Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of fmr1 protein isoforms

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Impaired expression of the FMR1 gene is responsible for the fragile X mental retardation syndrome. The FMR1 gene encodes a cytoplasmic protein with RNA-binding properties. Its complex alternative splicing leads to several isoforms, whose abundance and specific functions in the cell are not known. We have cloned in expression vectors, cDNAs corresponding to several isoforms. Western blot comparison of the pattern of endogenous FMR1 proteins with these transfected isoforms allowed the tentative identification of the major endogenous isoform as ISO 7 and of a minor band as an isoform lacking exon 14 sequences (ISO 6 or ISO 12), while some other isoforms (ISO 4, ISO 5) were not expressed at detectable levels. Surprisingly, in immunofluorescence studies, the transfected splice variants that exclude exon 14 sequences (and have alternate C-terminal regions) were shown to be nuclear. Such differential localisation was however not seen in subcellular fractionation studies. Analysis of various deletion mutants suggests the presence of a cytoplasmic retention domain encoded in exon 14 and of a nuclear association domain encoded within the first eight exons that appear however to lack a typical nuclear localisation signal.

INTRODUCTION

The fragile X syndrome, the most common cause of inherited mental retardation, is caused by an unstable expansion of a CGG repeat in the 5′ untranslated region of the FMR1 gene. The large expansions found in patients (>230 repeats) are abnormally methylated, and result in the transcriptional shut down of the FMR1 gene (1–4). A FMR1 knock out mouse model was established, that showed a rather similar phenotype and particularly impaired cognitive function (8). It is thus of importance to analyse the expression, and understand the role of the FMR1 gene. It is very widely, but not ubiquitously expressed, as shown by in situ hybridization (9,10) or by Western blot or immunohistochemical analysis of the FMR1 protein (FMRP) (11–13). The latter is detected in the cytoplasm, and is particularly abundant in neurones (11,14).

The 38 kb long FMR1 gene is composed of 17 exons, and is very widely, but not ubiquitously expressed, as shown by in situ hybridization (9,10) or by Western blot or immunohistochemical analysis of the FMR1 protein (FMRP) (11–13). The latter is detected in the cytoplasm, and is particularly abundant in neurones (11,14).

The 38 kb long FMR1 gene is composed of 17 exons, and is subject to alternative splicing that affects the presence of exon 12 and 14, and the choice of acceptor sites in exons 15 and 17 (15–17). On the basis of RT–PCR experiments, 20 protein isoforms would be predicted, and indeed 4–5 bands are detected in various tissues (11–14). In particular, exclusion of exon 14 alters the downstream reading frame, and potentially generates two very different types of carboxy-terminal regions, 12 or 24 and 88 or 105 amino acids long respectively (15,16), although there is yet no proof of the presence of such protein species in tissues. The major transcripts (that contain exon 14) code for proteins that contain two KH domains, and an RGG box. Such sequence motifs are found in many RNA binding proteins (18), and indeed an in vitro synthesised isoform, corresponding to the full-length protein, has been shown to bind RNA homopolymers (19) and some mRNAs (20). The significance of the KH domains have been further confirmed by the finding of a single patient with a very severe phenotype and a missense mutation (Ile367Asn) in a conserved position of the second KH domain (21). This mutation affects in vitro binding of RNA homopolymers (13,22). Both KH domains are present in all predicted isoforms but the RGG box is absent from those derived from transcripts lacking exon 14.

We have constructed eukaryotic expression vectors for several of the FMRP isoforms and used them in transfection studies. Unexpectedly, the isoforms that lack exon 14 sequences showed a nuclear localisation by immunofluorescence. Our results indicate that a domain encoded by exon 14 is required for cytoplasmic localisation of FMRP isoforms, and in its absence, a region contained in the N-terminal half directs the protein to the nucleus.

RESULTS

Human FMR1 protein isoforms

Extensive alternative splicing has been demonstrated for the FMR1 gene by RT–PCR on mouse and human RNA, that could potentially generate 20 protein isoforms ranging from 47 to 71 kDa (15–17) (see Fig. 1 and Table 1). The presence of at least four or five protein isoforms was detected in different human or mouse tissues although it could not be excluded that some of these correspond to post-translation modifications (11–14).

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We have cloned six of the splice variants described by Ashley et al. (15), including the ISO 1 form that includes all exons, by RT–PCR on RNA derived from human lymphoblasts. A seventh construct (FMR T), corresponding to ISO 17 (Table 1) was previously described (11). This confirmed the expression in lymphoblasts of mRNAs corresponding to the isoforms ISO 1, 4, 6, 7, 10 and 12. These isoforms show alternative splicing of exon 12 and/or of exon 14, using two different acceptor sites in exon 15 (see Fig. 1 and Table 1). The nomenclature in Table 1 is derived from that of Ashley et al. (15). In the latter paper, however, the alternate splicing in exon 17 is not discussed. We defined ISO 1 to 12 isoforms as containing the larger exon 17.

We constructed eukaryotic expression vectors expressing the various isoforms and transfected them in Cos cells. By Western blot analysis we compared the transiently expressed isoforms to the endogenous FMR1 proteins using a FMR1 specific monoclonal antibody (1C3, named 1a in ref. 11). Figure 2 shows the transfected isoforms ISO 1, 4, 6 and 7 compared to the endogenous FMRP bands in control untransfected Cos cells (lane 1) or in lymphoblasts (lanes 9 and 11). Due to multiple alternative splicing, different isoforms have a similar molecular weight (Table 1). The endogenous FMRP in Cos cells and in lymphoblasts showed one major band migrating at an apparent molecular weight of ~80 kDa, accompanied by three to four bands at 70–78 kDa (Fig. 2, lanes 1, 9, 11). The major band seen in the lymphoblast extracts, as well as in other cell types, showed the same electrophoretic mobility as ISO 7 (a form lacking exon 12 sequences), slightly faster than ISO 1 (compare lanes 11 and 12 in Fig. 2, see also Figs 5 and 6 below). The latter form was not detected among the endogenous proteins in the various cell types analysed. The smallest minor band migrates as ISO 6 (compare lanes 8 and 9).

The predicted MW of ISO 6, or its derivatives ISO 12 and 16 (that also lack exon 14) is 61, 58.8 and 59 kDa respectively and the exon 14 containing form with the nearest MW is ISO 19 at 64 kDa (Table 1). No endogenous bands in the range of ISO 4 were observed (provided that care had been taken to avoid proteolysis). It can be concluded that the exon 14 lacking forms with the short alternative C-termini (ISO 4, 5, 10, 11) are also undetected. Indeed, RT–PCR experiments have indicated that when exon 14 is excluded, use of the third exon 15 acceptor site (as in ISO 6, 12, 16) is predominant (15).

**Exon 14 is necessary for cytoplasmic localisation**

We have observed previously that a truncated FMRP construct deleted for the C-terminal half was targeted to the nucleus in
transfection experiments (11). We wondered if the differential splicing of FMR1 leading to different C-termini of FMRP, would target the protein to different cellular compartments. We analysed the intra-cellular localisation of various isoforms of FMRP by immunofluorescence after transfection of appropriate expression vectors in Cos cells. Whereas the ISO 1 and ISO 7 isoforms localised to the cytoplasm, as we have previously reported for endogenous FMRP (11), surprisingly the ISO 4, ISO 10, ISO 6, and ISO 12 isoforms displayed a nuclear localisation (Fig. 3 and results not shown). All the constructions showing this nuclear localisation splice out exon 14, thus creating a frame shift and either a short (ISO 4 and 10, first acceptor in exon 15) or long (ISO 6 and 12, third acceptor in exon 15) new C-terminal domain. This observation suggested that a sequence important for cytoplasmic retention might be situated in the C-terminal part, in the region coded by exons 14–17. To further analyse the sequence requirement for cytoplasmic localisation, we constructed C-terminal deletion mutants of FMR1. As a control we used the full length FMR T (11), (corresponding to ISO 17 in Table 1), which stained the cytoplasrn. Progressive deletions into FMR1 showed that exon 17 and 16, as well as exon 15 could be deleted without affecting the cytoplasmic location of the corresponding proteins (FMR N2 and FMR N3 constructs, Figs 1 and 3). However further deletions of exons 13 and 14 in the construct FMR N0 strikingly changed the staining to a nuclear localisation. Deleting more upstream sequences (up to exon 9 in FMR N) still kept the staining in the nucleus. Taken together, these results show that there is a determinant responsible for cytoplasmic localisation between exon 13 and exon 15. If we compare the data obtained for the natural isoforms ISO 4 and 6 with the data from deletion constructs, this narrows the relevant sequence down to exon 14.

From these results we conclude that there is a cytoplasmic retention domain encoded by exon 14, and a domain directing the protein to the nucleus in the N-terminal half (in exons 1–8). Simple inspection of the amino acid sequence encoded by exons 1–8 failed to reveal obvious nuclear localisation signals (NLS). The only putative consensus NLS in FMR1 consists of amino acids 597–601 KKEKP (3) and lies in the C-terminus that is
Figure 4. FMRP–β-galactosidase fusion proteins localise in the cytoplasm. The distribution of the chimeric protein in Cos transfected cells was assessed by histochemical staining using X-Gal. As a positive control, the nls of SV40 shows a nuclear localisation. N-gal is a construct extending from FMRP amino acid 1–284 and coordinates are given for the third construct.

deleted (or frame shifted) in the constructs showing nuclear localisation. Therefore further sequence(s) important for nuclear targeting have to be contained in the N-terminal part of the protein.

In order to search for an NLS like sequence, fragments of the N-terminus of the FMR1 gene were fused to the β-galactosidase gene (23). Indeed, in the region spanning exons 1–8, small basic signals such as amino acids RLR (111–113), KIK (128–130) in exon 5, KK (151–152) in exon 6, KR (179–180) and RTK (193–195) in exon 7 could be responsible for nuclear targeting. We transfected the β-galactosidase fusion constructs into three cell types (Cos, see Fig. 4, HeLa and NIH3T3, data not shown). The various portions of the N-terminal half, in particular the region encompassing exons 5–7, as in construct 97–218-gal, failed to direct the β-galactosidase into the nucleus (Fig. 4 and data not shown). The SV40 NLS used as positive control, gave a nuclear staining (Fig. 4). Thus we suggest that there is no functional NLS sequence in FMRP, but that an interaction of the N-terminus of FMRP with a nuclear component may direct the protein to the nucleus.

FMRP isoforms are present in both cytoplasmic and nuclear fractions

To further study the localisation of the different isoforms of FMRP, we fractionated cells into nuclear and cytoplasmic components that were analysed by Western blotting. In all cell types tested, FMRP could be detected in both cytoplasmic and nuclear fractions, while a control monoclonal antibody directed against a nuclear transcription factor (p62 subunit) (24) showed the expected nuclear localisation, with no signal in the cytoplasmic fractions (Fig. 5B,C). On the other hand, an anti-cytokeratine antibody (Sigma Immuno Chemicals C-1801) revealed a 56 kDa band highly enriched in the cytoplasmic fractions (not shown).

For each cell line a characteristic pattern of FMRP bands was reproducibly observed, that showed subtle differences between nucleus and cytoplasm. In all cell lines tested (monkey or human cells, Fig. 5A–C) the major band in total cell extracts, migrating just below ISO 1 was enriched in the nuclear fraction. Several of the minor bands were enriched in the cytoplasm. Presence of whole cells in the nuclear pellet cannot account for the observed level of FMRP bands, as such contamination, as judged by microscopic examination, was very low (<5%). In a lymphoblastoid cell line from a fragile X patient, no band was detected with the anti FMRP antibody, demonstrating that all bands observed in normal cells are indeed derived from the FMR1 gene (Fig. 5C).

We also used subcellular fractionation to analyse the localisation of the various transfected FMR1 isoforms. Surprisingly, all isoforms were found in both cytoplasmic and nuclear fractions, although the nuclear control antibody demonstrated a clean separation of the two fractions (Fig. 6). The immunofluorescence studies performed on a fraction of the same transfected cells confirmed the results shown in Figure 3. Similar results were obtained using a different method of nuclear preparation (see Materials and Methods, results not shown). We take this to mean that the association of FMRP with either cellular compartment is loose, allowing a leakage into the other fraction during extract preparation.

DISCUSSION

Extensive alternate splicing of the FMR1 gene has been described at the mRNA level in both man and mouse (15–17). Although 4–5 protein isoforms were indeed detected in various cells and tissues, the correspondence with the predicted mRNAs was not established. Furthermore, the functional significance, if any, of this multiplicity of isoforms remains unknown. It is therefore important to identify which isoforms are present in vivo, and whether they differ in some biological properties. To begin to address these questions, we have constructed expression vectors for seven isoforms, and have used transfection studies to compare them to endogenous FMR1 protein bands, and to analyse their intracellular localisation.
similar molecular weight ISO 12 or 16, may be present at exon 14 sequences. Nuclear staining was however observed in few mouse oesophageal cells (14). In subcellular fractionation studies, all isoforms were found in both compartments, suggesting that their association to nuclear or cytoplasmic components is loose, and disrupted by the fractionation method. The N-terminus of FMRP may be inducing nuclear targeting directly or via interaction with a nuclear component. The only putative consensus NLS was described in the C-terminus of FMR1 (KKEKP at position 597–601) (3), and is unlikely to be functional, as it is absent or frame shifted in constructs displaying a nuclear localization, and present in the cytoplasmic isoforms. In the N-terminal part of FMRP spanning exons 1–8, we noticed some putative bipartite basic signals (25), corresponding to amino acids RLR, KIK, KK, KR and RTK (see results), which could be responsible for nuclear targeting. Diverse N-terminal fragments were cloned in frame upstream of the β-gal coding sequence, but were unable to direct the fusion protein into the nucleus. Thus we suggest that there is no functional NLS sequence in FMRP, but rather that an interaction of the N-terminus of FMRP with a nuclear component may direct the protein to the nucleus. The KH domains that are involved in RNA binding (22) do not appear to be both necessary for nuclear localisation, as the second KH domain (but not the first) is deleted in the nuclear FMR P construct.

While for a long time it was believed that proteins were located in the cytoplasm by default, and that localisation in cell organelles depends on the presence or absence of a specific targeting signal, an increasing number of proteins have been shown to be held in detectable levels. A proposed identification as ISO 6, 12 or 16 would be in good agreement with Ashley et al. (15), who showed by RT–PCR that when exon 14 is spliced out, the third acceptor site of exon 15 is used much more than the first two. Definitive identification would require systematic 2D gel comparisons of endogenous proteins in different tissues, to the isoforms generated in transfection studies.

Our study demonstrates a striking difference between the isoforms encompassing exon 14, which are localised in the cytoplasm and those in which this exon has been spliced out, which are nuclear in immunofluorescence studies. The information necessary for nuclear localisation is present in the N-terminal half. The apparent contradiction with the cytoplasmic localisation of FMRP observed in various tissues by immunohistochemical analysis (11,14), may be due to the low level of isoforms lacking exon 14 sequences. Nuclear staining was however observed in few mouse oesophageal cells (14). In subcellular fractionation studies, all isoforms were found in both compartments, suggesting that their association to nuclear or cytoplasmic components is loose, and disrupted by the fractionation method. The N-terminus of FMRP may be inducing nuclear targeting directly or via interaction with a nuclear component. The only putative consensus NLS was described in the C-terminus of FMR1 (KKEKP at position 597–601) (3), and is unlikely to be functional, as it is absent or frame shifted in constructs displaying a nuclear localization, and present in the cytoplasmic isoforms. In the N-terminal part of FMRP spanning exons 1–8, we noticed some putative bipartite basic signals (25), corresponding to amino acids RLR, KIK, KK, KR and RTK (see results), which could be responsible for nuclear targeting. Diverse N-terminal fragments were cloned in frame upstream of the β-gal coding sequence, but were unable to direct the fusion protein into the nucleus. Thus we suggest that there is no functional NLS sequence in FMRP, but rather that an interaction of the N-terminus of FMRP with a nuclear component may direct the protein to the nucleus. The KH domains that are involved in RNA binding (22) do not appear to be both necessary for nuclear localisation, as the second KH domain (but not the first) is deleted in the nuclear FMR P construct.

As in previous studies, we observed 4–5 FMRP bands in various cells. The fainter bands were reproducibly observed, and are thus unlikely to be degradation products. The largest band we observed migrates as ISO 7 (deleting exon 12). No band at the level of ISO 1, the full-length FMR P, was detected in any of the cell types tested. It indicates that ISO 1 is very minor or absent. This confirms RT–PCR experiments showing that exon 12 is absent in at least 80% of the mRNAs, in both human and mouse (15,16). Splicing out exon 14 leads to a frameshift completely altering the C-terminal domain and generating either short or long alternate carboxy termini, according to the acceptor site used in exon 15. This alternative splicing is conserved between mouse and man, and should thus be biologically significant. The short isoforms were not detected in the cells studied. Of the isoforms in which exon 14 is deleted, only ISO 6, or its derivatives of similar molecular weight ISO 12 or 16, may be present at

Figure 5. Subcellular fractionation of three cell lines. (A) Analysis of cytoplasmic (C) and nuclear (N) fractions prepared from untransfected Cos cells. Lanes 2 and 4 are from one experiment and lanes 3 and 5 from a different preparation (20 µg of protein in each lane). Lanes 1, 6 and 7 show the transfected isoforms as size references (10 µg). (B) Fractionation performed on a human neuroblastoma cell line: SH-SY-5Y. Total cell extract (T, 10 µg), cytoplasm (C, 10 µg), wash (W, 10 µg) and nuclear fraction (N, 25 µg) are shown. (C) Lymphoblasts from normal (lanes 1–4) or fragile X (lanes 5 and 6) individuals are displayed after fractionation (50 µg protein in each lane). Symbols (T, C, W, N) as in panel B. Blots were probed with mAb 1C3; additionally in panels B and C a mAb directed against the 62 kDa subunit of transcription factor TFIIH (24) was used as a control, (np points to the nuclear protein).
the cytoplasm by specific sequences and we suggest that this may be the case for the exon 14 containing FMRP isoforms. Recent examples include Xenopus nuclear factor 7 (Xnf7) (26) and cyclin B (27). The subcellular localisation of Xnf7 is under developmental regulation: in oocytes and again after the midblastula transition it is transported in the nucleus by a classical NLS. However, upon oocyte maturation, phosphorylation exposes a cytoplasmic retention domain, which anchors the protein in the cytoplasm. Dephosphorylation on midblastula transition results in renewed nuclear entry (26). Cyclin B is cytoplasmic until the onset of mitosis and has recently been shown to possess a cytoplasmic retention domain in its N-terminus, which shows no sequence homology to that of Xnf7 (27). When we searched for sequence similarities between these cytoplasmic retention domains (CRD) and the FMR1 sequence, we found the best scores in exon 14: 45% similarity over 22 amino acids with the Xnf7 CRD, and 38% similarity over 45 amino acids with the cyclin B CRD (overlapping exon 14 and 15), (not shown). The significance of these similarities remains to be proven by experimental studies. A different mode of cytoplasmic retention is illustrated by the transcription factor NFκB whose nuclear localisation signal can be masked by the IκB protein, thus keeping the protein cytoplasmic and therefore inactive (reviewed by Thanos and Maniatis in ref. 28). The mode of action of cytoplasmic retention domains is unknown. They might mediate an interaction with a cytoplasmic protein or with components of the cytoskeleton. A putative interaction of FMRP with intermediating filament, which itself interacts with the nuclear membrane (29,30), might explain the presence of cytoplasmic isoforms of FMRP in nuclear fractions.

Differential interactions of the various isoforms with RNA or protein might account for the differential pattern seen in nuclear and cytoplasmic fractions. The exon 14 sequences that are necessary for cytoplasmic localisation are as well conserved as the KH domains in the newly described homologue of FMR1, the human FXR1 gene, and in Xenopus FMR1 (31). The FMR1 and FXR1 proteins were found to heterodimerise (R. Nussbaum, pers. comm.) and it will be interesting to see if exon 14 sequences play a role in such interaction.

The functional significance of isoforms lacking exon 14 sequences remains to be established. As they lack the RGG box often found in RNA binding proteins, they probably cannot bind RNA (13,19). Our studies suggest that some of the predicted isoforms indeed exist, albeit at low level (<10% of total FMRP in the cells analysed). Quantitative RT–PCR, or in situ analysis with specific oligonucleotide probes or antibodies may reveal whether exclusion of exon 14 is regulated during development, or is more important in some brain regions, and whether this exclusion is present also in the FXR1 gene (31). Experiments are underway (with Dr B. Oostra) to see if transgenic expression of the ISO 7 isoform may modify the phenotype of the FMR1 knock out mouse model of the fragile X syndrome (8).

**MATERIALS AND METHODS**

**Plasmid constructions**

Cloning of the different isoforms. RT–PCR was performed on total RNA isolated from a lymphoblastoid cell line. Different combinations of primers were used in order to cover the possible alternative spliced products. The HindIII(Ser277)-KpnI(Val399)-BamHI(Gly576) fragment allows the isolation of variants splicing out exon 14 as well as variants using different acceptor sites within exon 15. The HindIII(Gly576)-PstI (created by an appropriate PCR primer just downstream of the stop codon) fragment permits us to distinguish between the first and second acceptor site of exon 17. For each of these three fragments, the RT–PCR products were first cloned into pbBlueScript II. In a second step, different combinations of the fragments obtained in pbBlueScript were cloned together with the EcoRI-HindIII (Met1-Ser277) fragment from the previously described FMR T (ISO 17) (11) in the eukaryotic expression vector pTL1. The resulting isoforms ISO 1, 4, 6, 7, 10, 12 are represented in Figure 1.

Cloning of C-terminal deletion constructs. All constructs were cloned into the pTL1 vector. The shortest construct, FMR N has already been described (11). FMR N1 was generated by cloning of the EcoRI-KpnI fragment, corresponding to amino acids Met1 to Val399. FMR N2 and FMR N3 were prepared by co-cloning of an EcoRI-HindIII (Met1-Ser277) fragment and a PCR amplified fragment extending from HindIII (Ser277) to a BgIII created site at the end of exon 14 or 15 respectively. PCR was performed on the template ISO 1.

FMR-Δ14 was generated starting from ISO 7, by replacement of the HindIII (Ser277)-BamHI (Gly576) region with PCR products resulting in the deletion of exon 14 without altering the downstream reading frame. This was done by introducing an EcoRI site at the end of exon 13 and at the beginning of exon 15, which did not change the amino acid sequence (Leu-Lys425-Thr492-Asn-Ser) at the boundary of exons 13 and 15.

Cloning of FMR1 into a β-galactosidase expression vector. Various fragments of the N-terminus of the FMR1 gene were cloned in frame upstream of the β-galactosidase gene in the pCHK expression vector (23). PCR amplification was performed on the template ISO 1 with different combinations of primers in order to obtain overlapping fragments covering the FMR1 N-terminus. The fragment extending from Met1 to Asp284 (N-gal) was the equivalent of FMR N. Further constructs with N-terminal deletions of the first 97, 149, 213 amino acids and extending up to Asp284, were prepared. The region encompassing a putative bipartite NLS was also cloned, as a Ala97 to Arg218 segment comprising small stretches of basic amino acids in exons 5–7. As a positive control we used the SV40 NLS cloned in the same vector (gift of J. Ménissier-de Murcia) (23).

All constructions were verified by sequencing.

**Cell transfections and immunofluorescence detection**

Cos cells were transfected with 1–10 μg of various FMR1 constructs, as well as carrier DNA by the calcium phosphate co-precipitation technique (32). Forty hours after transfection cells were fixed according to three alternate procedures: (i) 2% paraformaldehyde for 4 min at room temperature; (ii) 100% methanol for 4 min at −20°C; (iii) methanol followed by acetone treatment for 2 min at −20°C. As no differences were observed, the first fixator (i) was used routinely and in Figure 3. After fixation cells were permeabilized with 0.1% Triton X100 for 15 min. After extensive washing in PBS, cells were incubated 2 h with 1:2 diluted hybridoma culture supernatant or with 1:5000 diluted ascites fluid at room temperature. The Cy3 conjugated anti mouse antibody diluted 1:200 and goat IgG were incubated
for 1 h. After washing with PBS, the nuclei were counterstained with Hoechst for 1 s.

**Subcellular fractionation**

The small volume procedure involves essentially the same steps required to make a nuclear extract according to the conventional protocol described by Dignam et al. (33). However, most centrifugation steps are performed in a microfuge and the lysis of the cells is done by rapidly pushing them through a hypodermic needle instead of using a Dounce homogenizer. The protocol described by Lee et al. (34) was used, but the final nuclear extract was not dialysed.

After incubation in ice-cold hypotonic buffer (see ref. 33) cells were lysed using a hypodermic needle and breakage of the cells was checked by microscopic examination. Centrifugation for 2 min at 3000 r.p.m. yielded the cytoplasmic fraction as supernatant. To avoid cytoplasmic contamination of the nuclear fraction, the pellet (nucleus and a few intact cells) was resuspended, treated as before and centrifuged a second time, giving a second cytoplasmic fraction. A 20 s centrifugation step at 10 000 r.p.m. was used to remove all the supernatant. After resuspension in a high salt buffer (see ref. 33) and incubation on ice, the nuclear debris were pelleted and the supernatant was collected as nuclear fraction.

Using a different buffer to swell cells in 10 mM Tris–HCl pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 0.3 M sucrose, breaking the cells and taking up the nuclear pellet in the above buffer without sucrose and with 400 mM KCl did not change the subcellular fractionation results.

**Histochemical staining of β-galactosidase fusion proteins**

FMR1-β-gal fusion proteins were expressed after transfection into three cell types (Cos, NIH3T3 and HeLa cells). At 48 h post-transfection, cells were washed, fixed and the β-galactosidase enzymatic activity was revealed by staining with X-Gal, and the reaction was carried out for 2–8 h at 37°C.

**Western blot analysis**

Whole cell extracts were prepared from transfected Cos cells, from lymphoblastoid cell lines or human neuroblastoma cell line SH-SY-5Y. Cell pellets were homogenised in lysis buffer 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM MgCl2, 0.5% NP40, 1 mM PMSF and protease inhibitors, left on ice for 15 min. Insoluble material was removed by centrifugation at 10 000 r.p.m. for 10 min at 4°C. Protein concentration of the supernatant was determined using a Bradford assay. Protein extracts (10–50 μg) were analysed on 7% SDS-polyacrylamide gels and immunoblotted onto nitrocellulose. The membranes were blocked in 3% nonfat dry milk and incubated with 1C3, a FMRP specific monoclonal antibody [previously described as 1a, (11)], diluting ascites fluid 1:10 000. The secondary antibody was coupled to peroxidase allowing revelation with the ECL kit (Amersham).

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**ABBREVIATIONS**

RT–PCR: reverse transcriptase–polymerase chain reaction; NLS: nuclear localisation signal.

**REFERENCES**