Bioinformatics analysis and identification of transcriptional regulation of human FOXG1 gene

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Abstract

Forkhead box G1 (FOXG1) is a transcriptional repressor with high expression in the nervous system. Most research focused on the biological function of FOXG1, however, the transcriptional regulation of the FOXG1 gene was rarely studied. Herein, we analyzed and cloned 2.3kb of the 5'-upstream sequences of the FOXG1 gene to better understand the regulation of the FOXG1 gene expression. The CpG islands and putative transcription factor binding sites were analyzed by bioinformatics methods. Then we defined a small region in the upstream 350bp area which was required for the promoter activity by constructing truncated mutants.

Introduction

The forkhead-box (FOX) protein is a class of transcription factors with a winged-helix DNA-binding domain, which consists of at least 43 members(Katoh M et al.2004). Deregulation of FOX family genes leads to congenital disorders, such as dysplasia, or carcinogenesis(Manoranjan B et al.2013;Li J et al.1993;Ariani F et al.2008;De Filippis R et al 2012;Mariani J et al.2015). Expression regulation of FOX family genes, as well as binding proteins and target genes of FOX transcription factors, should be comprehensively investigated to develop novel therapeutics and preventives for human diseases.

FOXG1, also known as brain factor-1 (BF-1), is a member of the FOX family located at human chromosome 14q12. FOXG1 is specifically highly expressed in the nervous system, where FOXG1 is involved in the development of the forebrain, retina and optic chiasm, olfactory epithelium, and inner ear(Kortüm F et al.2011). Defects of the FOXG1 gene in humans can lead to Rett syndrome and other forms of mental retardation, or microcephaly and other brain abnormalities(Martynoga B et al.2005;Ariani F et al.2008). Interestingly, abnormal expression of FOXG1 has been observed in various types of cancer, including hepatoblastoma, glioma, medulloblastoma, breast cancer, and ovarian cancer(Manoranjan B et al.2013;Chen J et al.2018;Adesina AM et al.2007;Li JV et al.2013).

As a transcription repression factor, FOXG1 plays a negative regulatory role mainly through interaction with other transcription factors. FOXG1 interacts with groucho and hes proteins to regulate progenitor cell differentiation in the telencephalon(Buscarlet M et al.2008). FOXG1 can also bind to FOXO/SMAD complex and decrease p21Cip1 expression to induce cell cycle progress(Wang L et al.2018). Despite the numerous and important advances in understanding the function of FOXG1 at the cellular and molecular level, very little is known about the molecular mechanisms that control its expression(Seoane J et al.2004). Two studies have shown that microRNA-9 and microRNA-200b could negatively regulate endogenous FOXG1 protein level(Garaffo G et al.2015). However, the mechanism of transcriptional regulation of the FOXG1 gene is still unclear.

Promoter is the most important regulatory sequence involved in gene expression. The activity of the promoter determines the expression level of the downstream gene. Thus far, there is no report available that investigated its specific expression and characterized its upstream promoter in the human FOXG1

gene. In this study, we firstly identified and characterized the promoter of the FOXG1 gene by using both bioinformatics methods and luciferase analysis and defined a small region as a core element required for the expression. These results provide a theoretical and experimental basis for subsequent research.

Materials And Methods

Cell Culture and Transient Transfection

HEK293 cell line was cultured in high glucose medium (DMEM)(HyClone,USA) containing 15% fetal bovine serum (FBS)(Gibco,USA)at 37◦C in an air atmosphere with 5% CO_2. Cells were plated in 96-well plates at a density of 10^4 cells/well and cultured overnight. Lipo8000™ Transfection Reagent (Beyotime,China)was used for transient transfection according to the manufacturer's protocol.

Bioinformatic Analysis Of The Promoter Region

The human FOXG1 gene sequence (NC_000014.9:28766787–28770277) was searched on the NCBI website(https://www.ncbi.nlm.nih.gov/).Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/) was used to predict the putative promoter region. EMBOSS CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot/index.html) was used to predict the CpGislands. ALGGEN-PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3/) was used to identify potential transcription factor binding sites (TFBS).

Generation Of Reporter Gene Constructs

A series of reporter gene plasmids containing truncated mutated regions upstream of the FOXG1 gene were constructed. The 5' flanking fragment of the human FOXG1 gene was amplified using the promoter-specific primers FOXG1-1984F and FOXG1 + 331R, which was then cloned into a pGL4.2-Basic vector (provided by Pro. Yu Zhang) with K*pn*I and X*ho* Irestriction enzyme sites to generate FOXG1-Pro (-1984/+331).All deletion promoter segments (-924/+331, -363/+331, -20/+331, and − 363/-11)were amplified by PCR using FOXG1-Pro (-1984/+331) as a template, and the corresponding primers were listed in Table 1. All plasmids were created by inserting the promoter fragment into the K*pn*I and X*ho* I sites of plasmid pGL4.2-Basic.

Table 1
Primer sequences used forplasmid construction

Primers	5’→3’
FOXG1-1984F	5’-GGGGTACCACAGTGGAAGGACATTTGAGG-3’
FOXG1 + 331R	5’-CCGCTCGAGGCTGGCAAGTCATGTAGCAA-3’
FOXG1-924F	5’-GGGGTACCTTCTCTCTGGGCACCTCTCA-3’
FOXG1-363F	5’-GGGGTACCAGGAGGAAGCCGGAAATGTG-3’
FOXG1-20F	5’-GGGGTACCCGCCTGTAGCTCGGAAAATTA-3’
FOXG1-11R	5’-CCGCTCGAGGCTACAGGCGCACACTAGG-3’

Luciferase Reporter Assay

The luciferase reporter constructs and pRL-TK (provided by Pro. Yu Zhang)were co-transfected into HEK293 cells with a ratio of 3:1. Twenty-four hours after transfection, cells were washed with phosphate buffered saline (PBS) and then used for the detection of the promoter activity according to the instruction of the Dual-Luciferase Reporter Assay System (Promega, USA). The firefly luciferase activities were normalized using the Renilla luciferaseactivity and the empty vector pGL4.2-Basic values were used as negative controls.Each experiment was repeated at least three times, and the results were presented as mean ± S.D.

Statistical analysis

Comparisons between thetreatment and control were conducted using two independent samples t-test. $P < 0.05$ was considered to be statistically significant.

Results

Bioinformatic analysis of the proximal 5 ’- flanking region of human FOXG1 gene

The gene sequence of human FOXG1 was obtained from NCBI, and a 2.3-kb fragment 5’-flanking region was selected for bioinformatic analysis.Promoter 2.0 Prediction Server analysis showed that there existed two potential promoter regions, and the highest score appeared in the 5' flank-800 bp region (Table 2). Meanwhile, the CpG island, a hallmark of the promoter region, was analyzed by EMBOSS CpGPlot software. Two CpG islands were detectedwhich located at -622bp to -931bp and- 58bp to -365bp upstream of the transcription start site, respectively (Fig. 1).

Table 2
Promoter position predicted by Promoter 2.0 Prediction Server software

Promoter	Position	Score	Likelihood
1	-800bp	0.671	Marginal prediction
2	-300bp	0.578	Marginal prediction

Molecular Cloning And Promoter Activity Assays Of Human Foxg1 Gene 5-flanking Regions

Next, we used a reporter system to test the transcriptional activity of the putative promoter. The human peripheral blood-originated genome was used as a template for amplification of promoter segment. 2.3kb upstream sequence of human FOXG1 gene was successfully amplified from human genomic DNA and cloned into a pGL4.2-Basic vector (Fig. 2A,B). The FOXG1-Pro(-1984/+331) or pGL4.2-Basic was transiently transfected into HEK293 cells with pRL-TK. The relative luciferase activity was presented by the ratio of activities of firefly luciferase to Renilla luciferase.The results showed that FOXG1-Pro(-1984/+331) presented strong promoter activity, at least 6 times higher than the control without apromoter, which was consistent with the results of bioinformatics analysis(Fig. 2C).

Identification Of The Core Promoter Of Human Foxg1 Gene

In order to find the core promoter of the human FOXG1 gene, we used FOXG1-Pro(-1984 /+331) as a template to construct a series of 5'-deletion fragments, including FOXG1-Pro(-924/+331), FOXG1-Pro(-363/+331), and FOXG1-Pro(-20/+331) (Fig. 3A,B). The 5'-deletion mutants were transiently transfected into HEK293 cells with pRL-TK. Luciferase reporting assay showed that promoter activities were detected in FOXG1-Pro(-924/+331), and FOXG1-Pro(-363/+331), but not in FOXG1-Pro (-20/+331). These results suggested that there was a potential core promoter within the region − 363 to -20 (Fig. 3C).

Next,we constructed the reporter vector FOXG1-Pro(-362/-11) containing expected fragment (Fig. 3A, B). The strong promoter activity was detected in FOXG1-Pro(-362/-11),which was even higher than FOXG1-Pro(-1984/+331).These results conformed that the core promoter of the human FOXG1 gene was located in the region − 362 to -11bp,which was consistent with the results of bioinformatics analysis.

Prediction Of Transcription Factor Binding Site

To assess the presence of transcription factor binding sites (TFBS) in the core promoter of the human FOXG1 gene, ALGGEN-PROMO software was applied. A total of 29 conserved cluster TFBS motifs were detected (Fig. 4). Except for the general transcriptional factors, we found that there were some binding sites of proliferation-related transcription factors in the FOXG1 promoter region, such as NF-kB signal,

PAX-5, and HNF-1A, which was consistent with the previously reported function of FOXG1 in cell proliferation.

Discussion

In this study, promoter analyses of the human FOXG1 gene were carried out to better understand the regulation of late gene expression. We cloned and functionally characterized the promoter of the human FOXG1 gene for the first time. Then a series of plasmids, containing truncated mutated regions upstream of the FOXG1 gene, were constructed to identify the core elements required for the promoter activity.

FOXG1 is an evolutionary conserved winged-helix transcription factor, which is highly expressed in the forebrain(Han X et al.2018).Results from different studies concurred that the main role of FOXG1 was to participate in telencephalon and neocortex development by regulating the proliferation and differentiation of neuronal progenitor cells(Hanashima C et al.2004). Recently, more and more studies indicated that FOXG1 expression was elevated in multiple cancers. In ovarian cancer, medulloblastoma, glioma and nasopharyngeal carcinoma, FOXG1 was shown to be up-regulated and promoted cell proliferation, cell cycle progression, and inhibited apoptosis(Xi H et al.2021;Chan DW et al.2009;Adesina AM et al.2007).As a transcriptional factor, most of the studies of FOXG1 mainly focused on the biological function, little research referred to gene expression regulation of FOXG1.

For gene transcription, the promoter is an important cis-regulatory element.In order to reveal the transcriptional regulatory mechanisms,cloning and analysis of the promoter are therefore important steps.In this study, we firstly analyzed 2kb of longfragmentsupstream of the human FOXG1 gene by using a bioinformatic approach. Two potential promoters have been identified in -300bp and − 800bp upstream of the transcription start site,respectively. Meanwhile,CpG analysis also showed that there were two CpG islands, a hallmark of the promoter region of genes, located in-622bp to -931bp and- 58bp to -365bp upstream of the transcription start site, respectively.

To functionally characterize FOXG1 promoter activities, a serial of deletion mutants for FOXG1 promoter were generated and their promoter activities were tested in the HEK293 cell line.The results showed that all the deleted mutants had transcriptional activities in comparison with the pGL4.2-basic vector,except FOXG1-Pro(-20/+331). FOXG1-Pro(-362/-11)showed the highest activities even compared with the plasmids containing the entire region FOXG1-Pro (-1984/+331),which suggested that the core transcriptional promoters were located in the − 362 to -11. These results were in good agreement with bioinformatic analysis. Meanwhile, we found that some inhibitory elements may exist in the region − 20 to + 331, which was a possible explanation for why FOXG1 could be detected in lower expression in other tissues except the brain, Next, the putative TFBS were analyzed. In addition to the transcriptional factors related to cell proliferation,we also found many factors related to immuno-inflammatory responses,such as:C/EBPbeta,Egr-1,which indicated that FOXG1 might be involved in immune regulation.We hope that this study may serve as a basis for further investigation of the detailed function of the FOXG1 core promoter and reveal the mechanism of gene expression regulation.

Conclusions

In this study, Promoter 2.0 Prediction Server software and EMBOSS CpGPlot software were used to predict the core promoter and CpG island of human FOXG1 gene. Next, by generating reporter gene constructs and performing luciferase activity assays, the core promoter of the human FOXG1 gene was confirmed to be located in the − 362 to -11bp region. We used ALGGEN-PROMO software to analyze the transcription factor binding sites of the core promoter sequence of the human FOXG1 gene. We found some proliferation-related transcription factor binding sites and some immune-inflammatory response-related factors in the FOXG1 promoter region. This study can provide new ideas for further research on the function of FOXG1.

Declarations

The authors declare that there are no conflict of interests.

Data Availability Statements

The date that support this study are avalible from the corresponding author upon resonble request.

Author contributions:

Ming-ming Lai designed research, Yu-Qing Wang performed research, analyzed data, and wrote the paper, Yan-Chen, Yong-Xin Liu and Cai-Ting Yang drawn tables and graphs.All authors reviewed the manuscript.

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Figures

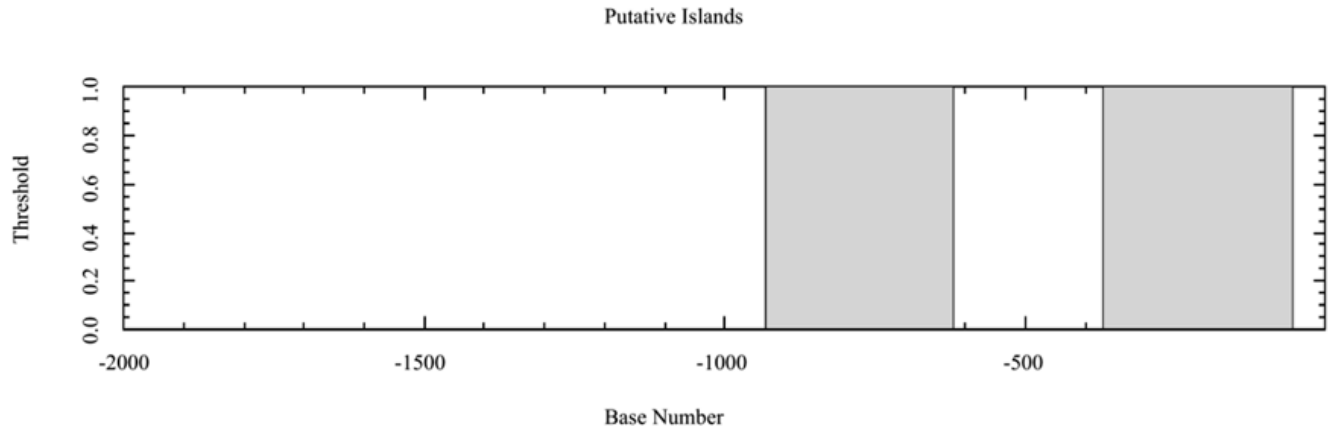

Figure 1

The result of EMBOSS CpGPlot software predicting the position of CPG island of FOXG1 gene

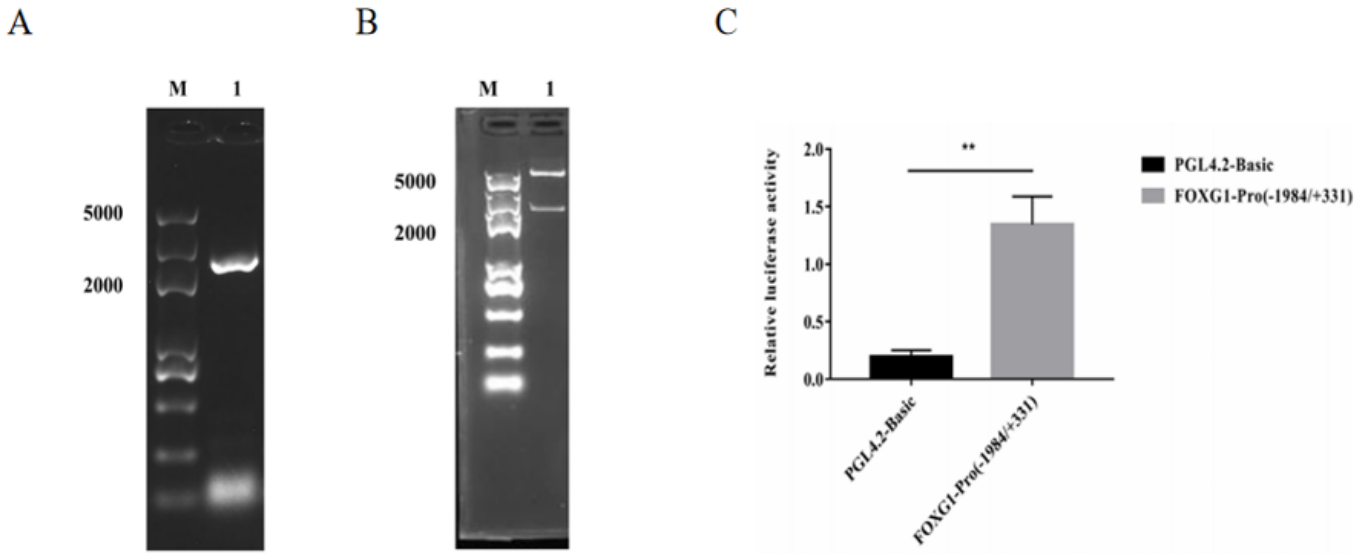

Figure 2

Cloning and luciferase activity assays of 2.3kb upstream sequence of human FOXG1 gene. (A)Amplification of 2.3kb promoter segment (M, DNA marker; 1, PCR fragment).(B)Identification of the cloned 2.3kb fragment of FOXG1 gene 5'-flanking region by restriction digestion (M,DNA marker; 1, FOXG1-Pro(-1984/+331)). (C)Luciferase activity assays in HEK293 cells.The reporter gene constructs FOXG1-Pro(-1984/+331) and the empty vector pGL4.2-Basic were transfected intocells. The relative luciferase activities were graphed as the fold increases over that of a pGL4.2-Basic vector.

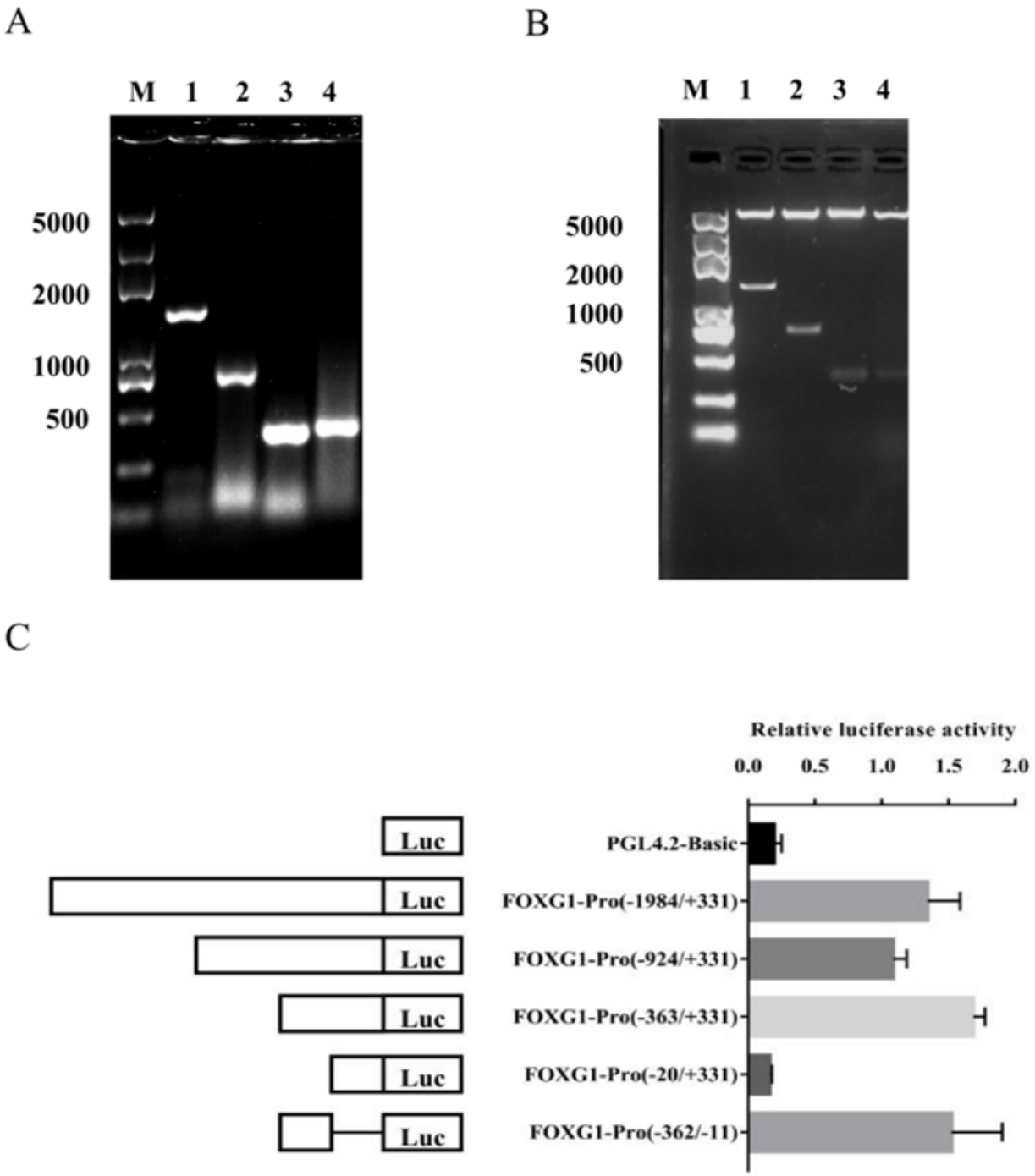

Figure 3

Identification of core promoter region in 5-flanking regions of human FOXG1 gene.(A) Amplification of the truncated promoter segments (M, DNA marker; 1-4, PCR fragments).(B)Identification of the cloned deletion mutants by restriction digestion (M, DNA marker; 1, FOXG1-Pro(-924 /+331); 2, FOXG1-Pro(-363/+331); 3, FOXG1-Pro(-20/+331); 4, FOXG1-Pro(-362/-11)).(C)Luciferase activity assays of human FOXG1 gene in HEK293cells. The different deletion constructs and the empty vector pGL4.2-Basic were transfected into HEK293 cells.The luciferase activities were graphed as the fold increases over that of a pGL4.2-Basic vector.

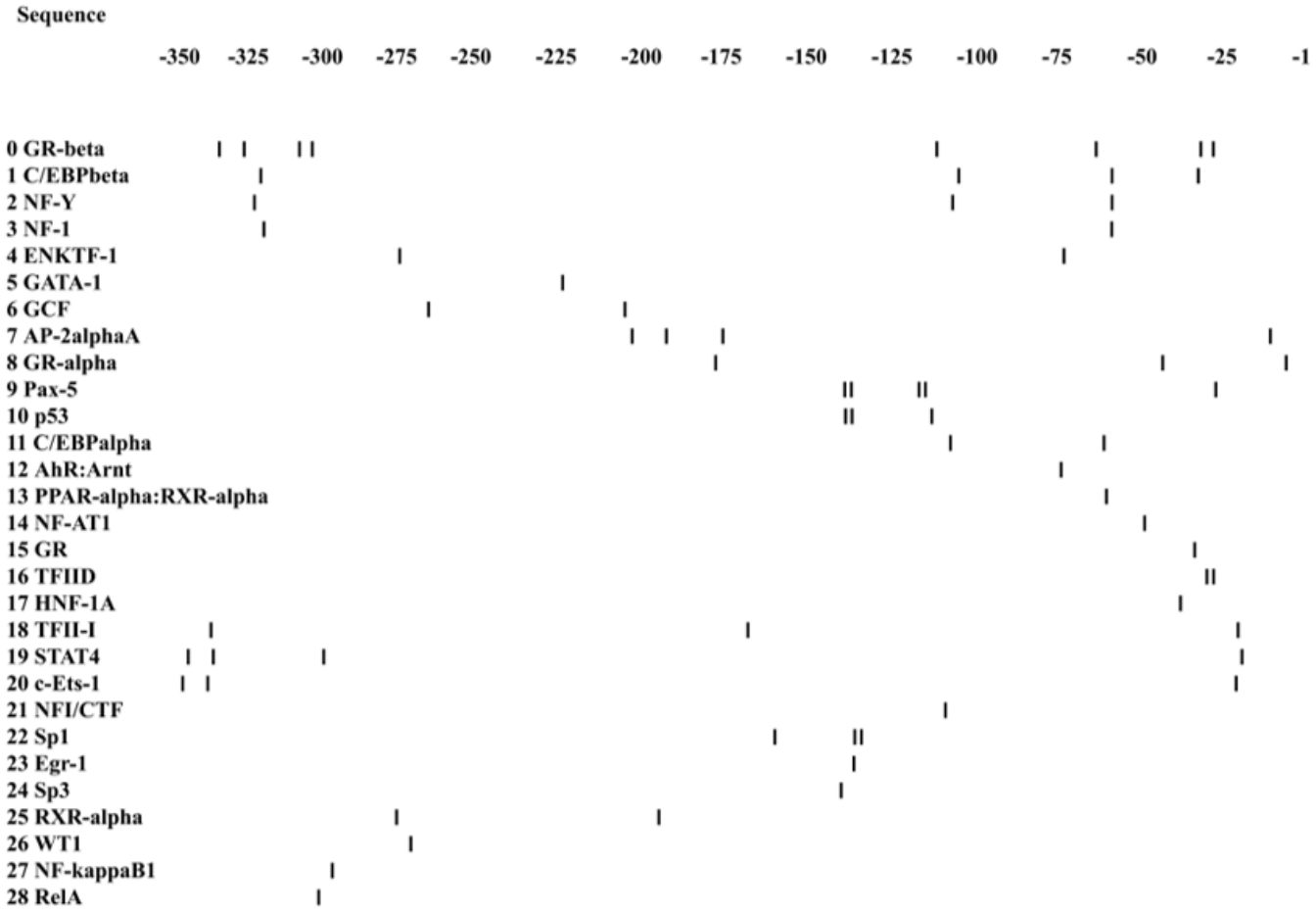

Figure 4

TFBS prediction in -350bp region upstream of the human FOXG1 gene.