E2F1-dependent methyl cap formation requires RNA pol II phosphorylation

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Gene expression is a process integral to cell proliferation. The E2F family of transcription factors upregulates expression of transcripts whose products are essential for cell cycle progression. Here, we report that E2F1 promotes gene expression by an additional mechanism, that is, formation of the methyl cap on RNA pol II transcripts. The methyl cap is required for mRNA maturation, expression and stability. We demonstrate that E2F1 increases RNA pol II phosphorylation, which promotes recruitment of the methyl cap synthetic enzymes. Upregulation of RNA pol II phosphorylation is required for E2F1-dependent methyl cap formation.

Introduction

Cell proliferation requires gene expression to drive the cell cycle and to provide sufficient protein synthesis for cell growth. Several transcription factors are key mediators of gene expression during the cell cycle, including E2F1, which regulates cell proliferation and apoptosis.[1] E2F1 has been demonstrated to upregulate both transcription and formation of the 7-methylguanosine cap or methyl cap.[2] The methyl cap is a structure added to the 5' end of RNA pol II transcripts that is essential for gene expression, mediating processes including transcript stabilization, splicing, nuclear export and translation initiation.[3,4] Regulation of methyl cap abundance has been observed in mammalian cells and yeast under conditions which influence cell growth and proliferation,[2,5] and mRNA cap methylation has been demonstrated to be rate-limiting for gene expression and cell proliferation.[6-8]

Methyl cap formation occurs early during transcription, and the enzymes which promote its formation, Capping enzyme (CE) and RNA guanine-7 methyltransferase (RNMT), are recruited to transcription initiation sites via an interaction with RNA pol II.[3,4] Transcripts are synthesized with a triphosphate group on the 5' terminus, and Capping enzyme catalyzes removal of the terminal phosphate and addition of an inverted guanosine cap to create the structure GpppX (X is the first transcribed nucleotide). RNA guanine-7 methyltransferase methylates the guanosine cap at the N-7 position to create the methyl cap, m7GpppX. RNA polymerase II is phosphorylated on the C-terminal domain (CTD) at the initiation of transcription, thus forming a docking site for CE and RNMT. The RNA pol II CTD has been demonstrated to be required for efficient methyl cap formation on transcripts produced from reporter constructs;[3,4] however, to our knowledge, RNA pol II phosphorylation has not been demonstrated to be required for methyl cap formation on endogenous transcripts.

Results and Discussion

In this study, we investigated regulation of methyl cap formation by E2F1 on its target transcript, the cyclin-dependent kinase, CDC2. E2F1 activity was regulated in fibroblasts by activation of E2F1-ER, a fusion protein of E2F1 and the estrogen receptor (ER).[9] E2F1-ER is retained in the cytoplasm until addition of the ER ligand, 4-hydroxytamoxifen, promotes its movement to the nucleus, where it binds E2F1 recognition motifs proximal to transcription initiation sites.[9] E2F1-ER was activated for 3 h; RNA was extracted, and RTPCR was used to demonstrate that the expression level of its target transcript, CDC2, was upregulated, whereas a control gene, GAPDH, was not (**Fig. 1A**). As had been observed previously, activation of E2F1 also resulted in an increase in the proportion of CDC2 transcripts with a methyl cap, as determined by anti-7-methylguanosine antibody immunoprecipitation followed by RTPCR (**Fig. 1B**). Methyl cap formation is dependent on recruitment of the methyl cap synthetic enzymes CE and RNMT to phosphorylated RNA pol II. Activation of E2F1 resulted in increased RNA pol II Ser-5 phosphorylation (**Fig. 1C**).
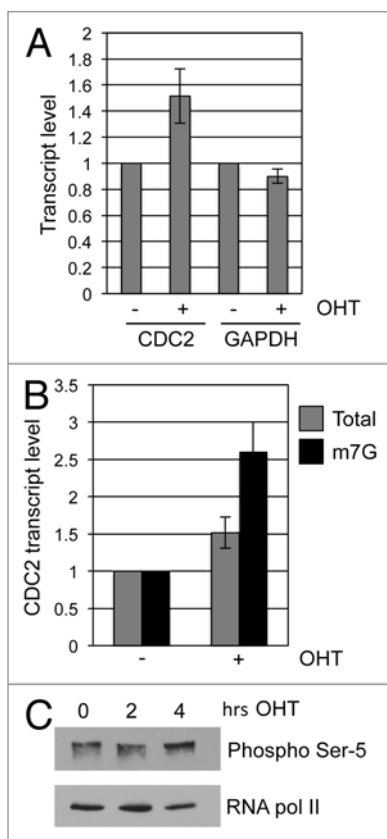
Figure 1. E2F1 regulates RNA pol II phosphorylation and cap methylation. E2F1-ER expressed in rat fibroblasts was activated by addition of 100 nM 4-hydroxytamoxifen (+) or vehicle control (-) for 3 h. (A) RNA was extracted and RT-PCR performed with primers specific for CDC2 and GAPDH. (B) RNA was oligo-dT-purified (total transcripts, gray bars) and subsequently anti-m7G-purified (m7G transcripts, black bars) and RT-PCR performed with primers specific for CDC2. (C) E2F1-ER was activated by incubation in 100 nM 4-hydroxytamoxifen (OHT) for the time course indicated. Western blotting was used to detect total RNA pol II and Ser-5 phosphorylated RNA pol II in nuclear extracts.

In order to determine the role of RNA pol II phosphorylation and transcription in the mechanism of methyl cap formation, cells were incubated with two inhibitors, Actinomycin D, a compound that forms a complex with DNA preventing movement of RNA polymerase, and DRB (Dichloro-1-β-D-ribofuranosylbenzimidazole riboside), an adenosine analog which inhibits RNA pol II kinases and, therefore, RNA pol II phosphorylation. The rate of RNA pol II transcription was determined by measuring the rate of ^{3}H-uridine incorporation into oligo-dT-purified RNA (predominantly mRNA). Incubating cells for 30 min with 175 nM Actinomycin D or 5 μM DRB inhibited RNA pol II-dependent transcription by approximately 90% (**Fig. 2A**). Following treatment with DRB or Actinomycin

D, CDC2 transcripts were depleted by approximately 50%, and CDC2 transcript levels became unresponsive to E2F1 (**Fig. 2B**).

The effect of the transcriptional inhibitors on E2F1-dependent methyl cap formation was determined by anti-7-methylguanosine antibody immunoprecipitation followed by CDC2 RTPCR. DRB treatment prevented the E2F1-dependent increase in the proportion of CDC2 transcripts with a methyl cap (**Fig. 2C**). This is consistent with the finding that CE and RNMT are recruited to phosphorylated RNA pol II at transcription initiation sites and validates the finding that RNA pol II phosphorylation is required for methyl cap formation on reporter construct transcripts. A surprising result was that Actinomycin D did not affect methyl cap formation on CDC2 transcripts. Since Actinomycin D and DRB inhibit transcription equivalently, whereas only DRB inhibits cap methylation, methyl cap formation is not dependent on full engagement of the transcription machinery.

Materials and Methods

Cell culture. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, at 37°C/5% CO_2. Rat fibroblasts were transduced with a retroviral expression vector directing expression of E2F1-ER.[9]

RNA analysis. RNA was extracted using Trizol reagent (Invitrogen) and further purified by phenol extraction. Oligo dT-cellulose (Ambion) purification was performed prior to anti-7-methylguanosine immunoprecipitation.[2] RTPCR was performed using gene-specific primers, all of which produced sequence-verified products.

RNA pol II detection. Cells were lysed in hypotonic lysis buffer and nuclear proteins extracted in Triton lysis buffer (10 mM Tris, pH 7.05, 50 mM NaCl, 30 mM Na_4 pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 10% glycerol, 0.5% Triton X-100, 10 μM Leupeptin, 1 μM Pepstatin and Aprotinin, supplemented with Phosphatase Inhibitor Cocktail I and II, Sigma). Twenty μg nuclear extract was resolved by SDS-PAGE and RNA pol II was detected by western blot (total RNA pol II SC-899, Santa Cruz Biotech, Ser-5 phosphorylated RNA pol II, H14 Convance).

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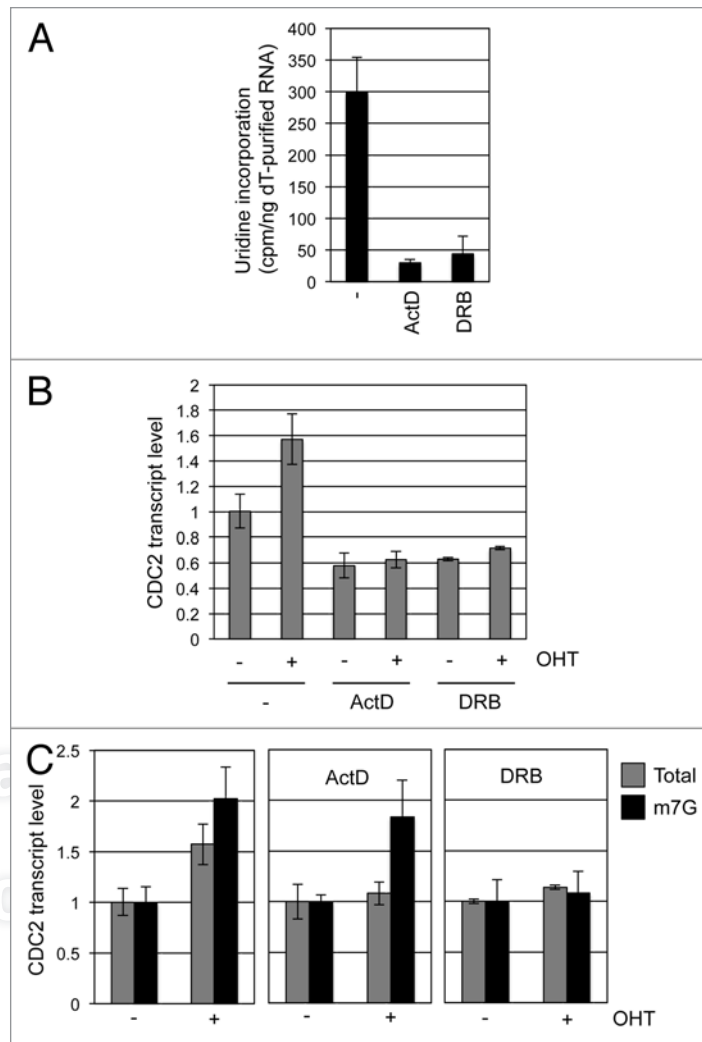
Figure 2. E2F1-dependent cap methylation requires RNA pol II phosphorylation. (A) Rat fibroblasts were incubated with 175 nM Actinomycin D (ActD), 5 μM DRB or vehicle control (-), for 30 min prior to addition of 10 μci/ml ^{3}H uridine for 15 min. RNA was harvested, oligo-dT-purified and ^{3}H uridine incorporation determined. (B) Following treatment with Act D or DRB, E2F1-ER was activated by addition of 100 nM 4-hydroxytamoxifen for 3 h (OHT). RNA was extracted, oligo-dT-purified and RT-PCR performed with primers specific for CDC2. (C) As in (b), RNA was oligo-dT-purified (total transcripts, gray bars) and anti-m7G-purified (m7G transcripts, black bars) and RT-PCR performed with primers specific for CDC2. Charts represent the average of three independent experiments and error bars indicate the standard deviation.