfection complexes contained 1.2 μL of Fugene 6 transfection reagent (Roche, Indianapolis, IN), 20 ng of the Renilla vector, pRL-CMV (Promega, Madison, WI), which was used to normalize data for transfection efficiency, and all wells received 400 ng total test vectors. In cotransfections, 200 ng of each p8 promoter vector and a total of 200 ng of the vector containing the *trans*-acting factor and/or empty-vector control were used as indicated. In dose-response cotransfections, 200 ng of each p8 promoter vector was added to each well. In addition, 0 to 200 ng ATF4-pCMV-Sport6 was added to wells as indicated, titrated to a total of 200 ng with empty pCMV-Sport6 vector. Cell cultures were incubated with the transfection complexes at 37°C in a humidified atmosphere with 5% CO_2. Approximately 24 h post-transfection, cells were lysed in passive lysis buffer (Promega) and a dual-luciferase assay was performed on each cellular lysate as per standard procedures (Promega) using a Turner Designs TD-20/20 luminometer with a 3 s delay and 15 s read time. To account for transfection efficiency, firefly luciferase was divided by renilla luciferase activity for each well. Then, values were normalized to the activity of the corresponding empty vector control(s). Transient transfections were performed a minimum of three times with at least two separate plasmid preparations for each construct that was tested.

ATF4 Western Blot Analysis

LβT2 cells were cultured and transfected with 0–200 ng ATF4 vector per 10^6 cells as described above. Approximately 24 h post-transfection, whole cell protein extract was harvested. Cells were washed twice and scraped into 1X PBS on ice, then pelleted at 3000*g* for 4 min at 4°C. Next, cells were lysed for 20 min at 4°C with rotation in RIPA lysis buffer [30 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 1 m*M* EDTA, 2.5 m*M* PMSF, 1% (v/v) Triton X-100] containing protease inhibitors (Roche). Cell lysates were then homogenized for 30 s using a Pellet Pestle® tissue homogenizer (Kimble/Kontes, Vineland, NJ), cellular debris was pelleted at 4000*g* for 20 min at 4°C, and cellular extract was stored at −80°C until use. Before use, protein concentration was determined using the standard DC protein assay protocol of Bio-Rad (Hercules, CA).

For Western blots, 100 μg protein was denatured for 4 min at 65°C in Laemmli Sample Buffer (Bio-Rad) supplemented with 2.5% (v/v) β-mercaptoethanol before loading and running on a 4–20% linear gradient polyacrylamide gel (Bio-Rad). Protein was transferred to Immun-Blot™ PVDF membrane (Bio-Rad) for 1 h in transfer buffer [25 m*M* Tris, 200 m*M* glycine, and 10% (v/v) methanol], rinsed in wash buffer [0.05% (v/v) Tween-20 in PBS], and blocked in blocking buffer [5% (w/v) nonfat dry milk in wash buffer] for 1 h at room temperature with rocking. Membranes were then incubated with a rabbit polyclonal ATF4 antibody (sc-200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in blocking buffer at a concentration of 1:500 for 16 h at 4°C with rocking. Following, membranes were washed and probed with peroxidase conjugated ImmunoPure® goat anti-rabbit IgG (Pierce Biotechnology, Rockford, IL) in blocking buffer at a concentration of 1:20,000 for 1 h at room temperature with rocking. After washing, membranes were incubated according to the standard Immun-Star™ HRP Substrate Kit (Bio-Rad) protocol and exposed to film for 3 h. To normalize for total protein, membranes were stripped in strip buffer [10% SDS (w/v), 1 *M* Tris-HCl (pH 7.0), and 0.7% (v/v) β-mercaptoethanol] at 50°C with rotation, then washed, incubated in blocking buffer for 1 h at room temperature with rocking, incubated with a mouse monoclonal peroxidase–conjugated GAPDH antibody (ab9482, Abcam Inc., Cambridge, MA) in blocking buffer at a concentration of 1:20,000 for 1 h at room temperature with rocking, washed, incubated with HRP substrate, and exposed as described above. Band intensity was quantified using ImageJ 1.36b image processing and analysis software (http://rsb.info.nih.gov/ij/).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extract was prepared from LβT2 cells as we have described previously *(48)*. Briefly, confluent 150 mm plates of LβT2 cells were washed twice in cold PBS. Cells were then scraped into 1 mL of a buffer containing 10 m*M* HEPES (pH 7.9), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* DTT, 0.5 m*M* PMSF, and protease inhibitors (Roche). Cells were incubated for 15 min on ice, NP-40 was added to a final concentration of 0.5%, cells were mixed using a vortex, and centrifuged at 4500*g* for 1 min at 4°C to pellet nuclei. After removal of supernatant, the pelleted nuclei were resuspended and stirred on ice for 30 min in 900 μL of a buffer containing 20 m*M* HEPES (pH 7.9), 1.5 m*M* $MgCl_2$, 420 m*M* NaCl, 0.2 m*M* EDTA, 25% glycerol, and protease inhibitors. Nuclear debris was pelleted by centrifugation at 15,300*g* for 10 min at 4°C, the supernatant was removed and stored at −80°C until use. Protein concentration was determined using the standard DC protein assay protocol of Bio-Rad.

The following 28-base oligonucleotides were used as probes for the gel shifts with underlined bases indicating sites of mutation compared to the wild-type sequence:

p8(−119/−92): 5'-AGGGCCAGATTGCATCAGACA GGGCTTG-3' and 5'-CAAGCCCTGTCTGATGCAATC TGGCCCT-3'.

p8(–91/–64), heterologous competitor: 5'-GAGGGCG GGGGCCAACCCCGTGGTTTAG-3' and 5'-CTAAACC ACGGGGTTGGCCCCCGCCCTC-3'.

p8(–119/–92)μ1: 5'-AGGGCCAGCTTGCATCAGAC AGGGCTTG-3' and 5'-CAAGCCCTGTCTGATGCAAG CTGGCCCT-3'.

p8(–119/–92)μ2: 5'AGGGCCAGAGTGCATCAGAC AGGGCTTG-3' and 5'-CAAGCCCTGTCTGATGCACT CTGGCCCT-3'.

p8(–119/–92)μ3: 5'-AGGGCCAGATGGCATCAGA CAGGGCTTG-3' and 5'-CAAGCCCTGTCTGATGCCA TCTGGCCCT-3'.

p8(–119/–92)μ4: 5'-AGGGCCAGATTTCATCAGAC AGGGCTTG-3' and 5'-CAAGCCCTGTCTGATGAAAT CTGGCCCT-3'.

p8(–119/–92)μ5: 5'-AGGGCCAGATTTAATCAGA CAGGGCTTG-3' and 5'-CAAGCCCTGTCTGATTCAA TCTGGCCCT-3'.

p8(–119/–92)μ6: 5'-AGGGCCAGATTGCCTCAG ACAGGGCTTG-3' and 5'-CAAGCCCTGTCTGAGGCA ATCTGGCCCT-3'.

p8(–119/–92)μ7: 5'-AGGGCCAGATTGCAGCAGA CAGGCTTG-3' and 5'-CAAGCCCTGTCTGCTGCAAT CTGGCCCT-3'.

p8(–119/–92)μ8: 5'-AGGGCCAGATTGCATAAGAC AGGGCTTG-3' and 5'-CAAGCCCTGTCTTATGCAAT CTGGCCCT-3'.

p8(–119/–92)μ9: 5'-AGGGCCAGATTGCATCCGAC AGGGCTTG-3' and 5'-CAAGCCCTGTCGGATGCAAT CTGGCCCT-3'.

p8(–119/–92)μ10: 5'-AGGGCCAGATTGCATCATA CAGGGCTTG-3' and 5'-CAAGCCCTGTATGATGCAA TCTGGCCCT-3'.

p8(–119/–92)μ11: 5'-AGGGCCAGATTGCATCAGC CAGGGCTTG-3' and 5'-CAAGCCCTGGCTGATGCAA TCTGGCCCT-3'.

p8(–119/–92)μ12: 5'-AGGGCCAGATTGCATCAGA AAGGGCTTG-3' and 5'-CAAGCCCTTTCTGATGCAA TCTGGCCCT-3'.

p8(–119/–92)μ13: 5'-AGGGCCAGATTGCATCAGA CCGGGCTTG-3' and 5'-CAAGCCCGGTCTGATGCAA TCTGGCCCT-3'.

EMSAs were performed essentially as described *(48)*. Briefly, double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP (PerkinElmer, Boston, MA) using T4 polynucleotide kinase. Binding reactions were carried out in 20 μL total reaction volume with 20 μg LβT2 cell nuclear extract incubated with 1X supershift buffer [10 m*M* $MgCl_2$, 25 m*M* KCl, 0.5 m*M* dithiothreitol, 12.5 m*M* HEPES (pH 7.9), and 0.5% Triton X-100], 10% glycerol, 200 ng salmon sperm DNA, 200 ng *E. coli* DNA, and 1 μg poly(dI-dC). Double-stranded oligonucleotide competitors were added as indicated. Upon addition of 25 fmol radiolabeled probe, reactions were allowed to incubate for 15 min at room temperature. Following this incubation, 1 μL of antibodies to ATF4 (sc-200 X, Santa Cruz), C/EBPα (sc-61 X, Santa Cruz), or TEF (a gift from Paul Herring, Indiana University School of Medicine, Indianapolis, IN) were added as indicated and reactions were allowed to incubate for an additional 15 min at room temperature and then 5 min on ice. Reactions were then separated on a 6% polyacrylamide gel.

Statistical Analysis

Luciferase activity was analyzed by one-way ANOVA and differences among groups were identified using Tukey's honestly significant difference multiple comparison test.

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