Cytoplasmic and Nuclear Retained DMPK mRNAs Are Targets for RNA Interference in Myotonic Dystrophy Cells*

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Small interfering RNA (siRNA) duplexes induce the specific cleavage of target mRNAs in mammalian cells. Their involvement in down-regulation of gene expression is termed RNA interference (RNAi). It is widely believed that RNAi predominates in the cytoplasm. We report here the co-existence of cytoplasmic and nuclear RNAi phenomena in primary human myotonic dystrophy type 1 (DM1) cells by targeting myotonic dystrophy protein kinase (DMPK) mRNAs. Heterozygote DM1 myoblasts from a human DM1 fetus produce a nuclear retained mutant DMPK transcript with large CUG repeats (~3,200) from one allele of the DMPK gene and a wild type transcript with 18 CUG repeats, thus providing for both a nuclear and cytoplasmic expression profile to be evaluated. We demonstrate here for the first time down-regulation of the endogenous nuclear retained mutant DMPK mRNAs targeted with lentivirus-delivered short hairpin RNAs (shRNAs). This nuclear RNAi-like phenomenon was not observed when synthetic siRNAs were delivered by cationic lipids, suggesting either a link between processing of the shRNA and nuclear import or a separate pathway for processing shRNAs in the nuclei. Our observation of simultaneous RNAi on both cytoplasmic and nuclear retained DMPK has important implications for post-transcriptional gene regulation in both compartments of mammalian cells.

Small interfering RNAs (siRNA(s)) have been shown to direct sequence-specific inhibition of gene expression in mammalian cells (1). siRNAs are RNA duplexes of 21–23 nucleotides with ~2 nucleotide 3’-overhangs that can induce degradation of their homologous target mRNAs without eliciting interferon responses in mammalian cells. The degradation of the target mRNAs occurs at the post-transcriptional level and is termed post-transcriptional gene silencing, one of the RNA interference (RNAi) pathways. RNAi requires incorporation of one of the short RNA strands into the RNA-induced silencing complex (RISC), wherein the sequence serves as a guide for identification of the targeted RNAs through base pairing. Argonaute 2 (Ago2) in RISC cleaves the target at a single site within the target mRNA through the PIWI domain (2–4), subsequently degrading the target. Because most protein components of RNAi, including Ago2 and Dicer, assemble and function in the cytoplasm (5–8) it is widely believed that RNAi only occurs in the cytoplasm.

Several lines of evidence have indicated that RNAi-like phenomena may also occur in the nucleus in addition to the well characterized cytoplasmic mechanism. siRNAs have been shown to initiate transcriptional gene silencing by targeting DNA sequences in the nucleus of fission yeast (9), flies (10), and human cells (11, 12). Moreover, in Caenorhabditis elegans, nuclear proteins are reportedly required for RNAi (13). Previous studies have also indirectly indicated nuclear RNAi-like pathways in animals (4, 10, 14, 15), and a recent study demonstrated that the nuclear 7SK RNA can be degraded by siRNAs (16).

Myotonic dystrophy type 1 (DM1) is a neuromuscular disorder caused by a large unstable CTG expansion in the 3’-UTR of the DMPK (myotonic dystrophy protein kinase) gene (17). Mutant (mt) transcripts harboring the large CUG repeats are fully transcribed and polyadenylated, but remain trapped in the nucleus (18). Complete nuclear retention of mt DMPK mRNAs with large CUG repeats is believed to be one of the most important pathological features of DM1 (19). Because DM1 cells express both a normal cytoplasmically localized DMPK mRNA and a mt nuclear retained version of this mRNA, this transcript represents a good target to determine whether or not the same siRNAs can target both transcripts for degradation. Using heterozygous DM1 myoblasts, we show reduction of both nuclear retained mt DMPK mRNAs containing long CUG repeats and cytoplasmic wt DMPK mRNAs that do not have long CUG repeats. The co-existence of nuclear and cytoplasmic RNAi in humans suggests that components of the RNAi machinery exist in both compartments. Finally, we demonstrate here that the same short hairpin RNA transcripts can function in both compartments, suggesting either nuclear Dicing or import of Diced siRNAs into the nucleus. These findings have important implications for applications of siRNAs in mammalian gene regulation.
MATERIALS AND METHODS

Primary Human Muscle Cell Cultures—DM1 and normal control myoblasts were obtained from the quadriiceps of 15-week-old aborted fetuses. Skeletal muscle biopsies were approved by Laval University and the CHUL ethical committees. Myoblasts were grown and differentiated as described previously (20).

In Situ Hybridization and Confocal Microscopy—Myoblasts grown on glass coverslips were hybridized with a PNA Cy3-(CAG), probe to detect mt DMPK mRNAs as described in the Singer laboratory protocols (20). Nuclei were visualized by inclusion of 4’,6-diamidino-2-phenylindole (DAPI) in the mounting solution. Samples were observed using confocal microscopy (Zeiss LSM 510), and optical sections were obtained at optical Z resolution. Amira software was used to process images and construct three-dimensional surface models of the confocal image data stacks.

Cloning of shRNAs into pHIV7, Their Transduction, and siRNA Transfection to DM1 Cells—shRNAs DM10, DM130, and DM1892 are directed to nucleotides 10–30, 130–150, and 1,892–1,912, respectively, of the human skeletal muscle DMPK mRNA (GenBankTM accession number NM_004409) (see Fig. 2A). shRNAs were cloned just downstream of a human U6 promoter using the following method. pTZ U6-1 was used as a template for PCR (21). The 5’-PCR primer contains the complementary sequences of the 3’-end of the U6 promoter (25-mer) and shRNA (21-nucleotide sense, 4-nucleotide loop (TGCC)), and 21-nucleotide antisense sequence; 46-mer) and six adenines (i.e. a total of 77-mer). PCR was performed using Taq polymerase according to manufacturer specifications (Invitrogen). PCR products containing 3’-A overhangs were digested with 3’-T overhangs. The shRNA clones were confirmed by DNA sequencing.

shRNA constructs in pCR2.1 vectors were digested with BamHI and NotI, purified, and ligated into the linearized pCR2.1 vectors (Invitrogen). Anti-DMPK polyclonal antibody (a gift from Dr. Lubov Timchenko, Baylor College) was used as a control protein (20).

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RESULTS

Heterozygous DM1 myoblasts have ~3,200 CTG repeats in one allele of the DMPK gene and 18 CTG repeats in the other (Fig. 1A). The size differences of the transcripts were verified by Northern (Fig. 1B) and Southern hybridization (data not shown). The DM1 myoblasts were isolated from the quadriiceps of a 15-week-old aborted, congenitally affected DM1 fetus (see “Materials and Methods”). Culturing of the myoblasts for 48 h in differentiation medium allowed DMPK expression but did not result in myoblast fusion (Fig. 1C). Fluorescent in situ hybridization using a Cy3-labeled DNA probe to the region upstream of the repeats shows that mt DMPK mRNAs are localized in the nucleus of DM1 myoblasts and normal (wt) transcripts are visible in the cytoplasm (Fig. 1C, right), whereas they are localized exclusively in the cytoplasm of normal myoblasts, as demonstrated previously by several groups (18, 25–27). To investigate the subnuclear localization of mt DMPK transcripts, confocal microscope imaging was performed following fluorescent in situ hybridization on the differentiated DM1 cells. DMPK mRNAs were localized inside the nucleus distal to the nuclear envelope (Fig. 1D). The localization of mt and wt DMPK mRNAs cannot be distinguished using fluorescent in situ hybridization, because they have the same base composition, but only DMPK mRNAs in the differentiated DM1 cells show nuclear signals. Therefore, DMPK mRNAs in the DM1 cells following differentiation represent an excellent model system to study nuclear RNAi.

A lentivirus-based vector (pHIV7) was used to deliver shRNAs targeting DMPK mRNAs under control of the human U6 Pol III promoter into the DM1 myoblasts (22). An eGFP gene driven by a CMV promoter was also incorporated as a selection marker (Fig. 2A). Four shRNAs were designed. Two are complementary to the coding sequences of the DMPK mRNA (DM10 and DM130), one is targeted to a site in the 3′-UTR previously shown to be accessible to ribozyme cleavage (DM1892) (21), and one is directed to an irrelevant sequence (22, CTRL IR). shRNA constructs were efficiently transduced into cultured DM1 myoblasts (Fig. 2B). High levels of transgene expression could be obtained throughout myoblast differentiation following their genome integration (28). The cells expressing eGFP were then sorted by flow cytometry. The expression of shRNAs was assessed by Northern hybridization using the total RNAs isolated from sorted cells with sense probes that hybridize to the antisense strands but not to targets. All shRNAs were expressed from the lentiviral vectors, and they were processed into ~21-nucleotide siRNAs as expected (Fig. 2B).

To investigate the function of shRNA constructs in DM1 cell pellets was washed twice in 2× SSC. The nylon filter was removed and UV light cross-linked for 3 min in preparation for nuclear run-on assays. For nuclear run-on assays, isolation of nuclei and synthesis of radio-labeled nascent RNAs were performed as follows. Transcriptions were carried out for 50 min in the presence of 200 μCi of [α-32P]ATP/sample to maintain similar specific activities between wt and mt DMPK transcripts. Total labeled RNAs were extracted from the samples described above. Hybridization was carried out overnight in the same Northern blot hybridization buffer as described previously (20), except for replacement of the salmon sperm DNA with 200 μg/ml of Escherichia coli tRNA. The CTG-80 probe specifically binds to mt DMPK mRNA but not to transcribed, as verified by Northern blotting (data not shown). All assays were performed in duplicate and carried out simultaneously with or without 400 μg/ml a-amanitin, which blocked transcription in all samples (data not shown). DMPK mRNAs were normalized with cyclophilin transcripts in each sample.

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myoblasts, the total RNA was extracted from transduced primary myoblast cultures, and the level of DMPK mRNA was determined by Northern hybridization using a DMPK cDNA probe (Fig. 3A). Normal DMPK mRNA contains between 5 and 37 CUG repeats and is easily distinguished from the mt DMPK transcripts containing long CUG repeats (Fig. 1B). Myoblasts were allowed to differentiate in the absence of growth factors for 48 h prior to RNA extraction to induce the expression of DMPK mRNA (29). Steady-state levels of mt transcripts were elevated in congenital DM1 cells, because mt DMPK transcripts with large CUG repeat expansions are more stable than the normal transcripts (data not shown). shRNAs DM10 and DM130 down-regulated the normal transcripts (26.5 ± 2.4 and 15.1 ± 3.3%). The irrelevant shRNA control did not elicit any significant change in total DMPK mRNA levels. Taken together our data suggest that an RNAi-like phenomenon occurs not only with cytoplasmic transcripts but also with nuclear transcripts.

To confirm that nuclear transcripts are affected by shRNAs, we performed nuclear and cytoplasmic fractionation of differentiated DM1 cells stably expressing the shRNAs (Fig. 3, C–E). The mt DMPK mRNAs were reduced effectively by both shRNAs DM10 and DM130 (51.5 ± 6.6 and 48.8 ± 3.9%) (Fig. 3). In addition, the normal transcripts remaining in the nuclear fraction were also reduced by these shRNAs (47.2 ± 5.7 and 46.2 ± 4.0%). As expected, shRNAs DM10 and DM130 down-regulated the normal transcripts found in the cytoplasm (77.8 ± 1.3 and 73.6 ± 1.5%). We could not detect any intermediate size cleavage products from the nuclear targeted transcripts, suggesting that the RNAs are rapidly degraded following cleavage. The changes in DMPK levels obtained from this fractionation experiment showed a similar trend as that seen with the Northern blot in (Fig. 3, A and B). The shRNAs DM10 and DM130 were the most effective and DM1892 had a weak but significant effect. These results show that shRNAs reduce both nuclear and cytoplasmic transcripts, demonstrating the existence of an RNAi(-like) process in both compartments of human cells.

Because only the normal DMPK transcripts can be translated in the cytoplasm of DM1 myoblasts, we performed a Western blot to measure the levels of DMPK protein translated from these normal transcripts. shRNAs DM10 and DM130 reduced DMPK protein levels by 55.9 ± 3.2 and 73.2 ± 3.0%, respectively, relative to the control (Fig. 3, F–H). The levels of DMPK protein were lowered by 30.8 ± 4.7% in DM1 cells expressing shRNA DM1892. These results are consistent with the reduction in mRNA levels determined with Northern blotting (Fig. 3, A and B).

To confirm nuclear RNAi(-like) pathway of shRNAs against mt DMPK mRNAs, we transfected DM1 cells with synthetic siRNA DM10 (ssiDM10) using two different methods. The first methodology relied on MPG, a fusion peptide derived from the HIV type-1 gp41 transmembrane protein and the SV40 nuclear localization peptide, which has previously been reported to facilitate the nuclear import of siRNAs (12, 30), whereas the second method relied on conventional Lipofectamine 2000,
which lacks nuclear specificity and has been reported to generally deliver siRNAs to target cells (31). To confirm delivery of ssiDM10 into the nucleus or cytoplasm using MPG or Lipofectamine 2000, we used Cy3-labeled ssiDM10. After transfection and differentiation, we performed confocal microscopy and then processed the image stacks to construct three-dimensional models using Amira software (Fig. 4A, B and C). Lipofectamine delivered ssiDM10 mainly to the cytoplasm (Fig. 4B), whereas MPG delivered siRNAs into both the nuclear and cytoplasmic compartments (Fig. 4C). To examine whether nuclear down-regulation of mt DMPK transcripts were affected by different delivery methods, we performed Northern hybridization analyses on RNAs from the DM1 cells transfected by MPG or Lipofectamine. Using a modified Lipofectamine 2000 to increase the transfection efficiency to DM1 cells (−65%), ssiDM10 effectively diminished the level of wt DMPK mRNAs (−70%) but not mt DMPK transcripts (Fig. 4A). However, ssiDM10 effectively diminished the level of mt DMPK mRNAs (−75%) (as well as wt DMPK mRNAs) in the DM1 cells transfected via MPG (Fig. 4A). A mutant form of ssiDM10 (mt ssiDM10), which contains four mismatches in the middle of the antisense strand, showed no down-regulation of wt or mt transcripts when either MPG or Lipofectamine was used, demonstrating sequence specificity for both the nuclear and cytoplasmic mechanisms. Using a mixture of MPG and Lipofectamine, in which the efficiency for ssiRNA delivery was made really poor by disrupting membrane organization (32), we did not detect the knockdown of either transcript (Fig. 4A, lanes 6 and 7). These data suggest that the initial nuclear expression of shRNAs produce an interference-like effect in the nucleus of primary human cells when the target message is generally retained in the nucleus.

An alternative explanation for the observed reduction in steady-state mRNA levels could be a decrease in transcription, because transcriptional gene silencing can be initiated by double-stranded RNAs (33), and it has recently been reported that transcriptional gene silencing can be induced by siRNAs and shRNAs in human cells (11, 12). We therefore carried out nuclear run-on experiments using nuclei isolated from transduced DM1 myoblasts expressing the shRNAs (Fig. 5). Cells expressing shRNAs DM10 and DM130 show reduced amounts of wt and mt DMPK transcripts (Fig. 5A), but transcriptional initiation appears normal for both mt and wt DMPK (Fig. 5, B and C). The reduced mRNA levels seen in both the nucleus and the cytoplasm of DM1 cells is thus not a result of altered transcription but is due to post-transcriptional gene silencing. Taken together our data strongly support RNAi mechanisms in both the nuclear and cytoplasmic compartments.

**DISCUSSION**

We were surprised to find a significant reduction in the nuclear retained mt DMPK mRNAs in DM1 cells, because existing evidence indicates that RNAi pathways operate primarily in the cytoplasm. Most protein components of the RISC are cytoplasmic (5–8). In addition, a separate study concluded that only cytoplasmic transcripts could be targeted by RNAi (8), although some reduction in nuclear transcripts was apparent, this was attributed to RNAi acting on transcripts in the process of being exported. The mt DMPK transcripts targeted in this study are located exclusively in the nucleoplasm (Fig. 1D and Ref. 18) and are clearly down-regulated by the appropriate siRNAs. Given our observations of the intracellular compartmentalization of the mt DMPK RNAs, we believe that the observed down-regulation is occurring in the nucleus and is not associated with transport of the mt DMPK through the nuclear pores. During the course of our work another study also demonstrated direct targeting of 7SK small nuclear RNA in human cells by siRNAs, and other siRNAs could also trigger RNAi on endogenous, nuclear retained U6snRNA (16). Moreover some RISC components could be found in the nuclear as well as cytoplasmic compartments (16). Taken together with our results, a nuclear RNAi(-like) pathway appears to operate in human cells.

A number of indirect studies have also indicated that RNAi(-like) phenomena may occur in the nucleus of human cells in addition to the well characterized cytoplasmic mechanism. A nuclear RNAi pathway was predicted by a study demonstrating that shRNAs under the control of the U6+27 promoter, although exclusively nuclear, are functionally active in silencing targeted transcripts (34). In *C. elegans*, the intron containing pre-mRNAs can be targeted by RNAi (14), siRNAs have been shown to initiate transcriptional gene silencing by targeting DNA sequences in the nucleus of fission yeast (9), flies (10), and human cells (11, 12). Moreover, in *C. elegans*, nuclear proteins are reportedly required for RNAi (13). Previous studies have also indirectly indicated nuclear RNAi(-like) pathways in animals (4, 10, 14, 15). These studies suggest that nuclear RNAi(-like) phenomena occur in a variety of animals.

There are some notable differences between our results and the Rana and co-workers (16) demonstration of nuclear RNAi, even though both used in human cells. The mt DMPK transcripts we targeted with shRNAs are large nuclear retained
mRNAs (12.4 kb) transcribed by RNA polymerase II (Pol II), whereas the 7SK or U6 RNAs targeted by Rana and co-workers (16) are non-coding small nuclear RNAs transcribed by Pol III. We demonstrated that the same shRNAs expressed from lentiviral vectors can simultaneously target both nuclear and cytoplasmic transcripts, whereas they observed knockdown of exclusive nuclear targets using synthetic siRNA. In addition, our observation used differentiated primary human cells rather than human cell lines. Taken together, our study along with their results (16) suggest that nuclear RNAi(-like) mechanism can target a variety of different transcripts in several types of human cells and may thus be a general phenomenon.

The demonstration that shRNAs can direct nuclear RNA destruction raises the question of whether the shRNAs are processed into siRNAs directly in the nucleus or are processed in the cytoplasm and transported back into the nucleus. The FIG. 3. Down-regulation of DMPK mRNAs in DM1 cells transduced with lentiviral vectors containing shRNAs. A, down-regulation of both normal (wt) and mutant (mt) DMPK mRNAs by shRNAs using Northern hybridization. Myoblasts were allowed to differentiate for 48 h in the appropriate media prior to RNA extraction to induce DMPK mRNA levels. Both transcripts are reduced by shRNAs DM10 and DM130. B, quantification of DMPK mRNAs normalized to cyclophilin mRNA. Standard error bars are based upon four independent experiments. C, Northern hybridization of nuclear (N) and cytoplasmic (C) extraction of differentiated DM1 myoblasts stably expressing shRNAs by lentiviral vectors. Approximately four times more volume of nuclear extracts than cytoplasmic extracts was loaded in the gel to achieve equal loading of total RNAs. D, hybridization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in A. E, quantification of DMPK mRNAs normalized to glyceraldehyde-3-phosphate dehydrogenase. Normalizations were also carried out relative to total 28 S rRNA intensities using ethidium bromide staining to confirm cell fractionation purity (data not shown). Standard error bars are based on three independent experiments. F, Western blotting of whole cell extracts using anti-DMPK antibody. Cells were allowed to differentiate for 48 h prior to harvesting. Only normal DMPK mRNA contributes to DMPK protein levels because the mutant mRNA is nuclear retained and not translated. G, Ponceau red staining of the membrane prior to blotting with a rabbit anti-DMPK antibody. Samples were normalized to total sample protein levels. H, quantification of DMPK protein normalized to Ponceau red staining. Standard error bars are based on four independent experiments.

FIG. 4. Effect of different delivery methods of siRNAs on down-regulation of wt and mt DMPK mRNAs in DM1 cells. A, expression of wt and mt DMPK mRNAs in DM1 cells transfected with wt or mt siDM10 using Lipofectamine 2000 (cytoplasmic (Cy)) or MPG (nuclear (Nu)). mt siDM10 contains four mismatches to the target in the middle of the antisense strand and was used as a control for specificity of RNAi. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. A combination of Lipofectamine and MPG was also used as a negative control as described in the text. An RNA marker (M) was used as a size indicator. One representative Northern assay from two independent experiments is depicted. GFP plasmids were co-transfected with the siRNAs to monitor transfection efficiency in the DM1 cells. B and C, localization of Cy3-labeled siRNAs by different transfection methods using fluorescent microscopy. Cytoplasmic delivery of siRNAs was accomplished by Lipofectamine 2000 (B). MPG can deliver some siRNAs to the nucleus (~35%) (C). DAPI staining was used to identify the nucleus. Scale bars = 10 μm.
processing of the shRNAs could take place via a Dicer cleavage mechanism or via endonucleolytic processing of the loop. At this time we have not been able to distinguish differences between these possibilities. Because siRNAs delivered to the nucleus by the MPG peptide can also trigger nuclear RNAi, this has important implications for future myotonic dystrophy treatment or other nuclear diseases.

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