ORIGINAL CONTRIBUTION

A Novel Central Nervous System–Enriched Spinocerebellar Ataxia Type 7 Gene Product

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Context: Polyglutamine-mediated neurodegeneration in spinocerebellar ataxia type 7 (SCA7) involves specific central nervous system structures despite widespread expression of the mutant ataxin-7 protein.

Objective: To determine whether expression of multiple gene products could contribute to selective neurodegeneration in SCA7.

Results: We identified a novel *SCA7* transcript and protein, both of which are enriched within the central nervous system. An isoform-specific antibody revealed that the novel ataxin-7 variant, in contrast with the previously described protein, localizes to neuronal cytoplasm and not to inclusion bodies present within the tissues of patients with SCA7.

Conclusions: In addition to expanding our understanding of *SCA7* gene expression, identification of a novel ataxin-7 protein enriched in the central nervous system suggests that expression of multiple polyglutamine-containing proteins may play a role in generating the neurodegenerative patterns characteristic of SCA7 and other polyglutamine expansion diseases.

Arch Neurol. 2003;60:97-103

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NINE INHERITED neurodegenerative diseases caused by the expansion of CAG repeat-encoded polyglutamine (polyQ) tracts are characterized by loss of specific neuronal populations despite widespread expression of the mutant proteins.[1,2] Several nonmutually exclusive hypotheses have been proposed to explain selective neurotoxicity in polyQ diseases and invoke involvement of neuronal intranuclear inclusion (NII) formation, alterations in gene expression, and disruption/promotion of normal/inappropriate protein-protein interactions involving mutant polyQ-containing gene products.[3-7] However, the possibility that multiple mutant polyQ proteins act in concert to generate the spatially restricted neurodegenerative patterns typical of CAG repeat disorders has received relatively little attention. Multiple distinct proteins are frequently produced from a single gene by alternative splicing, a mechanism of transcriptional regulation that exerts profound effects on normal and pathologic animal physiologic function.[8] Alternative splicing is particularly prevalent in the central nervous system (CNS), where recently conducted human genome-wide analyses estimate that greater than 60% of all genes are represented as multiple messenger RNA (mRNA) species.[9] Although alternate splice forms of several polyQ disease-associated genes have been identified, their unique contributions to pathology have not been thoroughly examined.[10-15] Spinocerebellar ataxia type 7 (SCA7) is caused by CAG repeat expansion within the *SCA7* gene and involves degeneration of cerebellar Purkinje cells, brainstem neurons, and retinal photoreceptors.[16,17] We hypothesize that multiple products of the *SCA7* gene contribute to this degeneration and report the identification of *SCA7b* and ataxin-7b, a novel *SCA7* splice variant and the ataxin-7 isoform it encodes. In contrast with the previously described ataxin-7 protein, ataxin-7b expression occurs predominantly within the CNS and may modulate the spatial distribution of SCA7-mediated neurodegeneration.

METHODS

BIOINFORMATICS

Spinocerebellar ataxia type 7 complementary DNA (cDNA) and ataxin-7 amino acid sequences were entered as GenBank queries using the basic local alignment search tool.[18] Sig-

nificant matches identified in the initial search were used as second-pass queries of GenBank. Significant matches were then used to search the Expressed Sequence Tags database for clones with high sequence similarities.[19]

GENOMIC STRUCTURE ANALYSIS

Human genomic DNA was amplified using the Expand Long Template Polymerase Chain Reaction (PCR) system (Roche, Indianapolis, Ind) and standard thermocycling conditions. Primer pairs B (5′-CCACTCACACACTCCTCTAGACAA-3′) and C (5′-CCTGTTCGTAAGCAAGGTGA GGAG-3′) were used to amplify intron 12 and atx7-2F (5′-CTCCTCACCTTGCT-TACGAAC AGG-3′) and D (5′-GTAACTTGTCCTCAAAAGT-GTCCGC-3′) to amplify the following intron. Agarose-embedded PCR products were purified with Prep-A-Gene (Bio-Rad, Hercules, Calif) and cloned into the pCRII TOPO vector (Invitrogen, Carlsbad, Calif). Recombinant plasmids were isolated with Qiaprep spin columns (Qiagen, Valencia, Calif) and subjected to automated cycle sequencing. Spinocerebellar ataxia type 7 genomic sequence was analyzed with DNAStar software (Madison, Wis).

REVERSE TRANSCRIPTASE PCR

Total RNA was prepared from whole human fetal brain with TriReagent (Molecular Research Center, Cincinnati, Ohio), and cDNA was generated with the SuperScript Preamplification System (Invitrogen) using an oligo dT primer. Spinocerebellar ataxia type 7b–specific fragments from fetal brain cDNA were amplified with Platinum Pfx DNA polymerase (Invitrogen) using primer pairs B and C, B and D, or A (5′-GGGCGCGAATTCAT-GTCGGAGCGG GCCGCG-3′) and SCA7b-R (5′-GGGTGATGTTGCTGAAATTCC-3′) using a thermocycling protocol recommended by the supplier. The provided enhancer solution was used at 3× concentration. Products were electrophoresed through 2% Tris/acetate–buffered agarose gels containing ethidium bromide, and UV light was used to visualize bands. Appropriately sized products were excised from the agarose, cloned into pCRII TOPO, and sequenced.

NORTHERN BLOTTING

Oligonucleotide SCA7b-R was end labeled by a standard procedure, and the β-actin control probe supplied by the manufacturer (Clontech, Palo Alto, Calif) was random hexamer-labeled with Rediprime II (Amersham Pharmacia, Piscataway, NJ).[20] Unincorporated radionucleotides were eliminated from the probe preparations using Sephadex G-25 fine Quick Spin columns (Roche). Hybridization of 10^7 cpm of each probe to Multiple Tissue Northern blots (Clontech) was performed overnight at 45°C for SCA7b-R or at 60°C for the β-actin control, and each blot was washed using a standard protocol.[20] Autoradiographic exposures were performed at –80°C between 2 intensifying screens.

ANTIBODY PRODUCTION

Generation and purification of polyclonal antiserum samples was performed as previously described using an oligopeptide corresponding to amino acids 928 through 943 (TFEDKLHLHSAL WTPR) of the predicted unique ataxin-7b C-terminus.[21]

IMMUNOBLOTTING

Glutathione S-transferase (GST)–ataxin-7b fusion proteins were expressed and purified as previously described except that the excision of a 1119–base pair (bp) *StuI/Xba*I fragment from the *SCA7b* cDNA resulted in the expression of ataxin-7b proteins lacking amino acids 455 through 828.[21] Tissue and cell extracts were prepared, electrophoresed on 8% polyacrylamide gels, and transferred to nitrocellulose membranes as previously described.[21] Immunoblots were blocked for 1 hour at room temperature in Tris-buffered saline with 0.04% Tween-20, containing 5% nonfat dried milk and incubated overnight at 4°C with either Ab161 or anti-GST diluted 1:1000 or anti–β-actin diluted 1:5000. Antibody A, preimmune serum, and Ab161 preadsorbed with 100μM oligopeptide antigen were used at 1:1000. After washing in TBS-T, blots were incubated with either horseradish peroxidase–conjugated donkey antirabbit (1:2000) or goat antimouse (1:3200) secondary antibodies for 1 hour at room temperature. Detection was performed with ECL-Plus (Amersham Pharmacia).

IMMUNOHISTOCHEMICAL ANALYSIS

Fixation, slide preparation, antigen recovery, and tissue staining were performed by standard methods.[22] Ab161 was diluted 1:50 and Monoclonal Antibody 1C2 (Chemicon, Temecula, Calif) was diluted 1:4000. Goat antirabbit and horse antimouse biotin-conjugated secondary antibodies were diluted 1:1000 and 1:300, respectively. Preadsorption of Ab161 was performed with 75μM oligopeptide antigen. Detection was performed with Vectastain Elite ABC and either the 3, 3′-diaminobenzidine or Novared substrate (Vector Laboratories, Burlingame, Calif).

RESULTS

To examine *SCA7* gene expression, portions of *SCA7* cDNA sequence were systematically searched against the National Center for Biotechnology Information Expressed Sequence Tags database using the basic local alignment search tool.[18,19] A single clone was identified from human testis that contains 549 bp of nonvector DNA corresponding to exons 12 and 13 of *SCA7* cDNA but that also harbors a novel intervening sequence of 67 nucleotides between the 2 exons. Alignment of *SCA7* genomic DNA with the Expressed Sequence Tags revealed that the 67-bp insertion lies within the 2881-bp *SCA7* intron 12 such that 1119 bp lies upstream of the insertion and 1762 bp lies downstream (**Figure 1**A). Canonical splice acceptor and donor sites flank the insertion, indicating that it likely represents an uncharacterized *SCA7* exon.[23] Polymerase chain reaction amplification of human fetal brain cDNA using an insertion-specific primer yielded a product migrating at the expected size of 221 bp, indicating that an *SCA7* mRNA containing the 67-bp sequence is expressed (Figure 1B, lane B/C). Primers flanking the insertion amplified 2 products, one containing and one lacking the insertion (Figure 1B, lane B/D). DNA sequence analysis of these amplicons confirmed that at least 2 distinct *SCA7* mRNAs are expressed within fetal brain tissue, the previously described *SCA7* mRNA (*SCA7a*) and a novel transcript (*SCA7b*) containing the additional exon. The entire 2.7-kilobase (kb) *SCA7b* coding region was amplified and DNA sequence analysis confirmed that, other than the 67-bp exon, *SCA7b* is identical to *SCA7a*, including the polymorphic CAG repeat tract near the 5′ end (Figure 1B, lane A/C).

Northern blot analysis with an *SCA7b*-specific probe revealed a 4.5-kb transcript migrating distinctly from pre-

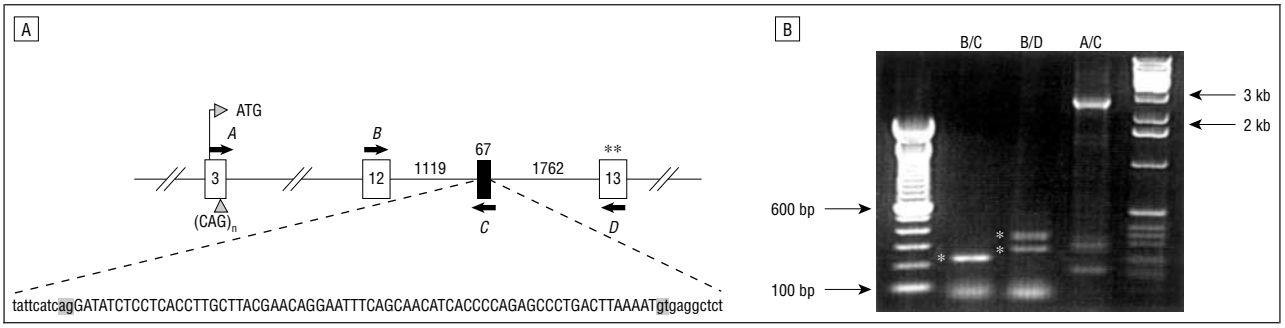

Figure 1. Identification of an alternate spinocerebellar ataxia type 7 (*SCA7*) exon. A, Genomic structure of *SCA7* (the figure is not drawn to scale). Previously designated exons 3, 12, and 13 are represented by open rectangles. The filled rectangle indicates the novel 67–base pair (bp) sequence. The asterisks denote the alternative TGA termination codons. Double slashes represent unillustrated genomic DNA. Arrows associated with capital letters *A* through *D* indicate the relative positions and orientations of primers *A* through *D*. Primer *A* contains the ATG start codon within exon 3 but not the polymorphic $(CAG)_n$ repeat tract. Base pair sizes of the novel exon and the introns flanking it are shown above the boxes designating these genetic elements. The uppercase letters below the dotted lines correspond to exonic sequence, and the lowercase letters represent flanking intronic sequence. The shaded boxes highlight consensus splice acceptor and donor sequences. Nucleotide sequences are deposited in GenBank (accession numbers: AA398030, Expressed Sequence Tags clone containing the novel exon; AF332956, *SCA7* genomic sequence between exons 12 and 13). B, Expression of 2 *SCA7* transcripts. Fetal brain RNA was amplified by reverse transcriptase polymerase chain reaction, with the primer pair indicated at the top of each lane. The asterisk in lane B/C indicates the 221-bp product corresponding to *SCA7b* messenger RNA (mRNA), and the asterisks in lane B/D mark the 331-bp and 264-bp products corresponding to the *SCA7b* and *SCA7a* mRNAs, respectively.

viously reported *SCA7* mRNAs and occurring in greater abundance in the brain than in non-CNS tissues, such as those of the heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas (**Figure 2**). The 4.5-kb transcript is expressed at similar levels within all CNS regions examined, including the cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal lobe, temporal lobe, and putamen (Figure 2). Reverse transcription PCR analysis of *SCA7b* distribution also demonstrated greater product abundance in the CNS than in peripheral tissues (data not shown). Taken together, these data indicate that *SCA7b* is a novel 4.5-kb transcript expressed throughout the CNS, where it occurs at significantly higher levels than in peripheral tissues.

Spinocerebellar ataxia type 7b is predicted to encode ataxin-7b, a 101-kd protein comprised of 945 amino acids. The N-terminal 887 amino acids of this protein, including the polymorphic glutamine repeat, nuclear localization sequence, and arrestin-like phosphoprotein–binding domain are identical to the previously described ataxin-7A protein, which is 892 amino acids in length (**Figure 3**A).[17,24] The 67-bp insertion and resultant frameshift within *SCA7b* mRNA encode 58 amino acids comprising the novel ataxin-7b C-terminus, which bears no significant homologic characteristic to described protein domains. To examine ataxin-7b expression, we raised an antibody (Ab161) against the unique C-terminus and confirmed specificity by demonstrating that Ab161 detects recombinant ataxin-7b proteins on immunoblots (Figure 3B). Examination of ataxin-7b tissue distribution with Ab161 revealed expression of a single 101-kd protein species, the signal intensity of which was consistently higher in the cerebellum, medulla, occipital pole, retina, heart, and pons compared with that in the liver, kidney, or pancreas (Figure 3C). Signal intensity was also relatively strong in the frontal lobe, skeletal muscle, and spinal cord but markedly reduced in the lung, lymphoblast, and intestine (data not shown). The 101-kd protein was neither detected by Ab161 preadsorbed with its oligopeptide antigen nor by rabbit serum collected prior to immunization, further demonstrating the specificity of Ab161 (data not shown). These data indicate that Ab161 specifically detects the *SCA7b* gene

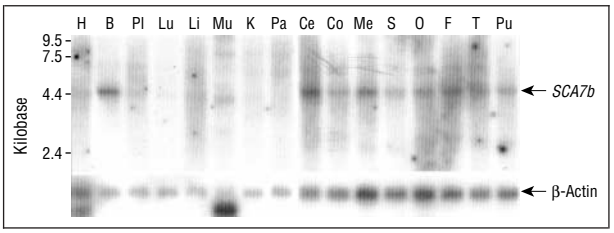

Figure 2. Spinocerebellar ataxia type 7b (*SCA7b*) tissue distribution. RNA blots containing poly A+ messenger RNA isolated from the human heart (H), brain (B), placenta (Pl), lung (Lu), liver (Li), skeletal muscle (Mu), kidney (K), pancreas (Pa), cerebellum (Ce), cerebral cortex (Co), medulla (Me), spinal cord (S), occipital pole (O), frontal lobe (F), temporal lobe (T), and putamen (Pu) were probed with a ^{32}P-labeled oligonucleotide specific for *SCA7b* as well as with a β-actin control probe to demonstrate equal sample loading.

product, ataxin-7b, a 101-kd protein expressed predominantly within tissues of the CNS.

Ab161 also allowed immunohistochemical analysis of ataxin-7b expression and revealed intense cytoplasmic staining predominantly within cerebellar Purkinje cells and basis pontis and inferior olivary neurons of the brainstem (**Figure 4**A-C). Neurons of the locus coeruleus and anterior horn also exhibited significant cytoplasmic staining (data not shown). In addition, omission of the hematoxylin counterstain revealed Ab161 immunoreactivity in cerebellar granule and molecular layer neurons (data not shown). Retinal staining with Ab161 revealed strong immunoreactivity within the photosensitive inner segments of photoreceptor cells as well as within large cell bodies residing within the ganglion cell layer, likely corresponding to displaced amacrine cells (Figure 4D). Significant staining was also detected within the cell bodies of the inner nuclear layer. In contrast, little to no Ab161 immunoreactivity occurred within photoreceptor outer segments or their cell bodies comprising the outer nuclear layer. Immunohistochemical specificity of Ab161 was demonstrated by a reduction of Purkinje cell staining intensity to background levels when cerebellar sections were probed with Ab161 preadsorbed with oligopeptide antigen (Figure 4E). These data indicate that ataxin-7b is a cytoplasmic component of neurons comprising major cerebellar and brainstem tracts

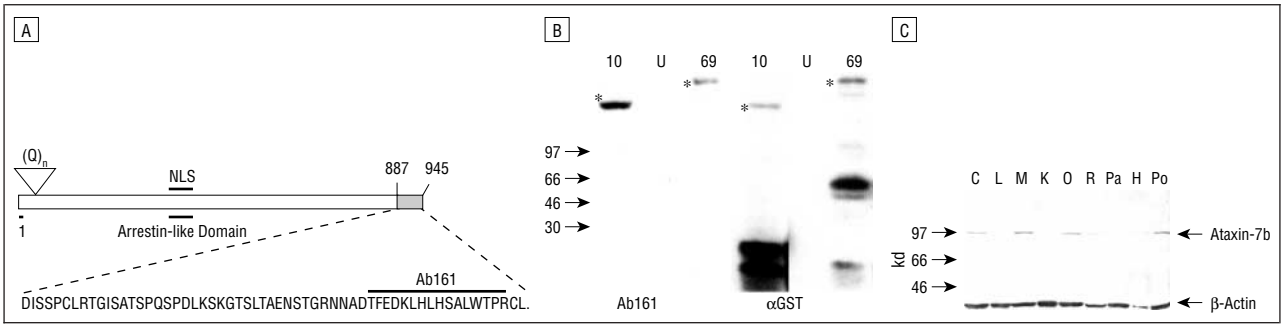

Figure 3. Ataxin-7b expression analysis. A, Schematic of the predicted ataxin-7b protein. Numerals correspond to amino acid number. Relative positions of the polymorphic glutamine tract $(Q)_n$, nuclear localization sequence (NLS), and arrestin-like phosphoprotein–binding domain are indicated. The shaded box represents the unique ataxin-7b C-terminus, the amino acid sequence of which is depicted in single-letter format framed by dotted lines. The oligopeptide consisting of 16 amino acids and against which ataxin-7b-specific Ab161 was raised is also indicated. B, Demonstration of Ab161 specificity. Immunoblots containing extracts from bacterial cultures expressing either an 88-kilodalton (kd) glutathione S-transferase (GST)-ataxin-7b-10Q (10) or a 96-kd GST-ataxin-7b-69Q (69) fusion protein, each lacking amino acids 455-828, were probed with Ab161 (left) or an anti-GST antibody (right). Immunoblots also contained extracts from uninduced bacterial cultures transformed with GST-ataxin-7b-10Q (U). Bands corresponding to full-length fusion proteins are indicated with asterisks. C, Tissue distribution of ataxin-7b. Immunoblots containing extracts of the human cerebellum (C), liver (L), medulla (M), kidney (K), occipital pole (O), retina (R), pancreas (Pa), heart (H), and pons (Po) were probed with Ab161 as well as with an anti-β–actin monoclonal antibody as a loading control.

and that this novel protein likely functions within the photosensitive machinery of the retina.

The formation of NIIs containing the expanded polyQ tract is a hallmark of CAG repeat disorders and has been reported in tissues of patients with SCA7, using antibodies directed at either the ataxin-7a N-terminus or the pathogenic glutamine repeat.[21,25,26] However, Ab161 failed to detect NIIs within inferior olivary neurons, neocortical pyramidal cells, and retinal photoreceptors of patients with SCA7 with either 66 (**Figure 5**A-C) or 41 CAG repeats (data not shown). Instead, the neuronal distribution of Ab161 immunoreactivity in each patient with SCA7 was predominantly cytoplasmic and resembled staining within neurons of control tissues. Staining with an N-terminal anti–ataxin-7 antibody, however, revealed numerous NIIs within sections of the same tissues from the patient with 66 CAG repeats (Figure 5D-F). Since Ab161 recognizes the ataxin-7b C-terminus, these data suggest that proteolytic cleavage of ataxin-7 proteins is involved in NII formation and/or their subsequent processing.

COMMENT

Since the defective gene products associated with polyQ expansion diseases are widely expressed, recognition of the molecular mechanisms underlying the restricted patterns of cell death characteristic of these disorders is not straightforward.[2] Several explanations have been proposed for this paradox but no conclusive evidence has been presented. The formation of polyQ-containing NIIs in specific neuronal populations of patients was initially thought to mark those cells for dysfunction and death. However, more recent studies have demonstrated that NII distribution in patients does not correlate well with the neurodegenerative patterns and further suggested that nuclear aggregation of expanded polyQ proteins may be benign or even beneficial.[27] In addition, numerous interactions between polyQ-containing proteins and tissue-specific partners have been identified, the disruption of which by polyQ expansion has been proposed to cause spatially restricted neuronal cell death.[4,6,28] However, most of such partnerships are not modulated by polyQ tract length, and the consequences of disrupting those that are polyQ-length–dependent are yet unclear. Finally, abnormal gene expression may be an important component of polyQ disease and may contribute to tissue-specific degeneration owing to the tendency of polyQ tracts to self-associate and promote inappropriate association of mutant proteins with polyQ-containing transcription factors, some of which may be neuron-specific.[5,29,30]

This report raises the additional possibility that cell-specific toxicity in polyQ diseases involves expression of multiple gene products with distinct tissue or cellular distributions. Our expression analyses indicate that CNS tissues contain significant levels of ataxin-7a and 7b isoforms, whereas cells within peripheral tissues predominantly express only ataxin-7a, potentially leading to cell-specific differences in toxic polyQ loads and increased degeneration of those neuronal populations that express multiple ataxin-7 isoforms. It is possible that threshold polyQ levels are required to initiate cell dysfunction and death, thus requiring only minor differences in expanded polyQ expression to produce profound cell-specific susceptibilities to polyQ toxicity. The fact that significant ataxin-7b expression is detected within cardiac and skeletal muscle, and that patients with SCA7 have been reported to suffer from defects within these tissues, is consistent with this model and further suggests that ataxin-7b expression may contribute to non-CNS degeneration in SCA7 (Ptacek, unpublished data, 1994; Huda Zoghbi, MD, unpublished data, 1998).[31,32]

Spatially restricted cell death in SCA7 may also involve the differences in subcellular localization of the ataxin-7 isoforms. Although previous studies have demonstrated that nuclear localization of mutant polyQ proteins is necessary for pathologic neuronal function, it is possible that simultaneous expression of expanded polyQ tracts in the cytoplasm exacerbates cytotoxicity.[33] In support of this hypothesis, significant levels of cell death were reported in cultured striatal neurons, wherein expression of mutant huntingtin was restricted to the cytoplasm.[34] Additionally, the immunohistochemical data presented in this report, together with previous reports of patients with SCA7 and murine models wherein ataxin-7a

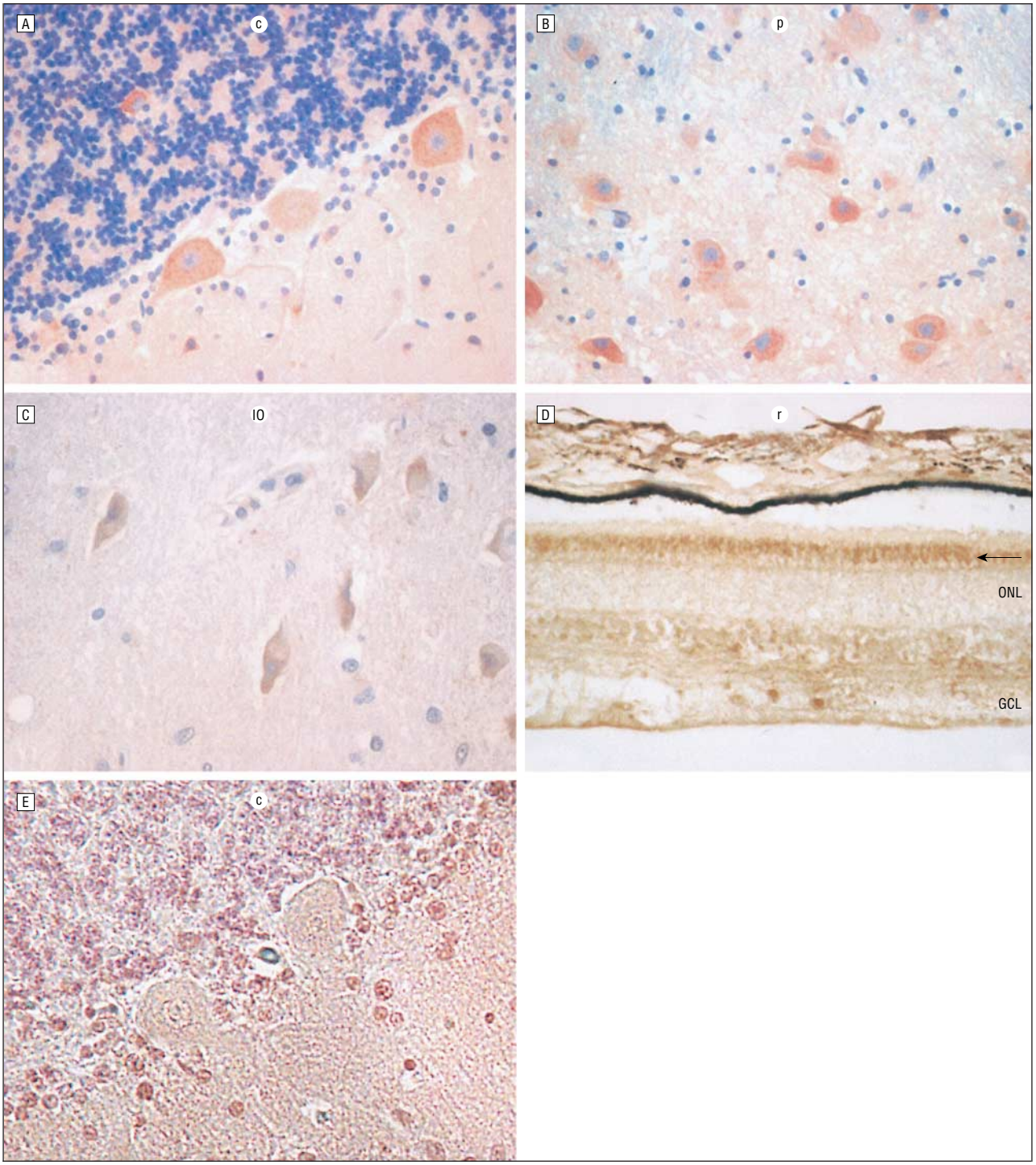

Figure 4. Ataxin-7b localization within control tissues. Representative cerebellar (A), pontine (B), inferior olivary (C) and retinal (D) sections (labeled c, p, IO and r, respectively, at top center of each panel) from a control individual were probed with either Ab161 (A-D) or Ab161 preadsorbed with oligopeptide antigen (E). In D, the locations of the outer nuclear layer (ONL) and ganglion cell layer (GCL) are indicated, and the arrow indicates the position of photoreceptor cell inner segments. A, B, and D, original magnification ×280; C and E, original magnification ×560.

N-terminal, but not C-terminal, epitopes were detected, suggest that ataxin-7 proteins undergo proteolytic cleavage.[35,36] Although we were unable to detect ataxin-7 fragments in human tissues, likely owing to sample degradation, we did observe cleavage events involving mutant ataxin-7 constructs in preliminary cell culture experiments and observed spatial distinctions between the ultimate cellular destinations of the N- and C-termini of each isoform (D.D.E., Said Bendahou, PhD, L.J.P., Y-H.F., unpublished data, 2001). Taken together, these data suggest that cleavage of ataxin-7 proteins is involved in SCA7 degeneration and raise the possibility that isoform-specific fragments exert distinct toxic influences that could contribute to selective neurodegeneration.

Recent investigation of *SCA7* genomic structure delineated intron-exon boundaries and estimated intron sizes

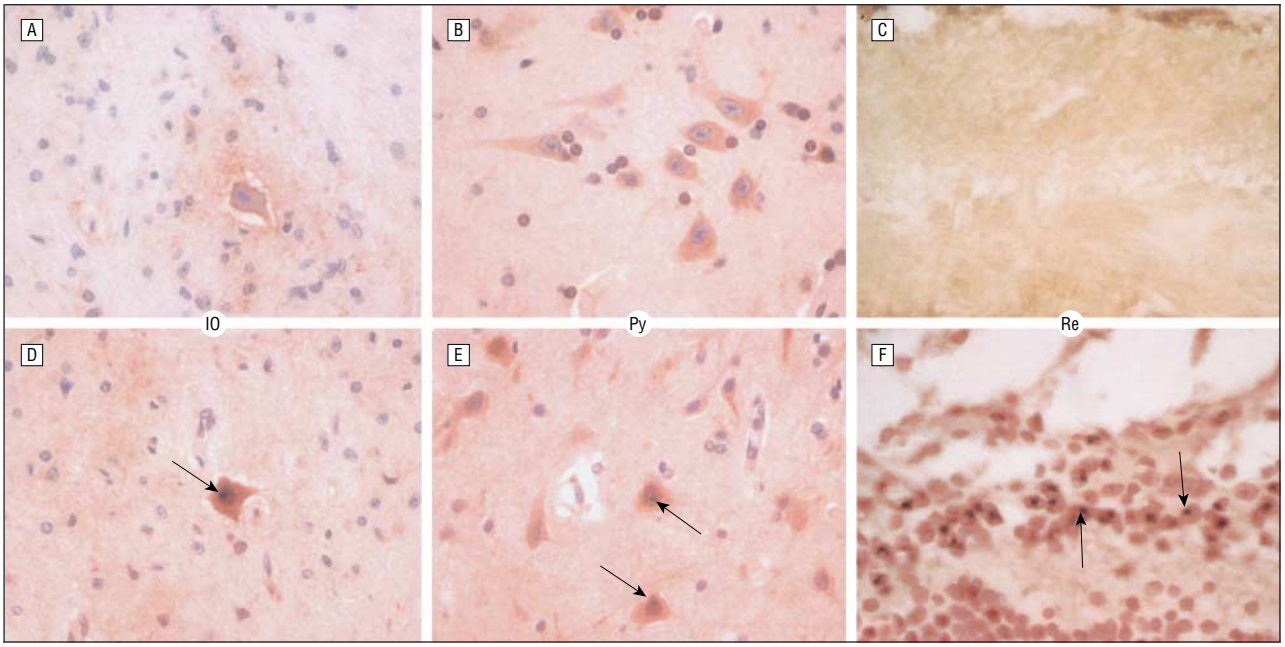

Figure 5. Ataxin-7b localization within the tissues of patients with spinocerebellar ataxia type 7 (SCA7). Representative inferior olivary (A and C), neocortical pyramidal (B and E), and retinal (C and F) neurons of a patient with SCA7 harboring a $(CAG)_{66}$ repeat at the *SCA7* locus were probed with either Ab161 (A-C) or a previously characterized N-terminal ataxin-7 antibody (D-F). The arrows indicate neuronal intranuclear inclusions (original magnification ×280).

but did not identify additional coding DNA.[37] This study furthers the current understanding of *SCA7* gene expression with the identification of *SCA7b*, a 4.5-kb CAG repeat–containing transcript with a coding sequence identical to that of the previously described *SCA7a* mRNA except for the inclusion of a 67-bp exon between exons 12 and 13.[17,38] Therefore, a nomenclature revision of the *SCA7* gene structure is proposed whereby the 67-bp sequence in *SCA7b* is denoted exon 12b and is flanked on the 5′ and 3′ ends by introns 12a and 12b, respectively. In summary, CNS-enriched expression of ataxin-7b fits well with neuropathologic findings in patients with SCA7 and may contribute to selective neurodegeneration in SCA7. Furthermore, the distinct subcellular localization of ataxin-7b raises the possibility that ataxin-7 isoforms contribute differently to the neurodegenerative pattern of SCA7. These findings suggest unique models to explain the restricted pattern of cell death in SCA7 and, since polyQ diseases involve similar genetic mutations and degeneration of specific neuronal populations, in polyQ diseases in general. Finally, these data clearly illustrate the importance of considering the complete expression profiles of CAG repeat-containing disease genes to gain a comprehensive understanding of the molecular mechanisms underlying polyQ-induced cell death.

Accepted for publication June 5, 2002.

This work was supported by Public Health Service research grant M01-RR00064 from the National Center for Research Resources, Bethesda, Md (Dr Ptacek), by grant NS41331 from the National Institutes of Health, Bethesda, Md (Dr Fu), and by a memorial fund generously established by a large Utah family with SCA7 (Salt Lake City).

Author contributions: *Study concept and design* (Drs Einum, Ptacek, and Fu); *acquisition of data* (Dr Einum); *analysis and interpretation of data* (Drs Einum, Clark, Townsend, Ptacek, and Fu); *drafting of the manuscript* (Dr Einum); *critical revision of the manuscript for important intellectual content* (Drs Einum, Clark, Townsend, Ptacek, and Fu); *obtained funding* (Drs Ptacek and Fu); *administrative, technical, and material support* (Drs Ptacek and Fu); *study supervision* (Drs Ptacek and Fu).

We are indebted to Kelley Murphy, BS, Huntsman Cancer Institute, Salt Lake City, for expert immunohistochemical assistance, and to Launce Gouw, MD, PhD, for bioinformatics support.

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