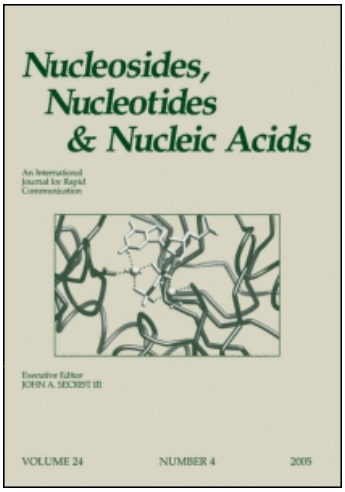

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Guanine Nucleotide Depletion Mediates Translocation of Nucleolar Proteins, Including RNA Helicase A (DHX-9)

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GUANINE NUCLEOTIDE DEPLETION MEDIATES TRANSLOCATION OF NUCLEOLAR PROTEINS, INCLUDING RNA HELICASE A (DHX-9)

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□ *DHX-9, a member of the DEXH family of RNA helicases, unwinds dsRNA/dsDNA by ATP or GTP-dependent hydrolysis. We asked whether DHX-9 played a role in the GTP depletion-induced inhibition of rRNA synthesis and/or nucleolar disruption. MPA, a specific inhibitor of inosine monophosphate dehydrogenase (IMPDH), induced a rapid translocation of DHX-9 from the nucleolus to the nucleus. EGFP-tagged DHX-9 mutated at the GTP binding site also localized to the nucleus. However, knockdown of DHX-9 by siRNA did not inhibit the rRNA synthesis or cause the nucleolar disruption. Thus, DHX-9 translocation found with IMPDH inhibition does not mediate the inhibition of rRNA synthesis.*

Keywords DHX-9; translocation; IMPDH; MPA; AVN-944

INTRODUCTION

RNA helicase (RHA, DHX-9), a member of the DEXH family of RNA helicases, regulates gene expression at various steps including transcriptional activation, RNA processing (eg. splicing) and export, and translation (polyribosomal association) through unwinding both double-stranded RNA (dsRNA) and DNA (dsDNA) by ATP or GTP-dependent hydrolysis.[1–3] A number of recent studies have documented the antiviral effects of small molecule inhibitors on viral or host NTPase/helicase. These inhibitors include amino-thiazolyphenyl[4] and thiazole amide derivatives,[5] inhibitors of the HSV helicase–primase; ribavirin, a nucleoside analogue inhibitor of IMPDH and also of HCV helicase;[6] and murabutide, a synthetic immunomodulator that was shown recently to suppress HIV-1 replication in macrophages and T cells by inhibiting the activity of host RNA helicase RH116.[7] Unlike HSV and HCV viruses, HIV-1 does not encode any RNA helicase,[8] and the host RNA helicase A was recently found to serve as a

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viral helicase to regulate the reverse transcription and transport of HIV-l viral mRNA.[8,9] Mechanistic studies suggest that RNA helicase A may exert its action through interaction with the 5′ proximal post-transcriptional control element (PCE) of HIV-1.[8,9] Additionally, RNA helicase A may play a role in HIV-1 particle assembly and reverse transcription.[10] RHA in association with PCE is also essential for reverse transcription and translation of other retroviral transcripts such as HTLV-1.[11]

We have recently demonstrated that total RNA synthesis as well as preribosomal RNA synthesis is potently inhibited by AVN944- or MPA-induced depletion of cellular guanine nucleotide pools.[12] While exploring the mechanism involved in the regulation of RNA transcription, we found that GTP depletion induces translocation of the nucleolar portion of DHX-9 to the nucleoplasm, and similar altered localization of DHX-9 was observed in two DHX-9 mutants in which the consensus sites for either ATP or GTP binding were mutated. The possibility that host cell helicase could serve as a therapeutic target for antiviral or anticancer chemotherapy lead us to further investigate this phenomenon.

MATERIALS AND METHODS

Cell Culture Conditions and Reagents

Uridine ([5,6-^{3}H], 35-50 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA, USA). Actinomycin-D was obtained from Calbiochem (La Jolla, CA, USA). 4′,6-diamino-2-phenylindol (DAPI) was purchased from Molecular Probes, Inc (Eugene, OR, USA). Antinucleolin monoclonal antibody (mAb) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-DHX-9 was from Cell Signaling Technology Inc. (Beverly, MA, USA). Fluorescein (FITC)-conjugated donkey anti-rabbit F(ab′)2 and rhodamine-conjugated goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

Generation of Expression Constructs

Isolation of total RNA and first-strand cDNA synthesis were performed as described previously.[12] The resulting first-strand cDNA was used for PCR amplification with high fidelity pfx DNA polymerase (Invitrogen). The primers used for DHX-9 are 5′-cttc***gaattc***tgatgggtgacgttaaaaattttctgtatg-3′ (forward primer) and 5′-cggt***ggatcc***ttaatagccgccacctcctcttccctgtcc-3′ (reverse primer). The italicized bases are the restriction enzyme sites (EcoR1 and BamH1) with three or four extra bases added at each end to promote efficient digestion by the restriction enzymes.

The DHX-9 PCR products were subcloned into the EcoR1 and BamH1 of p-EGFP-C3 (BD, biotech), and the resulting plasmids were sequenced.

K417R and G411A plus G416A mutant of DHX-9 was created by Quick Change Mutagenesis kit from Agilent, San Diego, CA, USA. The EGFP-DHX-9 construct and its mutant were introduced into U2OS cells by Superfect transfection (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction.

Immunocytochemistry

U2OS cells were grown on coverslips in 24-well plates in DMEM complete medium for 12 hours and were then treated with MPA (2 μM), Actinomycin D (5 nM or 500 nM), or vehicle control for the time indicated. Antibody concentrations are: primary antibody for DHX-9 diluted 1:50, and fluorescein isothiocyanate-conjugated secondary antibody diluted 1:100.

Measurement of Total RNA Synthesis and 45S Precursor rRNA Synthesis

[^{3}H] uridine incorporation into total RNA and determination of 45S precursor rRNA levels by semiquantitative RT-PCR were performed as described previously.[14]

Transfection of MCF-7 Cells with Double-Stranded RNA (siRNA) and Western Blot

The sequences of oligonucleotide for DHX-9 siRNA (5′-AAGAAGTGCAAGCGACTCTAG-3′) and control oligonucleotide (5′-AAAGTCATCGTGACTACGACG-3′) are described previously.[13] Oligonucleotides were synthesized by Qiagen, and electroporated into MCF-7 cells by using Amaxa Electroporation kit (supplemented Nucleofector). 5×10^6 cells/sample were resuspended in 100 μl of room temperature Nucelofector solution and then electroporated with 3 μg of siRNA (control siRNA, DHX-siRNA) or mock control. After electroporation, the cells were incubated at a density of 5×10^5/ml for additional 72 hours and were harvested for immunoblot analysis for DHX-9. Actin was used as a loading control to assess siRNA specificity. Western blots were performed as described previously.[14]

RESULTS

Effect of IMPDH Inhibition on Total and Pre-rRNA Synthesis

Both the total RNA synthesis and the pre-rRNA synthesis were markedly inhibited after MPA treatment of U2OS cells (Figures 1A and 1B). Similar inhibition was obtained with 500 nM Act-D, a dose that inhibits RNA polymerases I and II (Figure 2B).

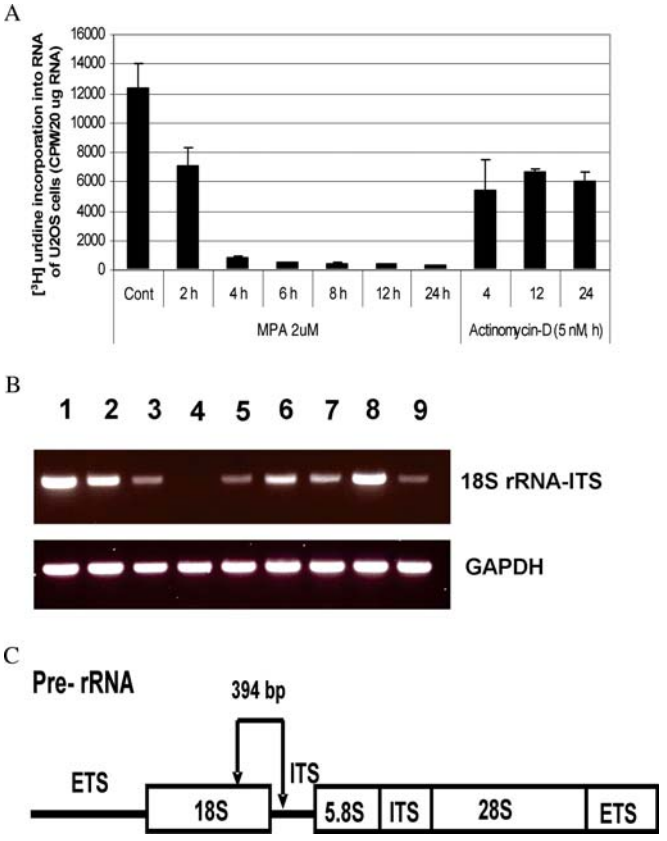

FIGURE 1 Effects of MPA and Actinomycin D on total RNA synthesis and pre-rRNA synthesis. U2OS cells were treated with MPA or Act-D at the concentrations and times indicated and [^{3}H]-uridine incorporation into RNA was measured (A); the effect of MPA on the expression of pre-rRNA levels was determined by RT-PCR (B). Lane 1, U2OS control; lane 2-5, U2OS cells treated with MPA for 2, 4, 8, and 24 hours; lane 6-7, U2OS cells treated with 5 nM Act-D for 0.5 and 4 hours; lane 8-9, U2OS cells treated with 500 nM Act-D for 0.5 and 4 hours. Primers aamplify a 394 bp fragment extending from the 3′ end of 18 S rRNA to the internal transcribed sequence (ITS) located between 18 S rRNA and 5.8 S rRNA of the intact pre-rRNA (shown in C).

Effect of GTP Depletion on the Translocation of Nucleolar DHX-9

As shown in Figure 2A, endogenous DHX-9 is predominantly localized in the nucleolus; MPA induces the translocation of DHX-9 to the nucleus after 2 hours of exposure.

Translocation of DHX-9 Induced by Actinomycin-D

U2OS cells were treated with with low (5 nM; specifically inhibits RNA polymerase I) or high (500 nM) dose actinomycin-D.[12]. As shown in Figure 2B, DHX-9 shifts in location from the nucleolus to the nucleus in

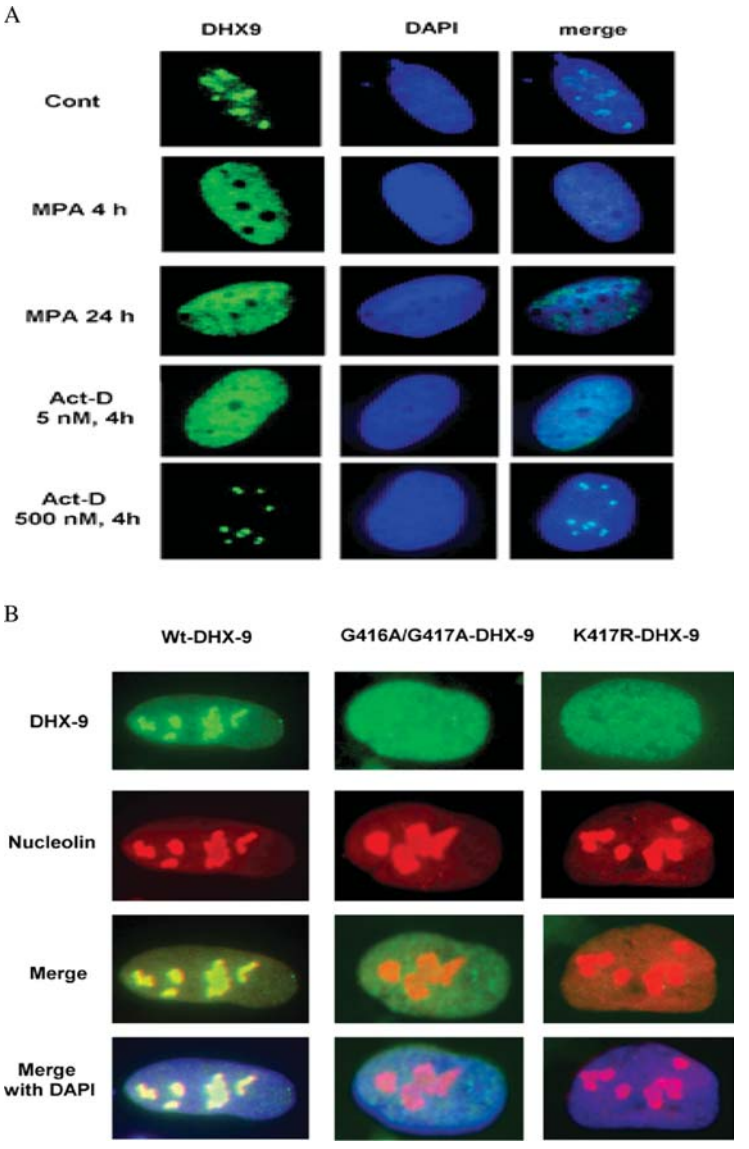

FIGURE 2 Effects of MPA, Actinomycin-D and mutations at the GTP or ATP binding sites of DHX-9 on its localization. U2OS cells were treated with 2 μM MPA for the time indicated, or 5 nM or 500 nM Act-D for 4 hours (A). DHX-9 was fused to EGFP and the K417R DHX-9 and G411A plus G416A DHX-9 mutations were created by site-directed mutagenesis. The resulting constructs were transfected into U2OS cells for 24 hours (B). Drug-treated or transfected cells were fixed, permealized, stained for DHX-9, and observed using fluorescent microscopy.

response to treatment with low dose actinomycin D, but moves to the nucleolar periphery (nucleolar cap) with high dose actinomycin-D. These data indicate that translocation of DHX-9 by GTP depletion and lower dose of actinomycin-D both correlate with inhibition of rRNA synthesis.

Localization of Wildtype EGFP-DHX-9 Fusion Protein and Its ATP or GTP Binding Site Mutants

Wildtype EGFP-DHX-9 fusion protein shows predominantly nucleolar localization, as does the endogenous protein (Figure 2C). In contrast, the G411A plus G416A mutations at the consensus GTP binding sites and a K417R mutant at the putative ATP binding site are localized in the nucleus (Figure 2C).

Effect of siRNA Knock-Down of DHX-9 by siRNA on RNA Synthesis and Nucleolar Localization

To further investigate the possible involvement of DHX-9 in the regulation of RNA Polymerase I activity, and subsequent RNA synthesis, MCF-7 cells were transfected with DHX-9 siRNA by electroporation. As shown in Figure 3A, a greater than 80% decrease of DHX-9 protein was achieved after 72 hours in MCF-7 cells. However, total RNA synthesis (data not shown) and nucleolar localization of nucleolin (Figure 3B) remained unchanged. Since Pol I-directed rRNA synthesis accounts for the majority of total RNA synthesis, the pre-rRNA synthesis is unlikely to be inhibited by reduced DHX-9 expression.

DISCUSSION

We have demonstrated that GTP depletion mediated by IMPDH inhibition induces rapid and complete shifts in location of nucleolar DHX-9 to the nucleoplasm in U2OS cells, which is in contrast to the nucleolar localization of both endogenous DHX-9 and the EGFP-tagged DHX-9 fusion protein. Nucleolar localization of DHX-9 has been shown previously in several cell types including NIH3T3, p388-D1, bovine oocytes, and MCF-7 cells[14–16] However, DHX-9 has also been shown to localize to the nucleolus in other cell types.[17] The mechanism whereby DHX-9 is enriched in one sub-cellular compartment versus another has not been studied. The fact that DHX-9 is enriched in the nucleolus of rapidly dividing as compared to resting cells suggests that the distribution of DHX-9 into various sub-cellular compartment may be influenced by the proliferation status of various cell types.

We have identified a distinct consensus GTP binding site within DHX-9 that overlaps partially with the ATP binding site (The ATP binding site motif: GCGKT at amino acids 414 to 418;[1] the GTP binding site motif: p-loop, GATGCGKT at amino acids 411 to 418 (GxxxxGK(S/T). The fact that GTP depletion induces translocation of nucleolar DHX-9 to the nucleus prompted us to evaluate whether the GTP and/or ATP binding motifs are involved in the regulation of nucleolar localization. Both endogenous

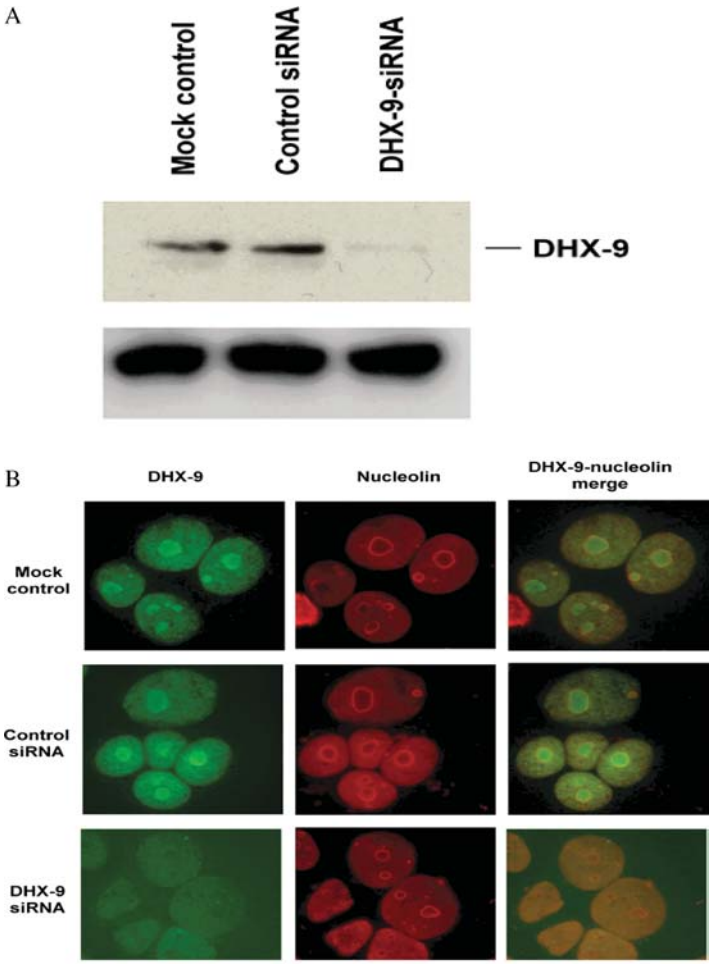

FIGURE 3 Effects of knockdown DHX-9 by siRNA on total RNA synthesis and nucleolin localization in MCF-7 cells. MCF-7 cells were introduced with DHX-9 siRNA by Amaxa electroporation as described in the Materials and Methods section. After 72 hours, cells were lysed and 30 μg of total protein were loaded for immunoblot analysis for DHX-9 and actin (A); Cells were fixed, permeabilized, and stained for DHX-9 and nucleolin (B).

DHX-9 and EGFP fusion DHX-9 (EGFP-DHX-9) are predominantly localized in the nucleolus; in contrast, the G411A plus G416A DHX-9 construct containing mutations at the consensus GTP binding motif, is located in the nucleus. A similar result was obtained with the K417R DHX-9 mutant containing a mutation at the ATP binding site. Mutation of this site has been shown to abolish helicase activity dependent on ATP binding.[1] It is possible that mutation of the ATP binding site may also alter GTP binding, as these sites overlap. Thus, it is reasonable to postulate that the direct binding of GTP to DHX-9 plays a role in the regulation of DHX-9 localization.

The significant reduction in DHX-9 protein levels induced by siRNA was not sufficient to inhibit pre-rRNA synthesis, nor was it associated with an alteration in the localization of other nucleolar proteins such as nucleolin and nucleophosmin. We therefore think it unlikely that the shift in localization of DHX-9 is primarily responsible for the cell cycle arrest and/or apoptosis that results from guanine nucleotide depletion.

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