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Research Paper

FMR1 RNA within the Intranuclear Inclusions of Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS)

Flora Tassone

Christine Iwahashi

Paul J. Hagerman*

Department of Biochemistry and Molecular Medicine; University of California; Davis School of Medicine; Davis, California USA

*Correspondence to: Paul J. Hagerman; Department of Biochemistry and Molecular Medicine; University of California, Davis, School of Medicine; One Shields Avenue; Davis, California 95616 USA; Tel.: 530.754.7266; Fax: 530.754.7269; Email: pjhagerman@ ucdavis.edu

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KEY WORDS

FXTAS, progenitor cells, inclusions, *FMR1* RNA, fragile X syndrome, neurodegeneration, premutation, intention tremor, toxic gain-of-function

ABBREVIATIONS

DMPK dystrophia myotonica-protein kinase gene
ZNF9 zinc finger protein 9 gene

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ABSTRACT

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a recently identified neurodegenerative disorder affecting older adult males with premutation alleles of the fragile X mental retardation 1 (*FMR1*) gene. The principal clinical features of FXTAS include progressive intention tremor, gait ataxia, parkinsonism, and autonomic dysfunction. The disorder affects at least one-third of carrier males over 50 years of age and, with an estimated carrier frequency of ~1/800 males, is likely to be one of the most common heritable forms of tremor and ataxia among older adult males in the general population. Brains from all FXTAS cases examined to date (10/10) possess numerous ubiquitin-positive intranuclear inclusions in broad distribution throughout the cerebrum and brainstem. The absence of either the neurodegenerative disorder or inclusions among adults with fragile X syndrome (who lack the *FMR1* protein), coupled with elevated *FMR1* mRNA with expanded CGG repeats in premutation carriers, has led us to propose an RNA toxic gain-of-function model for FXTAS. Consistent with this model, we have now identified *FMR1* mRNA within the intranuclear inclusions isolated from post-mortem (FXTAS) brain tissue.

INTRODUCTION

Fragile X syndrome is the most common inherited form of mental impairment, with carrier frequencies of approximately one in 800 males and one in 260 females.[1,2] The syndrome is caused by the expansion of a noncoding, repetitive trinucleotide (CGG) element in the fragile X mental retardation[1] (*FMR1*) gene beyond 200 repeats (full mutation), generally accompanied by transcriptional silencing.[3] Most carriers of smaller CGG expansions (55 to 200 repeats; premutation) had been thought to be clinically unaffected. However, at least one-third of older adult carriers are now known to develop a late-onset neurological disorder involving progressive intention tremor, gait ataxia, and parkinsonism;[4] this disorder has been designated fragile X-associated tremor/ataxia syndrome (FXTAS).[5-10]

FXTAS appears to be largely confined to the premutation range, where the *FMR1* gene is fully active.[11] The absence of FXTAS among adults with transcriptionally silent full mutation alleles, and elevated *FMR1* mRNA levels in premutation carriers,[12-14] has led us to propose that FXTAS is caused by a direct "toxic gain-of-function" of the *FMR1* mRNA itself.[4,8,10,15] A paradigm for such an RNA gain-of-function model is myotonic dystrophy, in which expansion of a noncoding CUG repeat in the 3′untranslated region (3′UTR) of the *DMPK* mRNA (DM1), or an intronic CCUG repeat in the *ZNF9* transcript (DM2), is responsible for both the disease itself and the associated intranuclear foci.[16-18] The central role played by the abnormal RNAs in DM1 and DM2 is reinforced by the presence of the respective RNAs within the intranuclear foci in both disorders. It has been suggested that the expanded C(C)UG repeat elements lead to the sequestration of one or more CUG-binding proteins, which prevents those proteins from carrying out their normal functions.[16,17,19,20]

An RNA-based mechanism for FXTAS is also consistent with the observations of Willemsen et al.,[21] who observed intranuclear neuronal inclusions in a "knock-in" mouse in which the endogenous CGG repeat was replaced by approximately 100 CGG repeats, and of Jin et al.,[22] who observed inclusion formation in *Drosophila* upon expression of a 90-repeat CGG element within a heterologous reporter gene. It should be noted that both the morphology and the (nuclear and cytoplasmic) localization of the inclusions in the fly are different than those of either human or mouse inclusions, perhaps reflecting differences in the manner that CGG repeats are recognized in the fly.

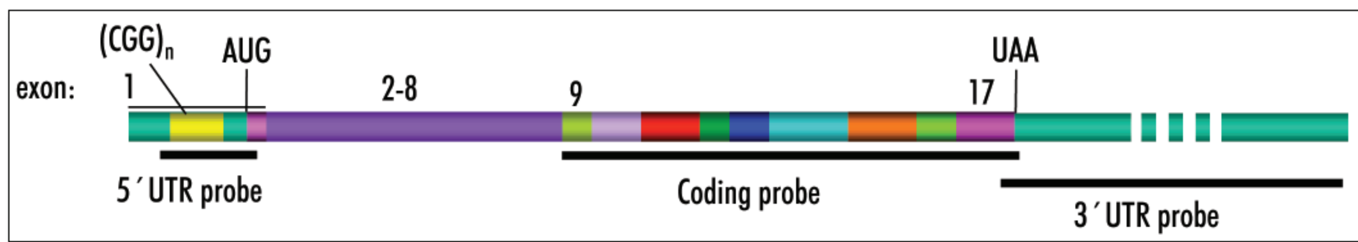

Figure 1. Diagram of the locations of the riboprobe sequences used for the fluorescence in situ hybridization experiments represented in Figure 2. 5′UTR probe: nt 13712–13992 (~0.3 kb) (*FMR1*: L29074), 30 CGG repeats; coding probe: nt 34486–50680 (~1.1kb of coding sequence), extending from exon 9 to approximately 40 nt downstream of the stop codon; 3′UTR probe: nt 50487–52756 (~2.3 kb), comprising most of the UTR sequence.

A central prediction of the proposed RNA gain-of-function mechanism for FXTAS is that *FMR1* mRNA would be present within the intranuclear inclusions. Although this prediction is not a strict requirement of the model, its verification would provide strong support for such a mechanism in the human disease process. To test this prediction, we have examined the inclusions found in postmortem brain tissue of a FXTAS patient for the presence of *FMR1* mRNA. Using biotinylated antisense and sense (control) riboprobes that are targeted to the regions within the 5′UTR (0.3 kb), the coding region (~1 kb), and the 3′UTR (~2 kb) of the *FMR1* mRNA (Fig. 1), we probed inclusion-bearing nuclei that had been isolated from the frontal cortex of a 70 year old male (Case 1 of Greco et al.;[15,11] 113 CGG repeats; mRNA 3.8 ± 0.13 times normal in peripheral blood leucocytes). As demonstrated in Figure 2, antisense riboprobes targeting both coding and noncoding (5′ UTR and 3′ UTR) portions of the *FMR1* message demonstrate the presence of *FMR1* mRNA within the intranuclear inclusions.

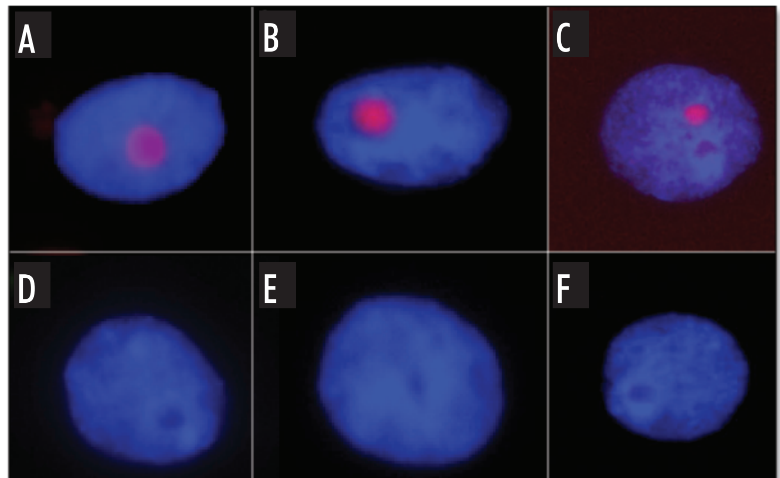

Figure 2. Riboprobe analysis of nuclei from the frontal cortex of a 70-year-old male who died with FXTAS (Case 1 of Greco et al.).[15] Riboprobe: (A) 5′UTR antisense; (B) coding antisense; (C) 3′UTR antisense; (D) 5′UTR sense; (E) coding sense; (F) b-actin antisense control. The clinical history and the neuropathological and molecular details of this case have been described.[11,15] The CGG repeat number was determined to be 113 CGG repeats, by both Southern blot and PCR analysis, in peripheral blood leucocytes and in several brain regions.[11]

As with the original counts in stained sections of the frontal cortex (Case 1, Greco et al.),[15] we observe inclusions in only a subgroup (~6–11%) of the isolated/probed nuclei (5′UTR: 28 antisense-positive inclusions among 507 nuclei; coding: 42 antisense-positive inclusions among 466 nuclei; 3′UTR: 50 antisense-positive inclusions among 471 nuclei). Under the same hybridization conditions, we do occasionally see fluorescence with the sense probes (~1–2% among 1563 nuclei for the three probes); however, the difference between antisense and sense probes is highly significant for each of the three antisense/sense pairs (5′UTR: χ^2 = 11.6, p = 0.0006; coding: χ^2 = 19.4, p < 0.0001; 3′UTR: χ^2 = 31.6, p < 0.0001). The background (sense) probe levels are comparable to antisense staining with a control (β actin) antisense probe (Lofstrand Labs) (~1 %; χ^2, 29.5, p < 0.0001 for comparison with the aggregate counts for the three antisense *FMR1* probes). The percent of nuclei bearing inclusions, identified with the three antisense probes, is comparable to the previous counts (6–8% of neurons) in the frontal/parietal cortex from Case 1 of Greco et al.[15] Note that we have never observed intranuclear inclusions in control brains.

Our results provide direct supporting evidence for an RNA-mediated mechanism for disease formation, at least at the level of association between the *FMR1* mRNA and inclusion formation. Since the latter is strongly associated with the development of FXTAS (10/10 brains examined to date possess intranuclear, neuronal inclusions), the current results provide strong evidence for an RNA gain-of-function mechanism for the development of FXTAS. As with myotonic dystrophy, the detailed mechanistic link between the clinical disorder and the RNA-bearing inclusions is likely to be revealed through the identification of the proteins that interact with, and are possibly sequestered by, the abnormal 5′UTR of the *FMR1* message.

METHODS

Nuclear Isolation and Slide Preparation. Nuclear isolation was performed at 5°C from tissue that had been stored at -80°C. One gram of frontal cortex was cut into 1 mm^2 pieces and homogenized in 4 volumes HB buffer (0.32 M sucrose, 50 mM Tris, 5 mM EDTA, 17 μg/ml phenylmethanesulfonyl fluoride containing protease inhibitor tablet, Complete, Roche) with 10 downward strokes of a loose fitting pestle followed by 10 downward strokes of a tight fitting pestle in a 15 ml Dounce homogenizer. The homogenate (5 ml) was filtered successively through 500 μ and 105 μ mesh nylon screens. Nuclei were pelleted from the filtered homogenate by centrifuging at 1500 g for 10 minutes at 5°C. Pelleted nuclei were washed three times in HB, followed by resuspension in 2.0 ml of BC buffer (20 mM HEPES, 400 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA; containing protease inhibitor tablet, Complete, Roche). 2 μl of the resuspended nuclei were smeared on a glass slide (Superfrost Plus; Fisher) and allowed to air dry. Slides were washed in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4mM KH_2PO_4, 0.2% polyoxyethylene (20)

sorbitan monolaurate, pH 7.3) for 10 minutes. Fixation was accomplished by immersion in Histochoice (Amaresco) for 10 minutes at room temperature followed by washing in PBS-T for 5 minutes. Slides were stored until staining in 70% ethanol at -20°C.

Preparation of Riboprobes. The 3′UTR region (~2 Kb), a portion of the 5′UTR containing the CGG element (~0.3 kb), and a portion of the coding region (~1.1 Kb) of the *FMR1* gene, all in the PCR-4-TOPO vector, were linearized by restriction enzyme digestion. RNA transcripts, including both sense and antisense, were synthesized from these DNA templates using T3 and T7 RNA polymerases and Biotin-UTP. DNA templates were removed by DNAse I treatment and phenol/chloroform extraction. The RNA transcripts were subjected to alkaline hydrolysis, followed by desalting on Sephadex spin columns (Lofstrand Labs).

RNA In Situ Hybridization. Following dehydration in 90% and 100% ethanol for 5 minutes, slides were dried at 50°C for several seconds. Hybridizations were performed overnight at 50°C in 30–50 µl of hybridization mix (1x hybridization solution, SIGMA; 50% deionized formamide/10% dextran sulfate, 0.1 mg/ml tRNA) containing 900 ng/ml of biotinylated riboprobe. Slides were covered with coverslips and sealed with rubber cement. Following hybridization, coverslips were removed and slides were washed twice in 4xSSC, once in 2xSCC, and once in 2xSCC/0.1% tween (5 min/wash). All washes were performed at 50°C. Slides were then air-dried and blocked with 200 µl of a blocking solution (3% BSA, 4xSSC, 0.5% tween) for 2 hours at 37°C. Detection was performed with avidin-Cy5 or EviTag (Jackson ImmunoResearch Laboratories; Antibodies, Inc.). Nuclei were counterstained with 200 µl of a 1 µM DAPI solution (Molecular Probes) for 5 minutes. Slides were then washed three times, 5 minutes each, in 1xPBS. Slides were mounted with a few drops of mounting media (Molecular Probe) containing anti-fade reagent, sealed with nail polish and stored at 4°C.

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