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Promoter paper

The characterisation of the human Wolfram syndrome gene promoter demonstrating regulation by Sp1 and Sp3 transcription factors

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Abstract

Wolfram Syndrome (DIDMOAD) is an autosomal recessive disorder characterised by insulin deficient diabetes mellitus and neurodegeneration. Mutations in a novel gene, *WFS1*, were found in nearly all patients and segregated with the disease. The *WFS1* gene is expressed in all tissue types studied and the 890aa protein product is localised to the endoplasmic reticulum (ER). In this study, we used a combination of reporter assays and in vitro and in vivo transcription factor binding assays to analyse the regulation of expression of the human *WFS1* gene in neuronal derived cells. A single transcription start site was mapped and a minimal promoter identified within 25 bp upstream of this site. This minimal promoter contains two DNA binding motifs (GC boxes) for the transcription factors Sp1/3/4 and binding of both Sp1 and Sp3 was demonstrated at both motifs in vitro and in vivo. The presence of intact GC boxes is essential for minimal promoter action. Thus, transcription factors of the Sp family are important regulators of the WFS1 promoter. A further up-regulating control region was identified containing three CCAAT box binding motifs; all demonstrated a reduction in expression after mutation. One CCAAT box represented part of a predicted ER stress response element.

Keywords: *WFS1*; Wolfram syndrome; Minimal promoter; Sp1; Sp3; CCAAT; ERSE

1. Introduction

Wolfram Syndrome (DIDMOAD) is an autosomal recessive disorder characterised by insulin deficient diabetes mellitus and neurodegeneration [1]. The *WFS1* gene is located at 4p16.1 and complete inactivation of the gene was shown to be the cause of Wolfram syndrome [2–4]. *WFS1* expression was ubiquitous throughout all tissues studied, though levels of expression differed between tissue types [3 4]. Mutation studies have identified a broad range of mutations, including nonsense, missense and splice variations, throughout the length of the gene [3–7]. No hot spots or genotype–phenotype relationships have been observed. The 890aa protein product localises to the endoplasmic reticulum [8]. It has been suggested that carrier status for mutated or polymorphic variants of *WFS1* could be associated with susceptibility to late onset diabetes, optic atrophy, psychiatric disorders and certain types of deafness [9–12].

Recent studies have demonstrated that the loss of *WFS1* expression leads to increased endoplasmic reticulum (ER)-stress and induced cell death, notably occurring in mouse pancreatic beta cells [13–16]. This suggests the *WFS1* protein product has a role in the ER-stress response of normal cells. Additionally, a role in programmed cell death, independent of ER-stress, has been suggested, but no clear function has yet been elucidated [17]. The mRNA expression levels of *WFS1* have been shown to be high in the tissues affected in the syndrome (neurons and pancreatic β-cells) and the transcriptional start site has been proposed at two different nucleotides 13 bp apart [3,4]. A further increase of *WFS1* expression has been reported following induction of the ER-stress response pathway, and this increase is dependant upon a putative 3 kb promoter upstream of exon 1 [14]. Recently, WFS1 expression has been demonstrated to be increased in response to XBP1, a key ER stress response transcription factor [18]. This study indicated the presence of a

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potential ER stress response element (ERSE) upstream of exon 1, but that this did not directly interact with XBP1 [18].

We aimed to characterise the *WFS1* promoter region by mapping the minimal promoter and its transcription start site and identifying transcription factors responsible for its regulation. Moreover, we aimed to confirm the differential expression of *WFS1* in cell lines representing different tissue types at the mRNA level and demonstrate this at the protein level.

2. Materials and methods

2.1. Cell lines and cultures

The SH-SY5Y and SK-N-AS human neuroblastoma cell lines, A549 lung cell line, JEG-3 placental cell line and SKRC45 kidney cell line were all grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin, non-essential amino acids and 2 mM L-glutamine (Invitrogen). The HEP-G2 liver cell line was grown in minimal Eagle's medium supplemented with 10% fetal calf serum, penicillin/streptomycin, non-essential amino acids and 2 mM L-glutamine (Invitrogen).

2.2. RNA preparation and Northern blot analysis

RNA was prepared from approximately 90% confluent cells grown in 75 cm^2 flasks using a single step guanidinium phenol–chloroform extraction procedure (TRIzol reagent-Sigma). The RNA was run on a 1.1% agarose gel with MOPS and transferred to a nylon membrane. Northern blot analysis was performed using Roche random priming kit with (α-^{32}P) dCTP and a *WFS1* cDNA probe produced from the WFS1 northern primers (Table 1). The blots were washed and exposed to phosphoimaging plates. The blots were stripped and re-probed with a GAPDH probe obtained using the GAPDH northern primers (Table 1) as a loading control.

2.3. Western blot analysis

Protein was extracted from 75 cm^2 flasks of 90% confluent cells using Ripa buffer (Pierce Biotechnology) containing a protease inhibitor mix (Roche). Samples of 25 μg protein extract for each cell line were run on a 10% polyacrylamide gel and transferred to a PVLF membrane. The primary rabbit α-Wolframin-N antibody ([19]—courtesy of Dr. Sabine Hofmann) was used at a dilution of 1 in 500. The blots were stripped and re-probed with a monoclonal β-Actin antibody (Sigma) as a loading control.

2.4. Primer extension

A P32-labelled WFS1 extension primer (Table 1) was used in an elongation reaction with 20 μg of total RNA using Thermoscript III (Invitrogen). The transcription start site was confirmed by primer extension reactions run alongside manual sequencing produced by the Sequenase II kit (Roche).

2.5. Computer based analysis

All analysis programs used are freely available on the Internet from the following websites:

http://www.ncbi.nlm.nih.gov/
http://genome.ucsc.edu/
http://www.hgmp.mrc.ac.uk/NIX/
http://www.genomatix.de/cgi-bin/promoterinspector/promoterinspector.pl
http://www.cbs.dtu.uk/services/promoter
http://www.cbrc.jp/research/db/TFSEARCH.html
http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl
http://www.ncbi.nlm.nih.gov/Blast

2.6. Plasmid construction

All reporter fragments were obtained through PCR using primers with built in restriction sites (Table 1). The mutated GC box fragments were obtained through PCR using primers incorporating required base pair substitutions incorporated (CCCGCCC→CTTGCCC). The mutated CCAAT box fragments were created using a site-directed mutagenesis kit (Stratagene) to alter the necessary base pairs (CCATT/CCACT→TCAGT). The fragments were cloned into the pGL3-basic plasmid (Promega) upstream of the *firefly* luciferase gene and the identity of the clones confirmed by restriction digestion and sequencing.

2.7. Transient transfection and the Luciferase assay

Transient transfection was performed on all cell lines using FuGENE6 transfection reagent (Roche). The cell lines were grown to 90% confluency and

Table 1
Primers

PCR Fragment	Forward	Reverse
WFS P1	GCAGGGTACCCGTTTGGAGTGGAGGGAGGCCGAAA	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACGC
WFS P2	GCAGGGTACCGTCCGCTCGGAAACTTTCGCTGTGG	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P3	GCAGGGTACCACGGGTCCAAACCACCTGTTACAGG	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P4	GCAGGGTACCATCCCACAGTCACCGTGTGAAAAGC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P5	GCAGGGTACCGAGGTGACTGACATGAACTACGCCG	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P6	GCAGGGTACCGGGCAAATAACTATGTCTGAGCCTC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P7 / ChIP FR	GCAGGGTACCCAAAGGGTTTGGGAAGTGCTTCAGC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P8	GCAGGGTACCGTGTTCTGATAGAGGCTCCTTAAGC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P9	GCAGGGTACCTATCATCAGAGTCCCTGGACATTGC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P10	GCAGGGTACCGCTAGCCGGCTCTTCAGCAGCGAGT	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS P11	GCAGGGTACCGTGCAGAAGGCCGCGCTAGC	GCAGCTCGAGAGTGCCTCAGGGCTCTTGCC
WFS P12	GCAGGGTACCTATCATCAGAGTCCCTGGACATTGC	GCAGCTCGAGAGTGCCTCAGGGCTCTTGCC
WFS P13	GCAGGGTACCGGGCAAATAACTATGTCTGAGCCTC	GCAGCTCGAGTTCGGCCTCCCTCCACTCCAGACGC
WFS wtwt	GCAGGGTACCGGCCCCGCCCCTGCCCCGCCCCCTC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS wtmt	GCAGGGTACCGGCCCCGCCCCTGCCTTGCCCCCTC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS mtwt	GCAGGGTACCGGCCTTGCCCCTGCCCCGCCCCCTC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS mtmt	GCAGGGTACCGGCCTTGCCCCTGCCTTGCCCCCTC	GCAGCTCGAGAAGTTGTTCGGGAGCAGCTGAACG
WFS primer extension	N/A	CCGCACGCGGCGCTGCGACGCCCTG
WFS1 northern	CACTAAGAAGTACGCCAAGGGCGT	GGAGGCCATGTCAATCAGGTACTC
GAPDH northern	CATCAATGACCCCTTCATTG	TGAGCTTGACAAAGTGGTCG