Internal Ribosome Entry Segment Activity of ATXN8 Opposite Strand RNA

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Abstract

Spinocerebellar ataxia type 8 (SCA8) involves the expansion of CTG/CAG repeats from the overlapping ataxin 8 opposite strand (ATXN8OS) and ataxin 8 (ATXN8) genes located on chromosome 13q21. Although being transcribed, spliced and polyadenylated in the CTG orientation, ATXN8OS does not itself appear to be protein coding, as only small open reading frames (ORFs) were noted. In the present study we investigated the translation of a novel 102 amino acids containing-ORF in the ATXN8OS RNA. Expression of chimeric construct with an in-frame ORF-EGFP gene demonstrated that ATXN8OS RNA is translatable. Using antisera raised against ORF, ATXN8OS ORF expression was detected in various human cells including lymphoblastoid, embryonic kidney 293, neuroblastoma IMR-32, SK-N-SH, SH-SY5Y cells and human muscle tissue. The biological role of the ATXN8OS ORF and its connection to SCA8 remains to be determined.

Introduction

The spinocerebellar ataxies (SCAs) comprise a heterogeneous group of disorders involving progressive degeneration of the cerebellum, brainstem, and spinal tract [1]. Of all SCAs, SCA type 8 (SCA8) presents a molecular trait that distinguishes it from other SCA type 8 (SCA8) involves the expansion of CTG/CAG repeats from the overlapping ataxin 8 opposite strand (ATXN8OS) and ataxin 8 (ATXN8) genes located on chromosome 13q21. Although being transcribed, spliced and polyadenylated in the CTG orientation, ATXN8OS does not itself appear to be protein coding, as only small open reading frames (ORFs) were noted. In the present study we investigated the translation of a novel 102 amino acids containing-ORF in the ATXN8OS RNA. Expression of chimeric construct with an in-frame ORF-EGFP gene demonstrated that ATXN8OS RNA is translatable. Using antisera raised against ORF, ATXN8OS ORF expression was detected in various human cells including lymphoblastoid, embryonic kidney 293, neuroblastoma IMR-32, SK-N-SH, SH-SY5Y cells and human muscle tissue. The biological role of the ATXN8OS ORF and its connection to SCA8 remains to be determined.

Although being apparently non-coding [3], a 102 amino acid-containing open reading frame (ORF) exists. The ORF is 446 nucleotides (according to NR_002717) or 1246 nucleotides (according to [10]) from the 5' end of ATXN8OS RNA (Fig. 1A). In eukaryotes, translation initiation involves recruitment of ribosomal subunits at either the 5' m7G cap structure or at an internal ribosome entry site (IRES). In cap-dependent mechanism, the initiation codon is located some distance downstream for most mRNAs, requiring ribosomal movement to this site, either linear or going around segments of the 5' leader to reach the initiation codon [11]. The cap-independent mechanism requires the formation of a complex RNA structural element termed IRES and the presence of trans-acting factors [12]. As a result, the ribosome entry window attains an unstructured conformation and in doing so facilitates ribosome recruitment. In addition, non-AUG triplets may be used as translation initiators for gene expression [13,14]. In this study we firstly examined the cap independent IRES activity in the ATXN8OS RNA using a dual luciferase reporter assay. Then we fused the ATXN8OS ORF in-frame with an EGFP tag to investigate if the ATXN8OS ORF could be translated using cell culture studies. The ORF expression was validated in human lymphoblastoid, neuroblastoma, embryonic kidney cells and muscle tissue using ORF antisense. The translation of ATXN8OS ORF was further examined by tandem MS determination.
IRES Activity of ATXN8OS RNA

Results

IRES Activity of ATXN8OS RNA

Despite being apparently non-coding [3], a 102 amino-acid ORF (AUG^{1247}) was noted in the ATXN8OS transcripts (Fig. 1B). To investigate if this ORF can be translated via a cap independent IRES activity, we constructed a dicistronic vector pRF in which IRES activity is expressed as percentages of the activity of the ECMV IRES, which was set at 100%. In addition, relative luciferase activities with a level of 33.7% and 19.6%, respectively, in HEK-293 and IMR-32 cells as compared to the directed firefly luciferase synthesis to a level of 100%. Each value is the mean ± SD of three independent experiments each performed in duplicate.

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Figure 1. IRES activity of the ATXN8OS transcript. (A) ATXN8OS organization with promoter (open arrow), exons (open boxes) and functional splice donor sequences (GT) of D exons (D5, D4, D, D’ and D”) indicated. The CTG repeat tract is located in exon A. Transcription start site of exon D5 and exon D are represented by +1 and +801, respectively. (B) ATXN8OS RNA (NR_002717) generated from the splicing events represented by the wavy lines. The putative ORF initiated from AUG^{1247} is indicated by the open boxes inside the RNA. The restriction enzymes and the cutting sites used to generate +801→+1195 cDNA fragment of ATXN8OS are shown on the bottom of the cDNA. (C) The dual luciferase reporter plasmid had Renilla luciferase and firefly luciferase genes between the TK promoter and polyadenylation signal. The locations of XbaI, Xhol and BamHI sites used for construction are shown on the top. (D) Relative luciferase activities generated by dual luciferase constructs with ECMV IRES and ATXN8OS +801→+1195 cDNA fragments were measured in HEK-293 cells, with IRES activity of +801→+1195 set at 100%. Each value is the mean ± SD of three independent experiments each performed in duplicate.

ATXN8OS ORF Expression

To investigate if indeed the ATXN8OS ORF could be translated, we cloned the ATXN8OS cDNA (NR_002717) and in-frame fused

an EGFP tag to the C terminal of the ATXN8OS ORF (Fig. 2A, pCMV/+801). The transcripts made from this construct will be initiated from exon D (+801). As the promoter region upstream of exon D5 was identified by comparing human and mouse genomic DNA sequences flanking the 5’ end of the transcripts [10], ATXN8OS gene sequence +1→+800 were included in construct pCMV/+1 so that transcripts made will be initiated from exon D5 (+1). In constructs pATXN8OS/−481 and pATXN8OS/−114, proximal ATXN8OS promoter fragments −481→−1 and −114→−1 were used to drive ATXN8OS expression to mimic the in vivo situation.

The constructs were transiently transfected into HEK-293 cells. After two days ORF-EGFP RNA levels were measured by real-time PCR quantification using ATXN8OS-specific probe C2/C1 and primers. As shown in Fig. 2B, when the expressed level in pATXN8OS/−481 cells was set as 1.0, ORF-EGFP RNA levels for transcripts initiated from +801 (pCMV/+801) versus transcripts initiated from +1 (pCMV/+1) and pATXN8OS/−481 and pATXN8OS/−114 were 3.4 and 1.0→2.7, respectively. Similar 1.0→2.9 range of ORF-EGFP RNA levels for transcripts initiated from +1 (pCMV/+1, pATXN8OS/−481 and pATXN8OS/−114) were also observed using ATXN8OS D5/D4 probe (Fig. 2B).

The EGFP fluorescence was evaluated by FACS analysis. As shown in Fig. 2C, compared to the pIRES2-EGFP (cap-independent EGFP expression, 100%), 196.5% EGFP fluorescence was seen in cells transfected with pEGFP-N1 (cap-
dependent EGFP expression). For the ORF-EGFP constructs, 1.4→59.0% EGFP fluorescence was seen as compared to the IRES-dependent EGFP fluorescence (pIRES2-EGFP). Transcripts initiated from +801 (pCMV/+801; 59.0% of pIRES2-EGFP) expressed 17.42 fold EGFP fluorescence compared to transcripts initiated from +1 (pCMV/+1, pATXN8OS/−481 and pATXN8OS/−114; 1.4→3.5% of pIRES2-EGFP).

To visualize the expression of ORF-EGFP protein, confocal microscopic examination of GFP fluorescence was carried out after transfection of pIRES2-EGFP, pCMV/+801 and pCMV/+1 construct, small and dispersed granules appeared mainly in the cytoplasm, in addition to showing diffuse cytoplasm expression. Cells transfected with pCMV/+1 or pATXN8OS/−481 gave sparse granules and weak, diffuse GFP fluorescence.

To examine the expressed ORF-EGFP protein, GST-ORF (S. japonicum GST from pGEX plasmid) fusion protein was prepared as antigen to raise antiserum in rabbit. Western blot immunostaining with GFP antibody or ORF antiserum was performed. As shown in Fig. 3B, similar proteins (40 and 30 kDa) were detected in cells transfected with pCMV/+801. Whereas the weakly expressed 40 kDa protein may represent the predicted ORF-EGFP protein (AUG+1247 start, 348 amino acids with MW of 39472; ExPASy: http://web.expasy.org/compute_pi/), the 30 kDa protein apparently differs from the predicted. The 30 kDa protein may be initiated from a downstream in-frame AUG codon (AUG+1490 start, 267 amino-acid fusion protein, MW 30061). A larger protein around 50 kDa was also noted by the Western blot either probing with GFP antibody or ORF antiserum. The existence of this 50 kDa protein indicated that ORF-EGFP protein may be translated from the sequence upstream of AUG+1247.

**ORF Immunodetection**

To validate if indeed ATXN8OS ORF is expressed in human cells, ORF antiserum was used to detect the possible endogenous ORF protein. As we hardly detected ORF protein in RIPA-soluble fraction and also the predicted 102 amino acids ATXN8OS ORF protein has a 62.9% chance of insolubility when overexpressed in E. coli (http://www.biotech.ou.edu/), urea lysis buffer was used for lymphoblastoid protein extraction since the average molecular weight of proteins that dissolve exclusively in urea buffer is up to 60% higher than in RIPA buffer [16]. On Western blot staining with ORF antiserum, while no specific polypeptide was detected with pre-immune serum, an unexpected 23 kDa protein was detected in soluble pellet fraction (Fig. 4A). The same 23 kDa protein was also observed in urea buffer-insoluble pellet fraction prepared from embryonic kidney 293 cells, neuroblastoma IMR-32, SK-N-SH, SH-SY5Y cells and human muscle tissue (Fig. 4B).

**ORF Identification**

To identify the endogenous ATXN8OS ORF protein, lymphoblastoid proteins from urea buffer-insoluble pellet fraction were subjected to 2D PAGE and 2D immunoblot (Fig. 5A). The identity of the three ORF-specific spots was determined using LC-MS/MS and Mascot data search in a database set up for the predicted ORF. As shown in Fig. 5B, six matched peptide with sequence coverage of 47% was obtained, including the N-terminal peptide VPCPGAPCCS LVATGSR which can only be generated from
translation start from GUG^{+953} due to the stop codon UGA existing upstream of GUG^{+953}.

Discussion

The ATXN8OS gene was isolated from a single sample directly, using the RAPID cloning method [3,17]. Sequence analysis revealed that the expansion consisted of a stretch of 11 CTA repeats followed by 80 CTG repeats. Analysis of this sequence did not reveal any possible spliced isoform possessing an ORF to extend through the expansion in either direction. Therefore, SCA8 was first proposed to be caused by an RNA gain-of-function mechanism [6]. In this study, we used dual luciferase assay to demonstrate that ATXN8OS RNA^{+801},^{+1195} had IRES activity (Fig. 1). As ATXN8OS ORF detected in human cells was predicted to be translated from GUG^{+953} (Fig. 5), the IRES activity of ^{+801},^{+1195} was compared with that of ^{+953},^{+1195}. To our surprise, the ^{+953},^{+1195} fragment showed higher IRES activity while less IRES activity was observed from ^{+801},^{+953} fragment (Fig. 1). The presence of a 12 amino-acid ORF (AU-G^{+890},UAG^{+926}) within ^{+801},^{+953} fragment may explain the reduced amount of translation that occurs from the downstream firefly luciferase cistron. Similar translation read-through of cellular transcripts can be seen with human angiotensin II type 1 receptor (AGTR1) mRNA (IRES name: AT1R_var3; http://www.iresite.org/IRESite_web.php?page=browse_cellular_transcripts) [18,19]. Accordingly, the enhancing IRES activity observed with ^{+953},^{+1195} fragment may be explained by the removal of inhibition derived from the small ORF's translation. As cap-independent mechanism requires the formation of a complex RNA structural element and the presence of trans-acting factors, it is also likely that some inhibitory factors may exist within ^{+801},^{+953} fragment that may be explained by the removal of inhibition derived from the small ORF's translation. As cap-independent mechanism requires the formation of a complex RNA structural element and the presence of trans-acting factors, it is also likely that some inhibitory factors may exist within ^{+801},^{+953} fragment and regulate ATXN8OS RNA IRES activity. The trans-acting factors are worthy to be further identified to investigate the translation mechanism of ATXN8OS RNA.

In our study, the predicted translation start GUG^{+953} was within the ATXN8OS IRES region ^{+801},^{+1195}, which is different from the general concept that putative IRES sequences are located in a close proximity to the 5' coding region of the genes. Nevertheless,
specific spots were analyzed and MS/MS data were searched in a database containing theoretical trypsinized fragments of 23-kDa ORF protein [20]. Translation initiation on such mRNAs results in the species, in addition to initiating at a downstream in-frame AUG [37]. Although not well conserved at the position, the downstream in-frame AUG codon has conserved A at the 2 position and C at the 7 position. In contrast, the AUG initiation sites showed conservation of G/C at the 2 position but less abundance U at the 4 position. The ATXN8OS ORF GUG initiation codon has conserved C at the −7 position but less abundance U at the −6 position, the downstream in-frame AUG codon has conserved A at the −3 position but also less abundance U at the −4 position. Among these properties, 5′-UTR of the alternative translation initiation sites showed conservation of G/C at the −6 position and C at the −7 position. In contrast, the AUG initiation sites showed consensus at position −3 for A/G and position +4 for G/A [24,25]. The ATXN8OS ORF GUG initiation codon has conserved C at the −7 position but less abundance U at the −6 position, the downstream in-frame AUG codon has conserved A at the −3 position but also less abundance U at the +4 position. Although not well conserved at the −6 position, the conserved C at the −7 position and other un-analyzed properties may support the use of the second most common alternative translation initiation GUG site [23] for the translation of ATXN8OS ORF protein.

In summary, our study indicated that the ATXN8OS putative ORF protein could be translatable and may be expressed via a naturally occurring non-AUG start codon. The biological role of ATXN8OS ORF and its connection to SCA8 are deserving of further investigation.

Materials and Methods

Ethics Statement

This study was performed according to a protocol approved by the institutional review boards of Chang Gung Memorial Hospital,
and all examinations were performed after obtaining written informed consents.

**Dual Luciferase Reporter Constructs**

The 1.3-kb \( ATXN8OS \) cDNA containing exons D, C2, C1, B, and A [26] (Fig. 1B) was cloned as described [8]. The \( ATXN8OS \) cDNA were then cloned into the EcoRI site of pEGFP-N1 (Clontech). To construct a dual luciferase reporter, a 76-bp \( Xho \)-BamHI polylinker region of pcDNA3 was first added between the \( Xho \) and BamHI sites of phiRL-TK vector (Promega) to introduce a \( Xho \) site as well as remove the SV40 late poly(A) region. Then a 1972-bp \( Xho \)-BamHI fragment containing the firefly luciferase gene and the SV40 late poly(A) signal from pGL3-Basic vector (Promega) was placed between the \( Xho \) and BamHI sites of the modified phiRL-TK vector. The resulting dual luciferase reporter plasmid had \( Renilla \) luciferase and firefly luciferase genes between the TK promoter and polyadenylation signal (Fig. 1C). The \( ATXN8OS \) cDNA in pEGFP-N1 was restricted with \( Xho \) and HindIII and the blunt cDNA fragment (+801 -+1195) was placed in the blunt \( Xho \) site between the two luciferase genes. The sense and antisense primers used for \( ATXN8OS \) amplification (+801 -+1195) were 5'-GGCGCCGATT-CATCCCTTTACCTTGTT 3' and 5'-CAGGAGGACATTCTCACG, respectively (EcoRI and HindIII sites underlined). The resulting PCR products were cloned, sequenced and restricted with EcoRI and HindIII to replace the +801 -+1195 fragment in dual luciferase reporter plasmid. The 632-bp blunt \( Xho \)-EcoRI IRES fragment from pIRES2-EGFP (Clontech) was inserted between the two luciferase genes as a positive control.

**Luciferase Reporter Assay**

Human embryonic kidney HEK-293 and neuroblastoma IMR-32 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS. Cells were plated into 12-well dishes (2 x 10^3/well), grown for 20 hr and transfected with the above ORF-EGFP constructs, pIRES2-EGFP and pEGFP-N1 (2 μg/well). Forty-eight hours later, total RNA was extracted using the Trizol (Invitrogen). The RNA was DNase treated, quantified, and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Using ABI PRISM 7000 Sequence Detection System (Applied Biosystems), real-time quantitative PCR was performed on a cDNA amount equivalent to 250 ng total RNA with TaqMan fluorescent probes Hs01382089-m1 (exon C2 and C1 boundary) (Applied Biosystems) was used for \( ATXN8OS \) mRNA quantification. Fold change was calculated using the formula 2^ΔΔCt, in which ΔCt indicates cycle threshold. Statistical analysis of differences between the groups was carried out using one-way analysis of variance (ANOVA).

**ATXN8OS ORF-EGFP Constructs**

The ORF translation termination sequence in C1 exon was removed and a \( SnaI \) restricted site (underlined) was added by PCR using primer 5'-GGCGCCGAGGACATTCTCACGAGG 3' (EcoRI) (in MCS of pEGFP-N1 Vector). The EcoRI fragment containing \( ATXN8OS \) ORF was in-frame fused to the EGFP gene in the pEGFP-N1 vector (between the EcoRI and BstUI sites). Portion of the Kozak consensus translation initiation sequence (ACCATG) in the EGFP gene was further removed by site-directed mutagenesis (primer 5'-GGCGCCGAGGACATTCTCACGAGG 3' (EcoRI)). Site-Directed Mutagenesis Kit, Stratagene). The resulting pCMV/+801 construct (where +801 represents transcription start site of exon D) (Fig. 2A) was verified by DNA sequencing. The construct was predicted to encode an ORF-EGFP fusion protein containing 346 amino acids.

To construct pCMV/+1 (+1 representing transcription start site of exon D), an \( SnaI \) site (underlined) was added to the 5’ end of \( ATXN8OS \) by PCR using primer 5’-GGCGCCGAGGACATTCTCACGAGG 3’ (EcoRI) and 3'-CAAGGGCAGAGGTGAATGCAG-GAGGACATTCTCACGAGG 5’ (BamHI). To construct pATXN8OS/-981, a 2.1-kb \( ATXN8OS \) gene 5’ fragment (AF252279 reversed complemented strand: 100533-110454) was cloned by PCR and sequenced. The 706-bp-SacI fragment containing \( ATXN8OS \) was cloned into the blunt backbone of the pGL3-Basic vector (Promega). Then cell lysates were prepared and luciferase activity was measured using a luminometer with a dual luciferase assay system (Promega). The resulting dual luciferase reporter plasmid contained the \( Renilla \) luciferase and firefly luciferase genes between the TK promoter and polyadenylation signal (Fig. 1C). The \( ATXN8OS \) cDNA in pEGFP-N1 was restricted with \( Xho \) and HindIII and the blunt cDNA fragment (+801 -+1195) was placed in the blunt \( Xho \) site between the two luciferase genes. The sense and antisense primers used for \( ATXN8OS \) amplification (+801 -+1195) were 5'-GGCGCCGATT-CATCCCTTTACCTTGTT 3' and 5'-CAGGAGGACATTCTCACG, respectively (EcoRI and HindIII sites underlined). The resulting PCR products were cloned, sequenced and restricted with EcoRI and HindIII to replace the +801 -+1195 fragment in dual luciferase reporter plasmid. The 632-bp blunt \( Xho \)-EcoRI IRES fragment from pIRES2-EGFP (Clontech) was inserted between the two luciferase genes as a positive control.

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**FACS Analysis of ORF-EGFP Expression**

HEK-293 cells were plated into 12-well dishes, grown for 20 hr and transfected with the above ORF-EGFP constructs, pIRES2-EGFP and pEGFP-N1 (2 μg/well). Cells were harvested for fluorescence activated cell sorting (FACS) analysis. The amounts of GFP expressed were analyzed in a FACStar flow cytometer (Becton-Dickinson), equipped with an argon laser operating at 350 nm. A forward scatter gate was established to exclude dead cells and cell debris from the analysis. 10^4 cells were analyzed in each sample.

**Confocal Microscopy Examination of ORF-EGFP Expression**

HEK-293 cells were grown on poly-L-lysine (100 μg/ml, Sigma) coated coverslips, grown for 20 hr and transfected with the above ORF-EGFP constructs, pIRES2-EGFP and pEGFP-N1 (2 μg/well). Cells were harvested for fluorescence activated cell sorting (FACS) analysis. The amounts of GFP expressed were analyzed in a FACStar flow cytometer (Becton-Dickinson), equipped with an argon laser operating at 350 nm. A forward scatter gate was established to exclude dead cells and cell debris from the analysis. 10^4 cells were analyzed in each sample.
Western Blot Analysis of ORF-EGFP Protein

Cells were lysed in RIPA buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% sodium deoxycholate, 1% NP-40 and 0.1% SDS) containing the protease inhibitor mixture (Sigma). After sonication and sitting on ice for 20 min, the lysates were centrifuged at 14,000 rpm for 30 min at 4°C. Protein concentrations were determined with the Bio-Rad protein assay kit, using albumin as standards. Proteins (25 µg) were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher and Schuell) by reverse electrophoresis. After being blocked, the membrane was stained with anti-EGFP (1:200 dilution, Santa Cruz Biotechnology) or ORF antiserum (1:200 dilution). The immune complexes were detected using horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch) or goat anti-rabbit (Rochland) IgG antibody (1:10000 dilution) and Immobilon™ Western Chemiluminescent HRP substrate (Millipore).

GST-ORF Construct and Antiserum

To construct GST-tagged ORF for antiserum production, BstI and EcoRI sites (italic) were added to the 5’ and 3’ ends of ATXN8OS ORF by PCR using primers 5’-GGCGCTTCGAGTGCTTCAACATGCAAGTC and 5’-CCTGGAGATCTCAACAGCTTACCTCATA (initiation and termination codons in boldface). The 317-bp BstI-EcoRI fragment containing ATXN8OS ORF sequences was then inserted between the AcI (location 928) and EcoRI (location 944) sites of pGEX-5X-3 (GE Healthcare). The location 928 AcI site (italic) used was added by site-directed mutagenesis using primer 5’-GATCTGATCGAGTTGACGGATCCCGAGATTC (mismatch nucleotides in boldface). The resulting pGST-ORF construct was verified by DNA sequencing and introduced into BL21(DE3)pLysS (Novagen). After IPTG induction, the 36-kDa antigen was purified using GST-BindTM resin (Novagen) and used to raise antiserum in rabbit (Litex Biolaboratories).

Lymphoblastoid and Neuroblastoma Cell Lines

Lymphoblastoid cells from a normal control were established (Food Industry Research and Development Institute, Taiwan) after obtaining informed consent. Cells were maintained in RPMI 1640 medium (GIBCO) containing 10% FBS. Human neuroblastoma SK-N-SH, SH-SY5Y and IMR-32 cells were cultivated in DMEM (IMR-32 and SK-N-SH) or 1:1 mixture of DMEM and 1640 medium (GIBCO) containing 10% FBS. Human neuroblastoma F12 medium (SH-SY5Y) containing 10% FBS. DMEM (IMR-32 and SK-N-SH) or 1:1 mixture of DMEM and 1640 medium (GIBCO) containing 10% FBS. The cells were centrifuged at 14000 rpm for 30 min at 4°C. The supernatants were transferred to new tubes. Pellets were then resuspended in SDS buffer (1.7% SDS, 20 mM Tris) by sonication. For Western blotting, proteins (30 µg) and aliquots of the supernatant were separated on 12% SDS-PAGE, blotted, stained with ORF antiserum (1:200 dilution) or actin antibody (1:10000 dilution, Chemicon) and immune complexes detected as described.

ORF Identification

For 2D PAGE and 2D immunoblot, 5 volume of 9.8 M urea lysis buffer was added and aliquots of the supernatant were first separated using Immobiline DryStrip (7 cm, pH 3–10) (GE Healthcare) and further separated by a 12.5% SDS-PAGE. The blotting membranes were stained with ORF antiserum (1:200 dilution) or actin antibody (1:10000 dilution, Chemicon) and immune complexes detected as described. The 2D gel was stained with SYPRO Ruby (Molecular Probe) and scanned on a Typhoon 9400 imager (GE Healthcare). The map was compared to the 2D immunoblot. The ORF-specific spots were punched out and subjected to reduction and alkylation by DTT/iodoacetamide, followed by in-gel digestion with freshly prepared Trypsin Gold (2.5 ng/µl, Promega) at 37°C for overnight. The obtained peptides were extracted with 50% acetonitrile containing 1% trifluoroacetic acid and tandem mass spectra were generated by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) at Proteomics and Protein Function Core Laboratory, Center of Genomic Medicine, National Taiwan University. MS/MS data were searched using the Mascot search engine (www.matrixscience.com) in a database containing theoretical trypsinized fragments of 23-kDa ORF protein initiated at GUG955 codon.

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Author Contributions

Conceived and designed the experiments: ICC HYL GJLC. Performed the experiments: ICC HYL YCH HCS YFS KSH. Analyzed the data: ICC YFS. Contributed reagents/materials/analysis tools: CMC YRW MTS HMHL. Wrote the paper: ICC GJLC.
13. Kozak M (1989) Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. Mol Cell Biol 9: 5073–5080.
14. Peabody DS (1989) Translation initiation at non-AUG triplets in mammalian cells. J Biol Chem 264: 5031–5035.
15. Gurtu V, Yan G, Zhang G (1996) IRES bicistronic expression vectors for efficient creation of stable mammalian cell lines. Biochem Biophys Res Commun 229: 295–298.
16. Ngoka LC (2000) Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers. Proteome Sci 6: 30.
17. Koob MD, Benzow KA, Bird TD, Day JW, Moseley ML, et al. (1996) Rapid cloning of expanded trinucleotide repeat sequences from genomic DNA. Nat Genet 18: 72–75.
18. Martin MM, Garcia JA, McFarland JD, Duffy AA, Gregson JP, et al. (2003) Translation of the human angiotensin II type 1 receptor mRNA is mediated by a highly efficient internal ribosome entry site. Mol Cell Endocrinol 212: 51–61.
19. Moskrej M, Vopalenský V, Kolnáry O, Masek T, Feketová Z, et al. (2006) IRESite: the database of experimentally verified IRES structures (www.iresite.org). Nucleic Acids Res 34(Database issue): D125–130.
20. Tournié C, Bornes S, Audigier S, Prats H, et al. (2003) Generation of protein isoform diversity by alternative initiation of translation at non-AUG codons. Biol Cell 95: 169–178.
21. Lock P, Ralph S, Stanley E, Boulet I, Ramsay R, et al. (1993) Two isoforms of murine hck, generated by utilization of alternative translational initiation codons, exhibit different patterns of subcellular localization. Mol Cell Biol 11: 4363–4370.
22. Hamn SR, Dixit M, Sears RC, Sealy L (1994) The alternatively initiated e-Myr proteins differentially regulate transcription through a noncanonical DNA-binding site. Genes Dev 8: 2441–2452.
23. Bruneing W, Pelletier J (1996) A non-AUG translational initiation event generates novel WT1 isoforms. J Biol Chem 271: 9646–9654.
24. Wegryn JL, Drudge FM, Valkatza F, Hook V (2000) Bioinformatic analyses of mammalian 5′-UTR sequence properties of mRNAs predicts alternative translation initiation sites. BMC Bioinformatics 9: 232.
25. Kozak M (1997) Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. Embry J 16: 2482–2492.
26. Nemes JP, Benzow KA, Moseley ML, Ramun L, Koob MD (2000) The SCA8 transcript is an antisense RNA to a brain-specific transcript encoding a novel actin-binding protein (KLHL1). Hum Mol Genet 9: 1349–1351.