Repeat Associated Non-AUG Translation (RAN Translation) Dependent on Sequence Downstream of the \textit{ATXN2} CAG Repeat

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Abstract

Spinocerebellar ataxia type 2 (SCA2) is a progressive autosomal dominant disorder caused by the expansion of a CAG tract in the \textit{ATXN2} gene. The SCA2 disease phenotype is characterized by cerebellar atrophy, gait ataxia, and slow saccades. \textit{ATXN2} mutation causes gains of toxic and normal functions of the \textit{ATXN2} gene product, ataxin-2, and abnormally slow Purkinje cell firing frequency. Previously we investigated features of \textit{ATXN2} controlling expression and noted expression differences for \textit{ATXN2} constructs with varying CAG lengths, suggestive of repeat associated non-AUG translation (RAN translation). To determine whether RAN translation occurs for \textit{ATXN2} we assembled various \textit{ATXN2} constructs with \textit{ATXN2} tagged by luciferase, HA or FLAG tags, driven by the \textit{CMV} promoter or the \textit{ATXN2} promoter. Luciferase expression from \textit{ATXN2}-luciferase constructs lacking the \textit{ATXN2} start codon was weak vs AUG translation, regardless of promoter type, and did not increase with longer CAG repeat lengths. RAN translation was detected on western blots by the anti-polyglutamine antibody 1C2 for constructs driven by the \textit{CMV} promoter but not the \textit{ATXN2} promoter, and was weaker than AUG translation. Strong RAN translation was also observed when driving the \textit{ATXN2} sequence with the \textit{CMV} promoter with \textit{ATXN2} sequence downstream of the CAG repeat truncated to 18 bp in the polyglutamine frame but not in the polyserine or polyalanine frames. Our data demonstrate that \textit{ATXN2} RAN translation is weak compared to AUG translation and is dependent on \textit{ATXN2} sequences flanking the CAG repeat.

Introduction

Spinocerebellar ataxia type 2 (SCA2) is an autosomal dominant cerebellar ataxia characterized by progressive degeneration of the cerebellum and parts of the brain stem. SCA2 is caused by CAG repeat expansion in the \textit{ATXN2} gene resulting in polyglutamine (polyQ) expansion in the ataxin-2 protein. The most common normal \textit{ATXN2} allele contains 22 CAGs and repeats of 33 CAGs or greater are pathogenic [1]. Patients with SCA2 are characterized by ataxia slowly...
progressing with age and slow saccadic eye movements [2], and SCA2 families are characterized by anticipation, whereby disease severity and age of onset correlate with CAG repeat length, which tends to increase generationally [3].

We previously characterized mechanisms of ATXN2 expression control to identify factors that may be exploited to reduce ATXN2 expression therapeutically [4]. The study was conducted with the hypothesis that lowering ATXN2 expression might be therapeutic because of a gene dose-phenotype relationship in polyQ diseases: SCA2 patients and mice homozygous for the mutated ATXN2 allele have more severe SCA2 phenotypes vs. heterozygous individuals [5,6], and phenotypes of other polyQ disease models are reversible [7–10]. We evaluated numerous ATXN2-luciferase (luc) constructs with unidirectional and interstitial deletions in the ATXN2 upstream region and determined that an ETS transcription factor binding site is required for ATXN2 expression. Our study also investigated the effect of CAG length on ATXN2 expression. One striking finding was that ATXN2-luc with only one CAG was low-expressing compared to any ATXN2-luc construct with longer CAGs. Therefore, we investigated this further because of a previous demonstration that expanded CAG repeats in the ATXN8 gene can initiate protein translation, by so-called repeat associated non-AUG (RAN) translation [11].

For repeat expansion genes, RAN translation is affected by repeat length whereby longer repeats are more susceptible to initiating translation, with no requirement for an AUG start codon [12–22]. RAN translation in all three reading frames (CAG, AGC, and GCA) was observed for CAG expanded ATXN8, by constructs lacking a start codon [11]. RAN translation is also initiated by hexanucleotide GGGGCC repeat expansion in intron 1 of the C9FTD/ALS gene C9orf72 [19,21,22], and by CGG repeat expansion in the 5′ UTR of FMR1, causing fragile X-associated tremor ataxia syndrome (FXTAS) [20]. RAN translation products may form high molecular weight aggregates that can be useful prognosticators of disease and very likely contribute to disease pathology. Accumulations of polyalanine and polyglutamine proteins were observed in disease tissues of SCA8 and DM1 patients [11], accumulations of poly-alanine-alanine and poly-glycine-proline peptides were observed in multiple CNS tissues from C9FTD/ALS patients [19,21,22], and polyglycine accumulations were observed in FXTAS patient brains [20]. Translation can also occur in the absence of an AUG start codon but not involving repeat expansion [23–29].

Understanding of ATXN2 RAN translation is important for developing therapeutics that reduce expanded CAG repeat-associated toxic gain of function associated with SCA2. In the present study we evaluated multiple ATXN2 constructs with varying CAG repeat lengths, with different ATXN2 sequences downstream of the CAG repeat, and different tags, for the ability to support RAN translation. We demonstrated that the structure of the ATXN2 sequence downstream of the CAG repeat significantly contributed to the ability for the RNA to undergo RAN translation. Constructs harboring the HA tag were more permissive to RAN translation than those harboring a luciferase tag, and additional ATXN2 sequence downstream of the CAG repeat abrogated RAN translation. We were not able to demonstrate significant RAN translation by the alternate polyalanine and polyserine frames of the ATXN2 CAG repeat.

Materials and Methods

Ethics Statement

No animal or human participants were used in this research.

Cloning of ATXN2-luc plasmids with start codon substitutions

Plasmid pGL2-5A3 includes a total of 1704 bp of ATXN2 upstream (1062 bp) and 5′-UTR (642 bp) sequence ahead of the ATXN2 start codon. Progressing downstream, the construct included
ATXN2 exon 1 encoded sequence through the first CAG of the CAG repeat, followed by the luciferase gene, followed by ATXN2 downstream sequence including the complete 3' UTR. pGL2-5B3, pGL2-5C3, and pGL2-5D3 are identical to pGL2-5A3 but include CAG lengths of 22, 57, and 101, respectively and include 108 bp downstream of the CAG repeat. Plasmids pGL2-5A3, pGL2-5B3, pGL2-5C3, and pGL2-5D3 were described previously [4]. Each of these plasmids were altered to include a ATG➔CTG (Met➔Leu) substitution in the ATXN2 start codon. The resultant constructs are referred to as ATG- or CTG- CAG1, CAG22, CAG57, and CAG101 or CAG102 (sequencing proved that the CTG-CAG102 construct gained 1 CAG relative to its ATG counterpart). The substitutions were made by amplifying the repeat region with forward primer CAG102 (sequencing proved that the CTG-CAG102 construct gained 1 CAG relative to its ATG counterpart) and reverse primer S2-B (5'-TGTATGGGCCCCTCACCCTGCTGAA-3') that includes an Apa I site for cloning and also the ATG➔CTG substitution, and reverse primer S2-A (5' -ccagctg gaggacgaggacgaggagac-3') that includes a Xho I site for cloning. The amplicon was excised from the non-mutant target plasmid with Apa I and Xho I and the mutant amplicon insert was ligated in place. All constructs were sequenced to verify the presence of the start codon ATG➔CTG substitution and the CAG length. Luciferase assays utilizing these plasmids were controlled with a promoterless luciferase plasmid lacking all ATXN2 upstream and exon 1 sequence but retaining the ATXN2 3'-UTR and downstream sequence after the luciferase gene. The control plasmid was created by excising the ATXN2 upstream and exon 1 sequence of pGL2-5A3 [4] with Hpa I and Xho I, filling the Xho I sticky end with T4 polymerase digestion, and ligating.

Cloning of CMV-ATXN2-luc plasmids

Plasmid pcDNA3.1-luc was first created by amplifying the luc insert with primer LucA (5'-GGCCCTCGAGAGGACG-3') and LucB (5'-TCGGGGCCCTCACCCTGCTGAA-3'), cutting the insert with Xho I and Apa I, and ligating into vector pcDNA3.1/Hygro (+) prepared with Xho I and Apa I digestion. CMV-ATXN2-luc plasmids possess 20 bp of the ATXN2 sequence immediately upstream of the CAG repeat, through 18 bp downstream of the repeat, and are modeled after plasmids used to study RAN translation in the HTT, JPH3 (HDL2), MJD1 and DMI genes in Zu et al. [11]. To prepare pCMV-ATG-ATXN2-luc the ATXN2 insert was amplified using primer Bam-A1 (5' -TGTATGGGCCCCTCACCCTGCTGAA-3') and primer Xho-B (5'-CTGGGGCCCTCACCCTGCTGAA-3'), the insert was digested with Bam HI and Xho I, and ligated into pcDNA3.1-luc prepared with Bam HI and Xho I digestion. Plasmid pCMV-CTG-ATXN2-luc was prepared in the same way except using forward primer Bam-A2 (5'-GTAGGGCTCTACCTTTGAGCAGGCGGCGGCGCGG-3'), which changes the start codon to a CTG. Both pCMV-ATG-ATXN2-luc and pCMV-CTG-ATXN2-luc were next modified by inserting a 6xStop cassette between the CMV promoter and the ATXN2 sequence. Two oligos (5'-CTAGCTAGTAGATAGATAGATAG-3' and 5'-GATCCCTACTATCTA-3') codon two stop codons in each reading frame were annealed and ligated in pCMV-CTG-ATXN2-luc prepared with Nhe I and Bam HI digestion, resulting in plasmid pCMV-6xStop-ATG-ATXN2-luc and pCMV-6xStop-CTG-ATXN2-luc. We then created two other plasmids from pCMV-6xStop-CTG-ATXN2-luc, one with luc shifted into the polyserine frame and the other with luc shifted into the polyalanine frame. To shift luc into the polyserine frame we amplified a fragment of ATXN2-luc with primer PolyS-For (5'-TGCCCTCGAGACGTGAAGACGAGC-3') and primer Luc-Rev (5'-TGCCCTCGAGACGTGAAGACGAGC3') and ligated into pCMV-6xStop-CTG-ATXN2-luc prepared by Xho I and Apa I digestion. We named the final construct pCMV-6xStop-CTG-ATXN2(polyS)-luc. Similarly, to shift luc into the polyA frame we amplified a fragment of ATXN2-luc with primer PolyA-For (5'-TGCCCTCGAGACGTGAAGACGAGC-3') and primer Luc-Rev and ligated into pCMV-6xStop-CTG-ATXN2-luc prepared by Xho I and Apa I digestion. We named the construct pCMV-6xStop-CTG-ATXN2(polyA)-luc. However, the luciferase activity was strongly reduced in the polyA frame, because luciferase translation is strongly reduced in the polyA frame.
Cloning of native promoter ATXN2-luc plasmids with luciferase in different frames

Constructs described here allowed for comparing RAN translation in the GCA (polyA), AGC (polyS), and CAG (polyQ) frames of ATXN2, upstream of luciferase, driven by the native ATXN2 promoter. To prepare these constructs we made modifications to pGL2-5D3 (containing 102 CAG repeats in ATXN2, previously described [4]) to place luc into either the polyA frame or the polyS frame. To prepare an insert for cloning the polyA frame we amplified a fragment of luciferase with forward primer Del1-A (5'-GGCCCTCGAGTGGAAGACGCCAAAAACATA-3') that includes an XhoI site for cloning and a CTG➔TG deletion in the luc CTG (the previously altered ATG➔CTG luc start codon), and reverse primer Rev-B (5'-CCAGAGGAATTCATTATCAGTGCAATTGTTTT-3'), that primes inside the luciferase gene across a unique EcoRI site for cloning. To prepare an insert for cloning the polyS frame we amplified from pGL2-5D3 a fragment of ATXN2 exon 1 including the CAG repeat using forward primer Ser-A (5'-GGCGTGCGAGCCGGTGTATG-3') that primes just before the ApaI site upstream of the CAG repeat and reverse primer Ser-B (5'-CCTCCTCGAGCGGGCTTGCGGACATTG-3') that primes downstream of the CAG repeat and includes an XhoI site for cloning. This shortens the 108 bp between the end of the CAG repeat and the beginning of the luciferase gene to 34 bp in order to exclude a stop codon in the polyS frame. The insert was ligated between the XhoI and ApaI sites of pGL2-5D3. Note that the resultant number of repeats in the completed GCA and AGC reporter plasmids is actually one less than the number of CAGs in the initial CAG102-ATXN2-luc construct (GCA101 & AGC101). All constructs were sequence verified.

Cloning ATXN2-3T plasmids

To assess RAN translation by western blotting we created ATXN2 expression plasmids each with three epitope tags (3T tag), with one epitope in each of the three frames. The 3T tag included the HA tag in the polyQ frame, the FLAG tag in the polyS frame, and the MYC tag in the polyA frame. To accomplish this, both pCMV-6xStop-ATG-ATXN2-luc and pCMV-6xStop-CTG-ATXN2-luc were modified by replacing luc with a 3T tag. Two oligos, 3T-For (5'-TCGAGTACCCATACGATGTTCCAGATTACGCTGGATTACAAGGATGACGACTATAAGAGAACAGAAACTGATCTCTGAAGAAGACCTGTAAGGGCC -3') and 3T-Rev (5'-CCAGAGGAATTCATTATCAGTGCAATTGTTTT-3'), that primes inside the luciferase gene across a unique EcoRI site for cloning. This shortens the 108 bp between the end of the CAG repeat and the beginning of the luciferase gene to 34 bp in order to exclude a stop codon in the polyS frame. The insert was ligated between the XhoI and ApaI sites of pGL2-5D3. Note that the resultant number of repeats in the completed GCA and AGC reporter plasmids is actually one less than the number of CAGs in the initial CAG102-ATXN2-luc construct (GCA101 & AGC101). All constructs were sequence verified.

Cloning of HA and FLAG-HA series of ATXN2 plasmids

Plasmids pATG-ATXN2-HA, pATG-ATXN2-FLAG-HA, pCMV-ATG-ATXN2-HA and pCMV-ATG-ATXN2-FLAG-HA with the ATXN2 ATG start codon and plasmids pCTG-ATXN2-HA, pCTG-ATXN2-FLAG-HA, pCMV-CTG-ATXN2-HA and pCMV-CTG-ATXN2-FLAG-HA with the start codon substituted with CTG were constructed in a stepwise manner described here. Both of pCMV-6xStop-ATG-ATXN2-3T and pCMV-6xStop-CTG-ATXN2-3T were modified to include a longer sequence of ATXN2 downstream of the CAG repeat, tagged with a single HA epitope. This was done by amplifying an insert fragment of ATXN2 with
primer ATX-for (5'-TGCCCTCGAGAATGTCGCAGCAGCCG-3') and primer ATX-HA-rev (5'-AAACGGGCCCTTAAGGTAATCTGGGAACATCGTATGGGTATTTGTACTGGGCACTTGACTC-3'), digesting the insert with Xho I and Apa I, and ligating the insert into either pCMV-6xStop-ATG-ATXN2-3T or pCMV-6xStop-CTG-ATXN2-3T prepared by Xho I and Apa I digestion, resulting in plasmids pCMV-ATG-ATXN2-3HA and pCMV-CTG-ATXN2HA. Sequencing demonstrated these plasmids contained 101 and 102 CAG repeats, respectively, and 801 bp of ATXN2 sequence downstream of the CAG repeat, exclusive of the Xho I site (originating from from pGL2-5A3). Plasmid pATG-ATXN2-HA was prepared by excising the CMV promoter in pCMV-ATG-ATXN2-3HA by digestion with Nru I and Nhe I and replacing it with the ATXN2 promoter obtained by PCR using primer pm1001b (5'–TGGCTTCGCGAGGCCCAGAGGCTGAGAC-3') and primer pm1002 (TCAGGCTAGCCTGAGGGGCCCATCAC), with template pGL2-5A3. This ATXN2 promoter fragment, designated ATXN2p, includes 96 bp of ATXN2 upstream sequence ahead of the 5'UTR transcription start site (a total of 738 bp upstream of the translation initiation site) and is longer than the minimal length required for ATXN2 expression and longer lengths can drive ATXN2 expression more weakly in HEK293 cells (see [4]). Plasmid pCTG-ATXN2-HA was prepared in the same way except the modification was to plasmid pCMV-CTG-ATXN2-3HA. Sequencing verified that both plasmids pATG-ATXN2-3HA and pCTG-ATXN2-3HA contained 101 and 102 CAG repeats, respectively. Next we modified each of pCMV-ATG-ATXN2-3HA, pCMV-CTG-ATXN2-3HA, pATG-ATXN2-3HA and pCTG-ATXN2-3HA by including an in-frame FLAG epitope 18 bp downstream of the CAG repeat. Because modifications made at this position resulted in CAG repeat contraction we did this stepwise. We prepared a double-stranded insert expressing the FLAG epitope by annealing oligo FLAG1A (5'-TCGAGCCTCGATTACAAGGATGAGCGATAAGC-3') and FLAG2B (5'-TCGAGCTTATCTGCTCATTCCCTGTAATCGGAGC-3') and ligating the annealed insert into the Xho I site of pCTG-ATXN2-HA downstream of the CAG repeat. By screening multiple constructs we obtained one containing 91 CAG repeats, designated pCTG-ATXN2-FLAG-HA. We then used pCTG-ATXN2-FLAG-HA as a template and amplified an ATXN2 fragment including the complete CAG repeat, using primers BamATG-For (5'-ATAGTAGGATCCTCACATGTCCGTGAAG-3') and Bam-REV (5'-CGAACATATTCTGGGAGTCCTCAATGGC1A-3'), by ligating the amplicon into pCMV-ATG-ATXN2-3HA prepared by Bam HI digestion, resulting in plasmid pCMV-ATG-ATXN2-FLAG-HA. To prepare pCMV-CTG-ATXN2-FLAG-HA we cut a CAG repeat containing fragment from plasmid pCTG-ATXN2-FLAG-HA with Bam HI and ligated it into plasmid pCMV-CTG-ATXN2-FLAG-HA prepared by Bam HI digestion. Finally, pATG-ATXN2-FLAG-HA was prepared by obtaining a CAG repeat containing fragment from pCMV-ATG-ATXN2-FLAG-HA with Nhe I and Aar I, and ligating it into pCTG-ATXN2-3HA cut with the same two enzymes. Each of the plasmids pATG-ATXN2-FLAG-HA, pCMV-ATG-ATXN2-FLAG-HA, and pCMV-CTG-ATXN2-FLAG-HA were verified by sequencing to contain 91 repeats like the ancestor plasmid pCTG-ATXN2-FLAG-HA.

**Luciferase assays**

HEK293T cells (ATCC) were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1X penicillin/streptomycin. Cells were transfected using Xfect Transfection Reagent following the vender’s protocol (Clontech). Transfections were conducted in triplicate wells of a 24-well plate. Transfections included 125 ng of luciferase reporter plasmid and 40 ng of pRL-SV40 (Promega). Assays were performed in triplicate per transfection after 24 or 48 h transfection using Dual-Glo Luciferase Assay System (Promega), recording relative light units (RLUs) from firefly luciferase and Renilla luciferase on a multimode plate reader (Beckman DTX880). Values were reported as the mean ± standard deviation (SD) of the
ratios of firefly luciferase / Renilla Luc (FLuc / RLuc), with n = 3 transfections for the calculation of SD. Each experiment was repeated at least three times.

Western blot assays
Proteins were separated on precast polyacrylamide gels (Bio-Rad), transferred to Hybond (Amersham) and detected by ECL (Amersham). Antibodies included goat anti-luciferase (Rockland Immunochemicals), mouse 5TF1-1C2 anti-polyglutamine antibody (Millipore), monoclonal mouse anti-β-actin-peroxidase (Sigma), monoclonal rabbit anti-c-Myc (Cell Signaling), mouse anti-HA-peroxidase (Roche), mouse anti-FLAG M2 (Stratagene), anti-hygro-mycin phosphotransferase (My Biosource Inc.). Secondary antibodies included peroxidase conjugated anti-mouse (Vector laboratories), and peroxidase conjugated donkey anti-goat and peroxidase conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories).

Real time PCR
HEK293T cells were cultured and transfected as above, in triplicate. Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer’s protocol. DNAse I treated RNAs were used to synthesize cDNA using ProtoScript cDNA synthesis kit (New England Biolabs Inc.). Two sets of primers were used for RT-PCR including luciferase primers GL2luc-2F (5’-ATCCGGAAGGACCAAGCCAGCC-3’) & GL2luc-2R (5’-GTGGAAGACTGCCCCACCC-3’) and GAPDH primers GAPDH-F (5’-GAAGGTGAAGGTCGAGTCAACG-3’) & GAPDH-R (5’-GAAGATGGTGATGGGATTTCC-3’). Quantitative RT-PCR (qPCR) was performed in Bio-Rad CFX96 (Bio-Rad Inc.) with the Power SYBR Green PCR Master Mix (Applied Biosystems Inc.). PCR reaction mixtures contained SYBR Green PCR Master Mix and 0.5 pmol primers. PCR amplification was carried out for 45 cycles. Cycling parameters were denaturation (95°C for 10 s), annealing (60°C for 10 s), extension (72°C for 40 s). The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. Luciferase expression level was normalized to GAPDH.

Results
Investigation of RAN translation using ATXN2 plasmids driven by native promoter
In order to evaluate the hypothesis that expanded CAG repeats in ATXN2 might initiate translation, we prepared multiple ATXN2-luc plasmids with different CAG length with and without the start codon (Fig 1A). The plasmids used in this experiment included 1062 bp of ATXN2 upstream sequence to drive expression because our initial intention was to describe RAN translation in the presence of the native ATXN2 promoter. We performed luciferase assays with these plasmids using HEK293T cells (Fig 1B). We observed increase in luciferase expression with increasing CAG length for plasmids with the ATXN2 ATG start codon. When the start codon was substituted (ATG➔CTG) the expression of ATXN2-luc with 1 CAG was only slightly higher than the vector control, but the expression level of ATXN2-luc constructs with 22, 57, or 102 CAG repeats were significantly higher, but still remarkably low compared to those constructs with the non-mutant start codon. This increase in expression might be attributed to RAN translation, but we did not see further increase in ATXN2-luc expression with increasing CAG repeat length (Fig 1D). We observed no corresponding RAN translation bands by western blotting using anti-luciferase antibody or anti-polyglutamine 1C2 antibody (Fig 1C).
Investigation of RAN translation using \textit{CMV-ATXN2-luc} plasmids

To make \textit{ATXN2} RAN translation more easily observed we increased transcript expression using the \textit{CMV} promoter, as was done by Zu et al. \cite{11}. Zu et al. studied RAN translation for

\begin{figure}
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\caption{\textit{ATXN2-luc} expression driven by the native \textit{ATXN2} promoter, dependent upon CAG length and the presence of a start codon. (A) Plasmid constructs used in luciferase assays. (B) Luciferase assays to evaluate \textit{ATXN2} expression driven by 1062 bp of its native upstream sequence, demonstrated increasing expression with increasing CAG length (ATG constructs). When the start codon was mutated, expression significantly higher than the control was observed only for \textit{ATXN2s} with CAG repeat lengths of 57 or 102 (CTG constructs). For the longest repeat expression was 25-fold reduced when the start codon was substituted with CTG. Values are mean±SD of three independent experiments. All constructs were cotransfected with SV40-Renilla luciferase and values are represented as meanFLuc / RLuc, the ratio of firefly luciferase to Renilla luciferase. (C) RAN translation products were not observed by western blotting using anti-luciferase (luc) or 1C2 antibodies. Note that polyglutamine proteins detected with the 1C2 anti-polyglutamine antibody are more easily seen as the length of the polyglutamine is increased. Loading was controlled by detecting actin. The mobilities of the smaller ataxin-2-luciferase bands are not consistent with RAN translation bands. (D) Analysis of the luciferase assay results for only the CTG-\textit{ATXN2-luc} constructs in B revealed significantly increased expression for constructs with 22 or greater CAG repeats but no increasing luciferase expression with increasing CAG repeat length. P<0.001 (**), Bonferroni post-hoc probability of significance. Assays utilized HEK293T cells with assays made 24 hrs after transfection.}
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HTT, HDL2, MJ1D1, and DM1, by creating plasmid constructs that included long CAG repeats preceded by 20 bp of gene-specific sequence, followed by antibody epitope tags. We followed this same approach for ATXN2 by creating CMV-ATXN2-luc plasmids with and without the ATG start codon, including only 20 bp ATXN2 sequence upstream of an expanded CAG (CAG101 or CAG102) repeat (Fig 2A). Luciferase assays using these constructs in HEK293T demonstrated strong ATXN2-luc expression in the presence of the ATG start codon that was reduced by 20 fold when the start codon was substituted with CTG (Fig 2B). When the start codon was mutated, ATXN2-luc expression remained 7 fold higher than background. This result was essentially the same as observed when using ATXN2-luc constructs driven by the native ATXN2 promoter. Despite use of a CMV promoter, putative RAN translation for CAG102 ATXN2-luc remained weak, and like for native-promoter-ATXN2-luc we were unable to detect the RAN translation protein products by western blotting, using anti-luciferase or anti-1C2 antibodies (Fig 1C). We also conducted quantitative PCR (qPCR) using RNAs from transfected HEK293T cells, to evaluate luciferase transcript abundance relative to GAPDH, demonstrating no evidence for the changes observed by luciferase assays or western blotting that could be accounted for by altered transcription (Fig 2D). One notable observation was that the fold-difference for CAG100 ATXN2-luc with vs. without a start codon driven by the native promoter was the same as the fold-difference for CAG101/102 ATXN2-luc with vs. without a start codon driven by the CMV promoter, at 20–25 fold reduction when the start codon was deleted (Fig 1B and Fig 2B). This indicated that there was little advantage of adding a CMV promoter to evaluating luciferase expression from these constructs.

Investigation of RAN translation using HA and FLAG HA epitope tagged ATXN2 plasmids

To further evaluate ATXN2 RAN translation we replaced the luciferase tag in our constructs with epitope tagged ATXN2 sequence. Following Zu et al. [11], we also inserted a 6X stop codon cassette (two stops in each frame) upstream of the ATXN2 start codon. Downstream of the CAG repeat included 801 bp of ATXN2 sequence with 101/102 CAG repeats tagged with a single C-terminal HA tag or an additional in-frame FLAG epitope 18 bp downstream of the ATXN2 sequence with 91 CAG repeats. Construct maps are provided in Fig 3A and 3B. We tested expression of these constructs in HEK293T cells by western blotting using anti-FLAG, anti-HA, and anti-1C2 antibodies with the ATXN2 start codon present or substituted with CTG, when driven by the CMV promoter or an ATXN2 promoter fragment (738 bp of upstream and 5'-UTR sequence). When constructs were driven by the CMV promoter expression was observed for all when the ATG start codon was present, including anti-HA, anti-1C2, and also for anti-FLAG when the FLAG epitope was present (Fig 3A). When the ATXN2 start codon was substituted by CTG we observed RAN translation using the 1C2 antibody (Fig 3A). The detected RAN translation bands were notably weaker than expression when the ATG start codon was present, and the RAN translation bands could not be visualized by western blotting using anti-FLAG, and when anti-HA was used we observed no RAN translation band for construct #2 in Fig 3A but there was an exceptionally weak band for construct #4. These results demonstrated that RAN translation is not favored vs AUG translation. Nearly identical results were obtained when the ATXN2 promoter was utilized to drive the expression of the otherwise identical constructs, except that no RAN translation bands could be observed when the ATG start codon was substituted to CTG (Fig 3B). Transfection and western blot loading was controlled by the use of anti-hygromycin phosphotransferase (HYG) (all plasmids contained the hygromycin resistance gene) and anti-Actin antibodies (Fig 3A–3C). Note that for each
A

Control

ATG-ATXN2

CTG-ATXN2

CTG

ATG

CMV

20 bp

CAG101

18 bp

luc

luc

luc

B

Luciferase Assay

Fluc / RLuc

Control

ATG-ATXN2

CTG-ATXN2

C

Control

ATG-ATXN2

CTG-ATXN2

luc

1C2

D

Q-PCR

luc / GAPDH

Control

ATG-ATXN2

CTG-ATXN2

NS
construct in Fig 3 we evaluated ≥ 5 independent plasmid preparations by western blotting and all were fully sequenced, to guard against CAG repeat mosaicism.

**ATXN2 RAN translation initiated in alternate ATXN2 reading frames**

We evaluated RAN translation in HEK293T cells in the alternative ATXN2 reading frames by luciferase assays. We used constructs with the ATXN2 start codon substituted to CTG and a
C-terminal luciferase gene in the polyglutamine (polyQ) frame or shifted into the polyserine (polyS) or polyalanine (polyA) frame, with expression driven by the CMV promoter or the ATXN2 promoter. When constructs were driven by the CMV promoter expression in either of the polyQ, polyS, or polyA frames was significantly higher than the vector control but remained low, at 4.5, 2.5 and 1.5%, respectively, of the expression observed in the polyQ frame when the ATG start codon was present (Fig 4A). When constructs were driven by the ATXN2 promoter expression greater than the control was only observed when luciferase was in the polyQ and polyS frames, and expression was only 3% of that observed in the polyQ frame when the ATG start codon was present (Fig 4B). Western blotting using HEK293T cell lysates and anti-luciferase antibodies revealed no bands for these constructs. All constructs that produced no expression were subjected to additional bidirectional full-length sequence verification.

We also investigated ATXN2 RAN translation in alternate reading frames by a western blot strategy using a CMV promoter to drive up expression of C-terminally tagged short ATXN2 proteins. The constructs that we used included the 6x stop cassette separating the CMV promoter and the ATXN2 start codon, and had 101 (or 102) CAG repeats followed by 18 bp of ATXN2 sequence, and a C-terminal 3T tag. The 3T tag was similar to that used in Zu et al. [11], except that the epitope order was HA (in frame with polyQ), FLAG (in frame with polyS), and MYC (in frame with polyA). Using the HA antibody reporting expression in the polyQ frame, we observed strong expression from CMV-ATXN2-3T plasmids, regardless of whether the start codon was substituted with CTG or not (Fig 4C). When the start codon was substituted to CTG a single RAN translation band was observed by expression in the polyQ frame only, at approximately 37 kDa. When the start codon was not substituted, however, we observed a predominant 40 kDa band, and the 37 kDa band was very weak. This indicated that translation initiation was highly favored from the AUG start codon vs RAN translation initiating from the CAG repeat. Western blotting of proteins expressed from CMV-ATXN2-3T plasmids using anti-FLAG reporting translation in the polyS frame, and anti-Myc reporting translation in the polyA frame revealed no bands above background, consistent with absence of detectable RAN translation in the polyS or polyA frames (Fig 4C).

Discussion

This study was initiated to determine whether RAN translation could be demonstrated for the ATXN2 gene. The motivation for this study was our observation that an ATXN2-luc construct with a single CAG repeat produced 50–75% less luciferase expression of an otherwise identical ATXN2-luc construct with 22 CAG repeats, the most common human wildtype allele (Fig 1 and [4]), without mRNA abundance explaining the difference [4]. We observed only weak evidence for RAN translation for ATXN2-luc constructs lacking the ATG start codon. We observed that ATXN2 sequences driven by the strong CMV promoter could undergo RAN translation, but RAN translation was inhibited when the ATXN2 sequence downstream of the CAG repeat was lengthened. The study demonstrated that RAN translation for ATXN2 was determined by the sequence flanking the CAG repeat and was not favored compared to AUG translation.

Evidence for ATXN2 RAN translation

Our study demonstrated a potential for the ATXN2 gene to undergo RAN translation. However, the conditions that permit RAN translation are only partly revealed by the results of this study. In seeking evidence for RAN translation for ATXN2, we undertook a number of strategies: increase of the CAG repeat length in constructs lacking the start codon, use of CMV and
Fig 4. RAN translation in ATXN2 alternate reading frames. (A and B) Luciferase assays performed using ATXN2 constructs with expression driven by (A) the CMV promoter or (B) the native ATXN2 promoter, with luciferase shifted into the PolyQ, PolyS, and PolyA frame. In all cases no ATXN2 ATG start codons...
endogenous ATXN2 promoters, alterations of the length of sequence downstream of the CAG repeat, and the use of luciferase vs small epitope tags.

We observed lack of increasing expression with increasing CAG repeat length for ATXN2–luc constructs with the start codon mutated, not supporting RAN translation from ATXN2-luc constructs. When we increased the length of the CAG repeat in ATXN2-luc constructs we observed progressively increased ATXN2-luc expression. Previously we observed no transcriptional differences among constructs with different CAG lengths and concluded that translational regulation might account for the observed expression differences [4]. The implication was that the expanded ATXN2 CAG repeat might enhance CAP mediated translation initiation or progression, or that the repeat might support RAN translation. But for the same constructs without the ATG start codon, we were unable to observe RAN translation bands by western blotting, and there was no progressively increasing ATXN2-luc expression (Fig 1).

Typically, RAN translation strength increases with the length of the CAG repeat, resulting in the production of multiple homopolymeric proteins [12]. The lack of increasing expression with CAG repeat length for ATG mutated ATXN2-luc constructs argues against RAN translation produced by these constructs, yet elimination of the start codon did not entirely abolish expression but reduced it to 5 times the background seen with transfections performed using the vector control. We then attempted to observe RAN translation by driving up the ATXN2-luc expression for the construct with 102 CAG repeats and by replacing the native ATXN2 promoter with the CMV promoter. The use of the CMV promoter resulted in an order of magnitude increase in ATXN2-luc expression, but once again when the ATG was eliminated the expression was reduced to about 5 times background. There was no evidence for RAN translation bands by western blotting (Fig 2).

ATXN2 RAN translation was observed when we used the CMV promoter to drive ATXN2 sequences tagged with C-terminal epitopes. CMV constructs of ATXN2 including -20 bp upstream of the CAG repeat thru 801 bp downstream of the CAG repeat with 101/102 CAG repeats expressed ATXN2 proteins. We tagged these constructs with the HA epitope and we also include an in-frame FLAG tag for some constructs with 91 CAG repeats in effort to not overlook RAN translation bands that might form incomplete proteins, although none such bands were observed. When the start codon was eliminated we observed ATXN2 bands that were detectable with the anti-polyglutamine antibody 1C2, but the expression of these RAN translation bands were considerably weaker than when driven by the ATG start codon and not detected using the anti-FLAG antibody, and only the faintest RAN-translation band was detected using anti-HA antibody (Fig 3A). We also prepared an identical series of epitope-tagged ATXN2 constructs driven by the ATXN2 promoter (738 bp of upstream and 5’-UTR sequence). When the ATXN2 promoter was utilized we were unable to observe evidence of RAN translation (Fig 3B). The inability to observe RAN translation when using the ATXN2 promoter was likely due to weaker expression (the strength of the CMV promoter vs the ATXN2 promoter is indicated by comparing Figs 1B and 2B). The overall result demonstrates that ATXN2 AUG translation is strongly favored over ATXN2 RAN translation.

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RAN translation in alternate reading frames

We evaluated RAN translation in the alternate reading frames in two ways, by luciferase assays using ATXN2-luc constructs in which luc had been shifted into the alternate reading frames, and western blotting using epitope tags. While luciferase expression from the CTG-ATXN2-luc constructs was low (Figs 1 & 2), this nevertheless provided an opportunity to investigate RAN translation driven by the ATXN2 promoter in alternate reading frames. When luciferase was shifted into either the polyQ, polyS, or polyA frame in CTG-ATXN2-luc constructs driven by the CMV promoter, expression for each of these constructs was significantly higher than for the vector control background (Fig 4A). CTG-ATXN2-luc expression was also significantly higher when luciferase was shifted into the polyQ and polyS frames but not the polyA frame when driven by the ATXN2 promoter (Fig 4B). However, apparent RAN translation from each of these constructs was weak compared to AUG translation (under 5% of AUG translation in the polyQ frame), and the relative differences of translation among the reading frames was not the same as that observed for constructs including a truncated ATXN2 sequence downstream of the CAG repeat, followed by a C-terminally positioned 3T epitope tag in place of luciferase. The 3T tag includes three epitopes positioned into each of the three reading frames, that has been used previously for the study of RAN translation [11]. For CMV-ATXN2-3T with 102 CAG repeats, western blot detection revealed strong RAN translation in the polyQ frame (Fig 4C). Expression from the CMV-ATXN2-3T construct in the polyQ frame was as strong as the complementary AUG translation. Additionally, a small quantity of the RAN translation product was observed even when the start codon was retained, indicating that the retention of the start codon does not prevent downstream RAN translation for the CMV-ATXN2-3T construct. This was not predicted based on a discussion of Kozak consensus sequence impact on translation initiation site codon usage indicating that it is unlikely that a preinitiation complex would bypass a strong upstream initiation codon in order to utilize a suboptimal one downstream [29]. The observation of RAN translation for the CMV-ATXN2-3T construct in the polyQ frame demonstrated that truncation of the ATXN2 sequence downstream of the CAG repeat is more permissive of RAN translation, or conversely that the ATXN2 sequence downstream of the CAG repeat inhibits ATXN2 RAN translation. We concluded that the minimal luciferase expression that we observed from CTG-ATXN2-luc constructs was not due to RAN translation because increasing CAG repeat lengths did not result in increasing RAN translation (Fig 1D), and expression was generally the same among the three frames (Fig 4A and 4B) unlike expression differences observed among the three frames when using the CMV-ATXN2-3T construct.

Ribosomal frameshifting in ATXN2

Translation artifacts observed by Toulouse et al. (2005) from expanded MJD-1 CAG repeats were attributed to ribosomal frameshifting [30]. The expanded CAG repeat of the MJD-1 gene, which causes SCA3, produced polyalanine and polyserine proteins in a repeat length-dependent manner. Consistent with RAN translation, polyalanine protein expression increased with longer repeat lengths, but polyserine protein expression was low. For MJD-1 the requirement for a start codon was not determined [30], therefore it remains unclear if the translation of MJD-1 in the alternative reading frames were initiated by RAN translation. However, treatment with anisomycin and sparsomycin, drugs that affect programmed ribosomal frameshifting, modulated expression of polyalanine and polyserine proteins expressed by MJD-1 [30]. Our study produced no evidence for frameshifting contributing to the translation of the expanded CAG repeat in ATXN2, because the ATXN2-luc constructs with luciferase in the polyS and polyA frames retained the native ATXN2 start codon in the polyQ frame, but only low
luciferase levels of expression were observed for those constructs (Fig 4B), and for the ATG-ATXN2-3T construct expression was only observed in the polyQ frame (Fig 4C).

Sequence factors influencing RAN translation
Initiation of RAN translation is influenced by secondary structure of sequences flanking the CAG repeat. The formation of secondary RNA structures appears to be critical, because hairpin-forming CAG repeats undergo RAN translation, while non-hairpin-forming CAA repeats of a similar length do not [11]. Hairpin structures are also important for CAG associated frameshifting, as hairpin-forming CAG repeats undergo frameshifting, constrained with non-hairpin-forming CAA repeats of a similar length [30]. Additionally, the GGGGCC hexanucleotide repeat of C9ORF72 forms hairpin structures that are even more stable than those formed from CAG repeats. GGGGCC hairpin structures become even more stable as repeat length increases [21]. G-quadruplex structures also contribute to RAN translation, are repeat length- and flanking sequence-dependent, and the hexanucleotide repeat of C9ORF72 and the CGG repeat of FMR1 both form G-quadruplex structures [20,31]. Constructs lacking an ATG initiation codon, containing an expanded CAG repeat with 20 bp of upstream sequences from the HTT, HDL2, MJ1, DMI genes, respectively, all produced RAN translation proteins, but with variable efficiency [11]. This is consistent with evidence that the flanking sequence of repeating units influences the threshold stability of hairpin structures [32]. In our study, the ability to observe ATXN2 RAN translation was promoted by close proximity of the short 3T epitope tag to the CAG repeat, but inhibited by inclusion of 801 bp of ATXN2 downstream of the CAG repeat. We conclude that inclusion of the 801 bp of ATXN2 downstream of the CAG repeat induced secondary RNA structures that inhibited RAN translation. The ATXN2 mRNA might undergo RAN translation in vivo that is regulated by RNA secondary structural shifts mediated by microRNAs or RNA binding protein interactions [33].

RAN Translation and Pathogenesis
RAN translation protein products are now known to contribute to pathogenesis in multiple diseases. ATXN8 RAN translation products accumulate in Purkinje cells of SCA8 patient brains [11]. C9ORF72 RAN translation products accumulate in ALS/FTD patient brains [19,22]. Expanded CGG repeat RAN translation products were identified in FXTAS patient brains [20]. For ATXN2, we demonstrated that RAN translation is possible when the gene is truncated. This raises the possibility that ATXN2 RAN translation might occur under the right circumstances, in vivo.

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Author Contributions
Conceived and designed the experiments: DRS SMP. Performed the experiments: WD MH TH LTP LWP KKT. Analyzed the data: DRS. Wrote the paper: DRS.

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