tryptic digestion and Vydac narrowbore C18 (300 Å, 2.1 mm by 150 mm) rpHPLC separation of tryptic fragments. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry was performed on a Finnigan Lasermat 2000. The putative lactacystin-modified residue on the primary lactacystin-binding protein was identified by adding a small amount of subunit X/MB1 isolated from [^{3}H]lactacystin-treated proteasome to a sample that had been treated with unlabeled lactacystin, and then isolating and sequencing radioactive tryptic fragments.
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Translational Suppression by Trinucleotide Repeat Expansion at *FMR1*

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Fragile X syndrome is the result of the unstable expansion of a trinucleotide repeat in the 5′-untranslated region of the *FMR1* gene. Fibroblast subclones from a mildly affected patient, each containing stable *FMR1* alleles with 57 to 285 CGG repeats, were shown to exhibit normal steady-state levels of *FMR1* messenger RNA. However, FMR protein was markedly diminished from transcript with more than 200 repeats. Such transcripts were associated with stalled 40S ribosomal subunits. These results suggest that a structural RNA transition beyond 200 repeats impedes the linear 40S migration along the 5′-untranslated region. This results in translational inhibition by trinucleotide repeat expansion.

Fragile X syndrome is a frequent cause of mental retardation that is inherited as an X-linked dominant with reduced penetrance (*1*). The mutational change in nearly all affected patients is the unstable expansion of a CGG trinucleotide repeat in the 5′-untranslated region of the *FMR1* gene (*2–4*). This repeat is normally polymorphic in length and content, exhibiting a mode of 30 cryptic repeats in the normal population, but the triplet is found in excess of ~230 repeats in affected patients, often approaching 1000 copies (*5, 6*). Male and most female carriers have an *FMR1* premutation with an intermediate number of repeats, between about 60 and 200 triplets. In most penetrant males with full-mutation alleles containing >230 CGG repeats, the *FMR1* gene is abnormally methylated and transcriptionally suppressed (*7–10*). The absence of the encoded protein, FMRP, a selective RNA-binding protein, is responsible for the clinical phenotype (*11*). About 15% of male patients do express *FMR1* mRNA and are termed mosaics, because they display a complex pattern of repeat size variation as well as incomplete methylation (*3, 7, 12*). A range of phenotypes from normal to penetrant, including severe mental retardation, has been found in this group. However, no clear correlation has emerged between the degree of hypomethylation, the extent of *FMR1* expression, and clinical involvement (*12, 13*). It has thus remained unclear if *FMR1* transcription is the sole determinant of penetrance or if other influences, such as translational suppression by lengthy CGG repeats in the *FMR1* transcript, affect FMRP levels.

A mildly affected 19-month-old male with near-normal cognitive and developmental abilities and slight physical features suggesting fragile X syndrome was studied (*14*). Polymerase chain reaction (PCR) analysis of the *FMR1* CGG repeat (Fig. 1A) showed a broad smear ranging in size from about 100 to 300 repeats in the patient (III-2), with a maternal premutation allele of ~70 repeats (II-3) and grandmaternal premutation allele of ~60 repeats (I-2), exhibiting the typical repeat instability found in fragile X syndrome kindred. We confirmed the PCR analysis by Southern (DNA) blot analysis (Fig. 1B) and demonstrated that the patient's gene was predominantly unmethylated (>90% by densitometry) by exhibiting cleavage with a methyl-sensitive enzyme, either Bss HII or Eag I. The broad 3.6-kb band, observed in both lymphocytes and fibroblasts of the patient, reflected a mean repeat length of ~290 triplets in an unmethylated state. Thus, this patient was atypical of most fragile X patients and carriers, because the repeat length was larger than that observed in nonpenetrant carriers, but in contrast to affected patients, including most mosaic males, nearly all cells exhibited the normal, unmethylated status of the *FMR1* gene.

Normal levels of *FMR1* mRNA were detected in the patient's lymphocytes and fibroblasts. Reverse transcriptase PCR across the repeat demonstrated concordance of repeat lengths between DNA and RNA. However, a protein immunoblot with a monoclonal antibody against FMRP (*15*) revealed only ~30% of normal FMRP levels relative to the control, β-tubulin. Instead of a general reduction in FMRP levels, immunofluorescent staining of FMRP in the patient's fibroblasts showed a mosaic pattern of variable reactivity (Fig. 2). This was in contrast to the more consistently positive cells of a normal male or the uniformly negative cells of a typically affected male, suggesting a FMRP level intrinsic to each cell of the patient.

To more fully evaluate this finding, we isolated individual fibroblast clones from low-density cultures. Seven clones were identified for further study by PCR amplification of the *FMR1* CGG repeat from DNA samples derived from independent fibroblast colonies. The clones displayed discrete repeat lengths of 57, 168, 182, 207, 266, 285, and 285 triplets, which spans the range of mosaicism in the patient's fibroblasts (Fig. 3A). Each clonal isolate contained hypomethylated *FMR1* alleles, which maintained a stable repeat length upon culture expansion. Steady-state RNA levels were evaluated in each clonal population by ribonuclease (RNase) protection of *FMR1* mRNA hybridized with ^{32}P-labeled antisense RNA and normalized to γ-actin signal as control. No significant differences in steady-state levels of *FMR1* mRNA were observed between or among the normal controls (30 and 32 repeats) and the patient's clonal cell populations (57 to 285 repeats) (Fig. 3B). Densitometric quantitation of *FMR1* signal (*16*) showed 101 ± 21.4 units for normals (n = 14) versus 124 ± 29 units for the patient's clones (n = 7). A typical fully penetrant male sample, with about 962 methylated repeats, showed no *FMR1* transcript, as expected (Fig. 3B, lane 9). These data therefore confirm and extend our previous demonstration of quantitatively similar levels of *FMR1* gene transcription between normal and premutation alleles of <100 CGG repeats (*16*).

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Despite the presence of normal levels of *FMR1* mRNA among these clones, a protein immunoblot with antibodies to FMRP showed a marked reduction in FMRP levels in clones with >200 CGG repeats (Fig. 3C). Clones with repeat lengths of 207 and 266 triplets showed FMRP levels that were 24% and 12%, respectively, of the normal. At 285 CGG repeats (lane 8), FMRP was nearly undetectable, whereas levels of β-tubulin remained unchanged. These results indicate that the lengthy trinucleotide repeat in the *FMR1* transcript influences FMRP translation and is consistent with experimental evidence at other loci where secondary structure in the 5′-untranslated region of mRNAs, particularly CG-rich regions, can impede translation (*17*). On the basis of the scanning model of translational initiation (*18*), it is therefore reasonable to predict that the 40S initiation complex is unable to proceed through the lengthy CGG repeat of the *FMR1* transcript, stalling translation.

To obtain direct evidence of stalled 40S ribosomal subunits, we fractionated cytoplasmic lysates on sucrose gradients, separating polysomes and their components (Fig. 4A), and then quantitated *FMR1* transcript in each fraction by RNase protection. In fibroblasts with 30 *FMR1* CGG repeats, ~90% of *FMR1* transcript was bound to approximately 4 to 12 assembled ribosomes (Fig. 4B), as determined by absorption peaks of gradient fractions at 254 nm (OD_{254}) (Fig. 4A), with undetectable stored transcript in the messenger ribonucleoprotein particle (mRNP) pools at the top of the gradient (in quiescent cells, mRNPs containing *FMR1* transcript sediment at ~13S). This finding is consistent with active *FMR1* translation. Comparable data was obtained with normal lymphoblasts, indicating that *FMR1* transcript is loaded with a similar number of translating ribosomes. In contrast, the cloned cells containing 266 CGG repeats, which produced only ~12% of the normal amount of FMRP (lane 7, Fig. 3C), revealed >70% of the *FMR1* transcript cosedimenting with 40 to 80S ribosomal subunits (fractions 17 and 19 in Fig. 4C). At 285 repeats, most *FMR1* transcript cosedi-

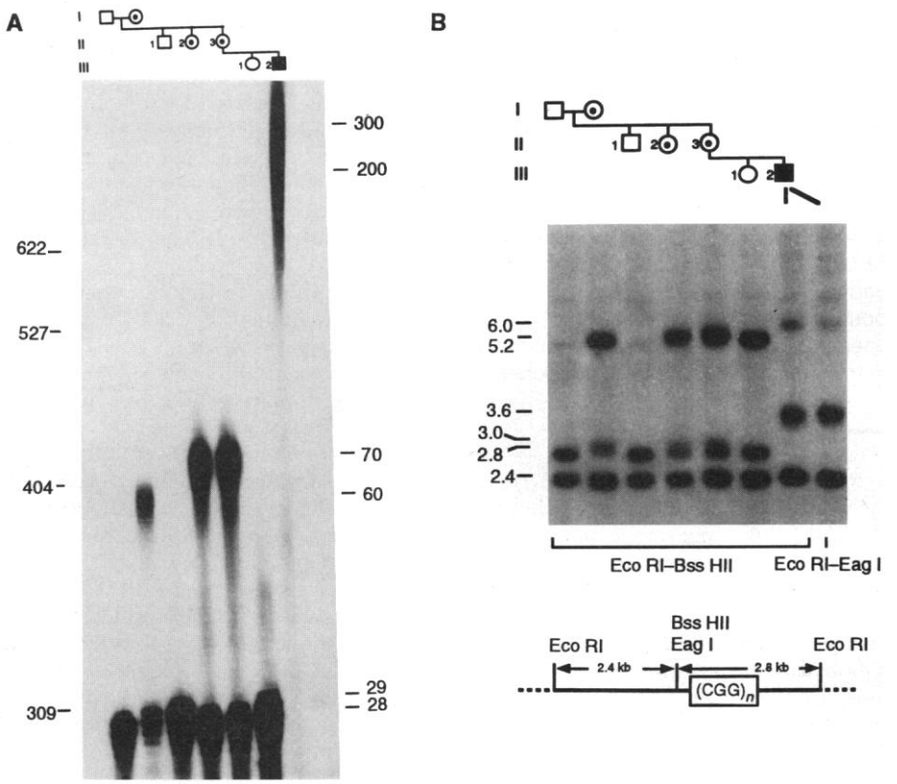

Fig. 1. DNA studies of the *FMR1* gene in the patient's family. The filled square is the patient, partially filled circles are female carriers, and open squares and circles are normal individuals. (**A**) Autoradiogram of polyacrylamide gel separating the products of PCR amplification of the *FMR1* CGG repeat from genomic DNA; primers flanking the repeat were used (*25*). Size markers on the left indicate base pairs as determined from Hpa II digests of pBR322; corresponding numbers of CGG repeats are shown on the right. Normal: 6 to 52 repeats; premutation carrier: ~60 to 200 repeats; full-mutation affected: >230 repeats (*1*). (**B**) Determination of *FMR1* CGG-repeat number and methylation status by Southern blot analysis of genomic DNA with probe pE5.1, as described (*2, 20*). Analysis of normal male DNA results in 2.4- and 2.8-kb bands, reflecting cleavage of a 5.2-kb Eco RI fragment (containing the 5′ portion of *FMR1*) by the methylation-sensitive enzyme Bss HII (or Eag I). Normal female DNA exhibits a similar pattern (2.4- and 2.8-kb bands) from the active X chromosomes plus a Bss HII–resistant, and therefore methylated, 5.2-kb band from the inactive X chromosomes. Female premutation carriers exhibit additional complexity with additional bands reflecting the CGG-repeat lengthening of the 2.8- and 5.2-kb bands, resulting in the shifting or broadening of the normal banding pattern. Full-mutation affected males typically show absence of cleavage by either Bss HII or Eag I, resulting in the substitution of the normal 2.4- and 2.8-kb bands with a large, diffuse smear in excess of 5.8 kb (*1*). In contrast, the DNA from the mosaic patient, after either Eco RI–Bss HII or Eco RI–Eag I digestion, showed <10% of the Eco RI fragments being methylated (and therefore resistant to either Bss HII or Eag I, showing only a faint band at ~6 kb). Unmethylated Eco RI fragments from this patient were cleavable by Bss HII or Eag I, resulting in the normal 2.4-kb fragment of the *FMR1* promoter region and, instead of the normal 2.8-kb fragment of exon 1 (containing the CGG repeat), a broad band of about 3.6 kb reflecting the increase in the CGG-repeat length by ~800 bp.

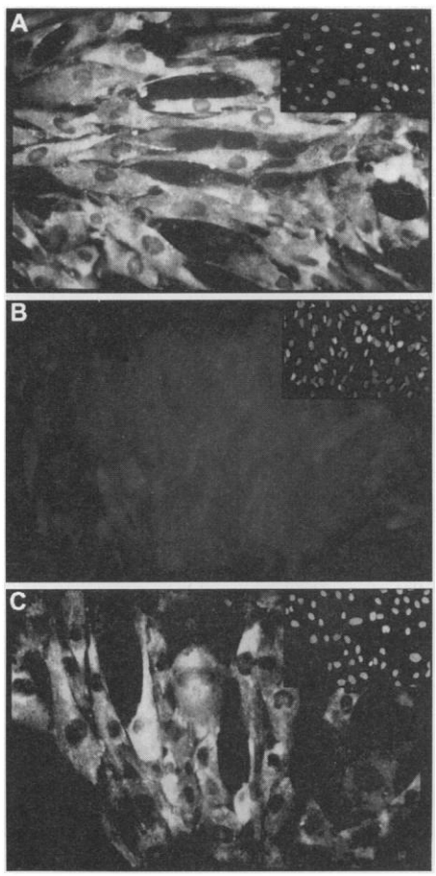

Fig. 2. Expression of FMRP in cultured fibroblasts of (**A**) a normal male, (**B**) a typical fragile X syndrome male without *FMR1* expression, and (**C**) the male patient investigated here. Immunofluorescence of FMRP (*26*) reveals the protein as cytoplasmic fluorescence in (A) and (C). (**Insets**) Diamidinophenylindole nuclear fluorescence of the respective fields to identify each cell, regardless of FMRP staining.

mented with 40 to 80S ribosomal subunits (Fig. 4D), whereas hypoxanthine phosphoribosyl transferase (HPRT) transcript, as a control, associated with polyribosomes (Fig. 4E). A similar profile of polysome-associated HPRT transcript was found among all clones studied, regardless of *FMR1* CGG-repeat lengths. Because multiple 40S initiation complexes can be as-

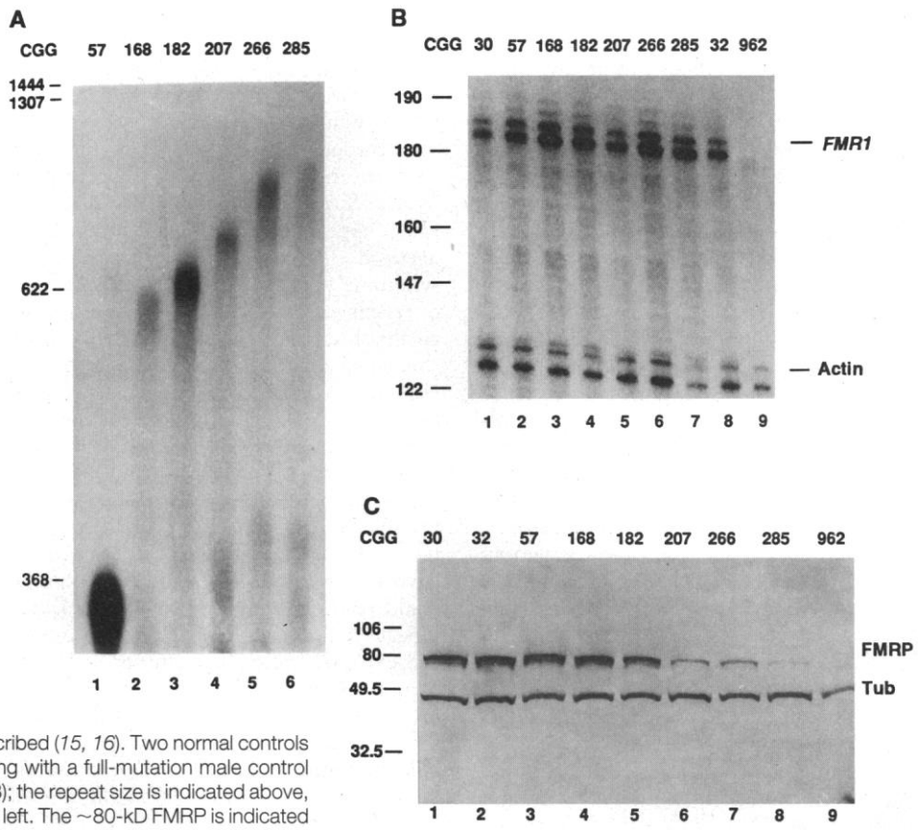

Fig. 3. *FMR1* characterization of independent fibroblast clones from the patient containing 57 to 285 CGG repeats (*27*). (**A**) CGG-repeat length in the cloned fibroblast cell lines. PCR amplification across the *FMR1* repeat was performed on genomic DNA from each clone with the presence of ^{32}P-α-deoxycytidine 5′-triphosphate (*25*). The estimated number of CGG repeats is shown at the top of each lane with size references on the left (in base pairs, determined from Hpa II and Taq I digests of pBR322). (**B**) Steady-state *FMR1* mRNA levels in the cloned fibroblast cell lines, as measured by RNase protection. The CGG-repeat length of the *FMR1* gene is shown above, with molecular weight size markers (in base pairs) on the left. RNase protection was performed as described (*16*) with a radioactively labeled 186-bp antisense RNA of exons 8 and 9 of *FMR1* and, as control, a labeled 128-bp antisense fragment of γ-actin. The multibanded patterns resulted from slight breathing of the RNA:RNA duplex (*16*). Fibroblast clones with 57 to 285 repeats are shown in lanes 2 through 7, with normal controls containing 30 and 32 repeats (lanes 1 and 8) and a typical penetrant male fragile X syndrome patient (~962 repeats) in lane 9. Absence of the *FMR1* signal in lane 9 demonstrates the specificity of the assay. (**C**) Protein immunoblot of FMRP in the cloned fibroblast cell lines. We resolved 7 μg of protein per lane through a 12% polyacrylamide gel using antibodies to FMRP and to tubulin, as previously described (*15, 16*). Two normal controls of 30 and 32 repeats (lanes 1 and 2) are shown along with a full-mutation male control (lane 9) and the six fibroblast clones (lanes 3 through 8); the repeat size is indicated above, and molecular weight markers (kilodaltons) are on the left. The ~80-kD FMRP is indicated on the right, as is the ~50-kD β-tubulin (Tub) control.

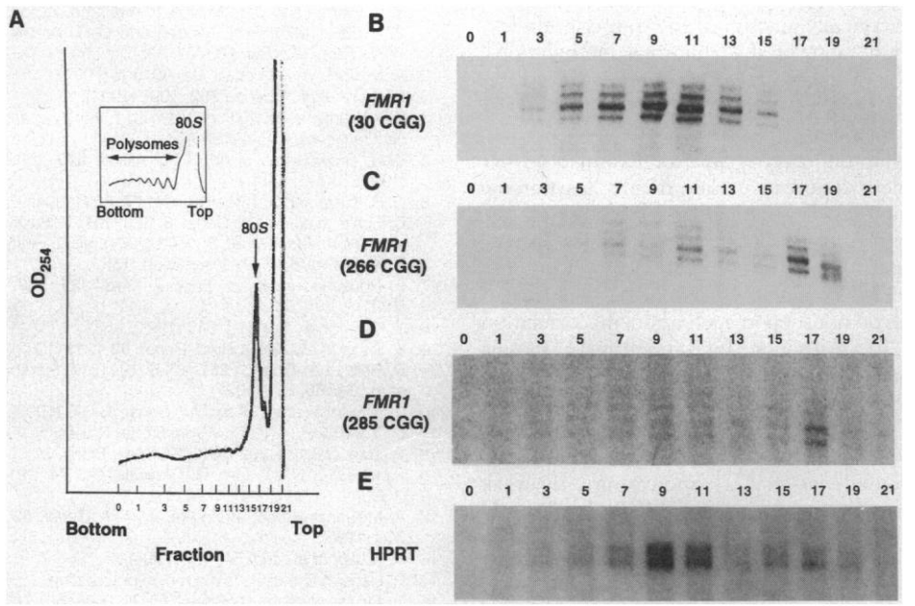

Fig. 4. *FMR1* transcript in fractionated polysomes and their components (*28*). (**A**) Sucrose gradient fractionation of polysomes and components. A 17.1 to 51% linear sucrose gradient of cytoplasmic extract was continuously monitored by absorption at 254 nm (OD_{254}) and was fractionated into 21 500- to 600-μl fractions. Parallel gradients were fractionated in the same way and subjected to RNA isolation and RNase protection. Fractions 19 through 21 contain free RNP particles followed by 40S (fractions 17 through 19) and 80S (fractions 15 through 17) ribosomal complexes. Monosomes and polysomes are found among fractions 1 through 15 and are more clearly shown at higher sensitivity of the absorbance scale (inset), where each peak from the large 80S fraction approximates *n* + 1 ribosomes. (**B**) *FMR1* transcript within gradient fractions from normal fibroblasts with 30 CGG repeats. Each gradient fraction was extracted, RNA isolated, and subjected to RNase protection, as described (*16*). All *FMR1* transcript is associated with polysomes. (**C**) *FMR1* transcript within gradient fractions from fibroblasts with 266 CGG repeats. The majority of transcript is associated with fractions 17 through 19, containing the 40S preinitiation complex with some transcript on polysomes, consistent with the observed 12% of normal FMRP level in the 266-repeat clone. (**D**) *FMR1* transcript within gradient fractions from fibroblasts with 285 CGG repeats. The majority of transcript is associated with fractions 17 through 19, containing the 40S preinitiation complex. Another independent clone with 285 repeats and little FMRP showed a similar profile. (**E**) HPRT transcript within gradient fractions (*29*) from fibroblasts with 285 CGG repeats showing the majority of transcript associated with polysomes. This pattern was seen in all the clones studied containing 30 to 285 *FMR1* repeats.

sociated with single mRNA molecules, especially for the mRNAs containing long 5′-untranslated regions (*19*), these data suggest the presence of stalled 40*S* initiation complexes that are unable to scan through >200 CGG repeats. Thus, the reduced FMRP levels observed in the patient's mosaic cell clones can be explained solely by the poor translational initiation efficiency caused by the expanded CGG-repeat segment.

These data support the scanning model of translation initiation and suggest that the 40*S* initiation complex does not hop over inhibitory RNA structure, in this instance, to reach the initiation codon (*18*). However, the 40*S* subunit can actually scan through as many as 182 CGG repeats. Previous in vitro evidence of sequence-dependent subunit stalling involved far fewer repetitive elements but with obvious secondary structure (*17*). In this regard, the finding of a rather narrow window of 25 repeats (Fig. 3C; 182 versus 207 repeats) within which a significant change in translation initiation occurs may indicate a structural RNA transition impeding 40*S* initiation complex scanning at ~200 trinucleotide repeats. Furthermore, this observation of translational inhibition by lengthy repeats may now be exploited as an experimental strategy to examine repeat expansion in cells or animals by placing fewer than 200 trinucleotide repeats in the 5′-untranslated region of a reporter or selectable transgene.

Although translational inefficiency caused by expanded CGG repeats is inconsequential to the majority of patients with fragile X syndrome (because the *FMR1* gene is typically inactive at the transcription level because of the abnormal methylation), this finding does have significant implication relating to the syndrome. The data explain discrepancies between the degree of symptom severity and the extent of *FMR1* methylation in mosaic fragile X syndrome patients. Despite earlier hopes that mosaic patients, who frequently exhibit partial transcription of the *FMR1* gene, would exhibit less clinical involvement, recent studies do not support such a general correlation (*20*). Although high-functioning mosaic male patients have been reported (*21*), a recent study has shown a wide range of FMRP levels (10 to 60% of normal) among penetrant mosaic males with >90% hypomethylation (*22*), indicating only weak correlation between FMRP levels and methylation. Because mosaic patients often exhibit widely variable degrees of both methylation and repeat-length mosaicism, it is apparent from the studies reported here that the repeat length of the hypomethylated alleles, and consequently the FMRP level, may be a critical determinant of disease severity among this group.

Therapeutic strategies have been proposed involving 5-azacytidine–induced hypomethylation of patients' *FMR1* genes (*7–9*). Because there are no coding changes in the *FMR1* gene (because the CGG repeat is in the 5′-untranslated region), release of the transcriptional inhibition by methylation reduction could theoretically restore FMRP production. However, the inability of the 40*S* initiation complex to scan through 285 CGG repeats, far fewer than the number observed in the typical fragile X patient [mean of 780 repeats (*20*)], appears to preclude this option. Indeed, these data suggest that fragile X syndrome could occur solely on the basis of repeat expansion, without the associated abnormal *FMR1* methylation. Likewise, this effect on translation could have consequence regarding trinucleotide repeat expansions elsewhere in the human genome. Although expansion of coding CAG repeats beyond 200 triplets has not yet been observed at loci such as that responsible for Huntington's disease (*23*), it would be of interest to determine whether or not an assembled ribosome could read through such a lengthy CAG repeat. If ribosome translocation through the expanded allele's transcript were impaired, reduced clinical involvement might be anticipated because the abnormal protein, containing a lengthened polyglutamine tract believed to cause altered properties (*24*), would be diminished in abundance.

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25. PCR amplification of the *FMR1* CGG repeat was performed under the conditions outlined in (*5*) with primers C and F described in (*8*).
26. Fibroblasts were established from a skin biopsy and cultured in Dulbecco's modification of Eagle's medium supplemented with 15% fetal bovine serum. Fibroblasts growing on chamber slides were reacted with murine anti-FMRP monoclonal antibody (80 μl of a 1:200 dilution of ascites fluid per slide) as described (*15*). Cy3-conjugated AffiniPure goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories) was used as the secondary antibody. After cover slip mounting with diamidinophenylindole (DAPI)–antifade solution (Oncor, Gaithersburg, MD), the cells were examined at ×400 magnification by epifluorescence with Zeiss filter cubes 02 and 15 for DAPI and Cy3 visualization, respectively.
27. Passage 4 cultures were trypsinized and plated at either 500 or 1000 cells per 100-mm dish, and well-demarcated colonies were isolated about 2 weeks later and expanded for study.
28. Cytoplasmic extract was prepared and fractionated on 17.1 to 51% linear sucrose gradient as described by H. B. J. Jefferies, G. Thomas, and G. Thomas [*J. Biol. Chem.* **269**, 4367 (1994)] except that a 2 M sucrose cushion was included at the bottom of the gradient. A total of 21 fractions containing 0.5 to 0.6 ml of the linear gradient were collected from bottom to top starting at the interface. Each fraction was subject to proteinase K digestion followed by phenol-chloroform extraction in order to isolate the total RNA. The ethanol-precipitated RNA in each fraction was subjected to RNase protection analysis (*16*).
29. A RT-PCR product relative to base pairs 222 through 608 of human HPRT mRNA [D. J. Jolly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 477 (1983)] was cloned into pCRII Vector (Invitrogen, San Diego, CA). The plasmid was linearized at the Xho I restriction site within the insert, and the antisense strand was synthesized from the *SP6* promoter by in vitro transcription in the presence of 60 μCi of α-^{32}P–uridine 5′-triphosphate. This riboprobe was used together with the antisense *FMR1* riboprobe in each hybridization, and the protected 358-bp HPRT fragment served as an internal control to represent the distribution of actively translated mRNA on the sucrose gradient.

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