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# The fragile X mental retardation protein has nucleic acid chaperone properties

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## ABSTRACT

**The fragile X syndrome is the most common cause of inherited mental retardation resulting from the absence of the fragile X mental retardation protein (FMRP). FMRP contains two K-homology (KH) domains and one RGG box that are landmarks characteristic of RNA-binding proteins. In agreement with this, FMRP associates with messenger ribonucleoparticles (mRNPs) within actively translating ribosomes, and is thought to regulate translation of target mRNAs, including its own transcript. To investigate whether FMRP might chaperone nucleic acid folding and hybridization, we analysed the annealing and strand exchange activities of DNA oligonucleotides and the enhancement of ribozyme-directed RNA substrate cleavage by FMRP and deleted variants relative to canonical nucleic acid chaperones, such as the cellular YB-1/p50 protein and the retroviral nucleocapsid protein HIV-1 NCp7. FMRP was found to possess all the properties of a potent nucleic acid chaperone, requiring the KH motifs and RGG box for optimal activity. These findings suggest that FMRP may regulate translation by acting on RNA–RNA interactions and thus on the structural status of mRNAs.**

## INTRODUCTION

The fragile X syndrome is the most common cause of inherited mental retardation in humans resulting from the absence of the FMR1 gene product, the FMR1 protein [fragile X mental retardation protein (FMRP)] (1,2). FMRP is a protein of 632 amino acids composed, in N-terminus to C-terminus order, of a protein-interacting domain (PPId), two K-homology (KH) motifs, a phosphorylation sequence and an RNA recognition sequence called the RGG box (1–3). FMRP is widely expressed, but most abundant in testes and brain, which are the organs strongly affected by the syndrome (4,5).

The cellular role of FMRP remains poorly understood and the current view is that it regulates mRNA transport and translation in a manner critical for the development of neurons (6,7). To achieve its function, FMRP is thought to be engaged in a number of interactions with cytoplasmic nucleic acid and protein partners (1,8–15) in association with messenger ribonucleoparticles (mRNPs) in actively translating ribosomes (16,17), however, the exact role of FMRP in translation remains elusive. Several studies have shown that FMRP can act as a negative regulator of translation *in vitro* and *in vivo* (18–20) and it has been proposed that in neurons a small fraction of FMRP acts as a repressor for the RNA to be transported and to be delivered at the budding dendrites (20). Indeed, while the great majority of FMRP has been observed in the neuron cell body, a small fraction was detected at distal locations such as neurites, dendrites and synaptosomes (5,21,22). A series of neuronal mRNAs have been isolated either by immunoprecipitation approaches (8), or by the use of a new technique called 'APRA' for antibody positioned RNA amplification (24). In addition, several target mRNAs have been isolated by differential display, by oligonucleotide (ODN)-based and by cDNA-SELEX (9,25,26). Also it has been reported that FMRP interacts with small noncoding RNAs such as BC1 and BC200, which in turn mediates their binding to specific mRNAs (27). These observations clearly indicate that FMRP has affinity to RNAs, however, it is not clear whether all these target mRNAs bind directly to FMRP or if FMRP protein interactors are required.

In view of the binding of FMRP to mRNAs and small noncoding RNAs, we addressed the question as to whether FMRP might be a nucleic acid chaperone protein. So-called nucleic acid chaperones bind in a cooperative manner to one or more nucleic acid molecules and promote the formation of the most stable structure while at the same time preventing folding traps that may preclude function (28,29). Importantly, nucleic acid chaperones do not require ATP to function and once the most stable nucleic acid structure is achieved, their continued binding is no longer required to maintain the structure (28,29).

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Canonical nucleic acid chaperones, such as retroviral NC proteins, are able to anneal, under physiological conditions *in vitro*, a specific primer tRNA to a complementary sequence at the 5′ end of genomic viral RNA; a prerequisite for the initiation of reverse transcription (30,31).

In the present study, we examined the ability of FMRP to chaperone the annealing of DNA with complementary sequences as well as strand exchange in a duplex nucleic acid structure *in vitro*. Furthermore, we investigated whether FMRP is capable of enhancing ribozyme-directed cleavage of an RNA substrate *in vitro*. Our results show that FMRP is a potent chaperone protein.

## MATERIALS AND METHODS

### Recombinant proteins

The full-length FMRP and six deleted variants ΔPPId, ΔKH1, ΔKH2, ΔKHT (ΔKH1 plus ΔKH2), ΔPhD and ΔRGG FMRP with a C-terminal (His)6-tag were produced in *Escherichia coli* and purified to homogeneity by affinity chromatography using Ni-NTA Probond beads (Invitrogen) according to the manufacturer's protocol as previously described (Fig. 1) (3).

HIV-1 nucleocapsid protein NCp7 and mutant NC(12–53) were synthesized by the fmoc/opfp chemical method and purified to >98% purity by high-pressure liquid chromatography (30). Proteins were dissolved at 1 mg/ml in buffer containing 30 mM HEPES pH 6.5, 30 mM NaCl and 0.1 mM $ZnCl_2$.

The YB-1/p50 protein (32) was a kind gift from Lev Ovchinnikov (Moscow, Russia) (31).

### DNA substrates

DNA ODNs corresponding to the HIV-1 TAR and the repeated R sequences (Mal isolate), in the sense and anti-sense orientation were purchased from Eurogentec (Belgium). TAR and R ODNs are 56 and 96 nt in length, respectively.

TAR(+) (sense) (30): 5′-GGTCTCTCTTGTTAGACC-AGGTCGAGCCCGGGAGCTCTCTGGCTAGCAAGGA-ACCC-3′; TAR(–) (anti-sense): 5′-GGGTTCCTTGCTAGC-CAGAGAGCTCCCGGGCTCGACCTGGTCTAACAAG-AGAGACC-3′; R(+).wt (sense): 5′-GGTCTCTCTTGTT-AGACCAGGTCGAGCCCGGGAGCTCTCTGGCTAGC-AAGGAACCCACTGCTTAAGCCTCAATAAAGCTTGC-CTTGAGTGCCTCCC-3′; R(-).wt (anti-sense): 5′-GGGAG-GCACTCAAGGCAAGCTTTATTGAGGCTTAAGCAGT-GGGTTCCTTGCTAGCCAGAGAGCTCCCGGGCTCGA-CCTGGTCTAACAAGAGAGACC-3′.

DNA ODNs corresponding to R(–) in which 7 nt were mutated at the 3′ end (underlined nucleotide): R(–).3′-modified: 5′.GGGAGGCACTCAAGGCAAGCTTTATTG-AGGCTTAAGCAGTGGGTTCCTTGCTAGCCAGAGAG-CTCCCGGGCTCGACCTGGTCTAACATCAGTCTCTA-3′; TAR(–) and R(+) ODNs were $^{32}$P-labelled with 50 μCi of [γ-$^{32}$P]ATP using T4 polynucleotide kinase. $^{32}$P-labelled DNAs TAR(–) and R(+) were purified by 10% PAGE, 7 M urea in 50 mM Tris-borate, 1 mM EDTA, pH 8.3 (0.5× TBE) before use.

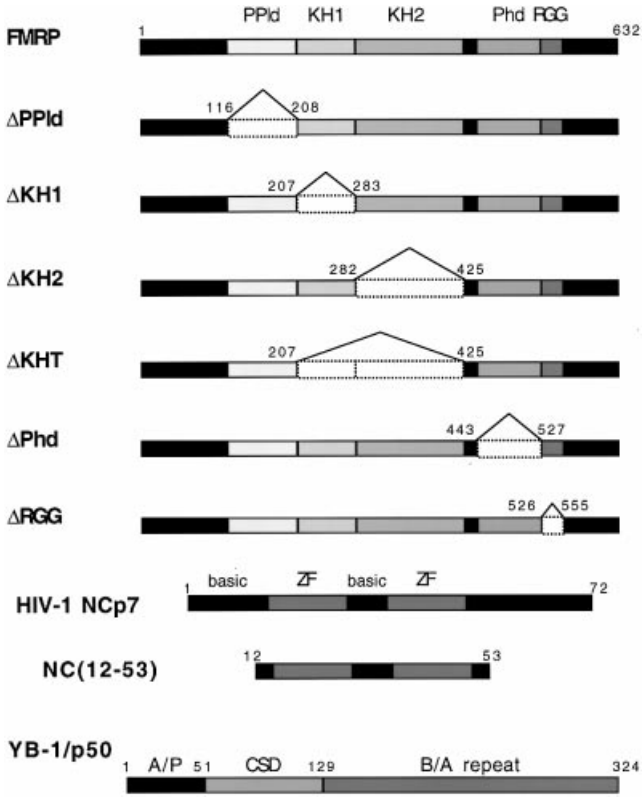



**Figure 1.** Scheme of the FRMP protein and deleted variants. Wild-type recombinant FMRP and its deleted variants used in the present study are schematically depicted here. Positions of the deleted regions, namely the protein interacting domain (PPId), the KH1 and KH2 domains, the phosphorylation domain (Phd) and the RGG box are shown. HIV-1 NCp7(1–72) and mutant NC(12–53) with the two CCHC zinc fingers (ZF) are shown. The complete YP-1/p50 protein is schematically shown with the N-terminal alanine- and proline-rich (A/P) domain, the cold shock domain (CSD) and basic and acidic amino acids (B/A) clusters domain. Amino acid positions are indicated.

### Plasmid DNAs

Plasmid DNAs pS14, pS20 and pR3 were kindly provided by E. Bertrand (33). All plasmid DNAs were amplified in *E.coli* 1035 (RecA-) and purified by affinity chromatography (Qiagen protocol).

### *In vitro* RNA synthesis

RNAs were labelled with [α-$^{32}$P]UTP during *in vitro* transcription, as previously described (31), using T7 RNA polymerase. Template DNAs pS14, pS20 and pR3 were digested with PstI, first treated by Klenow to remove the 3′ strand overhang and substrate RNA and the ribozyme generated by *in vitro* transcription with the following modifications: for substrate RNA (transcription of pS14 and pS20 DNA) the concentration of UTP was 10 μM and 50 μCi [α-$^{32}$P]UTP (Amersham, UK) were added. For the ribozyme (pR3 DNA template), the concentration of UTP was 100 μM and 10 μCi of [α-$^{32}$P]UTP were added.

Following *in vitro* RNA synthesis, the DNA template was removed by treatment with RNase-free DNase I (Promega) for 20 min at 37°C, followed by phenol and chloroform extractions, and ethanol precipitation. All $^{32}$P-labelled RNAs were

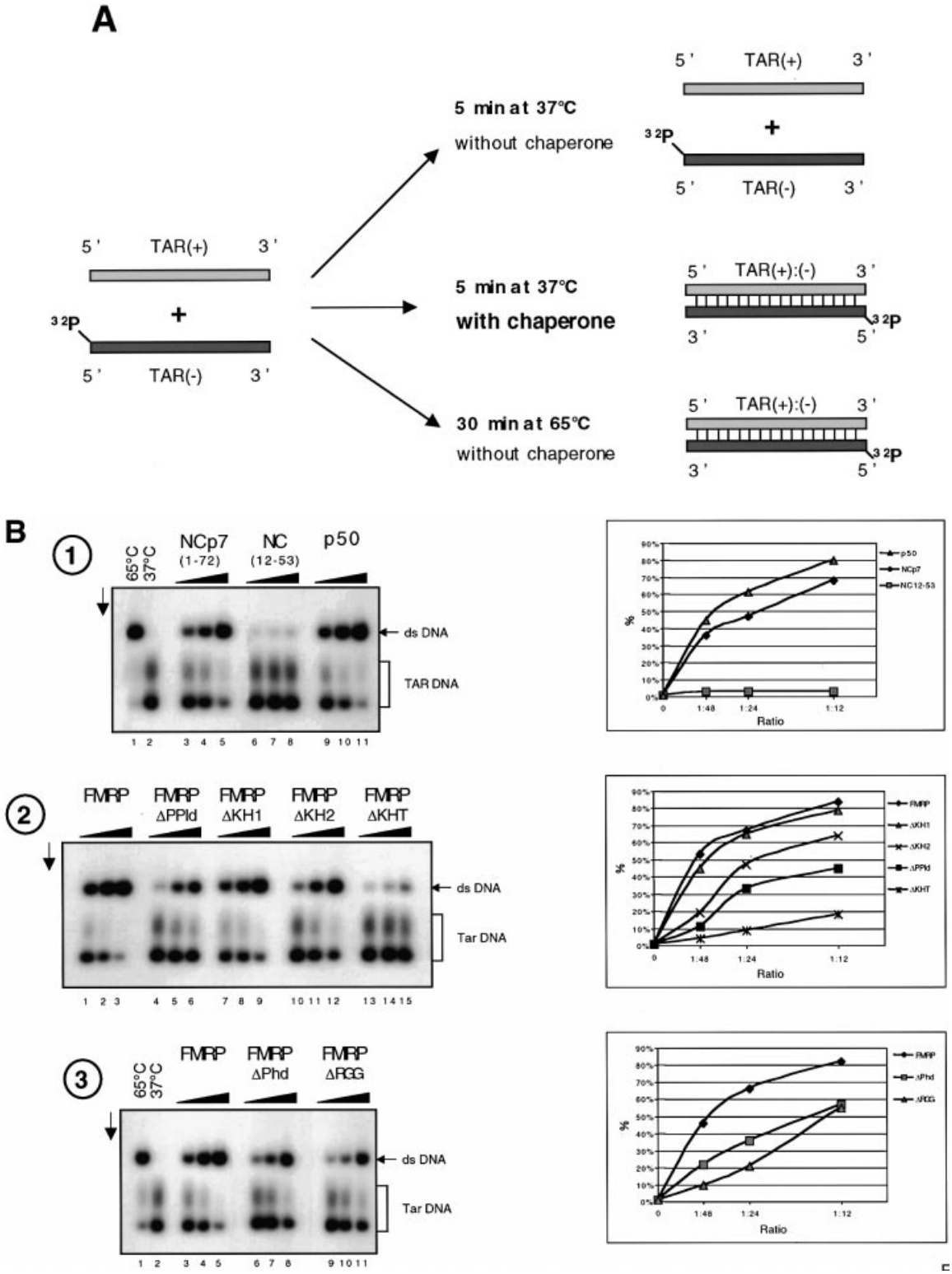



**Figure 2.** FMRP has nucleic acid annealing activity. (**A**) Schematic representation of the assay. HIV-1 TAR (+) and (–) DNA sequences are shown. 5′ $^{32}$P-labelled TAR(–) DNA is hybridized to TAR(+) upon heating at 65°C or following addition of a nucleic acid chaperone such as HIV-1 NCp7. (**B**) Annealing assays. Conditions were as described in Materials and Methods. Control hybridization was conducted for 30 min at 65 (lane 1) or 37°C (lane 2). Protein to nucleotide molar ratios were 1:48, 1:24 and 1:12, corresponding to a concentration of 0.35, 0.7 and 1.4 × $10^{-8}$ M, respectively. The vertical arrow shows the direction of electrophoresis. $^{32}$P-labelled TAR(–) DNA and double-stranded TAR DNA are indicated on the right. (1) NCp7, lanes 3–5; NC(12–53), lanes 6–8; p50, lanes 9–11. (2) FMRP, lanes 1–3; FMRP ΔPPId, lanes 4–6; FMRP ΔKH1, lanes 7–9; FMRP ΔKH2, lanes 10–12; FMRP ΔKHT, lanes 13–15. (3) FMRP, lanes 3–5; FMRP ΔPhd, lanes 6–8; FMRP ΔRGG, lanes 9–11. Curves shown on the right are quantitative assessments of the percentage of double-stranded DNA formed. Note that FMRP is as active as the canonical chaperone HIV-1 NCp7. Only FMRP mutant ΔKH1 was as active as wild-type protein, whereas mutants ΔRGG, ΔKH2, ΔPPId and ΔPhd were ~2.5–5 times less active and mutant ΔKHT was poorly active (see at molar ratio of 1:48).

purified by 8% PAGE, 7 M urea in 0.5× TBE. RNAs were recovered by elution in 0.3 M sodium acetate, 0.1% SDS, for 4 h at 37°C and ethanol precipitated. RNAs were dissolved in sterile $H_2O$.

### TAR(–)/TAR(+) annealing assays

HIV-1 TAR(+) and $^{32}$P-labelled TAR(–) ODNs were incubated (0.015 pmol, each equivalent to a DNA concentration of $3 \times 10^{-9}$ M) with or without protein in 10 µl containing 20 mM Tris–HCl (pH 7.0), 30 mM NaCl, 0.1 mM $MgCl_2$, 10 µM $ZnCl_2$ and 5 mM DTT. Reactions were performed at 37°C for 10 min except for the positive control which was kept at 65°C for 30 min under oil. Reactions were stopped with 5 µl of 20% glycerol, 20 mM EDTA pH 8.0, 0.2% SDS, 0.25% bromophenol blue and 0.4 mg/ml calf liver tRNA. Samples were analysed by 8% native PAGE in 0.5× TBE and, subsequently, gels were autoradiographed. Quantitation was by PhosphorImaging.

### DNA strand transfer and exchange assays

0.03 pmol of $^{32}$P-labelled R(+).wt, 0.03 pmol of R(–). 3′-modified and 0.03 pmol of R(–).wt were separately heat denatured for 2 min at 90°C and chilled on ice. All components were kept at 4°C. 0.03 pmol each of $^{32}$P-labelled R(+).wt and R(–).3′-modified, at a concentration of $6 \times 10^{-8}$ M, were mixed with reaction buffer to a final concentration of 20 mM Tris–HCl, pH 7.0, 30 mM NaCl, 0.1 mM $MgCl_2$, 10 µM $ZnCl_2$ and 5 mM DTT in 5 µl final volume, incubated for 30 min at 62°C under oil and chilled on ice. Then, 0.03 pmol of R(–).wt was added together with the protein using a protein to nucleotide molar ratio as indicated in the legends. Assays were for 5 min at 37°C, they were then chilled on ice and stopped with 2.5 µl of 20% glycerol, 20 mM EDTA pH 8.0, 0.2% SDS, 0.25% bromophenol blue and 0.4 mg/ml calf liver tRNA. Samples were resolved by 6% native PAGE in 0.5× TBE at 4°C and, subsequently, gels were autoradiographed. Quantitation was by PhosphorImaging.

### Hammerhead ribozyme cleavage assays

Ribozyme and substrate RNA were independently heated for 1 min at 90°C in $H_2O$. The reaction buffer was added to yield final concentrations of 5 mM $MgCl_2$, 100 mM NaCl, 20 mM Tris–HCl, pH 7.5. After slow cooling to 37°C, RNAs were further incubated for 5 min at 20°C. 0.1 pmol of ribozyme and 0.02–2 pmol of RNA substrate were then combined in a final volume of 10 µl, each protein was added to the final ratio indicated in the legend of the figures and incubation was for 25 min at 37°C. Reactions were terminated by adding 20 µl of stop solution (0.3% SDS, 15 mM EDTA), extracted with 30 µl of phenol and 15 µl of chloroform. The aqueous phase was precipitated with ethanol and the pellet resuspended in 45% formamide, 0.5× TBE, and 0.1% dye. $^{32}$P-labelled RNAs were analysed on 8% PAGE in 0.5× TBE and, subsequently, gels were autoradiographed. Quantitation was by PhosphorImaging.

## RESULTS

FMRP and the deleted versions (denoted ΔPPId, ΔKH1, ΔKH2, ΔKHT, ΔPhd and ΔRGG) were expressed in *E.coli* with a C-terminal (His)6-tag and purified by Ni-chelate chromatography (see Materials and Methods). HIV-1 NCp7 and its truncated version denoted ΔNC(12–53) were synthesized and purified according to a published procedure (30). YB-1/p50 was provided by Lev Ovchinnikov (32) (Fig. 1). To confirm that FMRP stably binds nucleic acids and RNA in particular, we examined by native gel electrophoresis the formation of nucleoprotein complexes between FMRP and either $tRNA^{Lys,3}$, BC1 or BC200, at concentrations of $10^{-8}$ M. Results of the gel shift experiments show that FMRP binds RNA (Supplementary fig. 1) and clearly has a high affinity for structured RNA molecules such as $tRNA^{Lys,3}$ (lanes 2–4 A), BC1 RNA (lanes 6–8 A) and BC200 RNA (lanes 10–12 A) in a manner similar to YB-1/p50 (Supplementary fig. 1E). Under the present ionic conditions, $K_d$ was estimated to be 25 nM. Deletion of the KH motifs caused a strong reduction of FMRP RNA-binding activity (Supplementary fig. 1B). Similar results were obtained with single-stranded DNA ODNs of 56–98 nt in length (data not shown and see below).

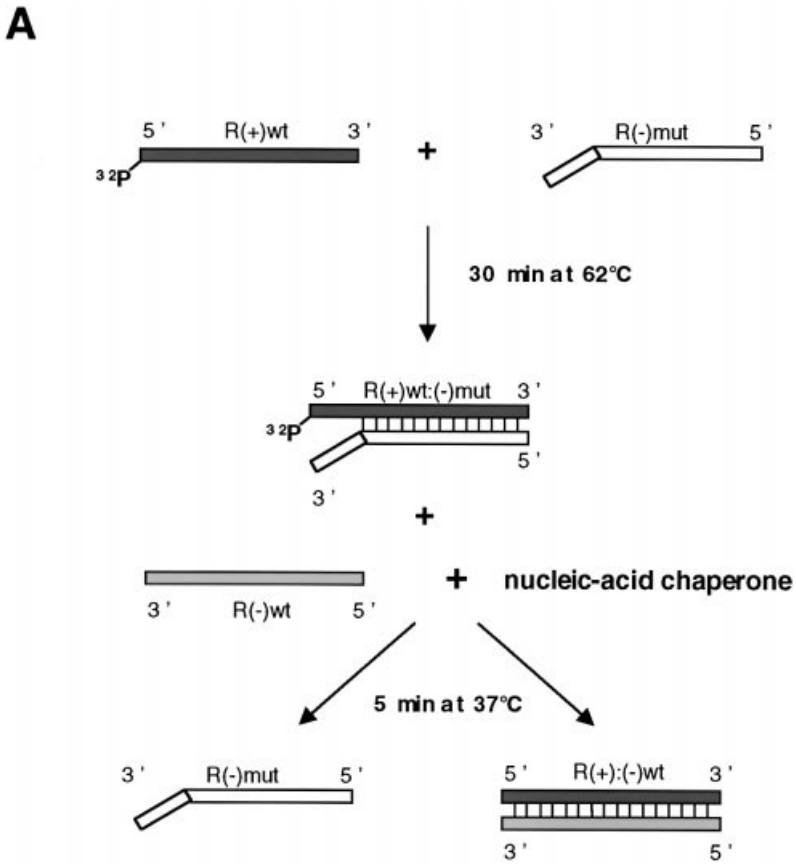
### FMRP has DNA annealing activity

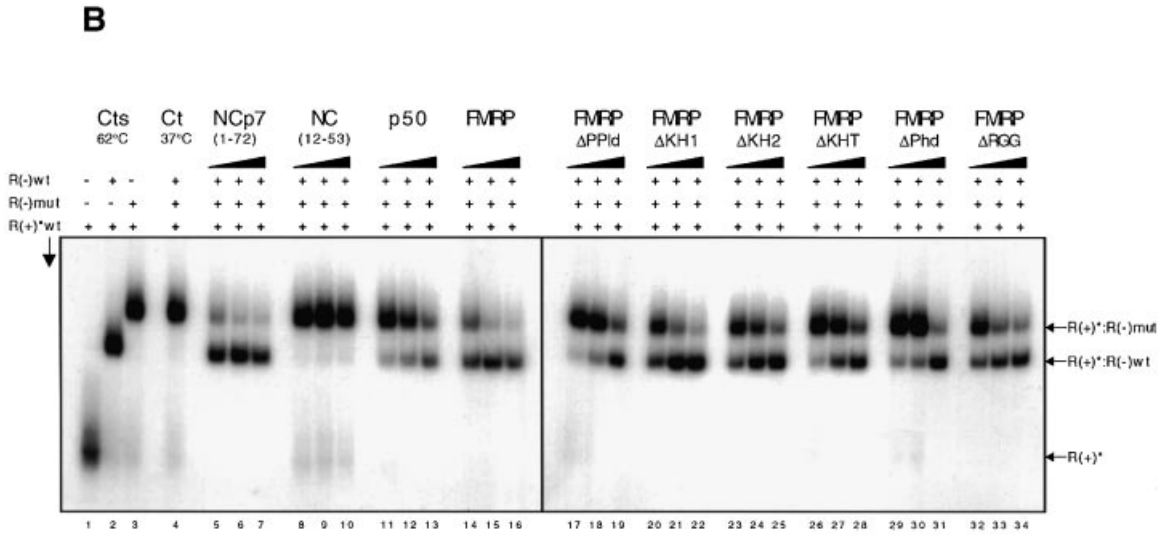
To assay for nucleic acid chaperone activity, FMRP was examined for its ability to enhance the annealing of complementary DNA ODNs (Fig. 2A). To this end, complementary $^{32}$P-Tar(–) and Tar(+) ODNs (56 nt) were incubated in the presence of increasing concentrations of FMRP, subsequently chased with a tRNA excess. Duplex formation was analysed by native gel electrophoresis.

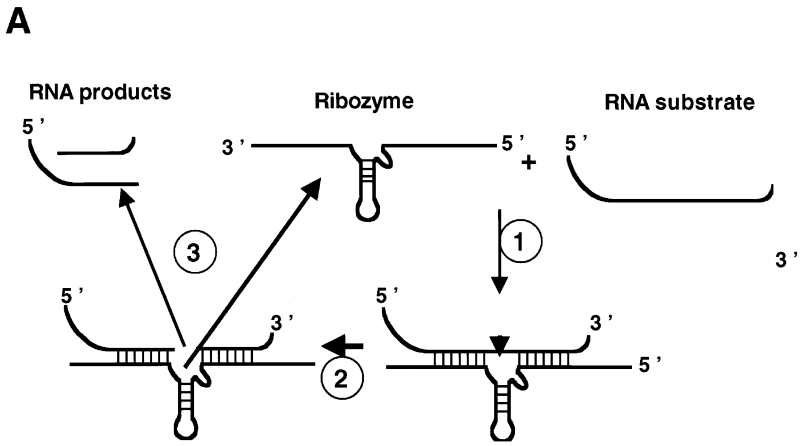
Results show that addition of FMRP caused nearly complete duplex formation [Fig. 2B, compare lanes 1–3 in (2) with lane 2 in (1)]. This effect is not simply due to enhanced molecular crowding, since at the same protein concentration, BSA did not enhance duplex formation (data not shown). Also, the propensity of FMRP to anneal the TAR substrates is not simply a general feature of DNA binding proteins, since at similar concentrations, the single-stranded

**Figure 3.** FMRP activates DNA strand exchange. (**A**) Schematic representation of the assay. DNA sequences corresponding to the HIV-1 R region of 96 nt in length. 5′ $^{32}$P-labelled R(+).wt was hybridized to R(–).mut to generate a double-stranded DNA with seven mismatches at one end (step 1). R(–).wt completely complementary to R(+).wt is added together with HIV-1 NCp7 or FMRP (step 2). Strand exchange is visualized by native 6% PAGE in 0.5 TBE. (**B**) Strand exchange assays. Conditions were as described in Materials and Methods. Proteins are indicated at the top of the figure. *R(+) is the $^{32}$P-labelled DNA. Double-stranded [*R(+):R(–).wt] and [*R(+):R(–).mut] are indicated on the right. Percentages of strand exchange were assessed by PhorphorImaging and are indicated in parentheses (see below). The vertical arrow shows the direction of electrophoresis. Lanes 1 and 2, *R(+).wt alone and annealed to R(–).wt. Lane 3, *R(+) hybridized to R(–).mut. Lane 4, [*R(+):R(–).mut] incubated with R(–).wt at 37°C for 30 min. Protein to nucleotide molar ratios were 1:8, 1:4 and 1:2 corresponding to a protein concentration of 1, 2 and $4 \times 10^{-7}$ M, respectively. NCp7, lanes 5–7 (64, 75 and 78%); NC(12–53), lanes 8–10 (4, 5 and 6%); p50, lanes 11–13 (22, 32 and 50%); FMRP wild type, lanes 14–16 (53, 70 and 78%); FMRP ΔPPId, lanes 17–19 (19, 31 and 62%); FMRP ΔKH1, lanes 20–22 (44, 61 and 73%); FMRP ΔKH2, lanes 23–25 (40, 54 and 65%); FMRP ΔKHT, lanes 26–28 (24, 39 and 53%); FMRP ΔPhd, lanes 29–31 (26, 32 and 60%); FMRP ΔRGG, lanes 32–34 (39, 53 and 60%). Note that FMRP displays greater DNA strand exchange activity than YB-1/p50 but is similar to HIV-1 NCp7. FMRP variants ΔPPId, ΔKHT and ΔPhd display a DNA exchange activity ~2.5-fold lower than wild-type FMRP.

DNA binding protein T4gp32 was unable to enhance duplex formation (33–37 and data not shown). FMRP ΔKH1 was as active as FMRP wild type (compare lanes 1–3 and 7–9; see also right panel), whereas other deleted variants were either ~3-fold less active (ΔPPId, ΔKH2, ΔPhd and ΔRGG) [Fig. 2B (2) and (3); see also right panels] or poorly active [ΔKHT; lanes 13–15 in Fig. 2B (2) and right panel]. FMRP was found to be as active as the canonical chaperone proteins HIV-1 NCp7 and YB-1/p50 [Fig. 2B (1), lanes 3–5 and 9–11 in comparison with lanes 1–3 in (2); see also right panels], while mutant NC(12–53) was inactive [lanes 6–8 in (1)] in agreement with previous reports (31).

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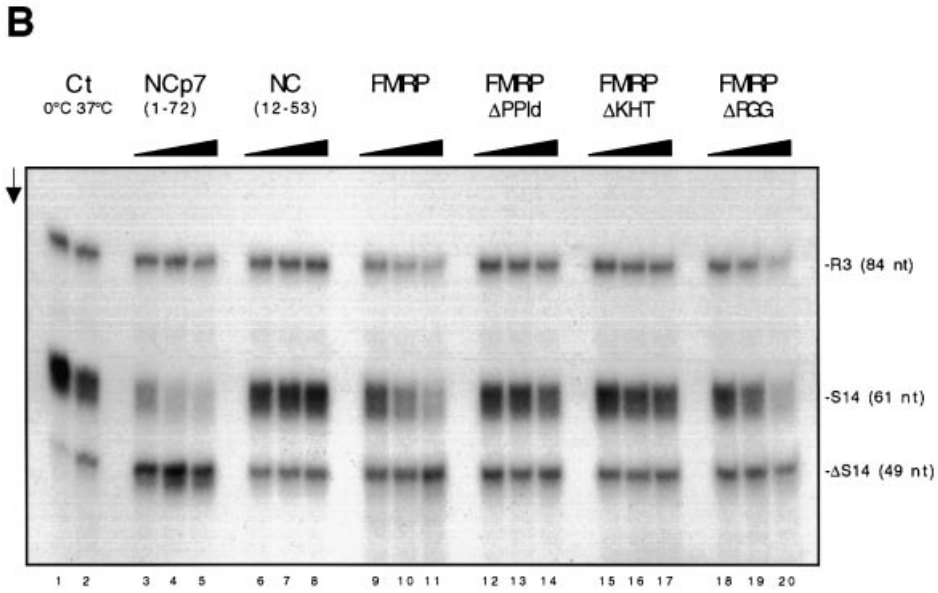
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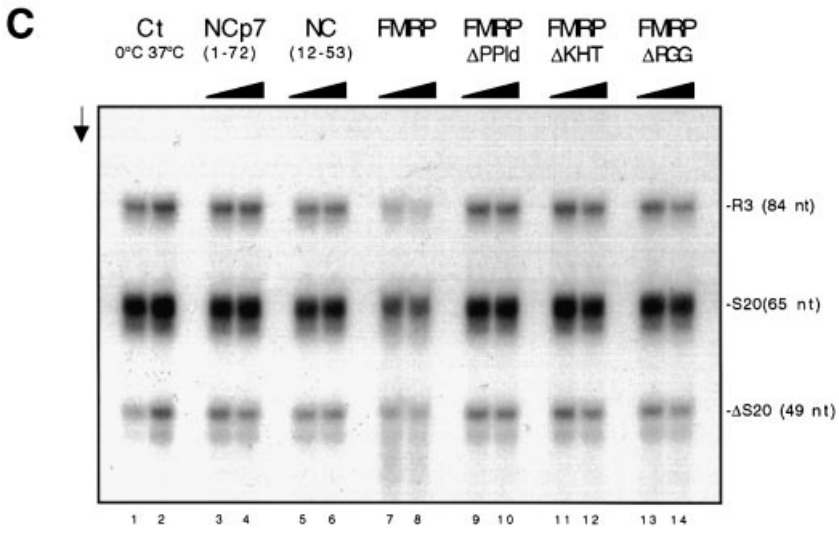
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We also examined the ability of FMRP to promote the hybridization of replication primer $tRNA^{Lys,3}$ to a complementary sequence at the 5′ end of the HIV-1 genomic RNA, called the primer binding site. Indeed, FMRP was able to hybridize the cellular tRNA to the viral RNA (Supplementary fig. 2; lanes 2–5).

## FMRP has DNA strand exchange activity

Another property of nucleic acid chaperones is to direct the formation of the most stable nucleic acid conformation. This can be assayed by examining the strand exchange activity of the protein (Fig. 3A). An imperfect DNA duplex with two partially complementary ODNs is formed by heating at 62°C. Then, the chaperone is added together with another ODN with the potential to form a perfect duplex with one of the two initial ODNs. After incubation under physiological conditions, the protein is removed and the ratio of the two DNA duplexes, the perfect duplex and the one containing seven mismatches, is assessed by native gel electrophoresis. As shown in Figure 3B (lanes 14–16), FMRP exchanged the matched strand [R(–).wt] for the mismatched strand [R(–).mut] as observed with HIV-1 NCp7 (lanes 5–7). FMRP was more active than p50 (lanes 11–13). In contrast, the level of the mismatched duplex remained unaffected by BSA, T4gp32 (30,31,34 and data not shown) or mutant NC(12–53) (compare lanes 8–10 and 14–16). The FMRP domains, such as PPId, KH and Phd, appeared to be critical for full activity (lanes 17–34).

Taken together, these results indicate that FMRP does indeed possess DNA annealing and strand exchange activities, which are properties expected for a nucleic acid chaperone (28,29), and that several domains, including the PPId, KH and RGG motifs, are important determinants for these activities.

## Enhancement of ribozyme cleavage of an RNA substrate by FMRP

Cleavage of an RNA substrate by a hammerhead ribozyme is a model system which allows examination of both the RNA annealing and unwinding activities of nucleic acid chaperone proteins. Chaperones can enhance the rate of ribozyme cleavage by activating the annealing of the substrate RNA to the hammerhead ribozyme (Fig. 4A, step 1) and the release of the RNA products (Fig. 4A, step 3), thus allowing cyclic reuse of the ribozyme (31,38). The ribozyme cleavage assay intends to investigate whether FMRP enhances ribozyme cleavage of an RNA substrate in a manner similar to NCp7 and hnRNP A1 chaperone proteins (see Fig. 4A) (33,38).

We selected the R3 hammerhead ribozyme and two RNA substrates, namely S14, with a 14 nt substrate ribozyme duplex length (7 nt either side of the cleavage site) and S20, with 10 nt either side of the cleavage site. The above RNA substrates were selected due to their likely biological relevance, as indicated by the similarity of data obtained *in vitro* and in cultured cells (33). $^{32}$P-labelled RNA S14, the hammerhead ribozyme and FMRP were mixed, incubated at 37°C for 25 min, protein removed by phenol extraction and RNA products recovered and analysed by PAGE under denaturing conditions. In the absence of a nucleic acid chaperone, ribozyme-directed cleavage of the RNA substrate occurred slowly at 37°C (Fig. 4B, top, lanes 1 at 4°C and 2 at 37°C; $^{32}$P-RNA substrate is S14 and upon cleavage it is ΔS14). In agreement with previous reports (33), HIV-1 NCp7 caused extensive ribozyme-directed cleavage of S14 RNA (lanes 3–5). On the other hand, NC(12–53) was poorly active (lanes 6–8). Interestingly, FMRP showed a clear enhancement of ribozyme-directed cleavage of S14 RNA (lanes 9–11). Deleted versions of FMRP, namely ΔPPId and ΔKHT were found to

**Figure 4.** Enhancement of ribozyme cleavage by FMRP. (**A**) Assay schematic. RNA substrate and hammerhead ribozyme, both $^{32}$P-labelled, were generated by *in vitro* transcription and purified by PAGE. Cleavage of the $^{32}$P-labelled RNA substrate by the hammerhead ribozyme first necessitates hybridization of the ribozyme to the substrate (step 1). After substrate cleavage (step 2, see arrow head), the RNA products must be released to allow recycling of the ribozyme (step 3). At the end of the reaction, protein is removed by phenol extraction and $^{32}$P-labelled RNAs analysed by PAGE under denaturing conditions to visualize the $^{32}$P-labelled RNA products. In the absence of a nucleic acid chaperone, annealing of the substrate to the ribozyme and release of the RNA products appear to be slow. Addition of a nucleic acid chaperone will accelerate hybridization of the substrate to the ribozyme and dissociation of the RNA products, and thus ribozyme turnover. Base pairing between the RNA substrate and ribozyme R3 are underlined on the substrate sequence. Ribozyme-mediated cleavage occurs on the 3′ side of A (space) for RNA S14, ...GAUUAAGUAGUA AGAGUGUCUGCA-3′, and for RNA S20, ...GAUUAAGUAGUA AGAGUGUCUGCA-3′. (**B**) Ribozyme-directed cleavage of RNA substrate. Percentages of ribozyme-directed RNA cleavage at 25 min were assessed by PhorphorImaging and are indicated in parentheses (see below). The vertical arrow shows the direction of electrophoresis. Ribozyme R3 (0.3 pmol) and RNA S14 (0.1 pmol) were incubated as described in Materials and Methods. $^{32}$P-labelled RNA substrate (S14) and product (ΔS14) were analysed by denaturing 8% PAGE in 0.5× TBE. Lanes 1–2, R3 and S14 at 4 or 37°C for 30 min (5% and 20%). Reactions with proteins were for 15 min at 37°C. Lanes 3–5, HIV-1 NCp7 at protein to nucleotide molar ratios of 1:20, 1:10 and 1:5, respectively (60, 73 and 75%), corresponding to a protein concentration of 0.5, 1 and 2 × $10^{-7}$ M, respectively. Lanes 6–8, NC(12–53) at molar ratios of 1:2.5, 1:1.2 and 1:0.6, respectively (25% for all ratios), corresponding to a protein concentration of 4, 8 and 16 × $10^{-7}$ M, respectively. Lanes 9–11, FMRP at protein to nucleotide molar ratios of 1:20, 1:10 and 1:5, respectively (33, 45 and 66%), corresponding to a protein concentration of 0.5, 1 and 2 × $10^{-7}$ M, respectively. Respective concentrations of the FMRP variants (lanes 12–20) were the same (see below). Lanes 12–14, FMRP ΔPPId at molar ratios of 1:20, 1:10 and 1:5, respectively (30, 35 and 39%). Lanes 15–17, FMRP ΔKHT at molar ratios of 1:20, 1:10 and 1:5, respectively (25, 28 and 32%). Lanes 18–20, FMRP ΔRGG at molar ratios of 1:20, 1:10 and 1:5, respectively (30, 40 and 57%). R3, S14 and the 5′ sequences of S14 (ΔS14) are identified on the right. Markers are on the left. Note that the RNA products rapidly accumulate in the presence of a chaperone like NCp7 (lanes 3–5) or FMRP (lanes 9–11), whereas they do so only slowly in the absence of a chaperone (lane 2). (**C**) Ribozyme-directed cleavage of RNA substrate S20. Ribozyme R3 (0.3 pmol) and RNA S20 (0.1 pmol) were incubated as described in Materials and Methods. $^{32}$P-labelled RNA substrate (S20) and product (ΔS20) were analysed by denaturing 8% PAGE in 0.5× TBE. Lanes 1–2, R3 and S20 at 4 or 37°C for 30 min (5 and 25%). Lanes 3–4, HIV-1 NCp7 at protein to nucleotide molar ratios of 1:10 and 1:5, respectively (15%). Lanes 5–6, NC(12–53) at molar ratios of 1:1.2 and 1:0.6, respectively (10%). Lanes 7–8, FMRP at protein to nucleotide molar ratios of 1:10 and 1:5, respectively (16%), corresponding to a protein concentration of 1 and 2 × $10^{-7}$ M, respectively. Respective concentrations of the FMRP variants (lanes 9–14) were the same (see below). Lanes 9–10, FMRP ΔPPId at molar ratios of 1:10 and 1:5, respectively (16%). Lanes 11–12, FMRP ΔKHT at molar ratios of 1:10 and 1:5, respectively (17%). Lanes 13–14, FMRP ΔRGG at molar ratios of 1:10 and 1:5, respectively (20%).

poorly enhance ribozyme-directed cleavage of S14 RNA (lanes 12–14 and 15–17, respectively). The ΔRGG deleted version of FMRP proved to be ~3-fold less active than complete FMRP (lanes 18–20).

Next, we examined the effects of FMRP using RNA substrate S20, which is able to form an extended duplex of 20 nt with the hammerhead ribozyme (Fig. 4A) and which prevents activation of ribozyme cleavage by HIV-1 NCp7 or hnRNP A1 (33,38). In the absence of a chaperone, only minimal ribozyme-directed cleavage of RNA S20 was observed at 37°C (Fig. 4B, bottom, lanes 1 and 2) in contrast to what was seen with RNA S14 (Fig. 4B, top). NCp7 did not enhance ribozyme cleavage of RNA S20, in agreement with previous data (lanes 3–4) (31). FMRP and deleted versions were also found to exhibit very little, if any, enhancing activity using RNA S20 (bottom, lanes 7–14).

## DISCUSSION

FMRP is not only an RNA-binding protein (Supplementary fig. 1), but is also a nucleic acid chaperone, thus providing nucleic acid remodelling properties for this cellular protein. This conclusion is supported by several lines of evidence. FMRP promotes the hybridization of complementary DNAs under low ionic strength conditions (Fig. 2), directs formation of the most stable duplex structure by achieving strand exchange and, once nucleic acid molecules have been refolded into their most stable structure, the protein is no longer required to maintain the structure (Fig. 3). FMRP enhances ribozyme-directed cleavage of an RNA substrate, most probably by activating substrate RNA annealing to the ribozyme in physiological relevant conditions (Fig. 4). In agreement with these findings, FMRP can promote the hybridization of replication primer $tRNA^{Lys3}$ to a complementary sequence at the 5′ end of the HIV-1 genomic RNA, called the primer binding site (Supplementary fig. 2) (34,35). According to these criteria, FMRP resembles the YB-1/p50 protein, which is a ubiquitous cellular chaperone (32) bound to mRNPs, involved in mRNA metabolism and in the regulation of translation (39) including its own transcript (40).

Deleted variants of FMRP, namely, ΔPPId, ΔKH1, ΔKH2, ΔKH1/KH2 (ΔKHT), ΔPhd and ΔRGG (Fig. 1), were examined for their ability to activate the annealing of complementary sequences, the strand exchange process and for their ability to enhance ribozyme cleavage of an RNA substrate (Figs 2–4). The results of these analyses on the chaperoning properties of the deleted variants of FMRP reveal that all the aforementioned domains are required for full activity. More precisely, deletion of only the PPId domain or the two KH motifs caused the strongest reduction in nucleic acid chaperoning directed by FMRP (Figs 3 and 4). These results suggest that several domains of FMRP may need to simultaneously make contact with a nucleic acid molecule to effectively direct its transconformation, in common with the viral nucleocapsid protein (35). Since the PPId domain is required for full activity, FMRP probably achieves nucleic acid chaperoning when it is in a dimeric form. If two or more RNA/DNA species with complementary sequences are present, binding of FMRP will generate a network of protein–nucleic acid and protein–protein interactions to induce refolding and hybridization of the RNA/DNA molecules involved (Figs 2–4).

FMRP is associated with mRNPs complexes, which shuttle between translating ribosomes and cytoplasmic granules in cells (20). The emerging picture proposes that FMRP modulates translation through a network of protein–RNA and protein–protein interactions. According to our findings, FMRP should also act on mRNA metabolism and translation by means of RNA–RNA interactions. These interactions might be under the control of non-coding RNA, as proposed for BC1 RNA (27), or by causing mRNA dimerization by intermolecular G-quartet formation (9,10,15). By analogy with YB-1/p50, a major cellular chaperone associated with mRNPs (32,39), it can also be envisioned that a low level of FMRP should stimulate translation, whereas a high level of FMRP should mask the mRNA and hence inhibit its translation (20,23,29). In support of this notion, recruitment of FMRP into mRNPs and polyribosomes *in vivo* is abolished upon deletion of either the PPId domain or the KH motifs (3). Interestingly, these very same domains are important determinants of the nucleic acid chaperoning activities of FMRP *in vitro*, suggesting that FMRP needs all its nucleic acid binding and chaperoning activities to exert its chaperone function *in vivo*. Our results open new perspectives on the functional role(s) of FMRP in RNA metabolism. Further analyses are required to investigate these functional aspects.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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