Nuclear localization of the spinocerebellar ataxia type 7 protein, ataxin-7

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Spinocerebellar ataxia type 7 (SCA7) belongs to a group of neurological disorders caused by a CAG repeat expansion in the coding region of the associated gene. To gain insight into the pathogenesis of SCA7 and possible functions of ataxin-7, we examined the subcellular localization of ataxin-7 in transfected COS-1 cells using SCA7 cDNA clones with different CAG repeat tract lengths. In addition to a diffuse distribution throughout the nucleus, ataxin-7 associated with the nuclear matrix and the nucleolus. The location of the putative SCA7 nuclear localization sequence (NLS) was confirmed by fusing an ataxin-7 fragment with the normally cytoplasmic protein chicken muscle pyruvate kinase. Mutation of this NLS prevented protein from entering the nucleus. Thus, expanded ataxin-7 may carry out its pathogenic effects in the nucleus by altering a matrix-associated nuclear structure and/or by disrupting nucleolar function.

INTRODUCTION

Spinocerebellar ataxia type 7 (SCA7) is one of several autosomal dominant ataxias characterized by neuronal loss, usually within the cerebellum and brainstem, in which the disease-causing mutation is the expansion of an unstable CAG trinucleotide repeat (1–3). This group of ataxias also includes SCA1, SCA2, SCA3 and SCA6 (4–9). In addition, the neurodegenerative disorders spinal and bulbar muscular atrophy (SBMA or Kennedy disease), dentatorubral-pallidoluysian atrophy (DRPLA) and Huntington’s disease (HD) are also caused by the expansion of a CAG repeat (10–13). Because in all of these diseases the CAG repeat is within the coding region of the disease gene, encoding a tract of glutamines, the expansion of an unstable CAG trinucleotide repeat (1–3) was used to search the dbEST database. This search identified a partial SCA7 cDNA clone that was obtained from the IMAGE Consortium cDNA library (Research Genetics). Using standard molecular biology and PCR techniques, a full-length SCA7 cDNA clone was constructed (Fig. 1). To alter the length of the CAG repeat, PCR primers flanking the repeat were used to amplify mutant tracts from genomic DNA of SCA7 patients (2). The PCR product was digested with AatII and NarI, and the resulting fragment, with an altered CAG tract, was subcloned back into the SCA7 cDNA. Sequence analysis was performed to confirm the integrity of the construct and the length of

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the CAG repeat tract. Finally, to verify the production of full-length ataxin-7 protein, a western blot of transfected COS-1 cell extract was probed with the monoclonal antibody 1F8 that recognizes proteins containing polyglutamine tracts larger than 37 repeats and revealed proteins of the appropriate size for intact ataxin-7 (data not shown).

Nuclear localization of ataxin-7 in transfected COS-1 cells

To assess the subcellular localization of ataxin-7, we transiently transfected COS-1 cells with SCA7 constructs containing different numbers of CAG repeats. Indirect immunofluorescence using the anti-Xpress antibody against the Xpress epitope located in the expression vector was performed. In transfected COS-1 cells, ataxin-7 with 52 glutamines localized throughout the nucleus (Fig. 2). In addition, numerous irregularly shaped protein accumulations were observed within the nucleus. These were small with concomitant diffuse staining throughout the nucleus, intermediate in size with less diffuse staining, or large with very little residual nuclear staining. Each of these types of cellular distribution of ataxin-7 was observed with approximately the same frequency. Interestingly, compared with mutant ataxin-7, ataxin-7 with 10 glutamines also formed irregularly shaped structures with similar types of cellular distribution (data not shown). Hence, the frequency and size of these accumulations did not correlate with the length of the expanded CAG tract in ataxin-7. In addition, we found no indication of toxicity in the transiently transfected COS-1 cells with either wild-type or mutant ataxin-7. This lack of toxicity is identical to our previous findings with COS cell transfections using ataxin-1 (17).

Solubility and ubiquitination of nuclear ataxin-7

To assess the biochemical nature of nuclear ataxin-7 in transfected COS-1 cells, we analyzed COS-1 cell extracts on western blots. No evidence of insoluble ataxin-7 complexes was found on western blots of lysates prepared from cells transfected with ataxin-7[10Q], ataxin-7[52Q] or ataxin-7[65Q] (Fig. 3). In all cases, ataxin-7 in the total cellular extract migrated to a position in the separating gel consistent with the amino acid length of the monomeric protein. No evidence of insoluble complexes was seen at higher molecular weights in the separating gel or in the stacking gel.

Immunofluorescent studies were undertaken to determine whether the ataxin-7 nuclear entities were ubiquitinated. This result indicated that, in contrast to the ataxin-1 nuclear aggregates seen in COS-1 cell nuclei, the ataxin-7 accumulations in transfected COS-1 cells were not ubiquitinated. Figure 4A depicts the deposition of ataxin-7[75Q] in the nucleus of a transfected COS-1 cell. Reactivity to an anti-ubiquitin antibody of the same COS-1 cell is shown in Figure 4B, and Figure 4C, depicting the merged images, reveals no evidence of co-localization. For comparison, a merged image of an ataxin-1[82Q]-transfected COS-1 cell nucleus stained with ataxin-1 (red) and ubiquitin (green) antibodies shows that the large ataxin-1 inclusions are positive for ubiquitin (yellow). Thus, nuclear mutant ataxin-7 in transfected COS-1 cells differs in its form from that seen with mutant ataxin-1 (17).

Ataxin-7 associates with the nuclear matrix and co-localizes with PML

Since ataxin-1 with an expanded CAG tract associated with the nuclear matrix (17), we sought to determine whether mutant ataxin-7 is also a nuclear matrix-associated protein. Nuclear matrix extracted from SCA7-transfected cells was stained with anti-PML, a protein known to associate with the nuclear matrix, and anti-Xpress antibodies for ataxin-7. Figure 5A shows that the nuclear matrix preparation was positive for ataxin-7. In Figure 5B, the same preparation was immunostained for PML. The merged image (Fig. 5C) reveals that several of the PML structures are positive for ataxin-7 (red) and ubiquitin (green) antibodies shows that the large ataxin-1 inclusions are positive for ubiquitin (yellow). Thus, nuclear mutant ataxin-7 in transfected COS-1 cells differs in its form from that seen with mutant ataxin-1 (17).

![Figure 2](https://via.placeholder.com/150x150)

Figure 2. COS-1 cells expressing ataxin-7 with 52 glutamines. Subcellular localization of ataxin-7 was determined by immunofluorescence using the anti-Xpress antibody. Ataxin-7[52Q] was expressed in the nucleus but exhibited different types of distribution. (A) Predominantly diffuse staining with small ataxin-7 accumulations. (B) Intermediate sized ataxin-7 accumulations with less diffuse nuclear staining. (C) Large ataxin-7 accumulations with little residual nuclear staining.
two ataxin-7- (green) transfected COS-1 cell nuclei, the ataxin-7 structures contain PML (yellow). Thus, analyses of both nuclear matrix preparations and intact cells indicate that in ataxin-7-transfected COS cells, a portion of the nuclear ataxin-7 localized to the PML structures.

Nucleolar localization

To assess further the distribution of mutant ataxin-7, we performed additional co-localization studies. Counterstaining of the transfected cells with propidium iodide identified the nucleic acid within the nucleus as well as the nucleolus (Fig. 6). Co-localization of the propidium iodide staining and mutant ataxin-7 expression indicated that in addition to the expression of mutant ataxin-7 throughout the entire nucleus, a portion of mutant ataxin-7 expressed in the nucleus localized to the nucleolus. Both the large ataxin-7[75Q] entities (Fig. 6C) and diffuse ataxin-7[75Q] staining (Fig. 6F) were observed in the nucleolus. Ataxin-7 with 10 glutamines also localized to the nucleolus.

Ataxin-7 contains an arginine–lysine NLS

During the cloning of the SCA7 gene, a putative arginine–lysine NLS was identified (2). This type of NLS generally is composed of a stretch of arginine and lysine residues that can be located anywhere within the primary sequence of a protein (27). To determine whether this ataxin-7 arginine–lysine region is a functional NLS, we fused the chicken muscle pyruvate kinase (CMPK) gene to a fragment of SCA7 containing the putative NLS. CMPK is normally a cytoplasmic protein that has been used previously in fusion constructs to identify NLSs (25,28).

Transfection of CMPK containing the FLAG epitope at the N-terminus into COS-1 cells resulted in exclusively cytoplasmic expression (Fig. 7A). However, when a fragment of SCA7 corresponding to amino acids 357–412 was fused to CMPK, the resulting fusion protein exhibited a nuclear localization (Fig. 7B). As seen for intact ataxin-7, amino acids 357–412 fused to CMPK localized to the nucleoli of transfected COS-1 cells (Fig. 7B). This indicates that amino acids 357–412 contain a functional NLS and possibly a nucleolar localization signal (N o L s ) . T h e p u t a t i v e N L S w a s m u t a t e d w i t h a base pair change replacing the lysine residue at position 380 with a threonine (Fig. 1). This amino acid substitution (K to T) has been shown to disrupt the SV40 NLS (29,30). A fusion of the mutated SCA7 fragment and CMPK was transfected into COS-1 cells. Indirect immunofluorescence using the anti-FLAG M5 antibody indicated that the fusion protein (ataxin-7 K380T–CMPK) localized to the cytoplasm (Fig. 7C). This further supports the presence of an SCA7 NLS residing in the fragment containing amino acids 357–412. To confirm that the NLS is functional within the full-length protein, ataxin-7 K380T was expressed in COS-1 cells. Indirect immunofluorescence using
the anti-Xpress antibody against the Xpress epitope was performed. Ataxin-7 (K380T) expressed in COS-1 cells localized to the cytoplasm (Fig. 7D). This is in contrast to the full-length wild-type SCA7 construct which exhibited nuclear expression (Fig. 2).

**DISCUSSION**

As seen previously for ataxin-7 in LCLs (26), ataxin-7 in transiently transfected COS-1 cells localized to the nucleus. Transport of ataxin-7 to the nucleus of COS-1 cells is dependent on a monopartate arginine–lysine NLS centered around Lys380. Mutation of Lys380 in ataxin-7 to a threonine completely blocked the transport of ataxin-7 into COS-1 cell nuclei. Within the nucleus, ataxin-7 was found associated with the nuclear matrix, PML bodies [PML oncogenic domains (PODs)] and nucleoli. These results indicate that ataxin-7 may have a role in multiple aspects of nuclear function.

The nuclear location of ataxin-7 in the transfected COS-1 cells is, to some extent, similar to that seen for another protein in which expansion of a polyglutamine tract is associated with an autosomal dominant spinocerebellar ataxia, ataxin-1 (17). Yet, there are several differences seen between ataxin-1 and ataxin-7 in their nuclear transport and localization that suggest that these two proteins have quite distinct nuclear functions. For both ataxin-1 and ataxin-7, nuclear transport in COS-1 cells is due to the activity of an arginine–lysine NLS. Mutating the NLS in ataxin-1 did not cause a complete redistribution of ataxin-1 to the cytoplasm of COS-1 cells (25), while the NLS mutation in ataxin-7 completely disrupted nuclear transport in COS-1 cells (Fig. 7). Thus, nuclear transport in COS-1 cells of ataxin-7 appears to be completely dependent on this NLS, while ataxin-1 transport in COS-1 cells is only partially dependent on the function of the NLS. Interestingly, the same NLS mutation in ataxin-1 had a much more dramatic effect on its subcellular distribution in Purkinje cells of the cerebellar cortex, resulting in an almost completely cytoplasmic distribution of ataxin-1 in Purkinje cells (25). Furthermore, the subcellular distribution of ataxin-1 varies with cell type. In most neurons, ataxin-1 is nuclear and in peripheral tissues it is cytoplasmic. In Purkinje cells, ataxin-1 is nuclear and cytoplasmic (22). It will be important to determine whether the subcellular distribution of ataxin-7 also varies with cell type and whether its nuclear transport is always completely dependent on its arginine–lysine NLS.

Ataxin-7 showed co-localization with another matrix-associated nuclear protein, PML. Thus, ataxin-7 is a component of PODs, which have been suggested to have an important cellular function. In acute promyelocytic leukemia, the POD structure is disrupted and treatment with retinoic acid restores POD structure and myeloid differentiation (31,32). In contrast to ataxin-7, ataxin-1 did not demonstrate co-localization with PML (17). Rather, expression of mutant ataxin-1 caused the redistribution of PML into the large ataxin-1 nuclear aggregates. Expression of mutant ataxin-1 in COS-1 cells causes a similar redistribution of transfected ataxin-7 (data not shown), further suggesting that ataxin-7 and PML are components of the same nuclear structure.

Another intriguing difference between nuclear ataxin-7 and ataxin-1 in transfected COS-1 cells is the distinctions in the...
large nuclear entities to which each localizes. In the case of ataxin-1, the formation of these inclusions is directly related to the length of the polyglutamine tract (17), and seems to contain an aggregated insoluble form of ataxin-1. In addition, the ataxin-1 aggregates stain with antibodies to ubiquitin (Fig. 4D) (33). In contrast, the formation of the ataxin-7 accumulation in COS-1 cell nuclei was not dependent on polyglutamine length, did not correlate with the presence of insoluble ataxin-7, and they were not ubiquitinated. Thus, the large nuclear entities formed in COS-1 cells by ataxin-7 and ataxin-1 are quite distinct in their biochemical properties.

An interesting aspect of the ataxin-7 nuclear distribution is the association of a portion of it with nucleoli. Nucleoli are composed of reiterations of rDNA that coalesce with specific proteins to form the sites of rRNA synthesis and processing. Recently, nucleolar sequestration of protein has been shown to be important in the regulation of mitosis (34,35). It was suggested further that nucleolar sequestration might be a means for the regulation of other biological processes. Thus, the localization of ataxin-7 to nucleoli raises the possibility that expansion of the polyglutamine tract in ataxin-7 may alter nucleolar function.

When the fragment of SCA7 corresponding to amino acids 357–412 was fused to CMPK, a portion of the resulting fusion protein localized to nucleoli within the nucleus (Fig. 7B). Thus, this 56 residue fragment of ataxin-7 not only contains the NLS but is important for targeting ataxin-7 to the nucleolus. Whether this portion of ataxin-7 contains an NLS or an element important for the binding of ataxin-7 to other nucleolar components remains to be determined. At present, there is no known consensus NLS sequence. Some NLSs appear to consist of a basic amino acid motif (36). Other proteins localize to the nucleolus by virtue of their interacting with other nucleolar components (37).

While the genetic basis of the polyglutamine diseases, including SCA7, is clearly an expansion of a CAG trinucleotide repeat encoding a polyglutamine tract, the molecular mechanism of pathogenesis remains uncertain. The results reported here further demonstrate that ataxin-7 is a nuclear protein and identify the region of the protein required for nuclear localization as well as association with nucleoli. The next step will be to determine whether one or both of these characteristics of ataxin-7 have a role in the SCA7 neuronal pathogenesis induced by polyglutamine expansion.

MATERIALS AND METHODS

SCA7 constructs

An Smal–EcoRV fragment containing the SCA7 cDNA with 10, 52 or 75 glutamines was cloned into the EcoRV site of the mammalian expression vector pCDNA1.1 or pCDNA3.1 HisA (Invit
rogen). Ataxin-7[K380T] was generated using a two-step PCR technique to change the AAA codon to ACA at amino acid 380. Two overlapping primers of opposite orientation containing the base change were used with flanking primers in two separate PCRs using the SCA7 cDNA as the template. These two PCR products which overlapped at codon 380 were then used as a template in a PCR with flanking primers to generate a large fragment that subsequently was recloned back into the SCA7 cDNA. Sequence analysis was performed to ensure the presence of the mutant codon and to verify the absence of additional mutations potentially introduced by PCR.

To generate the ataxin-7 (amino acids 357–412)–CMPK fusion protein, a construct described previously (25) that encoded the FLAG epitope (Kodak) and nine amino acids of ataxin-1 upstream of CMPK in pCDNA1.1 was used. PCR primers tailed with BglII restriction sites were used to generate a PCR product encoding amino acids 357–412. This PCR product was subcloned in-frame into the BglII site between the FLAG epitope and CMPK in the above-mentioned plasmid. Sequence and restriction digest analysis was performed to ensure that the fragment was in the proper orientation. A similar strategy was used to generate the plasmid containing the mutated NLS (K380T).

**COS-1 cell transfections and immunofluorescence**

COS-1 cell transfections were performed as previously described (17). Briefly, 1 × 10^6 cells in 60 mm dishes were transfected with 5 mg of plasmid DNA using DEAE–dextran. Cells were transferred to coverslips, fixed and stained 48 h post-transfection as described (38) with either M5 (anti-FLAG, 1:100; Kodak) or anti-Xpress (1:200; Invitrogen). Goat anti-rabbit or goat anti-mouse conjugated to Cy2 or lissamine rhodamine (Jackson Immunoresearch) were used at 1:200 as secondary antibodies. Propidium iodide at 1 mg/ml diluted in phosphate-buffered saline (PBS) or TO-PRO-3 (Molecular Probes) at 1 mM was used to stain nucleic acid. Nuclear matrix preparations from cells expressing ataxin-7[K380T] were produced as described (39). The nuclear matrix preparations were stained with anti-Xpress and anti-PML polysera (1:200) (40). Images were examined using a Bio-Rad MRC-1000 confocal microscope equipped with a krypton–argon laser. For the ubiquitin studies, COS-1 cells were stained with anti-Xpress for ataxin-7 or M5 for ataxin-1, and anti-ubiquitin (1:100; Dako).

**Western blot**

Protein extracts were prepared from 1 × 10^6 COS-1 cells transfected with ataxin-7 constructs that included a myc tag at the

![Figure 7. Ataxin-7 contains a monopartate arginine–lysine NLS.](image-url)
C-terminus. At 48 h post-transfection, cells were trypsinized, washed three times in PBS, resuspended in 100 µl of 0.25 M Tris pH 7.8 plus protease inhibitors (Protease inhibitor cocktail tablets; Boehringer Mannheim) and lysed using three cycles of freezing at −70°C and thawing at 37°C. Extracts were used whole (total fraction) or centrifuged at 12,000 g for 5 min, and the soluble fraction was taken from the supernatant. A portion of each fraction was electrophoresed on an 8% polyacrylamide gel, blotted (including the stacking gel) and probed with anti-myc (1:2500 3% milk in PBS and 0.1% Triton X-100; Zymed). After incubating with anti-mouse horseradish peroxidase conjugate, bands were visualized by chemiluminescence (NEN Renaissance kit).

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