

Defining early steps in mRNA transport: mutant mRNA in myotonic dystrophy type I is blocked at entry into SC-35 domains

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In myotonic dystrophy type 1 (DM1), triplet repeat expansion in the 3' untranslated region of *dystrophia myotonica protein kinase* (*DMPK*) causes the nuclear retention of mutant messenger RNA (mRNA). Although the *DMPK* gene locus positions precisely at the outer edge of a factor-rich SC-35 domain, the normal mRNA consistently accumulates within the domain, and this RNA is depleted upon transcriptional inhibition. In DM1, mutant transcripts detach from the gene but accumulate in granules that abut but do not enter SC-35 domains, suggesting that RNA entry into the domain is blocked. Despite their

exclusion from these compartments, mutant transcripts are spliced. MBNL1 (muscleblind-like protein 1) is an alternative splicing factor that becomes highly concentrated with mutant RNA foci. Small interfering RNA-mediated knock-down of MBNL1 promotes the accumulation or entry of newly synthesized mutant transcripts in the SC-35 domain. Collectively, these data suggest that an initial step in the intranuclear path of some mRNAs is passage from the gene into an SC-35 domain and implicate these structures in postsplicing steps before export.

Introduction

Nuclear mRNA transport is often thought of in terms of translocation through the nuclear pore, but mRNA export also requires intranuclear progression of transcripts from the gene to the nuclear pore. In some genetic diseases, failed export of a mutant mRNA is critical to the phenotype, yet typically it is not well understood how nuclear export is impeded or whether mutant mRNA accumulates at a specific point within the nuclear structure. In fact, the examination of mRNA blocked at a specific step in export may help illuminate the path whereby mRNA normally transits from the gene to the nuclear pore. The analysis of human disease gene mutations that impact nuclear metabolism of the mRNA provides an avenue to study both disease pathogenesis and the interrelationship between nuclear structure and steps in mRNA biogenesis. In addition, the study of naturally occurring disease alleles in patient cells provides the advantage that the mutant mRNA is expressed in a normal structural and physiological context.

This study examines the intranuclear fate of normal and triplet repeat-expanded transcripts in myotonic dystrophy type 1

(DM1) to define the point in the nuclear structure where the progression of normal and mutant transcripts diverge, which, in turn, provides insight into the step at which mutant mRNA transport is blocked. In DM1, expansion of a CTG triplet repeat occurs in the 3' untranslated region of the gene encoding *dystrophia myotonica protein kinase* (*DMPK*), a serine/threonine protein kinase (Aslanidis et al., 1992; Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). Normal alleles have 5–35 repeats, whereas DM1 alleles have 50 to >1,000. It has been shown that the mutant *DMPK* genes encode mRNAs containing expanded repeat sequences and that these RNAs are sequestered in the nucleus, where they accumulate in discrete nuclear foci (Taneja et al., 1995; Hamshere et al., 1997; Liquori et al., 2001). However, it is not known whether normal *DMPK* mRNA associates with any defined intranuclear nuclear structure after its transcription and whether this differs for the mutant RNA.

In addition to the defect in mRNA transport in DM1, it is hypothesized that the expanded repeat RNAs alter the nuclear distribution or activity of specific CUG-binding proteins, which, in turn, affects the alternative splicing of other pre-mRNAs (Day and Ranum, 2005; Osborne and Thornton, 2006). Multiple proteins such as CUG-binding protein (Timchenko et al., 1996, 2001; Phillips et al., 1998), heterogeneous nuclear RNP H (hnRNP H; Kim et al., 2005), double-stranded RNA-binding proteins

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Abbreviations used in this paper: *DMPK*, *dystrophia myotonica protein kinase*; hnRNP H, heterogeneous nuclear RNP H; HSMM, human skeletal muscle myoblast; MRG, mutant RNA granule.

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(Tian et al., 2000), or transcription factors Sp1 and retinoic acid receptor γ (Ebraldize et al., 2004) have been identified as interacting with CUG repeat RNA. MBNL1 (muscleblind-like protein 1), a known regulator of alternative splicing (Pascual et al., 2006), is an especially strong candidate. In DM1 cells, MBNL1 is sequestered in mutant *DMPK* RNA foci (Miller et al., 2000; Fardaei et al., 2001; Mankodi et al., 2001), and the loss of MBNL1 results in a phenotype that has similarities to DM1 (Kanadia et al., 2003).

Understanding how mutant *DMPK* RNA transport is blocked requires an appreciation of the relationship of the normal RNA to nuclear structure. The mammalian nucleus is comprised of several nonmembrane bound compartments, each containing specific subsets of macromolecules. Of particular relevance here, a host of pre-mRNA metabolic factors concentrate markedly in 10–30 irregularly bordered domains (Nyman et al., 1986; Carter et al., 1991; Spector et al., 1991; Blencowe et al., 1994; Hall et al., 2006), which are defined here by staining for the spliceosome assembly factor SC-35 (Fu and Maniatis, 1990). These SC-35 domains (also known as speckles or splicing factor compartments) are also highly enriched in poly(A) RNA (Carter et al., 1991; Xing et al., 1993). SC-35 domains are associated with a specific subset of active genes (Jolly et al., 1999; Smith et al., 1999; Moen et al., 2004), with many genes and gene-rich R-band DNA clustering around their periphery (Shopland et al., 2003). Importantly, the genes position at the immediate edge of an SC-35 domain but not within them, whereas several pre-mRNAs have been shown to detach from the gene and enter the domains (Xing et al., 1995; Smith et al., 1999), which contain multiple distinct mRNAs (Shopland et al., 2002).

Several observations suggest that the egress of specific gene transcripts from a peripheral gene into the domain may be linked to postsplicing steps rather than just splicing itself. For *COL1A1* (collagen type 1 $\alpha 1$) RNA, it was shown that most introns are removed at the gene just outside the domain, which is consistent with most splicing being cotranscriptional (Johnson et al., 2000). Some genes lacking introns become associated with domains upon activation (Jolly et al., 1999). More recently, several factors linked to postsplicing complexes required for mRNA export have also been shown to be concentrated within these domains (Zhou et al., 2000; Masuda et al., 2005; Schmidt et al., 2006).

In osteogenesis imperfecta type I, a splicing defect in intron 26 of the collagen 1 $\alpha 1$ gene results in an abnormal accumulation of the mutant collagen RNA within SC-35 domains, indicating an impediment to the egress of the RNA from the domain (Johnson et al., 2000). This study suggested that SC-35 domains are a screening point for properly processed mRNA, a concept supported by a study that described the movement of poly(A) RNA through SC-35 domains (Molenaar et al., 2004). We have proposed that nuclear export of some specific mRNAs begins by passage through the SC-35 domain neighboring the gene. This process may consist of two distinct steps: entry of the newly formed mRNA into the domain and exit of export-ready mRNA from the domain. The study of osteogenesis imperfecta type I provides evidence for the second step, as it was shown that the exit of mutant mRNA from domains was blocked or impeded. However, it is not clear whether entry into the domain from the peripheral gene is also a discrete step.

In this study, we examine the status of normal and mutant *DMPK* RNA relative to intranuclear structure in both normal myoblasts and myoblasts from individuals with DM1. Although both normal and mutant transcripts detach from the gene, they show distinct distributions relative to SC-35 domains. Normal *DMPK* transcripts consistently accumulate within the SC-35 domain, emanating from a gene precisely positioned at the outer edge of the domain. In contrast, mutant transcripts detach from the gene but no longer progress within the SC-35 domain and accumulate in multiple round foci that accumulate at the outer boundary of SC-35 domains. We provide direct visual evidence that mutant *DMPK* RNA in foci is spliced despite its exclusion from the splicing factor-rich domains. We also examine the role of the MBNL1 protein in the sequestration of *DMPK* RNA carrying the repeat expansion and demonstrate that loss of MBNL1 restores the ability of the mutant *DMPK* RNA to accumulate in domains. Our results suggest that egress of *DMPK* transcripts from the gene into an SC-35 domain is a normal step in the nuclear progression of this mRNA, and transcripts from the mutant allele are blocked from normal entry into SC-35 domains. This block correlates with the failed cytoplasmic export of mutant *DMPK* RNA, suggesting that export is not blocked at the nuclear pore but at a very early step in nucleoplasmic transport.

Results

DMPK mRNA accumulates within SC-35 domains

Although several active genes and their mRNAs associate closely with SC-35 domains, this association is locus specific, as has been observed for slightly more than half of the 25 active endogenous genes studied (Hall et al., 2006). To determine whether the normal *DMPK* gene and its RNA associate with SC-35 domains, a sequential hybridization technique (Xing et al., 1995; Johnson et al., 2000) was used to detect the *DMPK* mRNA in one color using a cDNA probe (2.3 kb) and the gene in a second color using a cosmid probe (~40 kb). As shown in Fig. 1 A, after hybridization to normal differentiated myotube nuclei, *DMPK* RNA is usually seen in two irregular tracklike accumulations that are larger than and juxtaposed to the *DMPK* locus signal. RNA hybridization with the *DMPK* cDNA probe and costaining with an antibody to the spliceosome assembly factor SC-35 demonstrate that these RNA accumulations are found within SC-35 defined domains (Fig. 1, C–J), with >84% ($n = 316$) of signals examined (see Fig. 3) clearly overlapping part or most of the domain. 3D analysis (Fig. 1, K–P and Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200706048/DC1>) confirms that the *DMPK* RNA lies largely within the domain.

DNA hybridization of the *DMPK* gene locus also shows that 70–90% associate with an SC-35 domain ($n = 136$ signals; Fig. 1 B). We initially defined association to indicate that two entities appear to be in direct contact as viewed by light microscopy, with no visible separation (Xing et al., 1993). By this strict criterion, 70% of *DMPK* loci associate with a domain, but an additional 20% show just a minute hairline separation (Fig. 1 B, left inset); thus, up to 90% of *DMPK* genes localize to the region of active genes that surrounds an SC-35 domain. This contrasts with

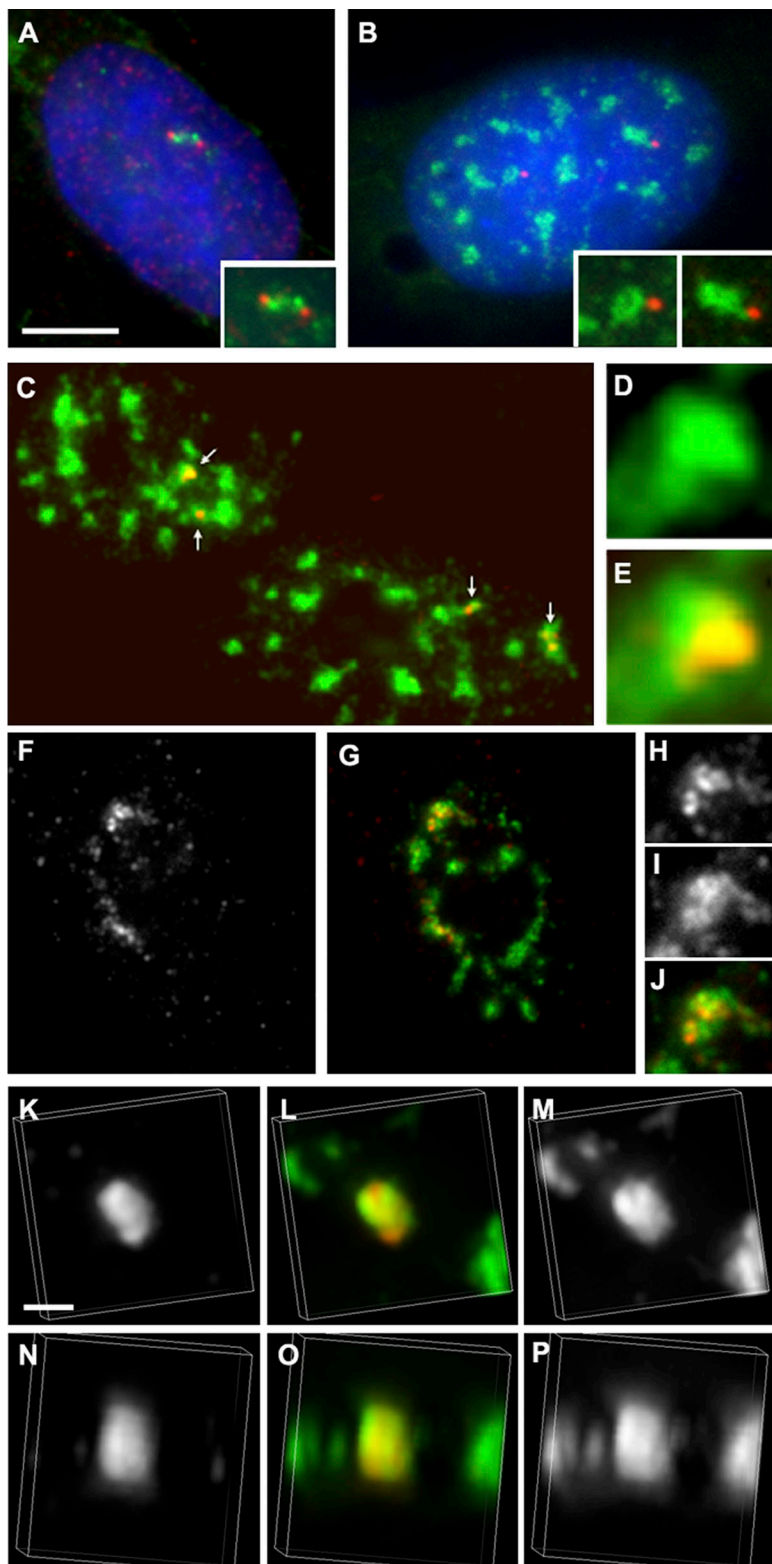
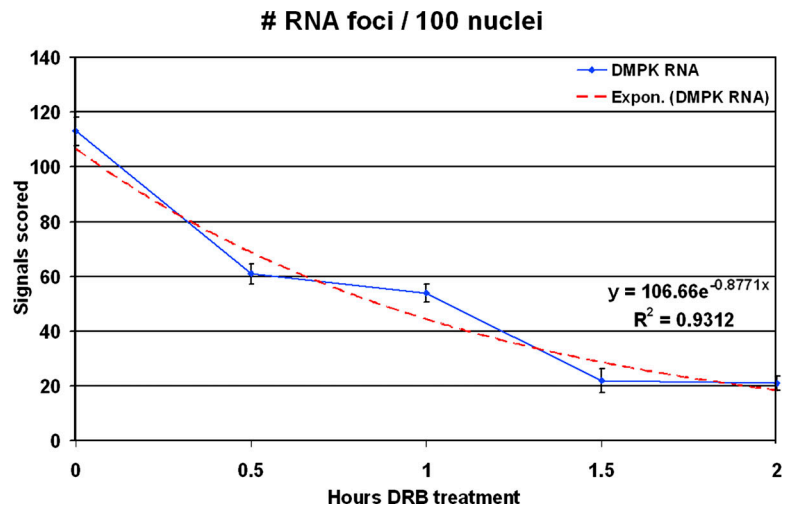


Figure 1. *DMPK* gene and RNA association with SC-35 domains in normal muscle cells. (A) In normal myotube nuclei, the two *DMPK* gene loci (red) have larger, somewhat diffuse tracks of *DMPK* RNA (detected with a cDNA probe; green) juxtaposed to them. Although the two RNA tracks are not usually in contact with each other, this image shows the two tracks intermingling as they extend from the gene loci. The inset is a magnification of genes and RNA. (B) The majority of *DMPK* gene loci (red) are associated with the edge of SC-35–defined domains (green) in myoblast nuclei. The inset is a magnification of genes and SC-35 domains. (C) 50MB-1 normal myotube nuclei showing an overlap of *DMPK* RNA (red; arrows) with SC-35 domains (green). (D and E) Magnification of domain showing that *DMPK* RNA enters but does not fill the domain. Magnification of domain (green; D) and SC-35 + RNA (E). Overlap appears yellow. (F–J) *DMPK* RNA tracks in normal HSMM myotube nuclei. (F) *DMPK* RNA signals alone. (G) *DMPK* RNA signals (red) overlapping SC-35 speckles (green). Magnifications of the *DMPK* RNA track (H), SC-35 domain (I), and merged image (J) with RNA in red and SC-35 in green show that the *DMPK* RNA diffuses throughout most of the domain. (K–P) Top (K–M) and side (N–P) views of 3D rendered image of *DMPK* RNA (red; K and N) and SC-35 (green; M and P) shows that *DMPK* RNA is diffused through the SC-35 domain. Bars (A), 5 μm ; (K) 1 μm .

the much lower theoretically and empirically derived random association rates; based on the volume occupied by SC-35 domains ($\sim 3\text{--}10\%$; Carter et al., 1993; Xing et al., 1993) and on association frequencies of many nonassociated loci, the random association frequencies are $\sim 5\text{--}25\%$ (Xing et al., 1995; Smith et al., 1999; Moen et al., 2004). Most importantly, although the loci are

clearly associated with the domain edge, they are not within it like the RNA signal. Thus, although this may not represent all of the *DMPK* mRNA, an accumulation of *DMPK* transcripts emanates from one side of the gene, which is positioned at the domain boundary, into the SC-35 domain. Although *DMPK* is not as complex and highly expressed, this structural arrangement is very

Figure 2. **Loss of DMPK RNA foci after transcription inhibition.** Detection of DMPK RNA accumulations was counted at different time intervals after transcription inhibition with dichlorobenzimidazole ribofuranoside (DRB) in normal differentiated muscle. 100 nuclei were scored at each time point. Red curve is the exponential least squares fit through points using the equation $y = 106.66e^{-0.8771x}$. This equation allows the calculation of the half-life or time at which half as many RNA foci are present as 43.5 min. Error bars represent SD.



similar to that shown for mRNAs such as *COL1A1* and myosin heavy chain, which were shown to represent posttranscriptional mRNA that has detached from the gene and accumulates within an adjacent SC-35 domain (Xing et al., 1995; Smith et al., 1999).

SC-35 domain-associated DMPK RNA foci are lost upon transcription inhibition

Although SC-35 domains are enriched for poly(A) RNA, much of this RNA remains in the domain long after transcription inhibition (>6 h), leading to suggestions that poly(A) RNA in the domains is comprised of nuclear RNA that is not exported to the cytoplasm (see Discussion). Thus, because DMPK RNA is consistently within splicing factor-rich domains, a question may remain as to whether this focus of RNA is trapped in the SC-35 domain or whether it can be chased from domains after transcription inhibition, as may be expected for an exported mRNA. To approach this question, we visualized DMPK RNA in normal myotube nuclei after transcription inhibition using dichlorobenzimidazole ribofuranoside. Loss of discernible mRNA foci in myotube nuclei was scored (Fig. 2), and a half-life of these RNA accumulations was calculated to be ~43 min. This half-life is similar to the export rate found for a fluorescently labeled *ftz* mRNA that also associated with nuclear speckles (Tokunaga et al., 2006) and is far shorter than the >15-h half-life reported for the expanded CUG repeat-containing DMPK RNA foci that are retained in the nucleus in DM1 cells (Davis et al., 1997).

The collective aforementioned results define the nuclear distribution of normal DMPK mRNA, in which recently synthesized transcripts associate with a specific nuclear compartment, laying a foundation for investigating any potential reorganization of the mutant mRNA. Our results demonstrate that accumulation within SC-35 domains is a normal part of the mRNA biogenesis and suggest that it is linked to the export of some (if not all) DMPK mRNA.

Mutant DMPK RNA accumulates in foci at the periphery of SC-35 domains

We next determined the relationship of nuclear-retained DMPK RNA containing triplet repeat expansion to SC-35 domains.

A previous study briefly examined the relationship of mutant DMPK mRNA foci to SC-35 domains and concluded that there was no substantial association (Taneja et al., 1995). Hybridization to DMPK mRNA in DM1 myoblasts from a homozygous DM1 patient (Fig. 3, A–C) shows that the mutant mRNA containing expanded CUG repeats is typically seen within nuclei in numerous round, discrete spots, which we refer to as mutant RNA granules (MRGs) as opposed to the two more irregular tracks seen for the DMPK mRNA in normal muscle cell nuclei (Fig. 1 A). The numerous MRGs in DM1 myoblasts are not the result of several copies of the DMPK gene as confirmed by DNA hybridization (Fig. 3, G, I, and K). In DM1 nuclei, 92% of the numerous MRGs (on average more than four per nucleus in homozygous muscle cells) associate with SC-35–defined domains (Fig. 4). However, this association is markedly different than the RNA foci in normal muscle nuclei, which are reproducibly found within the SC-35 domain (Fig. 1, C–E). As quantified in Fig. 4, the bulk of the associations of MRGs with SC-35–defined domains shifts from internal (complete overlap) in normal muscle cells to an association with the edge of the domains in DM1. This edge association is apparent in most cases by simple 2D analysis; in cases in which the DMPK RNA signal initially would be scored to overlap the SC-35, further 3D analysis found that it was most typically either in a hole of lower density SC-35 signal or actually above or below the SC-35 domain (Fig. 3, D and E). 3D visualization of DM1 nuclei (Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200706048/DC1>) supports the localization of these RNA foci predominantly at the outer edge of SC-35 domains.

It has previously been demonstrated that MRGs do not colocalize with other nuclear structures, such as PML bodies, exosomes, and the perinucleolar compartment (Jiang et al., 2004). However, the presence of multiple MRGs in close association with SC-35 domains suggested the possibility that the RNA could be in paraspeckles, which are novel nuclear domains that abut SC-35–defined speckles (Fox et al., 2002). Simultaneous detection of the mutant RNA foci and the paraspeckle protein PSP1 clearly demonstrated that the signals were not overlapping (unpublished data).

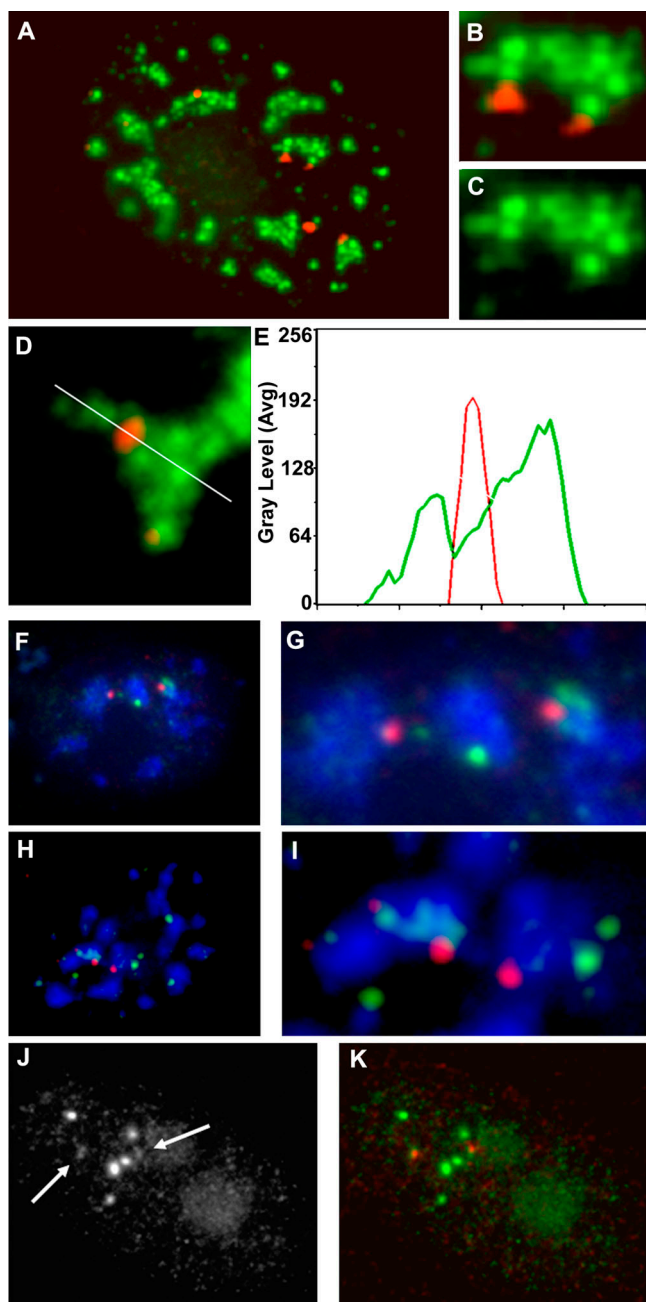


Figure 3. Distribution of *DMPK* mRNA and SC-35 in DM1. (A) Myotube nuclei from a DM1 homozygote shows numerous *DMPK* RNA foci (red) abutting but not overlapping SC-35 domains (green). (B and C) Magnifications of domain (B) with *DMPK* mRNA (C) demonstrating the tight association of RNA foci with a domain, but only with the domain edge. (D and E) *DMPK* RNA foci are often in SC-35 holes. (D) *DMPK* RNA signal (red) in the DM1 myoblast sits in a region of decreased SC-35 staining (green). Signal intensities along the line in the image are shown in the linescan graph (E). (F and H) Three color images of DM1 heterozygote myotube nuclei. *DMPK* genes (red) are associated with the edge of SC-35 domains (visualized with an antibody to SRm300; blue). *DMPK* RNA hybridization (green) shows the RNA track emanating from one allele into the adjacent domain, whereas the other allele (presumably the mutant) has very little RNA associated with it, but there are RNA foci that are associated with the neighboring domain. (G) Magnification of F to show detail. (I) Magnification of H to show detail. (J and K) *DMPK* RNA foci in the homozygous DM1 myoblast nucleus. Most RNA signals are in brighter foci (J and green signals in K), but small RNA signals are seen (J; arrows) that are associated with *DMPK* gene signals (K; red), likely representing nascent transcripts.

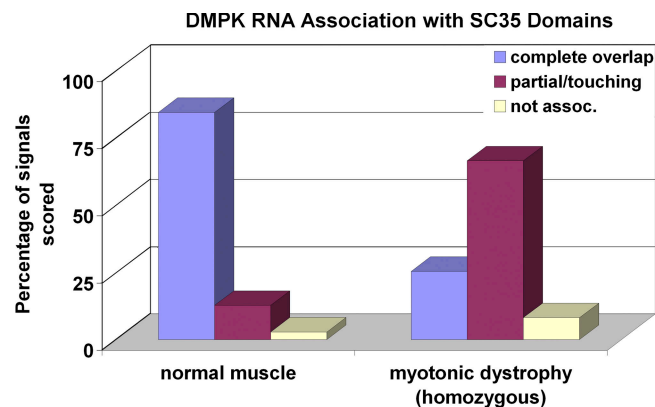


Figure 4. Graph indicating the frequency of RNA distributions seen in normal (50MB-1) myotube nuclei and myotube nuclei derived from muscle of a DM1 homozygote. Myotube nuclei, $n = 316$; nuclei derived from muscle of a DM1 homozygote, $n = 209$.

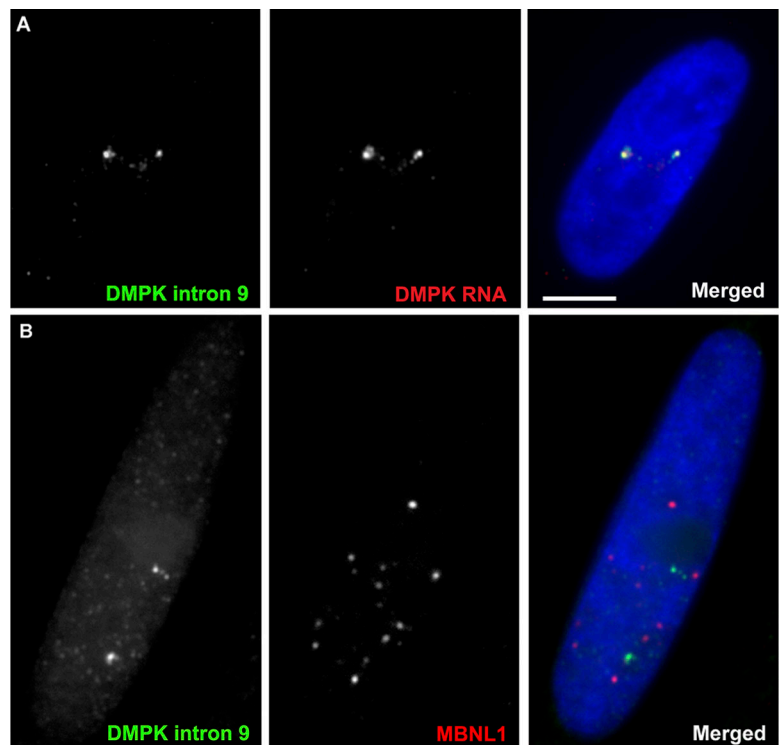
We then combined simultaneous DNA, RNA, and SC-35 detection in heterozygous DM1 myotube nuclei. As in normal muscle nuclei, *DMPK* genes in DM1 myoblasts show similar positioning at the edge of SC-35 domains. Thus, the change in *DMPK* RNA association is not caused by a shift in gene localization relative to SC-35 domains. Fig. 3 (F–I) shows that the two *DMPK* alleles are positioned at the edge of splicing factor domains. One allele has an irregular dispersed track of *DMPK* RNA extending from it into the adjacent domain, which is clearly characteristic of transcripts released from the normal allele into the SC-35 domains in normal muscle cells (Fig. 1, C–J). The other allele is distinct from the normal allele in that it has only a minute RNA signal (nascent transcripts) that does not extend into the domain, similar to *DMPK* DNA and RNA signals seen in homozygous mutant DM1 nuclei (Fig. 3, J and K). This relationship of the *DMPK* genes, RNA, and SC-35 is apparent in 3D as seen in Video 3 (available at <http://www.jcb.org/cgi/content/full/jcb.200706048/DC1>).

In Fig. 3 (F–I), we also see that MRGs associate with domains other than the ones adjacent to the DNA loci. This suggests that MRGs have drifted from the transcription site but clearly still show an affinity for the SC-35 domains. However, unlike the normal *DMPK* RNA, these MRGs are restricted to the periphery and do not enter the domains. This shift in localization relative to the SC-35 compartment in DM1 demonstrates that the normal path of *DMPK* RNA from the gene into the domain is blocked for RNA carrying the triplet repeat expansion. In contrast to mutant *COL1A1* RNA in osteogenesis imperfecta type I, in which mutant transcripts entered the domain normally but failed to exit properly (Johnson et al., 2000), some block to export in DM1 occurs at the entry of the repeat-expanded transcripts into the domain. Although we cannot exclude that some normal transcripts are exported without entering SC-35 domains, these results show that the block in export is correlated with a failure of mutant *DMPK* RNA entry into the domain.

Mutant *DMPK* transcripts within nuclear foci are spliced

The failure of the mutant *DMPK* RNA to enter SC-35 domains raises the question as to whether splicing occurs normally despite

Figure 5. RNA foci in DM1 contain spliced *DMPK* RNA. (A) In HSMM myoblast nuclei, *DMPK* intron 9, which was detected with a PCR-generated probe (green), detects an RNA focus that overlaps the focus of RNA detected with the *DMPK* cDNA probe (red). The right panel shows the three-color merged image. (B) In DM1 differentiated muscle cells, signals seen with the *DMPK* intron 9 probe (green) do not overlap DM1 RNA foci detected with MBNL1 (red). MBNL1 overlap with *DMPK* RNA is seen in Fig. 6 B. The right panel is a merged three-color image. Bar, 5 μ m.



the repeat expansion in the 3' untranslated region, which prevents their accumulations within these splicing factor-rich structures. Using Northern analysis of extracted cellular mRNA, an earlier study (Davis et al., 1997) was unable to detect intronic sequences in *DMPK* RNA derived from DM1 or normal cells, suggesting that splicing was unaffected. We directly addressed whether the mutant RNA foci still contain introns by visualizing RNA in intact DM1 cells using a PCR-generated probe encompassing the *DMPK* intron 9 simultaneously detected with a cDNA probe. In normal muscle nuclei that have just two high concentration sites of largely unspliced pre-mRNA emanating from each normal allele, intron 9 signals intermingle with much of the localized *DMPK* cDNA signal (Fig. 5 A). In contrast, the numerous bright foci of mutant *DMPK* RNA in DM1 cells (visualized by their enrichment of MBNL1 protein; Fig. 6 A) lack the intron 9 signal (Fig. 5 B). Similar results are seen when using a *DMPK* cDNA probe to detect RNA foci (unpublished data). This result illustrates that the mutant RNA in the MRGs of DM1 cells is spliced. Thus, although blocked entry into the SC-35 domain could still relate to failed export of the mRNA, it does not appear to affect splicing. This is consistent with other evidence that collagen pre-mRNA splicing occurs primarily at the edge of the SC-35 domain, whereas more mature mRNA is within the domain (Johnson et al., 2000).

Repeat transcripts in DM1 form structural accumulations with MBNL1

Evidence indicates that DM1 pathogenesis is caused, in large part, by a deleterious effect of the nuclear RNA accumulations, particularly on alternative splicing of specific mRNAs. Sequestration of the alternative splicing factor MBNL1 has emerged as key to these splicing alterations (see Introduction).

In normal myotube nuclei, MBNL1 is seen in a broadly distributed, somewhat punctuate pattern by immunostaining (Fig. 6 A). Interestingly, MBNL1 does not appear particularly concentrated in splicing factor-rich SC-35 domains but does show sites of punctuate concentration, some of which abut domains in normal cells. In DM1 muscle cells, the distribution of MBNL1 changes markedly. As shown in Fig. 6 B, we find that the MBNL1 nucleoplasmic signal is dramatically reduced in DM1 myotube nuclei and that MBNL1 is concentrated with mutant *DMPK* RNA in the very bright MRGs. These results corroborate evidence from Jiang et al. (2004) and Lin et al. (2006) that MBNL1 becomes sequestered with the repeat RNA accumulations coincident with MBNL1-dependent changes in alternative splicing patterns in muscle.

Myotonic dystrophy type 2 (DM2) has phenotypic similarities to DM1 (Day et al., 2003), and, in DM2 cells, we also see that MBNL1 protein becomes sequestered with nuclear foci of mutant repeat RNA (unpublished data), which is consistent with the idea that overlapping clinical features in DM1 and DM2 may be caused by the common sequestration of MBNL1 (Day and Ranum, 2005). However, in DM2, the repeat expansion is in a quadruplet repeat (CCTG) in the first intron of a zinc finger protein, *zfn9*, on chromosome 3 (Liquori et al., 2001). Although DM2 cells contain repeat RNA foci, unlike DM1, the foci contain only intron sequences, and the intronic DM2 repeat does not impede *zfn9* mRNA export or translation (Botta et al., 2006; Margolis et al., 2006). Thus, because the repeat RNA foci in DM2 do not contain mRNA blocked from export, their relationship to SC-35 domains was of less interest. We did note that there was some association of the MBNL1/*Znf9* intronic RNA foci around SC-35 domains, although to a much lesser extent (51%; $n = 604$; unpublished data) than the DM1 MRGs (92%).

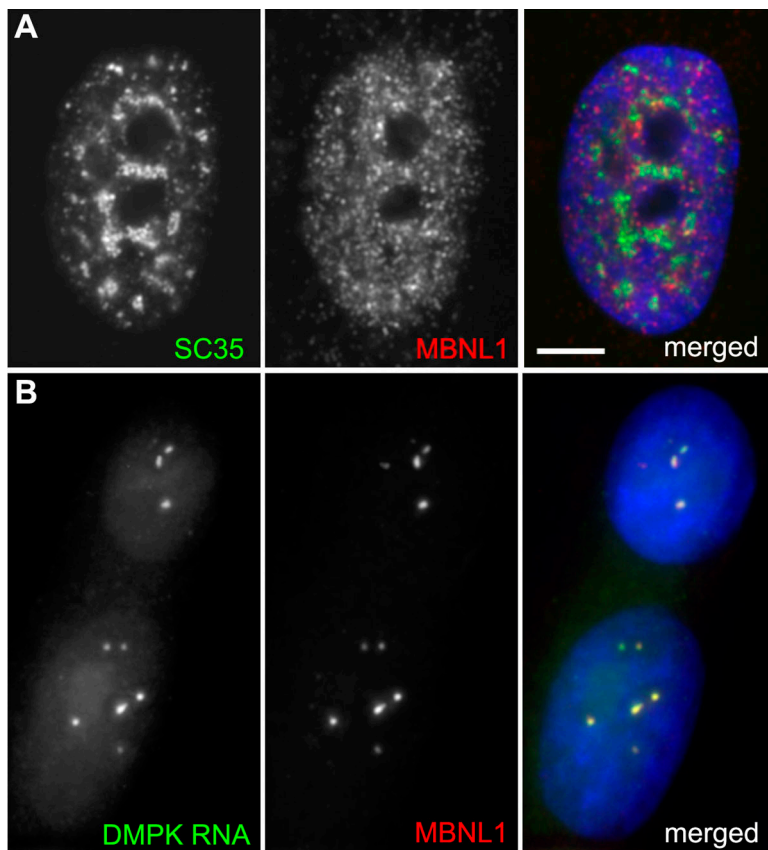


Figure 6. **MBNL1 is sequestered in mutant RNA foci in DM1.** (A) In normal muscle, MBNL1 (red) is present in a diffuse nucleoplasmic pattern that neither enriched nor depleted in SC-35 domains (green). (B) In DM1 muscle nuclei, *DMPK* RNA foci (green) overlap foci of MBNL1 (red). Bar, 5 μ m.

Whether the aggregation with MBNL1 is partially responsible for the collection of MRGs around SC-35 domains is not known, but the important point is that MBNL1 sequestration is common to both DM1 and DM2, whereas blocked export of the mutant mRNA only occurs in DM1, possibly contributing to the unique clinical features of DM1.

MBNL1 down-regulation alters repeat RNA localization

Recent publications have suggested that MBNL1 and hnRNP H actually have roles in the formation of repeat RNA foci and RNA sequestration in DM1. Dansithong et al. (2005) have demonstrated that reduction of MBNL1 levels by RNAi disrupts repeat RNA foci in proliferative DM1 myoblasts. Similarly, Kim et al. (2005) have found that siRNA-mediated knockdown of hnRNP H restores the export of repeat-containing RNA out of the nucleus. These two proteins interact, as MBNL1 is responsible for hnRNP H association with repeat foci (Paul et al., 2006). Thus, MBNL1 would appear to play a key role in the retention of mutant *DMPK* RNA in the nucleus by forming the MRG aggregates.

If the normal biogenesis and export of *DMPK* RNA are accomplished via SC-35 domains, these reports raise the possibility that knockdown of MBNL1 might alter the localization of repeat-containing RNA relative to SC-35 domains so that it is within domains, as seen for the normal *DMPK* RNA. To address this possibility, we performed siRNA-mediated knockdown of MBNL1 in differentiated DM1 muscle cell cultures. To determine the effectiveness of the RNAi in individual cells, we performed RNA in situ hybridization on siRNA and control

coverslips using CAG repeat oligonucleotides and stained for MBNL1. Coverslips were scored for differentiated nuclei that had repeat RNA foci with and without MBNL1 (Fig. 7, A and B). As seen in the graph in Fig. 7 C, 27% of differentiated muscle nuclei treated with siRNA ($n = 148$) had CUG repeat RNA foci (MRGs) without substantial levels of MBNL1, whereas CUG RNA colocalized with MBNL1 foci in all of the control nuclei ($n = 142$). This in situ approach demonstrates effective transfection and siRNA depletion of MBNL1 expression in a substantial subset of differentiated cells.

Although Dansithong et al. (2005) reported that MBNL1 was necessary for mutant RNA focus integrity, we found intact RNA foci in cells in which MBNL1 expression was down-regulated by RNAi 5 d after transfection. We believe there is a difference in results because our analysis was performed on nonproliferative cultures under low serum differentiation conditions, whereas the cultures used by Dansithong et al. (2005) were dividing during the 5 d after transfection. We suggest that in their experiments, mutant *DMPK* RNA foci may have broken down during mitosis and not reformed afterward as a result of dilution and depletion of previously made MBNL1. Our evidence is in agreement with the statement by Dansithong et al. (2005) that the absence of MBNL1 is sufficient to prevent nucleation of the mutant RNA foci.

A triple label experiment in which CUG RNA, MBNL1, and SC-35 were detected was used to investigate changes in CUG RNA localization upon MBNL1 knockdown (Fig. 8 A). In nuclei in which MBNL1 expression was suppressed, we noted that there was commonly a single SC-35 domain that contained

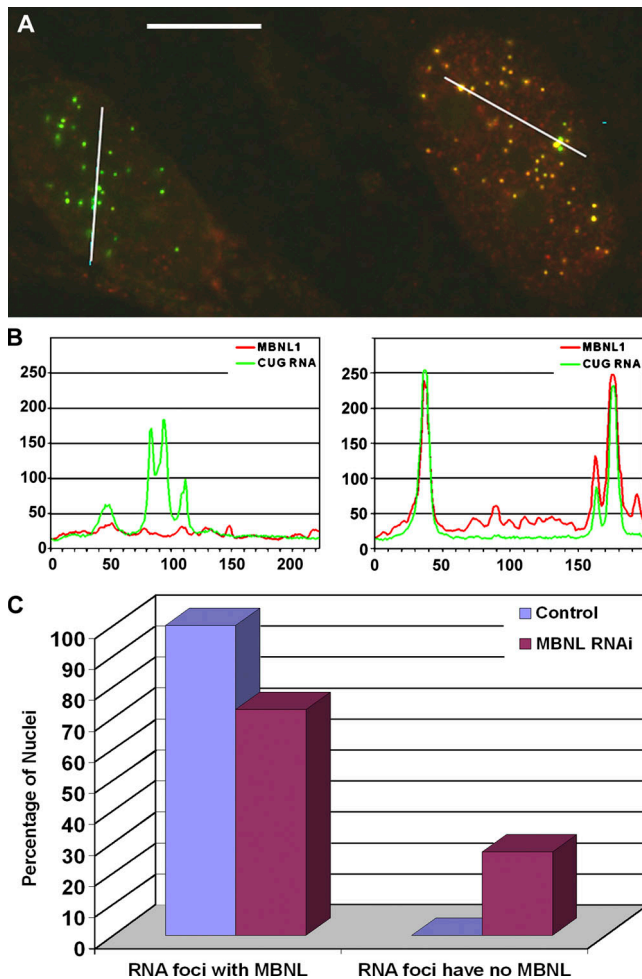


Figure 7. **siRNA-mediated down-regulation of MBNL1.** (A) Two nuclei in the same image frame showing the accumulation of MBNL (red) with CUG (*DMPK*) RNA (green). The left nucleus demonstrates the loss of MBNL1 relative to the nucleus on the right. (B) Linescan graphs of signal intensities along the lines drawn through each nucleus in A show the loss of MBNL1. (C) Quantitation of MBNL1 signal with CUG repeat foci in control ($n = 142$) and siRNA-treated ($n = 148$) nuclei. Bar, 10 μm .

an irregular, rather diffuse accumulation of repeat RNA that was distinct from the round MRGs (Fig. 8 B; and see 3D visualization in Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200706048/DC1>). Based on scoring by two independent investigators, these signals were far more common in the cells in which MBNL1 was knocked down (63% of nuclei; $n = 39$) than in control cells or in untransfected cells from the same experiment (8% of nuclei; $n = 37$; Fig. 8 C). These signals were reminiscent of diffuse *DMPK* RNA accumulations from the normal allele detected with a cDNA probe (Fig. 1, C–J; and Fig. 3, G and I). Thus, we suspected that these signals did not arise from the pre-existing RNA/protein aggregates (MRGs) but are new repeat-containing transcripts arising from the affected allele, which would not have any newly synthesized MBNL1 with which to complex as a result of the siRNA-mediated inhibition.

To address the possibility that the diffuse CUG RNA signals were new transcripts emanating from a *DMPK* gene locus, we performed another triple label experiment on MBNL1 knock-down cells. In this experiment, we detected SC-35 followed by

hybridization for CUG RNA with an oligonucleotide probe and a DNA hybridization with a *DMPK* genomic probe. Analysis of this experiment provided numerous clear examples of *DMPK* gene loci abutting more diffuse CUG RNA (mutant *DMPK* RNA) signals within SC-35 domains (Fig. 9). In nuclei in which the diffuse RNA signal and two *DMPK* gene loci were visible, the dispersed RNA signal was always adjacent to one of the genes. These results demonstrate that depletion of MBNL1 by RNAi allows newly transcribed *DMPK* RNA containing expanded CUG repeats to enter the SC-35 domain abutting the gene locus.

Discussion

Findings presented here further our understanding of nuclear structure and mRNA transport and, at the same time, provide insight into the cellular pathogenesis of DMI. We show that newly synthesized *DMPK* RNA accumulates within the interior of an SC-35–defined domain in normal muscle nuclei, having emanated from a gene positioned at the domain’s edge. In contrast, the mutant *DMPK* transcripts in DMI detach from the gene but are not within the domain; rather, they accumulate in multiple granules that gather at the edge of domains. This change in domain association occurs in concert with mutant *DMPK* RNA retention in the nucleus. Although the transcripts do not appear to enter the SC-35 domain, they are nonetheless spliced. Down-regulation of MBNL1 changes the distribution of newly transcribed mutant *DMPK* RNA such that it is now found within the SC-35 domain.

Our studies examine the detailed relative distributions of gene/RNA/protein associated with normal versus mutant alleles but in static images that capture a window of time. A major advantage of this approach is that it provides information about the real endogenous gene and RNA, which are expressed in a native structural as well as physiological context. Although this does not provide direct visualization of molecules in live cells, our findings collectively provide evidence of the route occupied by at least a substantial fraction of these native transcripts. Because transcripts clearly emanate from the gene, for the sake of this Discussion, we consider the gene point A and will consider any accumulation of transcripts at a resolvable distance from the corresponding gene to have moved with respect to it.

Collectively, these findings suggest several important points regarding the relationship of the SC-35 domains to mRNA metabolism and transport. (1) The consistent positioning of both homologous *DMPK* genes at the outer edge of an SC-35 domain adds to the body of evidence that specific active genes are organized relative to SC-35 domains, providing an example of an associated gene that is not particularly complex or highly expressed. (2) We interpret the accumulation of transcripts within the SC-35 domain (to one side of the gene at the domain edge) to indicate a normal step in the path of *DMPK* mRNA for at least a substantial fraction of *DMPK* transcripts. The fact that mutant transcripts do not enter these domains further supports the idea that entry into the domains is a step that can be blocked by mutation. (3) Because MRGs contain spliced mRNA, passage into the splicing factor-rich domain does not appear to be required for splicing, which is consistent with other evidence that most splicing occurs at the domain periphery (Johnson et al., 2000). These findings are

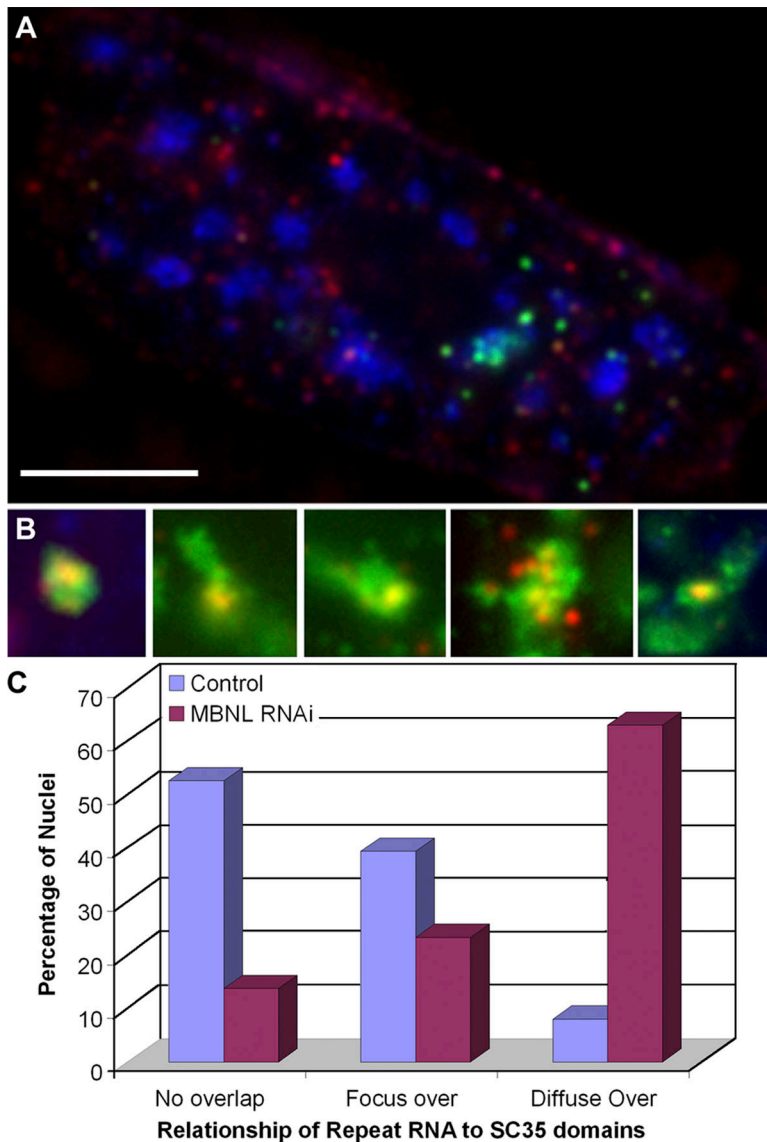


Figure 8. Down-regulation of MBNL1 changes CUG repeat RNA association with SC-35 domains. (A) Three-color image of myotube nucleus showing CUG repeat RNA (green) without MBNL1 accumulation (red) in foci and in a diffuse pattern that overlaps an SC-35 domain (blue). (B) Several images of CUG repeat RNA (red) coincident with SC-35 domains (green) in different nuclei. Overlap appears yellow. (C) Frequency of CUG repeat RNA distributions relative to SC-35 domains. Bar, 5 μ m.

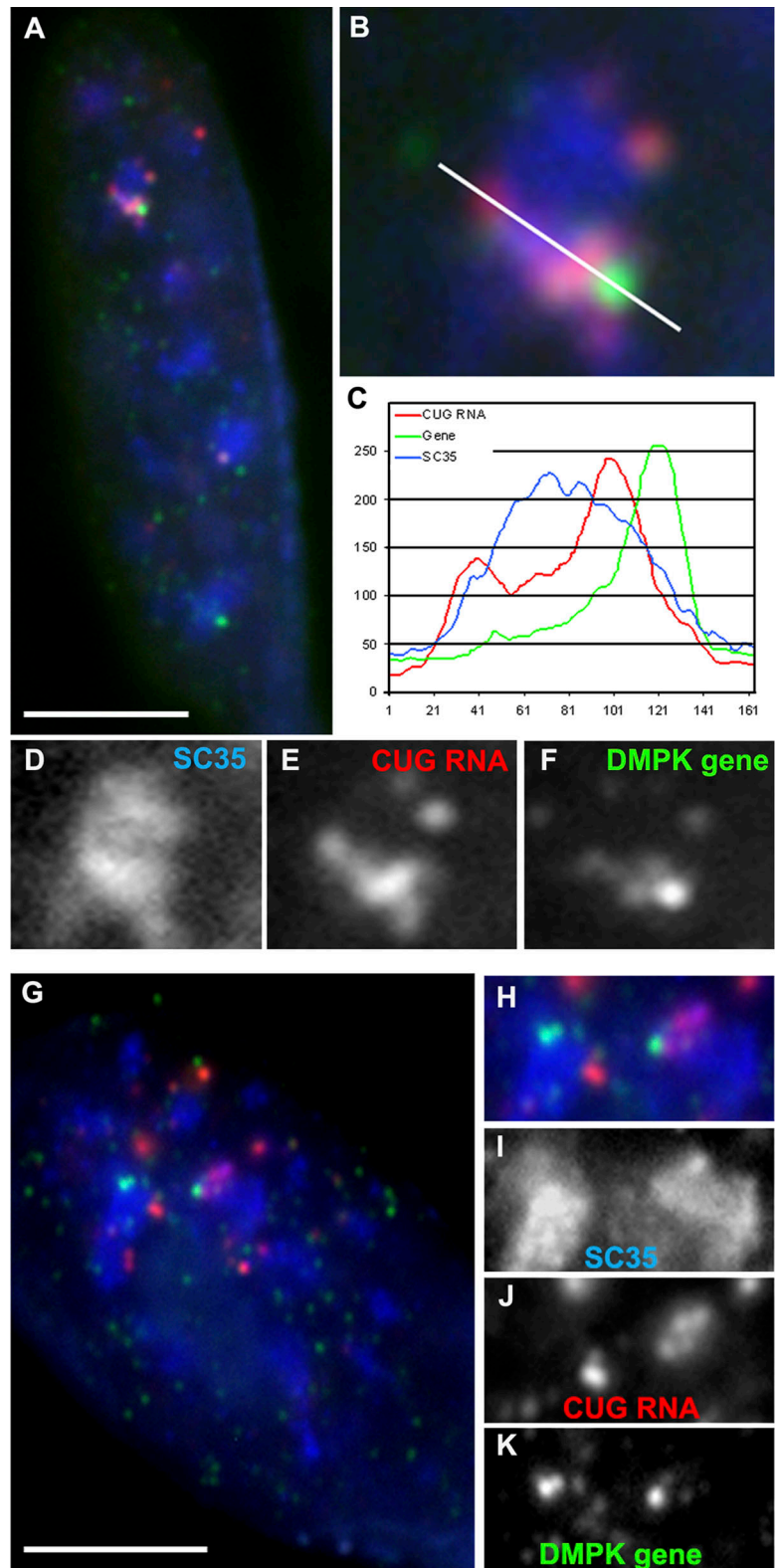
consistent with the idea that postsplicing steps linked to export may occur within the domain. (4) Blockage of mutant transcripts before entering SC-35 domains provides further evidence for the structural integrity of the SC-35 domain (with which normal and mutant alleles remain associated), which exists independently of the presence of *DMPK* mRNA within it. Consistent with this, the normal *DMPK* mRNA does not occupy the whole domain of splicing factors, as might be expected if the domain was merely factors bound to this pre-mRNA. (5) Loss of *DMPK* RNA signals upon transcription inhibition demonstrates that the normal *DMPK* RNA accumulation in SC-35 domains does not represent RNA just trapped within the domain but is chased as would be expected for a transported mRNA. (6) Remarkably, of the two mutant pre-mRNAs studied thus far, both have shown abnormal accumulations at or within the SC-35 domains and not at other sites such as the nuclear envelope or nucleolus. Whereas mutant *COL1A1* RNA in osteogenesis imperfecta accumulates to abnormal levels within the domain, mutant *DMPK* transcripts accumulate outside the domain. This supports a model in which

passage into and release from these domains are distinct steps in the normal path of some pre-mRNAs.

Although we demonstrate that many *DMPK* transcripts are not randomly dispersed upon initial release from the gene, these results should not be misinterpreted as excluding the possibility that export of these mRNAs may also involve multidirectional diffusion, which is consistent with other models of RNA trafficking (Politz et al., 2003; Gorski et al., 2006). These collective findings support a model of early steps in mRNA export and maturation as presented in Fig. 10.

This study provides evidence of a relationship between *DMPK* mRNA and SC-35–defined speckles that is disrupted when a CUG expansion in DM1 prevents normal mRNA export. Despite numerous studies describing the association of specific mRNAs with SC-35–defined speckles (Xing et al., 1995; Jolly et al., 1999; Smith et al., 1999; Shopland et al., 2002), the idea that SC-35 domains contain mRNAs and/or play a role in mRNA export remains somewhat controversial (Hall et al., 2006). Therefore, we will briefly address the two major pieces of evidence that

Figure 9. **Diffuse CUG RNA signals are associated with the *DMPK* gene locus.** (A) Three-color image of SC-35 (blue) *DMPK* DNA (green) and CUG RNA (red) show two gene signals associated with SC-35 domains, but only one gene is juxtaposed to the diffuse CUG RNA signal overlapping SC-35. (B) Magnification of *DMPK* gene, CUG RNA, and SC-35 with the line representing the linescan in C. (D–F) Blue (D), red (E), and green (F) channels of the image in B. (G) Three-color image of SC-35 (blue) *DMPK* DNA (green) and CUG RNA (red) shows two gene signals associated with SC-35 domains, but only one gene is juxtaposed to the diffuse CUG RNA signal overlapping SC-35. (H) Magnification of the relevant region of nucleus in G. (I–K) Separated channels of the image in H. Bars, 5 μm .



generate this uncertainty. First, the belief that SC-35 speckles do not contain appreciable short-lived pre-mRNAs is based on early findings that tritiated uridine incorporation labels the speckles (interchromatin granule clusters) very little relative to the surrounding nucleoplasm (Fakan and Bernhard, 1971; Fakan, 1994; for review see Spector, 1993). However, a study did find newly

synthesized BrUTP-labeled RNA concentrated within these domains (Wei et al., 1999). Most importantly, labeling methods such as [³H]uridine or bromo-UTP are wholly nonspecific and do not necessarily reflect the distribution of pre-mRNA. These methods primarily label unknown heterogeneous nuclear RNA (Salditt-Georgieff et al., 1981) and introns (most of pre-mRNA mass) that

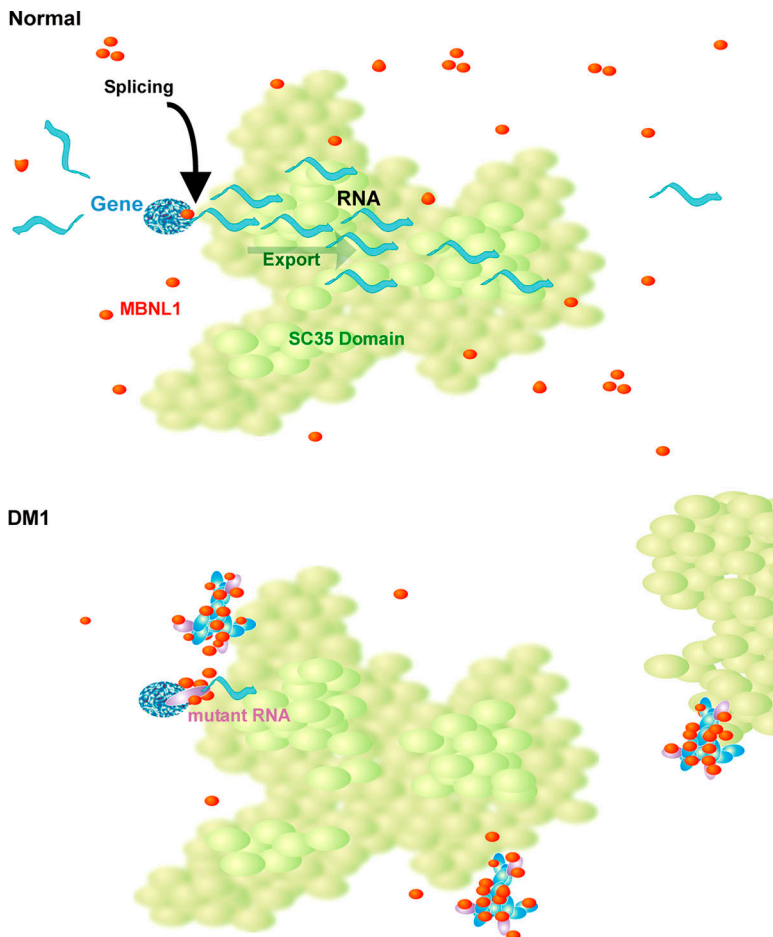


Figure 10. **Model of SC-35 domain function.** In this model, *DMPK* genes are positioned at the edge of SC-35 domains. The RNAs produced from these loci are primarily spliced at the edge of the domain. These mRNAs then transit into the domain (step 1). In the interior of the domain, the bound splicing factors, such as small nuclear RNPs and serine-arginine proteins, are removed and recycle back to the domain edge. These are replaced by export factors such as tip-associated protein and Aly. The export-ready mRNAs then leave the domain (step 2) and are transported to the nuclear envelope. In DM1, the RNA is spliced, but expanded repeat sequences in the RNA are bound by MBNL1 (and perhaps other factors), forming aggregates (MRGs) that do not enter the domain and impeding export of the RNA.

are rapidly removed and disperse during the labeling period (Xing et al., 1993; for review see Moen et al., 1995). Evidence in support of this concern has been strengthened in recent years by findings that transcription of nongenic DNA is far more widespread throughout the genome than previously anticipated (Cheng et al., 2005; Johnson et al., 2005). Furthermore, copious amounts of RNA throughout the nucleoplasm are detected by RNA hybridization using Cot-1 DNA, composed largely of repetitive elements such as Alu and long interspersed nuclear elements (Hall et al., 2002), which recent findings suggest is largely nongenic transcription (Hall et al., 2002; Chaumeil et al., 2006; Clemson et al., 2006). In short, uridine incorporation detects a great deal of RNA that is not mRNA and, as such, does not accurately represent the distribution of pre-mRNA in the nucleus.

Second, the demonstration that the poly(A) RNA signal often remains in SC-35 domains upon transcription inhibition (Lawrence et al., 1993; Huang et al., 1994) is often interpreted to indicate that this poly(A) RNA is not mRNA but a putative long-lived, polyadenylated, structural RNA. However, transcriptional inhibition has complex effects on nuclear RNA distributions and transport that varies with the RNA. In a dramatic example of this, we recently identified an abundant noncoding polyadenylated nuclear RNA, NEAT1 (Hutchinson et al., 2007), that actually enters the SC-35 domains upon treatment with certain transcription inhibitors (unpublished data). This illustrates the difficulty of interpreting the nature of the poly(A) RNA in

domains from transcription inhibition experiments. Our study demonstrates that at least some specific mRNAs, such as *DMPK* RNA, do leave the SC-35 domain upon transcription inhibition. In addition, recent studies of labeled RNAs introduced into live cells indicate that poly(A) RNA (Molenaar et al., 2004) and specific mRNAs (Tokunaga et al., 2006) passage through domains and that export-ready mRNA is present in speckles (Schmidt et al., 2006). Thus, although our study takes a different approach to study naturally occurring mutations of an endogenous RNA, our findings complement and substantially extend approaches that seek to understand the behavior of endogenous molecules by examining labeled, microinjected RNAs in live cells.

In addition to its relevance for fundamental nuclear structure, this study contributes insight into the cellular pathogenesis of DM1. (1) We have identified a point in nuclear structure at which the paths of normal and mutant *DMPK* mRNA diverge. (2) As this difference correlates with cytoplasmic mRNA export, it defines the intranuclear step at which the block in transport of many or all mutant *DMPK* transcripts likely occurs. (3) We provide evidence that the formation of MRGs mediated by MBNL1 is responsible for blocking the mutant RNA from entry into the domain. Our analysis indicates that depletion of newly synthesized MBNL1 allows newly transcribed *DMPK* RNA carrying CUG repeat expansions to accumulate within SC-35 domains in a manner similar to that seen with normal *DMPK* RNA. MBNL1 may act in concert with other factors, such as hnRNP H, to bind

CUG repeat RNA and form the RNA/protein aggregates. Thus, both the nuclear retention of mutant DMPK RNA as well as sequestration of specific nuclear factors like MBNL1 and hnRNP H are likely involved in the complex DM1 phenotype.

Materials and methods

Cell culture

The normal muscle cultures used were human skeletal muscle myoblasts (HSMMs; Clonetics) and 50Mb. Myoblast strain 50Mb (normal myoblast preparations flow sorted to substantially remove contaminating fibroblasts) was provided by H. Blau (Stanford University, Palo Alto, CA; Webster et al., 1988). It was obtained from the vastus lateralis muscle of a 10-year-old male with no known muscle pathologies. For propagation, cells were cultured at subconfluent density in serum-rich medium with medium changes every other day. To induce muscle differentiation, cultures were grown to near confluence and maintained in low serum medium without further medium changes until the appearance of myotubes. Propagation medium was Ham's F-10 supplemented with 20% FCS, 1% vol/vol chick embryo extract (60 Å ultrafiltrate; Invitrogen), 1 mM insulin, and 1 mM dexamethasone (Sigma-Aldrich), whereas differentiation medium contained DME low and 2% horse serum. Both contained 200 U/ml penicillin and 200 mg/ml streptomycin. HSMMs were grown in SkGM BulletKit media (Cambrex) with an additional 1.5% FBS. The differentiation media was DME-F-12 with 2% horse serum added when the cells reached ~60% confluence.

Homozygous DM1 myoblasts (DM(SW)) were derived from a severely affected homozygote (provided by L. Timchenko, Baylor College of Medicine, Houston, TX; Caskey et al., 1996). In our hands, this cell line ceased to differentiate after a few passages. Heterozygous DM1 myoblast cultures F1D and myoblast C were obtained from C. Thornton (Wellstone Muscular Dystrophy Cooperative Research Center, Rochester, NY). These myoblasts were grown in Ham's F-10 with 0.12% NaHCO₃, 15% FBS, 5% defined supplemental calf serum, and 2 mM L-glutamine and penicillin/streptomycin. Fusion medium was described in the previous paragraph. DM2 fibroblasts were provided by L. Ranum (University of Minnesota, Minneapolis, MN). DM2 fibroblasts were grown in DME with 10% FBS, penicillin/streptomycin, and L-glutamine.

Probes and antibodies

DMPK RNA was detected using a 2.3-kb DMPK cDNA clone pRMK (Timchenko et al., 1995). A genomic clone, cosmid F18894 (L08835) overlapping the DMPK gene, which was obtained from the Human Genome Center (Lawrence Livermore National Laboratory, Livermore, CA), was used for DNA detection. The 2-kb DMPK intron 9 probe was generated by PCR amplification from the F18894 clone using primers GAGTTGCAGGATCA-GTCTTGGG and TGCTGATCTCTGGTGGGAAAC. *Znf9* gene and RNA detection were performed using PCR-generated probes encompassing exons 2–5 of the *znf9* gene. Probes were generated by PCR amplification of a bacterial artificial chromosome (Rp11-814L21; BACPAC Resource Center, Children's Hospital Oakland Research Institute, Oakland, CA) using primers AAACCTTTGCCATCACCATC and GCCTTGCAAATTGTCTGGAT. Biotin or fluorescein end-labeled oligonucleotide probes (CAGCAGCAGCAGCAG-CAG and CAGGCAGGCAGGCAGGCAGGCAGGCAGG) were used to detect DM1 (CUG) and DM2 (CCUG) repeat RNAs, respectively. All probes except direct-labeled oligonucleotides were labeled using nick translation as described previously (Johnson et al., 1991; Tam et al., 2002).

SC-35 domains were detected with an antibody to the spliceosome assembly factor SC-35 (Sigma-Aldrich; Fu and Maniatis, 1990) or to an antibody to the splicing coactivator SRm300 (provided by B. Blencowe, University of Toronto, Toronto, Canada; Blencowe et al., 1998). MBNL1 was detected with monoclonal antibodies 3B10 and A2764, which were obtained from C. Thornton. An antibody to hnRNP H was obtained from D. Black (Howard Hughes Medical Institute/University of California, Los Angeles, Los Angeles, CA).

In situ hybridization

The methods used in this study, including procedures for nonisotopic probe preparation and FISH, have been published in detail previously (Johnson et al., 1991; Tam et al., 2002). Two-color detection of RNA and genes simultaneously was performed by sequential hybridization and detection of RNA followed by fixation, NaOH treatment, and DNA hybridization (Xing et al., 1995). In some experiments, before or after hybridization, samples were stained with antibodies as described previously (Xing et al., 1993, 1995).

MBNL1 RNAi

RNAi was performed by methods adapted from Dansithong et al. (2005). Double-stranded siRNA to MBNL1 (5'-CACUGGAAGUAUGUAGAGA-dTdT-3') was obtained from Dharmacon. Fresh cultures of myoblasts from DM1 patients (myoblast C) were cultured and differentiated as described in the Cell culture section. 5-d differentiated myoblast cultures on coverslips in six-well plates were rinsed twice with Opti-MEM (Invitrogen). For each coverslip, 5 µl Oligofectamine (Invitrogen) and 375 pmol MBNL1 siRNA in a 1-ml total volume of Opti-MEM were preincubated for 20 min at room temperature and added to the cells. Controls had no siRNA. Transfection was incubated at 37°C overnight and replaced with differentiation media (DME + 5% horse serum). Cells were extracted and fixed at 5 d after transfection.

Microscopy and analysis

Images presented in this study are either single plane or extended focus images from z stacks (step size = 100 nm) and were acquired using an Axiovert 200 microscope or an Axiophot microscope (Carl Zeiss Microimaging, Inc.) equipped with a 100× NA 1.4 planApo objective using Axiovision (Carl Zeiss Microimaging, Inc.) or MetaMorph (Universal Imaging Corp.) imaging software. The multiband pass dichroic and emission filter sets (model 83000; Chroma Technology Corp.) were used with excitation filters set up in a wheel to prevent optical shift. Images were captured on an Orca-ER camera (Hamamatsu) or a CCD camera (200 series; Photometrics). Z stacks were further processed using constrained iterative deconvolution in Axiovision 4.1 (Carl Zeiss Microimaging, Inc.) and displayed as extended focus projections in some cases. Rendered images are maximum value projections. Results shown are from multiple experiments and were scored by several investigators.

Online supplemental material

Video 1 demonstrates the colocalization of normal DMPK RNA and an SC-35 domain in 3D. Video 2 is a 3D representation showing that mutant DMPK RNA forms foci that are predominantly at the edge of SC-35 domains in DM1 muscle cell nuclei. Video 3 demonstrates in 3D that both DMPK gene loci associate with SC-35 domains in heterozygous DM1 muscle but only one locus has visible RNA accumulating in the adjacent domain. Video 4 is a 3D representation showing that upon siRNA-mediated knockdown of MBNL1, CUG RNA (representing mutant DMPK RNA) localizes within an SC-35 domain adjacent to a DMPK gene locus. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200706048/DC1>.

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