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A nuclear role for the Fragile X mental retardation protein

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Fragile X syndrome results from lack of expression of a functional form of Fragile X mental retardation protein (FMRP), a cytoplasmic RNA-binding protein of uncertain function. Here, we report that FMRP contains a nuclear export signal (NES) that is similar to the NES recently identified in the Rev regulatory protein of human immunodeficiency virus type 1 (HIV-1). Mutation of this FMRP NES results in mis-localization of FMRP to the cell nucleus. The FMRP NES is encoded within exon 14 of the *FMR1* gene, thus explaining the aberrant nuclear localization of a natural isoform of FMRP that lacks this exon. The NES of FMRP can substitute fully for the Rev NES in mediating Rev-dependent nuclear RNA export and specifically binds a nucleoporin-like cellular cofactor that has been shown to mediate Rev NES function. Together, these findings demonstrate that the normal function of FMRP involves entry into the nucleus followed by export via a pathway that is identical to the one utilized by HIV-1 Rev. In addition, these data raise the possibility that FMRP could play a role in mediating the nuclear export of its currently undefined cellular RNA target(s).

Keywords: Fragile X syndrome/HIV-1/nuclear export/RNA binding

Introduction

Lack of expression of a functional form of the Fragile X mental retardation protein (FMRP) results in Fragile X syndrome, the most common form of inherited mental retardation (Verkerk *et al.*, 1991; reviewed by Oostra and Williams, 1995). In general, this condition results from the expansion of a trinucleotide repeat sequence present in the 5′ non-coding region of the *FMR1* gene, which in turn leads to the suppression of *FMR1* transcription and, hence, FMRP expression (Oberlé *et al.*, 1991; Verkerk *et al.*, 1991; Yu *et al.*, 1991). However, in rare cases, Fragile X syndrome has been shown to result instead from deletions or missense mutations within *FMR1* (Gedeon *et al.*, 1992; Wohrle *et al.*, 1992; De Boulle *et al.*, 1993).

Despite the evident importance of FMRP, little is known about the physiological role of this novel gene product. FMRP is expressed in many human tissues, including particularly the brain, and is found predominantly in the cell cytoplasm, although occasional nuclear staining has been reported (Devys *et al.*, 1993; Hinds *et al.*, 1993; Verheij *et al.*, 1993). As a result of alternative splicing of the 17 exons present in the *FMR1* gene, several isoforms shorter than the full-length, 614 amino acid form of FMRP have been observed *in vivo* (Ashley *et al.*, 1993a; Verkerk *et al.*, 1993; Sittler *et al.*, 1996). While the significance of these isoforms remains unclear, both the exon structure of the *FMR1* gene and the primary sequence of FMRP are highly conserved across species boundaries (Verkerk *et al.*, 1991; Ashley *et al.*, 1993a). In addition, two human genes closely related to *FMR1*, termed *FXR1* and *FXR2*, have recently been identified (Siomi *et al.*, 1995; Zhang *et al.*, 1995). FMRP has been reported to form specific homomultimers, as well as heteromultimers with the *FXR1* and the *FXR2* gene products, when expressed in yeast (Zhang *et al.*, 1995). However, the significance of this finding is again unclear.

One clue as to the function of FMRP derives from the identification of sequences homologous to two motifs observed in several RNA-binding proteins (Ashley *et al.*, 1993b; Siomi *et al.*, 1993; Musco *et al.*, 1996). In particular, FMRP contains two sequence elements that show homology to the heterogeneous nuclear ribonucleoprotein K homology (KH) domain and one sequence element that is similar to the so-called RGG box (Figure 1A). While data have been presented that demonstrate that FMRP can indeed bind RNA with some degree of sequence specificity *in vitro*, no *in vivo* RNA target for FMRP has yet been identified (Ashley *et al.*, 1993b; Siomi *et al.*, 1993). However, the identification of a severe Fragile X syndrome patient with a missense mutation in the second FMRP KH domain that blocks *in vitro* RNA binding (De Boulle *et al.*, 1993; Siomi *et al.*, 1994), is clearly consistent with the hypothesis that RNA binding plays an important role in mediating FMRP function. This latter hypothesis, combined with the observed cytoplasmic localization of FMRP in expressing cells, has led to the suggestion that FMRP might play a role in regulating the translation of specific target mRNA species *in vivo* (Ashley *et al.*, 1993; Siomi *et al.*, 1993). The finding that FMRP is associated with cytoplasmic ribosomes (Khandjian *et al.*, 1996) is clearly consistent with this hypothesis.

Recently, a novel, minor isoform of FMRP has been reported that lacks exon 14 sequences (Sittler *et al.*, 1996). Unexpectedly, this FMRP variant was found to localize to the cell nucleus, thus leading to the proposal that exon 14 might contain a cytoplasmic retention domain (Sittler *et al.*, 1996). Here, we report that exon 14 instead contains a protein nuclear export signal (NES) that is functionally identical to the NES recently identified in the Rev regulatory protein encoded by human immunodeficiency virus type 1 (HIV-1) (Fischer *et al.*, 1995; Wen *et al.*, 1995;

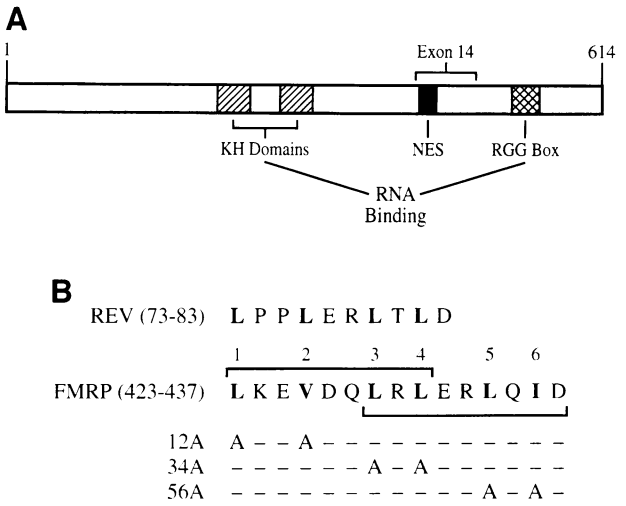

Fig. 1. Location and sequence of a candidate FMRP nuclear export signal. (**A**) Domain organization of FMRP. The KH domains and RGG box, which are believed to mediate specific RNA binding by FMRP, are indicated. In addition, the figure shows the location of the candidate NES sequence analyzed in this manuscript and the extent of FMRP exon 14, the lack of which has been reported (Sittler *et al.*, 1996) to result in the aberrant nuclear localization of FMRP. (**B**) The 10 amino acid NES of HIV-1 Rev is shown (top), with the four critical leucine residues highlighted. The M10 mutation substitutes aspartic acid/leucine for Rev residues 76 and 77 (leucine/glutamic acid) and inactivates this NES (Malim *et al.*, 1989a; Fischer *et al.*, 1995; Wen *et al.*, 1995). The two overlapping candidate FMRP NES sequences are indicated by brackets above the FMRP sequence, with potentially critical large hydrophobic residues highlighted and numbered. Three alanine substitution mutants of these FMRP NES residues are described (bottom).

Fridell *et al.*, 1996; Meyer *et al.*, 1996). In HIV-1 Rev, this NES fulfills a critical role in the Rev-mediated nuclear export of RNA molecules that are bound by the Rev RNA-binding domain (Malim *et al.*, 1989a, 1991). Overall, these data provide evidence for an unexpected nuclear role for the normally cytoplasmic FMRP and raise the intriguing possibility that FMRP, like HIV-1 Rev, might function to permit the sequence-specific nuclear export of target RNA molecules in expressing cells.

Results

Identification of a nuclear export signal in FMRP

Although nuclear localization signals (NLSs) have been known for some time, signals that mediate the efficient nuclear export of proteins were only recently identified (Fischer *et al.*, 1995; Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996). The prototype NES is the ~10 amino acid NES found in HIV-1 Rev (Figure 1B), which in turn is similar to NES sequences found in several other retroviral Rev proteins (Malim *et al.*, 1991; Fischer *et al.*, 1995; Meyer *et al.*, 1996). In addition, NES sequences have been identified in two cellular proteins, i.e. protein kinase inhibitor and transcription factor IIIA (Wen *et al.*, 1995; Fridell *et al.*, 1996). Mutational analysis of the HIV-1 Rev NES, as well as sequence comparisons to other NESs, suggest that a functional NES consists of four critically spaced, large hydrophobic amino acids in a generally hydrophilic sequence context (Malim *et al.*, 1991; Bogerd *et al.*, 1996). While leucine is the preferred large hydrophobic residue, isoleucine, methionine and valine are acceptable in at least some NES sequence contexts. Based on these characteristics, we initiated a search of available human databases to identify additional cellular NESs. This search identified two overlapping candidate NES sequences within FMRP. As shown in Figure 1B, the first of these extends from residue 423 to 432 within FMRP while the second extends from 429 to 437.

In order to demonstrate that this FMRP sequence indeed formed a functional NES, we used a previously described assay that directly visualizes NES activity (Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996). For this purpose, wild-type or mutant forms of a 20 amino acid segment of FMRP, consisting of residues 418–437, were expressed in bacteria as fusions with the carboxy terminus of glutathione-*S*-transferase (GST). The mutant FMRP sequence used in this experiment, termed 34A, contains alanine residues in place of the leucine residues normally found at 429 and 431 within FMRP (Figure 1B). Based on previous work, it is predicted that substitution of alanine for any two leucines within an NES will inactivate NES function (Malim *et al.*, 1991; Wen *et al.*, 1995; Bogerd *et al.*, 1996). The 34A mutation should therefore inactivate both candidate FMRP NES sequences (Figure 1B).

The recombinant GST:FMRP and GST:34A proteins were purified by affinity chromatography, mixed with rabbit IgG and then microinjected into the nucleus of HeLa cells. After 30 min of incubation at 37°C, the injected culture was fixed and subjected to double-label immunofluorescence to localize both the fusion protein and the IgG in the injected cells. Both IgG and GST lack an NLS and an NES and both will therefore normally remain in the cellular compartment into which they are microinjected (Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996). The IgG serves here as a control to ensure that microinjected proteins were indeed introduced exclusively into the nucleus (Figure 2B and D). If the sequence fused to GST lacks NES function, then this fusion protein will also remain nuclear, as is indeed seen with the mutant GST:34A fusion (Figure 2C). In contrast, if the attached sequence is a functional NES, then the GST fusion protein will be exported to the cytoplasm (Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996). This is, in fact, readily seen with the wild-type GST:FMRP fusion protein, which has become almost entirely cytoplasmic (Figure 3A) even though the co-injected IgG has remained entirely nuclear (Figure 3B). Therefore, it is apparent that the FMRP sequence shown in Figure 1 indeed contains a functional NES.

As noted above, it was recently reported that an alternatively spliced minor isoform of FMRP, lacking exon 14 sequences, shows an aberrant, predominantly nuclear localization in expressing cells (Sittler *et al.*, 1996). While this result led to the proposal that exon 14 contained a cytoplasmic retention domain, the alternative possibility, i.e. that the nuclear localization of this FMRP isoform might reflect a block to its nuclear export, was not considered. In this context, we were therefore intrigued to note that loss of FMRP exon 14, which encodes residues 425–489, would result in the deletion of the FMRP NES sequence (Figure 1).

To test the hypothesis that the aberrant subcellular

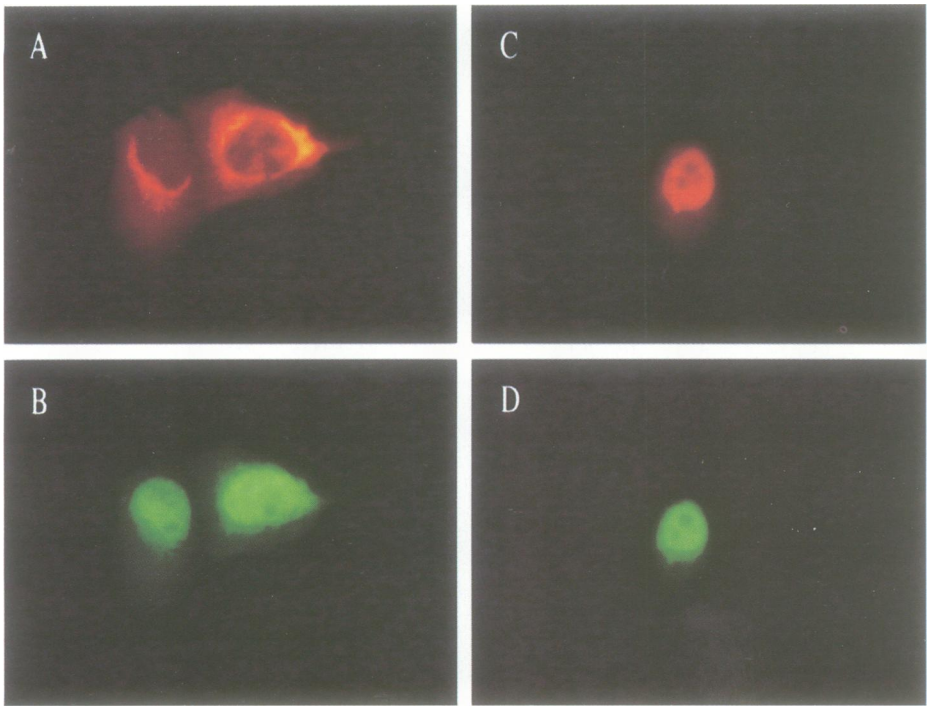

Fig. 2. Microinjection assay for FMRP NES function. Purified recombinant proteins consisting of GST fused to wild-type (**A** and **B**) or 34A mutant (**C** and **D**) forms of residues 418–437 of FMRP were microinjected into HeLa cell nuclei along with rabbit IgG. After incubation at 37°C for 30 min, the cells were fixed and the subcellular location of the GST fusion proteins (A and C) and IgG (B and D) determined by double label immunofluorescence.

localization of this FMRP isoform indeed reflected the loss of a functional NES, we attached an epitope tag to the amino-terminal end of a cDNA clone encoding wild-type or 34A mutant forms of the full-length, 614 amino acid form of murine FMRP, which is 97% identical to human FMRP at the amino acid sequence level. Both wild-type FMRP and the 34A mutant were then expressed in human cells and their subcellular localization determined by indirect immunofluorescence. As shown in Figure 3A, full-length wild-type FMRP is, as previously reported (Devys *et al.*, 1993; Verheij *et al.*, 1993; Verkerk *et al.*, 1993), localized primarily to the cell cytoplasm. In contrast, the 34A missense mutant of FMRP was found to be predominantly localized to the nucleoplasm, with nucleolar exclusion (Figure 3B). While some perinuclear and weak cytoplasmic localization could also be observed, the intense nuclear fluorescence seen with the 34A FMRP mutant was clearly in sharp contrast to the nuclear exclusion characteristic of wild-type FMRP. It is therefore apparent that the 34A missense mutant of FMRP reproduces the aberrant nuclear localization previously reported (Sittler *et al.*, 1996) for the FMRP isoform that lacks all of exon 14. The normally cytoplasmic steady-state localization of FMRP is therefore clearly dependent on the integrity of the exon 14 NES sequence shown in Figure 1B.

Rev and FMRP contain functionally equivalent NESs

The HIV-1 Rev protein is required for the nucleocytoplasmic transport, and hence translation, of the incompletely spliced, late class of HIV-1 mRNAs that encodes the virion structural proteins (Felber *et al.*, 1989; Malim *et al.*, 1989b; Fischer *et al.*, 1994). In the absence of Rev function, these RNAs are retained in the cell nucleus and either spliced to completion or degraded. Rev function is therefore essential for HIV-1 structural protein expression and replication. Extensive mutational analysis has identified two distinct functional domains in Rev (Malim *et al.*, 1989a, 1991; Hope *et al.*, 1991). The first of these is the NES shown in Figure 1B, which is located towards the Rev carboxy terminus. Although the precise mechanism of action of the Rev NES remains unclear, evidence has been presented demonstrating a specific interaction with a nucleoporin-like cellular cofactor, termed Rab or RIP, that is believed to target the Rev–RNA complex to nuclear pores (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995). The second Rev functional domain is fully contained within the amino-terminal 66 amino acids of Rev and mediates both specific binding to the Rev response element (RRE) RNA target and Rev multimerization (Malim and Cullen, 1991).

Because the RNA-binding/multimerization domain and the NES domain of Rev are functionally autonomous, it is possible to examine whether an NES is functionally equivalent to the Rev NES by simply attaching it to the Rev RNA-binding/multimerization domain and testing for Rev function (Hope *et al.*, 1991; Fridell *et al.*, 1996). To examine this question for the FMRP NES, we constructed expression plasmids that encoded a fusion protein consisting of the first 66 amino acids of Rev linked to residues 418–437 of wild-type FMRP (M9/FMRP). In addition, we generated plasmids that would express similar Rev fusion proteins containing the mutant FMRP sequences indicated in Figure 1. These mutations were designed to address which of the two candidate FMRP NES sequences

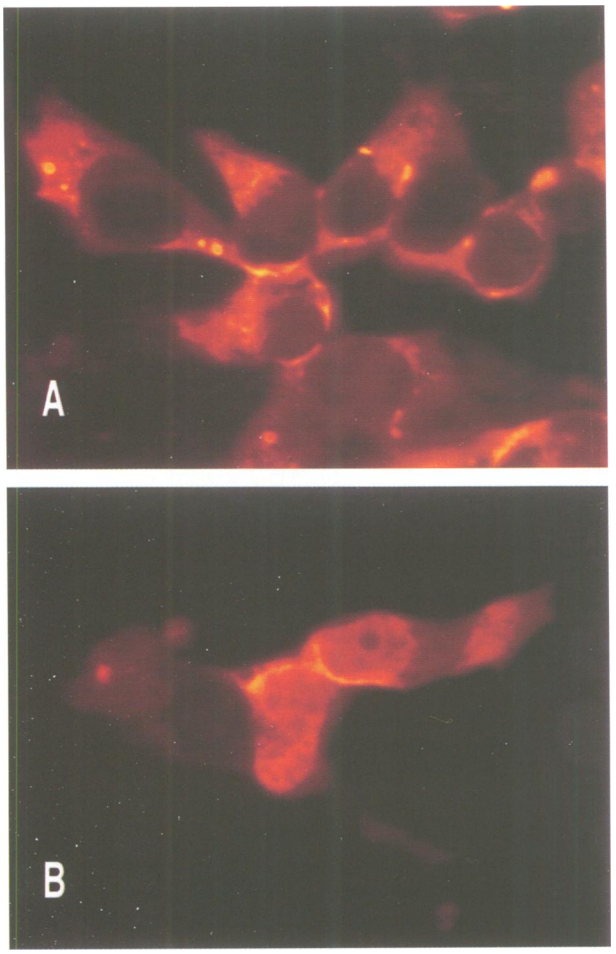

Fig. 3. The FMRP NES is required to maintain cytoplasmic localization. Human 293T cells were transfected with expression plasmids encoding full-length wild-type (**A**) or 34A mutant (**B**) forms of FMRP. The encoded proteins were amino-terminally tagged with an influenza HA epitope tag and were visualized using a monoclonal antibody specific for this viral sequence. Both proteins were expressed at equivalent levels, and migrated at the same relative size, as determined by Western blot analysis.

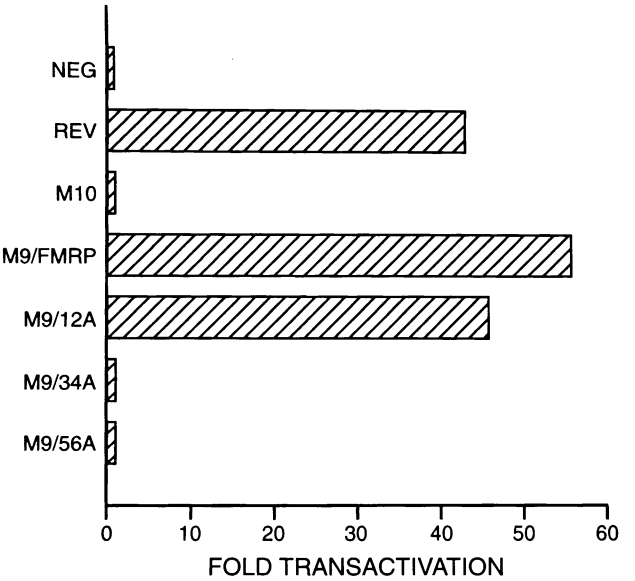

Fig. 4. The FMRP NES is functionally equivalent to the Rev NES. Relative transactivation of the CAT indicator gene present in the pDM128/CMV indicator plasmid (Hope *et al.*, 1991; Malim *et al.*, 1991), induced by the indicated wild-type or mutant Rev or Rev/FMRP fusion proteins, was measured at 48 h after transfection of the cell line COS. The indicated M9 fusion protein consists of residues 1–66 of HIV-1 Rev linked to wild-type or mutant forms of residues 418–437 of FMRP.

was functional. The 12A mutation, which substitutes alanine at FMRP positions 423 and 426, is predicted to inactivate the candidate NES located between 423 and 432, while the 56A mutation, which substitutes alanine at FMRP positions 434 and 436, would inactivate the candidate FMRP NES located between 429 and 437. As noted above, the 34A mutation is predicted to inactivate both candidate NES sequences.

The previously described pDM128/CMV indicator construct (Hope *et al.*, 1991; Malim *et al.*, 1991) used in this experiment encodes both the chloramphenicol acetyl transferase (CAT) indicator gene and an RRE RNA target sandwiched between two splice sites. In the absence of Rev, the unspliced RNA encoded by pDM128/CMV is spliced prior to nuclear export, and little CAT activity is detected. In contrast, in the presence of Rev, the unspliced form of this RNA is efficiently exported to the cytoplasm and translated to yield functional CAT protein (Hope *et al.*, 1991; Malim *et al.*, 1991). This result is confirmed in Figure 4, which shows a minimal level of CAT activity in cells transfected with pDM128/CMV alone that is boosted ~45-fold by co-transfection with a Rev expression plasmid. The Rev M10 mutant, which is mutated at Rev residues 76 and 77 (Malim *et al.*, 1989a) (Figure 1B), is unable to activate CAT expression, thus confirming the known requirement for a functional NES (Malim *et al.*, 1989a, 1991; Fischer *et al.*, 1995; Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996). However, the chimeric M9/FMRP fusion protein, consisting of Rev residues 1–66 linked to FMRP residues 418–437, activated pDM128/CMV dependent CAT expression at least as effectively as wild-type Rev. Therefore, it is apparent that the FMRP NES is fully capable of functionally substituting *in cis* for the HIV-1 Rev NES. The data presented in Figure 4 also demonstrate that the mutant M9/12A FMRP fusion retains essentially full activity while the M9/34A and M9/56A fusions are inactive. As all four FMRP fusion proteins were found to be expressed at comparable levels in transfected cells by Western blot analysis (data not shown), this maps the NES to FMRP residues 429–437 and demonstrates that the other candidate NES sequence, located between FMRP residues 423 and 432, is not functional.

As noted above, an important property of the Rev NES is its ability to interact specifically with the nucleoporin-like cellular Rab cofactor (Bogerd *et al.*, 1995; Fritz *et al.*, 1995). We therefore used the yeast two-hybrid assay for *in vivo* protein–protein interactions (Fields and Song, 1989) to test whether the FMRP NES could also bind Rab. We have previously described (Bogerd *et al.*, 1995) yeast plasmids that express the GAL4 DNA binding domain alone (pGAL4) or fused to wild-type (pGAL4/REV) or mutant NES (pGAL4/M10) forms of the HIV-1 Rev protein. Similar plasmids expressing the GAL4 DNA binding domain linked to wild-type (GAL4/FMRP) or mutant (GAL4/34A) forms of the FMRP NES were prepared and all five plasmids introduced individually into

Table I. The NES of FMRP and HIV-1 Rev bind the same cellular cofactor

	β-Gal activity	
	+pVP16/RAB	+pVP16
pGAL4	<1	<1
pGAL4/REV	792	<1
pGAL4/M10	<1	<1
pGAL4/FMRP	615	<1
pGAL4/34A	<1	<1

Wild-type forms of the Rev and FMRP NES were expressed in yeast as fusions with the GAL4 DNA binding domain (Bogerd *et al.*, 1995). Coexpression of a fusion protein containing the human Rab cofactor linked to the VP16 transcription activation domain resulted in activation of a *lacZ* indicator gene linked to GAL4 DNA binding sites (Fields and Song, 1989; Haper *et al.*, 1993). This activation was not observed when mutant forms of the FMRP (34A) or Rev (M10) NES were used.

the yeast indicator strain Y190 (Harper *et al.*, 1993), along with a second plasmid encoding the VP16 transcription activation domain either alone (pVP16) or fused to the human Rab protein (pVP16/RAB) (Table I) (Bogerd *et al.*, 1995). A specific interaction between any of these GAL4 hybrid proteins and the VP16/RAB fusion will activate the *lacZ* indicator gene present in the Y190 yeast strain by recruiting the VP16 transcription activation domain to flanking GAL4 DNA binding sites (Fields and Song, 1989; Harper *et al.*, 1993; Bogerd *et al.*, 1995).

As shown in Table I and previously reported (Bogerd *et al.*, 1995), co-expression of the VP16/RAB hybrid with the wild-type GAL4/REV fusion results in a marked activation of *lacZ* expression. This activation is dependent on the integrity of the Rev NES, as shown by the lack of activity of the M10 Rev NES mutant. Similarly, the GAL4/FMRP fusion also gave rise to readily detectable levels of β-galactosidase (β-gal) activity in the presence of the VP16/RAB fusion, but not when an unfused form of VP16 was co-expressed. This interaction was blocked by mutational inactivation of the NES present in FMRP, as seen by the lack of activity of the GAL4/34A fusion. We therefore conclude that the FMRP NES is able to specifically bind to the same cellular NES cofactor utilized by the HIV-1 Rev NES.

Discussion

The identification of *FMR1* as the genetic locus underlying the inherited condition Fragile X syndrome represented an important first step towards understanding and, it is hoped, eventually ameliorating this highly prevalent form of mental retardation. However, efforts to define the role and mechanism of action of FMRP have so far met with relatively little success, with the primary achievement being the definition of FMRP sequences that are similar to RNA-binding domains seen in other cellular proteins and that can mediate *in vitro* RNA binding by FMRP (Ashley *et al.*, 1993; Siomi *et al.*, 1993) (Figure 1A). Unfortunately, neither the *in vivo* RNA target(s) for FMRP, nor the role played by RNA binding in mediating FMRP function, have as yet been defined.

In this manuscript, we report the identification of a second functional domain within FMRP, i.e. a leucine-rich NES that is closely similar to the NES recently identified in the Rev regulatory protein of HIV-1 (Fischer *et al.*, 1995; Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996) (Figure 1). Indeed, the FMRP NES appears functionally identical to the Rev NES in that it can fully substitute for the latter in mediating the nuclear export and expression of a Rev responsive RNA target molecule (Figure 4) and specifically binds the same nucleoporin-like cellular cofactor that has been shown to mediate the function of the Rev NES (Table I) (Bogerd *et al.*, 1995; Fritz *et al.*, 1995).

The identification of an NES within FMRP explains the puzzling recent finding that a naturally occurring isoform of FMRP, lacking a 65 amino acid sequence normally encoded by exon 14, is localized to the nucleus while other FMRP isoforms are predominantly cytoplasmic (Sittler *et al.*, 1996). The FMRP NES mapped in this report is entirely located within exon 14 and, as shown in Figure 3, inactivation of this NES by introduction of a missense mutation results in the same aberrant nuclear localization seen with the naturally occurring FMRP isoform lacking exon 14. Together, these findings provide strong support for the hypothesis that FMRP enters the cell nucleus as part of its normal mechanism of action and that re-entry into the cytoplasm is dependent on the identified NES sequence. The fact that the full-length, ~71 kDa form of FMRP significantly exceeds the ~60 kDa exclusion size for passive diffusion through nuclear pores (Görlich and Mattaj, 1996) clearly implies that not only nuclear export but also import of FMRP must be an active process. While it therefore appears probable that FMRP must also contain some form of NLS, initial efforts by Sittler *et al.* (1996) to define such a sequence proved unsuccessful. These authors therefore suggested that FMRP might be directed to the nucleus by an interaction with a 'nuclear component'.

Of interest, FMRP NES is fully conserved in the two recently identified human homologs of *FMR1*, named *FXR1* and *FXR2* (Siomi *et al.*, 1995; Zhang *et al.*, 1995). The *FXR1* and *FXR2* gene products, which are ~60% identical to each other and to FMRP at the primary sequence level, have also been reported to be localized in the cytoplasm at steady-state (Zhang *et al.*, 1995). However, the fact that both of these proteins contain intact copies of the NES defined here in FMRP raises the possibility that FXR1 and FXR2 may also play some role in the cell nucleus.

In the absence of any real information about the normal role of FMRP in the cell, any mechanistic proposals based on the data presented in this manuscript must be viewed as speculative. Nevertheless, and given the assumption that the physiological role of FMRP is closely tied to its reported ability to bind RNA (Ashley *et al.*, 1993; Siomi *et al.*, 1993), what then does the identification of an NES suggest about this protein? In considering this question, we have been strongly influenced by our observation that the FMRP NES is functionally equivalent to the Rev NES, which is known to play an important role in the nuclear export of specific HIV-1 RNA species (Malim *et al.*, 1989a, 1991; Fischer *et al.*, 1995; Wen *et al.*, 1995; Fridell *et al.*, 1996; Meyer *et al.*, 1996). It is also intriguing that FMRP and Rev are not only similar in containing an NES and an RNA-binding domain but also share the ability to

form homomultimers *in vivo*, an activity which is known to be critical for Rev function (Malim and Cullen, 1991). We therefore suggest that FMRP might also play a role in the sequence-specific nuclear export of target RNA species. One possibility is that FMRP might function exactly like Rev, i.e. that FMRP may continuously shuttle into and out of the cell nucleus while mediating nuclear RNA export (Meyer and Malim, 1994). However, the fact that FMRP is normally predominantly cytoplasmic (Devys *et al.*, 1993; Verheij *et al.*, 1993), while Rev is normally predominantly nuclear (Malim *et al.*, 1989a; Meyer and Malim, 1994), may argue against this hypothesis. Alternatively, FMRP might enter the nucleus and there assemble into a ribonucleoprotein particle that is then exported to the cell cytoplasm under the influence of the FMRP NES, where it then remains. This hypothesis envisions a role for FMRP that is somewhat similar to that of ribosomal RNA-binding proteins, which are synthesized in the cytoplasm, enter the nucleus to be assembled into ribosomes in the nucleolus, and are then returned to the cytoplasm as a ribosomal RNP. The recent finding that FMRP is, in fact, loosely associated with cytoplasmic ribosomes (Khandjian *et al.*, 1996) may be consistent with this latter proposal. Clearly, future efforts to validate either of these two tentative hypotheses, and to more completely understand the cellular role of FMRP, will require far more insight into the properties of this enigmatic protein, including, at minimum, the clear identification of an RNA target for FMRP function.

Materials and methods

Molecular clones

A full-length murine FMR1 cDNA clone was kindly provided by S.Warren (Ashley *et al.*, 1993). To construct an influenza hemagglutinin (HA) tagged FMRP expression plasmid, the *FMR1* cDNA was first amplified by polymerase chain reaction (PCR) using oligonucleotide primers that introduced a unique *Nco*I restriction site at the translation initiation codon and a *Sal*I site just downstream of the coding region. This PCR product was inserted 3′ to the cytomegalovirus immediate early promoter in pBC12/CMV (Malim *et al.*, 1989b) and a DNA fragment encoding three copies of the HA epitope YPYDVPDYA (a gift from N.Landau) was introduced at the *Nco*I site. The 34A mutation (Figure 1B) was introduced into the full-length HA-tagged FMRP by replacing an Asp718–*Xba*I fragment within the *FMR1* coding region with a PCR-generated DNA fragment containing the desired mutation.

The pcRev, pcRevM10 and pcRevM9 expression plasmids have been described (Malim *et al.*, 1989a). pcRevM9 contains an introduced *Bgl*II restriction site overlying amino acids 67 and 68 of Rev and also contains a unique *Xho*I site downstream of the Rev coding region. The plasmids pM9/FMRP, pM9/12A, pM9/34A and pM9/56A were made by replacing the *Bgl*II–*Xho*I fragment in pcRevM9 with synthetic oligonucleotides encoding FMRP amino acids 418–437, either wild-type or with the mutations shown in Figure 1B. These plasmids are therefore predicted to encode the amino-terminal 66 amino acids of Rev fused to FMRP residues 418–437.

The prokaryotic expression plasmids pGST/FMRP and pGST/34A were constructed by transferring *Bam*HI–*Xho*I fragments from pM9/FMRP and pM9/34A into pGEX4T-1 (Pharmacia). These plasmids are predicted to express GST fused to wild-type or mutant FMRP amino acids 418–437. In addition, an eight amino acid linker consisting of Rev amino acids 59–66 is retained between the GST and FMRP residues.

The pVP16/RAB yeast expression, which contains a full-length Rab cDNA fused in-frame to sequences encoding the transcription activation domain of VP16, has been described (Bogerd *et al.*, 1995). pGAL4/REV contains a cDNA encoding Rev amino acids 2–116 (*Bgl*II–*Xho*I) inserted into the *Bam*HI and *Sal*I sites of pGBT9 (Clonetech). pGAL4/M10 is identical except for the presence of the M10 mutation of the Rev NES (Bogerd *et al.*, 1995). These plasmids encode fusion proteins consisting of Gal4 amino acids 1–147 fused to Rev amino acids 2–116. Likewise, pGAL4/FMRP and pGAL4/34A were constructed by inserting *Bam*HI–*Xho*I fragments derived from pM9/FMRP and pM9/34A (see above) into the *Bam*HI and *Sal*I sites of pGBT9. The pGAL4/FMRP and pGAL4/34A plasmids are predicted to encode proteins consisting of Gal4 amino acids 1–147 fused to FMRP amino acids 418–437. As with the GST fusion proteins, a linker consisting of Rev amino acids 59–66 is present between the Gal4 and FMRP sequences.

For all clones, PCR amplifications were performed with PFU DNA polymerase (Stratagene) and mutations were verified by DNA sequencing using Sequenase version 2 (United States Biochemical). In addition, expression of all clones was confirmed by Western blot analysis.

Mammalian cell microinjection

All mammalian cell lines were maintained in Iscove's modified Eagle's medium supplemented with 10% fetal calf serum and gentamicin. GST fusion proteins were purified using standard procedures in the absence of detergent (Fridell *et al.*, 1996; Meyer *et al.*, 1996). For injection, purified proteins were diluted to 3 mg/ml in phosphate buffered saline and each preparation supplemented with 3 mg/ml rabbit IgG (Jackson ImmunoResearch) so that the site of injection could be determined. Two days prior to injection, 2×10^5 HeLa cells were plated onto gelatinized 35 mm dishes. After injection, cells were returned to 37°C for 30 min and then fixed with 3% paraformaldehyde. GST fusion proteins were detected with an anti-GST monoclonal antibody (Santa Cruz Biotechnology) followed by a Rhodamine-conjugated goat anti-mouse antibody (Cappel). IgG was directly visualized with a fluorescein conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch). Cells were visualized at ×400 using a Leica DMRB fluorescence microscope.

Cellular localization of wild-type and mutant FMRP

Human 293T cells were transfected with 1 μg of an HA-tagged FMRP expression plasmid and 15 μl of lipofectamine, as described in the protocol provided with the lipofectamine reagent (Gibco-BRL). At 48 h post transfection, 10^5 cells were transferred to poly-D-lysine treated chamber slides and returned to 37°C overnight. The cells were then fixed and the HA-tagged proteins detected using the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) followed by a rhodamine-conjugated goat anti-mouse antibody (Cappel).

Rev activity assay

The Rev activity of the M9/FMRP fusion proteins was determined using a published assay based on the pDM128/CMV indicator plasmid (Hope *et al.*, 1991; Malim *et al.*, 1991). COS cells were transfected with 300 ng of a Rev or negative control (pBC12/CMV) expression plasmid, 200 ng of carrier DNA, 20 ng of pDM128/CMV and 20 ng of the internal control plasmid pBC12/CMV/β-Gal. At 48 h post transfection, cells were harvested and assayed for CAT and β-gal activity as described (Bogerd *et al.*, 1995). CAT activities were adjusted for slight variations in transfection efficiency, as revealed by the observed β-gal activities.

Two-hybrid interaction analysis

The ability of the Rev and the FMRP NES to interact with the human Rab cofactor was assayed using the yeast two hybrid system (Fields and Song, 1989; Bogerd *et al.*, 1995). Briefly, the yeast strain Y190 (Harper *et al.*, 1993) was transformed with the appropriate Gal4 and VP16 hybrid protein expression plasmids. After 3 days of selection on culture plates, double transformants were transferred to selective medium. The following day, cultures were assayed for β-gal activity as described (Bogerd *et al.*, 1995).

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