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Identifying serum-inducible sequence elements on the mouse ZFP36 promoter

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Zinc finger protein 36 (ZFP36) is an RNA-binding protein that downregulates the translation of certain gene products by inducing the degradation of mRNA. ZFP36 is an immediate-early gene: its transcription is activated directly following appropriate cellular stimuli, without any need for intervening protein synthesis. Prominent mRNA targets of ZFP36 are tumor necrosis factor-□ (TNF-□), epidermal growth factor (EGF) and c-fos. As defects in the c-fos and EGF pathways have been consistently found in cancers, ZFP36 is thought to be a tumor-suppressor. ZFP36 also is responsible for the suppression of TNF-□, giving it an additional role in the control of the inflammatory immune response. Thus, studying the regulation and action of ZFP36 may yield insights into mechanisms underlying cancer and inflammatory syndromes. To examine the regulation of ZFP36, two conserved regions upstream of the start of transcription were assayed for their ability to confer serum induction. Using a luciferase reporter gene system in HeLa cells, we show that neither region examined is induced by serum.

Key words: electron transfer, photosynthesis, quinones, UV-Vis spectroscopy

Introduction

Immediate-early genes are characterized by immediate transcriptional activation upon appropriate stimulation, including treatment of cells with mitogen-containing serum, without any need for intervening protein synthesis (Johansen, 1995). As they are among the first genes to be activated in many signaling pathways, immediate-early genes provide a gateway to study the cellular response. Zinc finger protein 36 (ZFP36) is an immediate-early gene product that regulates the expression of some genes by destabilizing their mRNA. The protein binds to AU-rich sequences on mRNA and catalyzes removal of the polyadenylated tail, reducing the half-life of the RNA (Blackshear, 2002). The zinc finger motif is common in DNA and RNA binding proteins, though ZFP36 is unusual in that it has a Cys3-His zinc-coordinating domain, as opposed to the more common Cys2-His2 domain (Tchen, 2004). This slight difference in peptides might subtly impact the nucleotide binding dynamics of ZFP36. Notable genes regulated in part by ZFP36 include tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF) and c-fos, a transcription factor that promotes proliferation (Amit, 2007) Mice deficient in ZFP36 develop an excess of TNF-α, and the overabundance of this cytokine results in autoimmune complications such as arthritis (Lai, 1998). As EGF and c-fos are both implicated in proliferation, ZFP36 is also thought to act as a tumor-suppressor (Amit, 2007)

The ZFP36 gene is regulated in several ways. Tchen and colleagues et al. have shown that the ZFP36 gene is mitogen-inducible via protein kinase p38 (Tchen, 2004). This result is consistent with qPCR data demonstrating that ZFP36 is induced (upregulated) strongly by serum after one hour **(Figure 1)**. However, the upregulation seems to be independent of serum response factor (SRF), the transcription factor that primarily mediates the serum response (Prywes, unpublished data). Lai and colleages showed that there are important regulatory elements downstream of the start of ZFP36 transcription, and at least one of those regulatory elements is on an intron (Lai, 1995; Lai, 1998). Given that ZFP36 is induced by serum one hour after induction, we hypothesized that there may be additional serum-dependent regulatory elements upstream of the start of transcription. We identified two conserved regions in the promoter region of mouse ZFP36 and used a luciferase reporter gene assay to test the ability of each region to confer serum induction in HeLa cells. However, we ultimately ascertained that neither region was able to confer serum induction of ZFP36 in HeLa cells. This study and other studies of the regulation and action of ZFP36 will provide vital information about this protein, and further research will play a significant role in gaining a better understanding of the biological pathways for proliferation and inflammation.

MOLECULAR BIOLOGY

Received Dec. 29, 2009. Accepted Feb. 8, 2009. Published April 5, 2009.
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Materials and Methods

Plasmid construction

Two plasmids, pZFP-2791-FGL3 and pZFP-1835-FGL3, were constructed from a plasmid called pZFP-3.7-FGL3 that had been made previously. The insert was excised with two different enzyme combinations: KpnI and HindIII in one case, and KpnI and BglII in the other. This resulted in linearized plasmids that carried an ampicillin resistance gene, a minimal promoter from the human c-fos gene, and a firefly luciferase gene. The two promoter regions of interest were obtained from PCR amplification of mouse genomic DNA. The primers delineated regions from -2591 to -1810 and from -1835 to -901 base pairs relative to the start of transcription, with regions chosen based on mammalian conservation patterns **(Figure 3)**. Each primer was designed with overhangs to insert the restriction sequences into the PCR products. Each pair of plasmid and PCR product was ligated at 14 °C for four hours using the T4 DNA ligase enzyme.

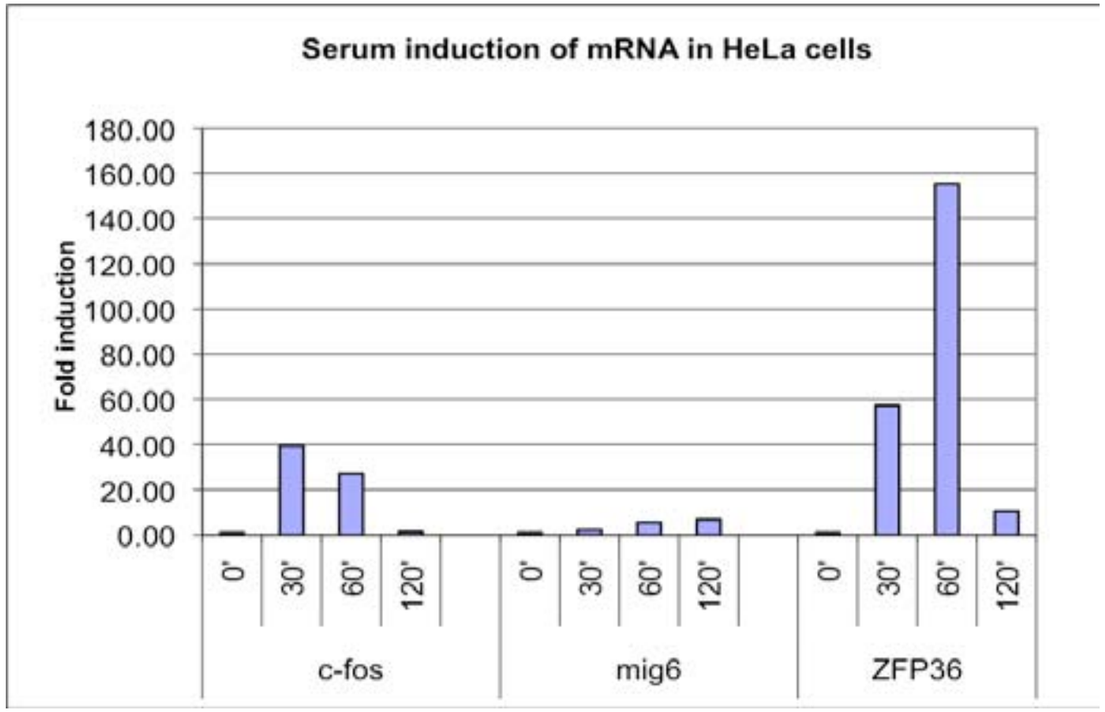
The resultant plasmids were transformed into E. coli for amplification. Sequencing revealed that the pZFP-1835-FGL3 plasmid had only a single-base change in the c-fos minimal promoter region. The pZFP-2590-FGL3 sequence showed an extra, non-contiguous piece of mouse ZFP36 DNA that had been inserted due to the presence of an internal BglII site in the mouse gene. This piece was excised and the plasmid amplified to give the correct sequence.

The other plasmids transfected were pCyr61, a control for which moderate serum induction was expected (Babic et al.), pOFGL3, which contains only the c-fos minimal promoter and luciferase, and two other plasmids (pZFP-3.7-FGL3 and pZFP-932-FGL3), which incorporate regions of the mouse promoter at -3700 and -932 bases respectively.

Transfection

Transfection was carried out using a calcium phosphate protocol. In this method, a fine calcium phosphate precipitate introduces genetic material into cells. At the appropriate pH, DNA binds weakly to the calcium phosphate particles, and cells which endocytose the precipitate may take up the genetic material.

Figure 1 *Results from qPCR for c-fos, mig6, and ZFP36 in HeLa cells.*



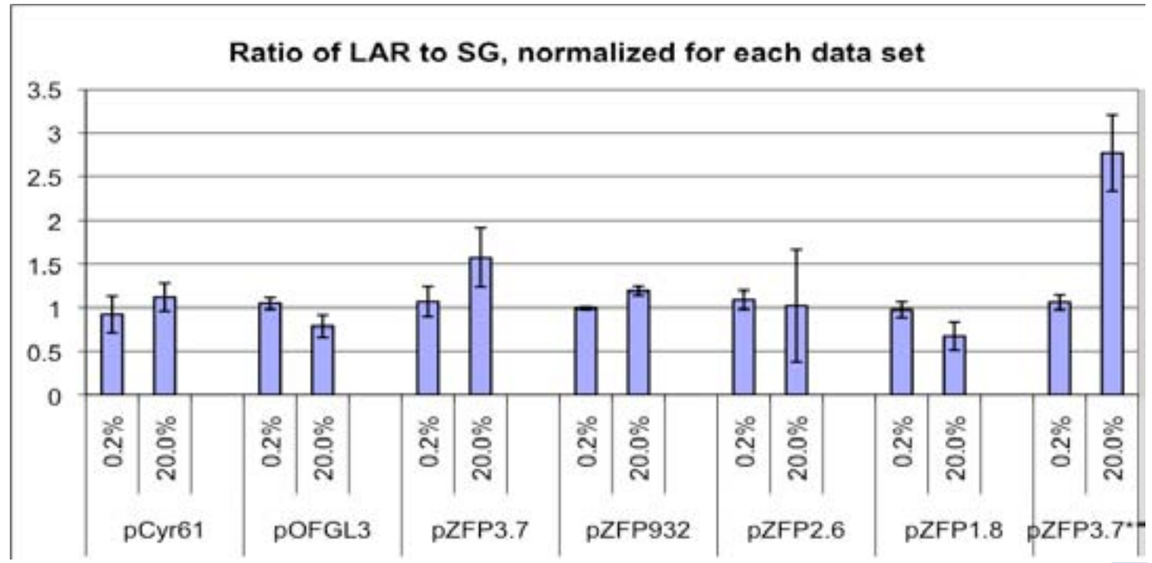


Figure 2 *Graph illustrating the induction of each reporter gene by serum. The raw data consisted of duplicate pairs of 0.2% and 20% serum conditions, given as ratios of LAR to SG luminescence. The values were calculated by normalizing each data set to one of the 0.2% ratios, then averaging together the duplicates and, if available, other data sets from different dates. No significant serum induction is seen, except in the ZFP3.7** condition, which used RL-TK in place of RL-SV40 as the renilla plasmid.*

Each transfection comprised a reporter plasmid, an RL-SV40 plasmid, and the non-luciferase plasmid pCAN, thus keeping the total DNA quantity constant at approximately 1 µg/µL. The RL-SV40 plasmid carries the enzyme renilla luciferase, which is coupled to a promoter from the SV40 virus. Since renilla luciferase can be measured independently of firefly luciferase, and the SV40 promoter is expressed constitutively, this plasmid was used to control for variable transfection efficiencies. In one experiment, the alternate renilla plasmid RL-TK was used, which has a tyrosine kinase promoter in place of the SV40 promoter.

Cells were plated onto 24-well plates the evening prior to transfection at a density of 6.5 x 104 cells per well, in Dulbecco's Modified Eagle Medium (DMEM) containing 10% NCS. Transfection mixtures were prepared using 1.75 µL of 2.5 M calcium chloride, the appropriate plasmids, and water to a total volume of 16 µL. The reporter plasmids were added at a concentration of 125 ng per well, and the renilla plasmid at a concentration of 66 ng per well. For each reporter gene, four identical wells were prepared to allow for duplicates. Each transfection mixture had a total volume of 64 µL. To each tube, 64 µL of HBS (a pH-adjusted mixture containing soluble phosphate salts) was added. The tubes were vortexed briefly and allowed to sit for 10 minutes at room temperature to allow the calcium phosphate to precipitate. Then 32 µL of each mixture was added to the appropriate well. The following morning, the media was replaced with 10% NCS in DMEM.

Serum induction and luciferase assay

On the afternoon of the day following transfection, the media in all of the wells were changed to 0.2% neonatal calf serum (NCS). The following morning, the cells in half of the wells were induced by adding 125 µL of pure NCS, raising the serum concentration to 20%. Three hours after serum induction, all cells were washed with sterile PBS and treated with 100 µL of Passive Lysis Buffer, which keeps cytosolic proteins intact during lysis. The cells were left at room temperature for 20 minutes on a slowly rotating shaker.

A bioluminescence detector was used to assay luciferase activity. Two reagents were used for the assay: Luciferase

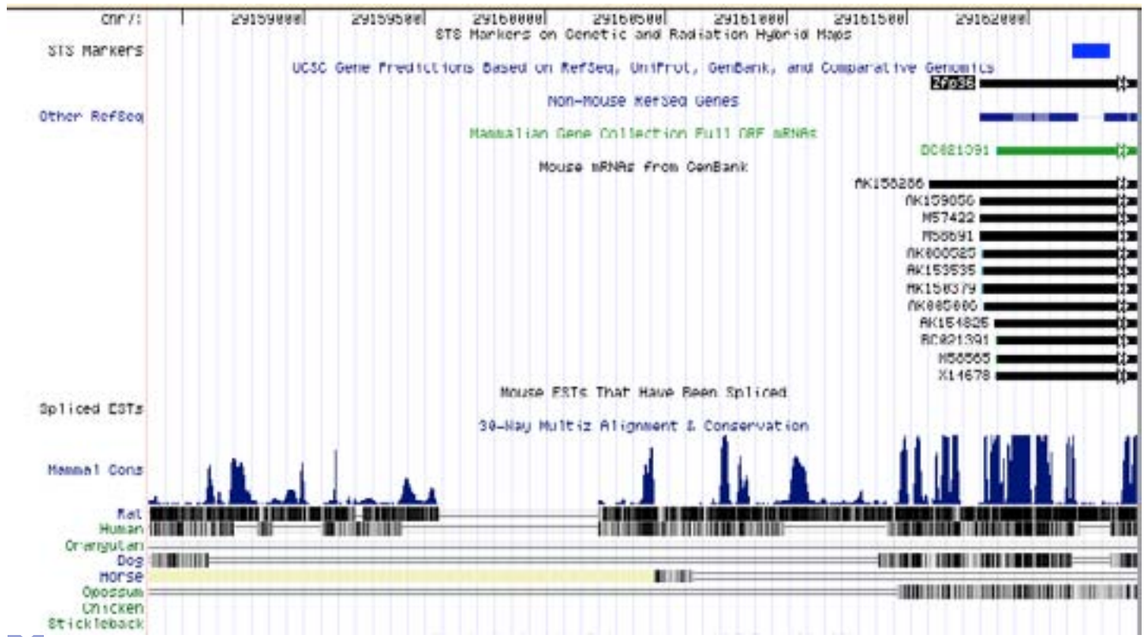


Figure 3 *Upstream ZFP36 promoter region, showing mammalian conservation regions. From UCSC Genome Biogenetics, http://genome.ucsc.edu/*

Activating Reagent (LAR) and Stop & Glow reagent (SG). LAR is the substrate of the firefly luciferase enzyme, and SG is the substrate of renilla luciferase. Each well was measured in two steps. First, 5 µL of cell lysate is added to 25 µL of LAR, and the luminescence was measured. Second, 25 µL of SG was added to the mixture and the luminescence is measured again. When analyzing the data, each measurement was normalized by computing the ratio of LAR to SG luminescence.

Results

ZFP36 mRNA was significantly induced by serum

Quantitative PCR was run on cDNA prepared from HeLa cells that were stimulated with serum for durations between 0 and 120 minutes **(Figure 1)**. ZFP36 showed an induction pattern similar to c-fos, with strongest induction between 30 minutes and 1 hour. Mig6, a gene that is not induced by serum, was used as a negative control.

None of the reporter genes tested was significantly induced by serum

Figure 2 shows a summary of the results of the luciferase assays performed on transfected HeLa cells. None of the reporter genes showed an appreciable increase in expression upon induction with 20% serum. Minimal serum induction was observed when a different renilla plasmid was used (see Discussion).

Discussion and Conclusion

The data do not support the hypothesis that conserved regions between -2150 and -901 confer serum induction of ZFP36. This result was reproducible through several rounds of transfections. This suggests that the observed serum induction of ZFP36 is mediated entirely by elements that are downstream of the start of transcription.

One issue that arose in the luciferase data was the inconsistency of renilla measurements. The SG values tended to vary, and previous experiments using a similar protocol indicated that renilla itself may be induced by serum. To test if serum induction of the renilla gene was affecting the results, an alternate renilla gene with a tyrosine kinase promoter (RL-TK) was substituted for RL-SV40 in one

trial. The calculated data do indeed show that pZFP-3.7-FGL3 was induced slightly less than threefold in this trial, but both the LAR and SG measurements from this trial were so different from those in other trials that no conclusions could be drawn from this result. The great variation in LAR measurements indicates a non-trivial relationship between two transfected genes: that is, the presence of one gene in the transfection mixture can affect the efficiency of the transfection of other genes. Another possible confounder could have been the plasmid preparations themselves, as calcium phosphate transfection is pH-sensitive. Using a different transfection method could more conclusively resolve this issue. Nevertheless, the significant serum induction observed in the qPCR results does not appear to be mediated by any of the upstream regions that were examined.

In conclusion, neither region tested was induced by serum when expressed on a reporter gene in HeLa cells. Future studies may further investigate the downstream regulatory elements characterized by Lai et al., or attempt to find other cellular stimuli that regulate ZFP36.

References

Amit, Ido, Ami Citri, Tal Shay et al. "A module of negative feedback regulators defines growth factor signaling." Nature Genetics. 2007, 39(4): 503-512.

Babic, Alexander M, Maria L Kireeva, Tatiana V Kolesnikova & Lester F Lau. "CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth." Proceedings of the National Academy of Sciences United States of America. 1998, 95(11): 6355-6360.

Blackshear, Perry J. "Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover." Biochemical Society. 2002, 30(6): 945-952.

Cen, Bo, Ahayla Selvaraj & Ron Prywes. "Myocardin/MKL Family of SRF Coactivators: Key Regulators of Immediate Early and Muscle Specific Gene Expression." Journal of Cellular Biochemistry. 2004, 93:74-82.

Johansen Finn-Eirik & Ron Prywes. "Serum response factor: Transcriptional regulation of genes induced by growth factors and differentiation." Biochimica et Biophysica Acta. 1995, 1242:1-10.

Lai, Wi S, Michael J Thompson & Perry J Blackshear. "Characteristics of the Intron Involvement in the Mitogen-induced Expression of Zfp-36." The Journal of Biological Chemistry. 1998, 273: 506-517.

Lai, Wi S, Michael J Thompson, Gregory A Taylor, Yi Lui & Perry J Blackshear. "Promoter Analysis of Zfp-36, the Mitogen-inducible Gene Encoding the Zinc Finger Protein Tristetraprolin." The Journal of Biological Chemistry. 1995, 270: 25266-25272.

Tchen Carmen R, Matthew Brook, Jeremy Saklatvala & Andrew R Clark. "The Stability of Tristetraprolin mRNA is Regulated by Mitogen-activated Protein Kinase p38 and by Tristetraprolin Itself." The Journal of Biological Chemistry. 2004, 279: 32393-32400.