Published in final edited form as:
Oncogene. 2009 March 5; 28(9): 1169–1175. doi:10.1038/onc.2008.463.

Specific regulation of mRNA cap methylation by the c-Myc and E2F1 transcription factors

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Abstract

Methylation of the mRNA 5′ guanosine cap is essential for efficient gene expression. The 5′methyl cap binds to eIF4E, which is the first step in the recruitment of mRNA to the 40S ribosomal subunit. To investigate whether mRNA cap methylation is regulated in a gene-specific manner, we established a method to detect the relative level of cap methylation on specific mRNAs. We found that two transcription factors, c-Myc and E2F1, induce cap methylation of their transcriptional target genes, and therefore, c-Myc and E2F1 upregulate gene expression by simultaneously inducing transcription and promoting translation. c-Myc-induced cap methylation is greater than transcriptional induction for the majority of its target genes, indicating that this is a major mechanism by which Myc regulates gene expression.

Keywords

E2F1; gene expression; mRNA cap methylation; Myc; translation

Introduction

Nascent RNA polymerase II transcripts are processed by a series of modifications, including addition of the 5′ methyl cap, splicing and polyadenylation (Gingras *et al.*, 1999; Jacobson and Peltz, 1996; Shatkin and Manley, 2000). These modifications are essential for gene expression since they stabilize mRNA, promote mRNA nuclear export and permit efficient mRNA translation. The mRNA cap is 7-methylguanosine linked to the first transcribed nucleotide through a 5′-5′ triphosphate bridge, and is formed by a series of reactions (Shatkin, 1976; Shuman, 2002). Initially, the 5′ triphosphate of the nascent RNA is hydrolysed to diphosphate, and the diphosphate RNA is “capped” by the addition of GMP to create 5′GpppX, where X is the first transcribed nucleotide. In mammals, both the RNA triphosphatase and RNA guanylyltransferase are found in a single, bi-functional peptide called Capping Enzyme (CE). Prior to capping, nascent mRNA is readily degraded, and the addition of the cap stabilizes mRNA from attack by exoribonucleases (Schwer *et al.*, 1998). A separate enzyme, the RNA (guanine-7-) methyltransferase (RNMT), methylates the guanosine cap at the 7-position to produce 5′m7GpppX. mRNA cap methylation further protects the mRNA against attack by exonucleases and is essential for efficient cap-dependent translation of mRNA (Schwer *et al.*, 2000; Shatkin, 1976). The translation initiation factor, eIF4E, which is the initiating component of the eIF4F complex that recruits

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mRNA to the 40S ribosomal subunit, only binds efficiently to the mRNA cap when it is methylated (Gingras *et al.*, 1999; Gu and Lima, 2005).

Both mRNA capping and mRNA cap methylation occur co-transcriptionally at the initiation of transcription (Bentley, 2005; Sims *et al.*, 2004). The C-terminus of RNA polymerase II consists of approximately fifty repeats of the amino acid sequence YSPTSPS, which is phosphorylated on Ser-5 as transcription initiates. CE and RNMT bind to Ser-5 phosphorylated RNA polymerase II and are thus recruited proximal to their substrate, the 5′ end of the nascent transcript (McCracken *et al.*, 1997). In mammalian cells, CE and RNMT are found in distinct complexes, and therefore, it is possible that mRNA capping and cap methylation are differentially regulated in a gene-specific manner. Furthermore, CE and RNMT have been demonstrated to act with different kinetics on in vitro transcription reactions (Moteki and Price, 2002). In this report, we demonstrate that two transcription factors, c-Myc and E2F1, promote increased cap methylation of their transcriptional target genes, thus simultaneously inducing transcription and promoting translation. Furthermore, the majority of c-Myc transcriptional target genes are the target of both mechanisms of gene regulation, indicating that mRNA cap methylation is a major effector of c-Myc function.

Results

Synthesis of anti-7-methylguanosine antibodies

Efficient translation of mRNA is dependent on the methylation of the 5′ guanosine cap (Gingras *et al.*, 1999; Shatkin, 1976). To investigate the level of mRNA cap methylation on specific gene products, antibodies were raised and affinity-purified on 7-methylguanosine (m7G) (see methods). In order to determine the specificity of the antibodies, immunoprecipitations were performed on an excess of in vitro transcribed mRNA, which was synthesized with a guanosine cap (G-mRNA) or a 7-methylguanosine cap (m7G-mRNA). The anti-m7G antibodies bound to ten-fold more m7G-mRNA than G-mRNA (Fig. 1A and B). As a negative control, the same reactions were carried out with either unpurified preimmune serum, preimmune serum affinity-purified on 7-methylguanosine, or affinity-purified, polyclonal anti-EIF4E or Mnk1antibodies. None of the control antibodies bound significantly to either G-mRNA or m7G-mRNA (not shown) and the results for preimmune serum are shown in Fig. 1A and B.

The specificity of the interaction between the anti-m7G antibodies and m7G-mRNA was further tested by performing the immunoprecipitations in the presence of a titration of free 7-methylguanosine triphosphate (m7GTP) or guanosine triphosphate (GTP). One hundred-fold more GTP than m7GTP was required to inhibit the m7G-mRNA-antibody interaction by 50% (Fig 1C, see methods). Since the anti-m7G antibodies bound specifically to m7G-mRNA, we proceeded to use the antibodies to determine the relative level of cap methylation on specific cellular mRNAs.

c-Myc induces cap methylation of its target genes

c-Myc is a cellular transcription factor which promotes cell proliferation and is essential for development (Cowling and Cole, 2006; Dang *et al.*, 2006). Previously we demonstrated that N-Myc (a homologue of c-Myc) promotes increased translation of specific mRNAs (Cowling and Cole, 2007). We had found a subtle increase in the binding of an anti-2,2,7 trimethylguanosine antibody to these mRNAs when extracted from N-Myc expressing cells compared to vector control, indicating that N-Myc was upregulating mRNA cap methylation. With the production of anti-7-methylguanosine antibodies, the hypothesis that Myc proteins promote mRNA cap methylation could be tested more rigorously.

c-MycWT, c-MycΔMBII and vector control were expressed in *myc*-/- fibroblasts (Fig.2a). c-MycΔMBII has a deletion of Myc Box II (MBII), a domain essential for the maximal biological and biochemical response to Myc (Cowling and Cole, 2006). To investigate the relative cap methylation of specific transcripts, anti-m7G antibody immunoprecipitations were performed on total cellular RNA purified from log-phase cells. The relative mRNA levels and m7G-mRNA levels were determined for ten previously established c-Myc transcriptional target genes, RuvBL2, HSP60, EIF4A1, EIF4E, Nol5a, FBL, MINA, EBNA1BP2, EIF2B1 and CDK4 (Zeller *et al.*, 2003). The relative total mRNA and m7G-mRNA levels were normalized to GAPDH mRNA, which was unresponsive to c-Myc expression (data not shown), and further normalised to vector control mRNA and m7G-mRNA, respectively. c-Myc expression resulted in increased mRNA levels (from 1.4 to 2.2-fold) for nine of these Myc target genes, with MINA being the exception (Figure 2b). c-Myc expression also resulted in increased mRNA cap methylation for all ten mRNAs (from 1.5-fold to 8.9-fold). Notably, there was a 8.9-fold c-Myc-dependent induction of MINA m7G-mRNA, in the absence of increased total MINA mRNA. For all genes except CDK4, the Myc-dependent increase in mRNA cap methylation was substantially higher than the Myc-dependent increase in mRNA expression level (Figure 2b). CDK4 mRNA did not exhibit enhanced c-Myc-dependent mRNA cap methylation, and c-Myc expression resulted in 1.5-fold increases in CDK4 mRNA and CDK4 m7G-mRNA. For all genes, mRNA binding to unpurified preimmune serum, preimmune serum affinity-purified on 7-methylguanosine and other affinity-purified polyclonal control antibodies was negligible and not c-Myc-regulated (data not shown).

Myc Box II (MBII) has been demonstrated previously to be necessary for maximal Myc-dependent transcription of most Myc target genes, although it remains unclear why the transcription of individual Myc target genes is differentially dependent on MBII (Cowling *et al.*, 2006; Nikiforov *et al.*, 2002). We also found that the expression level of Myc target genes was differentially responsive to the Myc Box II deletion (Fig 2b). Furthermore the level of Myc-dependent mRNA cap methylation of individual mRNAs was also differentially responsive to the MBII deletion (Fig 2b). Cap methylation of the MINA mRNA was completely MBII-dependent, whereas cap methylation of EIF4A1 and EIF4E mRNAs was only partially MBII-dependent. From the small number of genes investigated here, there did not appear to be a correlation between the MBII-dependence of mRNA expression level and mRNA cap methylation level, which indicates that transcription and mRNA cap methylation may be MBII-dependent due to distinct mechanisms.

All of the genes analyzed above are activated by c-Myc. We wanted to test if Myc-repressed genes were also regulated at the level of cap methylation. Transcription of the *c-myc* gene is repressed by c-Myc protein. In *myc* -/- fibroblasts, one copy of the c-*myc* coding region has been replaced by the Neo gene coding region and therefore Neo mRNA provides a readout for c-*myc* promoter activity (Bush *et al.*, 1998). Expression of c-MycWT repressed the Neo mRNA levels and Neo m7G-mRNA levels equivalently (Fig.2). Therefore, Myc repressed genes do not appear to exhibit differential cap methylation.

In summary, for nine out of the ten Myc target genes tested, expression of c-MycWT elevated the level of cap methylation more than it elevated the total mRNA levels. Therefore, c-Myc-induced cap methylation is probably a significant effector of c-Myc function.

c-Myc promotes mRNA polysome loading

Methylation of the mRNA cap is required for efficient binding to eIF4E and formation of the eIF4F complex that recruits mRNA to the 40S ribosomal subunit (Gingras *et al.*, 1999; von der Haar *et al.*, 2004). Therefore, the mRNAs that are cap methylated in response to c-Myc

expression are predicted to have an increased number of ribosomes loaded. mRNA-ribosome complexes were extracted from cells expressing c-Myc and vector control, and complexes were resolved on a sucrose gradient which separates heavily ribosome-bound mRNA from that which has fewer ribosomes bound. Fractions were collected from the sucrose gradient and analysed for RNA content by measuring the absorbance at 260nm (Figure 3A). Extracts from both the c-Myc-expressing and vector control cells exhibited classical polysome profiles, including peaks for hnRNPs, free ribosomal subunits, and polysomes. Fine resolution of individual polysome peaks was not possible since fractions were collected by hand. The 260 nM absorbance profile exhibited qualitative changes in response to c-Myc expression. Compared to the vector control cells, the peak of ribosomal subunits and free ribosomes was reduced in extracts resolved from c-Myc-expressing cells, whereas the polysome peak was increased. This suggested that the c-Myc-expressing cells have increased mRNA translation potential. In addition to upregulating mRNA cap methylation, c-Myc has previously been demonstrated to increase expression of translation factors including eIF4E and eIF4G (Schmidt, 2004), to increase expression of ribosomal and nucleolar proteins (Boon *et al.*, 2001; Guo *et al.*, 2000; Kim *et al.*, 2000; Schuhmacher *et al.*, 2001), and to increase expression of RNA polymerase I and III transcripts (Gomez-Roman *et al.*, 2006). These events may collectively increase the translational potential of the cell.

To assess the relative polysome loading of individual mRNAs, the migration of Nol5a, FBL, HSP60 and CDK4 mRNA was analysed across the sucrose gradient (Figure 3B). c-Myc expression induced a shift in the migration of Nol5a, FBL and HSP60 into more dense fractions, indicating that these mRNAs had more ribosomes bound. This correlated with the finding that c-Myc expression enhances cap methylation of these mRNAs. In contrast, the distribution of CDK4 mRNA was unchanged by c-Myc expression, consistent with the lack of enhanced cap methylation (Fig.2). As a negative control, the migration of GAPDH mRNA was analysed (Figure 3B). GAPDH had been found previously to be unresponsive to c-Myc and, as expected, the migration of GAPDH mRNA did not change significantly in response to c-Myc expression.

An unexpected finding was that even in the vector control cells, the Nol5a, FBL and HSP60 mRNAs were largely present in the light polysome fractions. Since Myc induced a 2-4-fold increase in mRNA cap methylation above the increase in mRNA level for all three genes, a large proportion of the mRNAs are unmethylated in the vector control cells and are not predicted to be ribosome bound. To confirm that the less dense polysome fractions contained mRNA that had reduced mRNA cap methylation compared to the denser fractions, RNA from fractions 26 and 32 was used as a substrate for an immunoprecipitation with anti-m7G antibodies (Fig.3c). Nol5a and FBL mRNA from c-Myc-expressing cells was found in fractions 26 and 32 (Fig.3b), but the proportion of cap methylated mRNA was significantly higher in fraction 32 than 26, which correlated with fraction 32 containing the more densely ribosome-loaded mRNA (Fig.3c). The fact that fraction 26 from the c-Myc-expressing cells has reduced cap methylation reveals that there is still a fraction of mRNA without methylated caps in cells containing high levels of c-Myc. Nol5a and FBL mRNA from vector control cells was not detectable in fraction 32, and the proportion of m7G-mRNA in fraction 26 was similar to that found in fraction 26 for the c-Myc expressing cells (Fig. 3b).

Therefore, although mRNA cap methylation is required for efficient EIF4E binding, mRNA which is not cap methylated is probably recruited to ribosomes, albeit inefficiently. Such mRNAs may be recruited by internal ribosome entry or by weak binding to EIF4E.

E2F1 induces cap methylation of its target genes

We were interested to extend the analysis of differential cap methylation to other transcription factors. We chose to analyse E2F1 which shares many properties with c-Myc. E2F1 is a member of the E2F family, which regulates transcription, cell proliferation and apoptosis (Blais and Dynlacht, 2007; Iaquinta and Lees, 2007). The response to E2F1 was investigated using E2F1-ER, a fusion protein of E2F1 and a modified oestrogen receptor (Muller *et al.*, 2001). E2F1-ER and vector control were expressed in rat fibroblasts, and E2F1 expression was detected by Western blot (Fig 4A). Following activation of E2F1-ER for 2 and 4 hours using the modified oestrogen receptor ligand, 4-hydroxytamoxifen, RNA was harvested and cap methylated mRNA was immunoprecipitated using anti-m7G antibodies (Fig 5B). Five genes previously determined to be E2F1 target genes, MCM2, CDC2, CDC25a, PCNA and CDK2, were investigated (Muller *et al.*, 2001). MCM5, CDC2 and CDK2 exhibited E2F1-dependent increases in mRNA cap methylation significantly above the increase in mRNA levels, similar to the findings for Myc. E2F1 activation resulted in equivalent increases in total PCNA mRNA levels and PCNA m7G-mRNA levels. cdc25a was unresponsive to E2F1 activation in our cell system and therefore served as a negative control. The finding that some E2F target genes are not significantly induced by E2F1 in this cell system supports the previous observation that E2F target genes are cell type-dependent (Muller *et al.*, 2001). 4-hydroxytamoxifen incubation with vector control cells, and vehicle incubation with E2F1-ER expressing cells did not result in induction of transcription or cap methylation (data not shown). For all genes, mRNA binding to unpurified preimmune serum and preimmune serum purified against 7-methylguanosine was negligible and not E2F1-regulated (data not shown). In summary, E2F1 induces mRNA cap methylation of a subset of its target genes.

Discussion

In this report we demonstrate that mRNA cap methylation can be regulated in a gene-specific manner by cellular transcription factors. We found that two transcription factors which regulate cell proliferation, c-Myc and E2F1, increase mRNA cap methylation of their target genes. In the case of c-Myc, it is probable that mRNA cap methylation is a major mechanism by which the transcription factor regulates gene expression because, for nine out of the ten transcriptional target genes investigated, the expression of c-MycWT elevated the level of cap methylation more than it elevated the total mRNA levels.

We found that E2F1 and c-Myc stimulated different levels of mRNA cap on different transcripts, and the stimulation of mRNA cap methylation was not obviously related to the level of transcription stimulation (Fig.2 and 4). Furthermore, for each Myc target gene investigated, deletion of MBII inhibited transcription and mRNA cap methylation differentially. This suggests that although mRNA cap methylation occurs predominantly at the initiation of transcription, it may also be partially uncoupled from transcription. Regulation of mRNA cap methylation is likely to be dependent on gene-specific features, including promoter sequences, promoter chromatin modifications, or the 5′UTR structure, all of which may influence RNMT recruitment and/or RNMT activity.

Simultaneous promotion of transcription and mRNA cap methylation is a method of upregulating gene expression, which will produce a greater net protein induction than upregulation of either mechanism alone. We previously demonstrated that protein levels correlate with the dual influences of increased transcription and translation for two other Myc target genes, Nucleolin and RUVBL1 (Cowling and Cole, 2007). One could speculate that having two mechanisms to upregulate gene expression may ensure that E2F1 and c-Myc target genes/proteins are induced efficiently to enhance the signal to proliferate.

Methods

Anti-m7G antibody production and purification

7-Methylguanosine was conjugated to Human Serum Albumin (HSA) via the periodate-oxidised nucleoside resulting in approximately 23 molecules of 7-methylguanosine per molecule of HSA. Rabbits were immunised 4 times, at three week intervals with 0.5 mg antigen in Freud's adjuvant. Rabbits were bleed at week 10. Serum was purified on 7-methylguanosine agarose (Amersham), eluted with 0.2 M Glycine, pH 2.0 and dialysed overnight against phosphate-buffered saline. Further details of the synthesis, immunisation and purification protocols are available on request.

Cell line establishment and culture conditions

TGR fibroblasts and the *myc*-/- derivative line HO 15.19 were cultured in DMEM/10% FCS. Cells were infected with retroviruses expressing the following constructs, LXSH (vector), LXSH-c-Myc, LXSH-c-MycΔMBII (Δ129-145), pBabe-puro and pBabe-puro E2F1-ER. E2F1 activity was induced by incubation of cells expressing E2F1-ER with 100 nM 4-hydroxytamoxifen (Sigma).

RT-PCR

RT-PCR was carried out using the One-Step RTPCR kit (Invitrogen) according to manufacturer's instructions. Products were separated by gel electrophoresis and quantified by phospho imaging. Typically 15-25 cycles were used and reactions were determined to be in the linear range by titration of input. Primer sequences are available on request.

Protein Detection

Cell extracts were made using a 0.5% Triton buffer and analysed by Western Blot using antibodies raised against c-Myc, HA(E2F1) and gamma-Tubulin (all Santa Cruz Biotech).

Immunoprecipitations

G-mRNA and m7G-mRNA was in vitro transcribed from pBS c-Myc using the MaxiScript kit (Ambion). RNA was extracted from cells by Trizol (Invitrogen) extraction, followed by phenol-chloroform extraction.

5 μl purified anti-m7G antibody or control antibody was prebound to 25 μl Protein A/G Sepharose in PBS, 0.01%Triton, 0.1 mg/ml BSA, 0.1 mg/ml polyU and 1 mM DTT for 30 min at room temperature followed by 2 washes. IPs were carried out using 2 μg cellular RNA in 200 μl of the same buffer plus 1μl RNAguard (Amersham) for 1 hr at room temperature followed by three washes. When used, 0.001 ng in vitro transcribed RNA was added to the IPs. Input RNA was incubated under the same conditions as the immunoprecipitations. RNA was purified from the IPs and inputs by phenol-chloroform extraction and precipitated using 4 μg of tRNA as a carrier. Competition experiments were performed by the co-incubation of a titration of 2 μM to 0.2 nM GTP or m7GTP (Ambion) with the immunoprecipitations. Inhibition of 50% m7G-mRNA binding was achieved by 2 nM m7GTP or 200 nM GTP.

Polysome purification

Log-phase cells (typically 3-6 × 15 cm plates) were treated with 100 μg/ml cycloheximide for 3 min, washed in PBS/100 μg/ml cycloheximide, and lysed in 4 pellet volumes of lysis buffer (1%Triton, 300 mM NaCl, 15 mM MgCl2, 10 mM Tris pH 7.5, 100 μg/ml cycloheximide). Extracts were centrifuged at 14K for 5min, twice. Supernatant was layered over a 10 ml gradient of 10-50% sucrose, 140 mM NaCl, 15 mM MgCl2, 10 mM Tris pH

7.5, 100 μg/ml cycloheximide. Gradients were centrifuged at 164K g for 2hrs. 250 μl fractions were collected by hand, and absorbance at 260 nM was measured. RNA was purified from every other fraction by phenol chloroform extraction, and used as a substrate for RT-PCR. Total RNA measurements and RT-PCR signals were normalized to the total input RNA for each cell line.

Acknowledgments

We thank Klara Acs for much helpful advice. We thank Kristian Helin for the E2F1-ER construct. This work was funded by NIH grants to MDC and an MRC grant to VHC.

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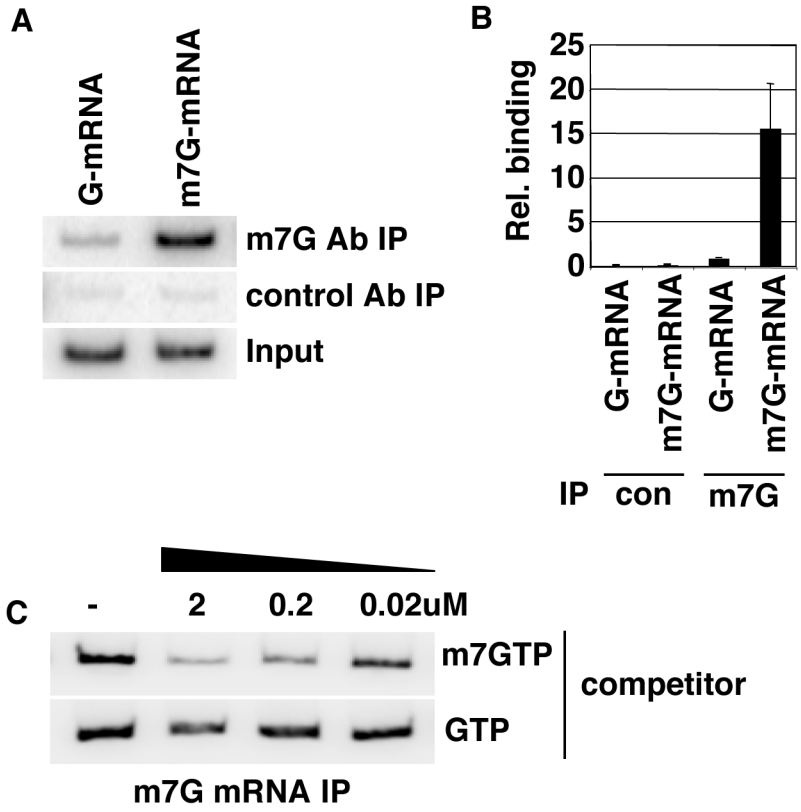

Figure 1. m7G-mRNA immunoprecipitation

A) In vitro transcribed mRNA synthesized with a 5′ guanosine cap (G-mRNA), or a 7-methylguanosine cap (m7G-mRNA) was offered as a substrate in an immunoprecipitation assay performed using polyclonal affinity-purified, anti-m7G antibodies and preimmune serum. The immunoprecipitated RNA and inputs were amplified by RTPCR. B) The immunoprecipitated mRNA detected in (A) was quantified, expressed as values relative to input material and normalised to the anti-m7G immunoprecipitation of G-mRNA. Values represent the mean of three experiments and error bars indicate the standard deviation. C) Immunoprecipitation of m7G-mRNA with anti-m7G antibodies was carried out in the presence of 2, 0.2 or 0.02 μM m7GTP or GTP, and bound RNA was detected as above. Experiment was performed three times with similar results.

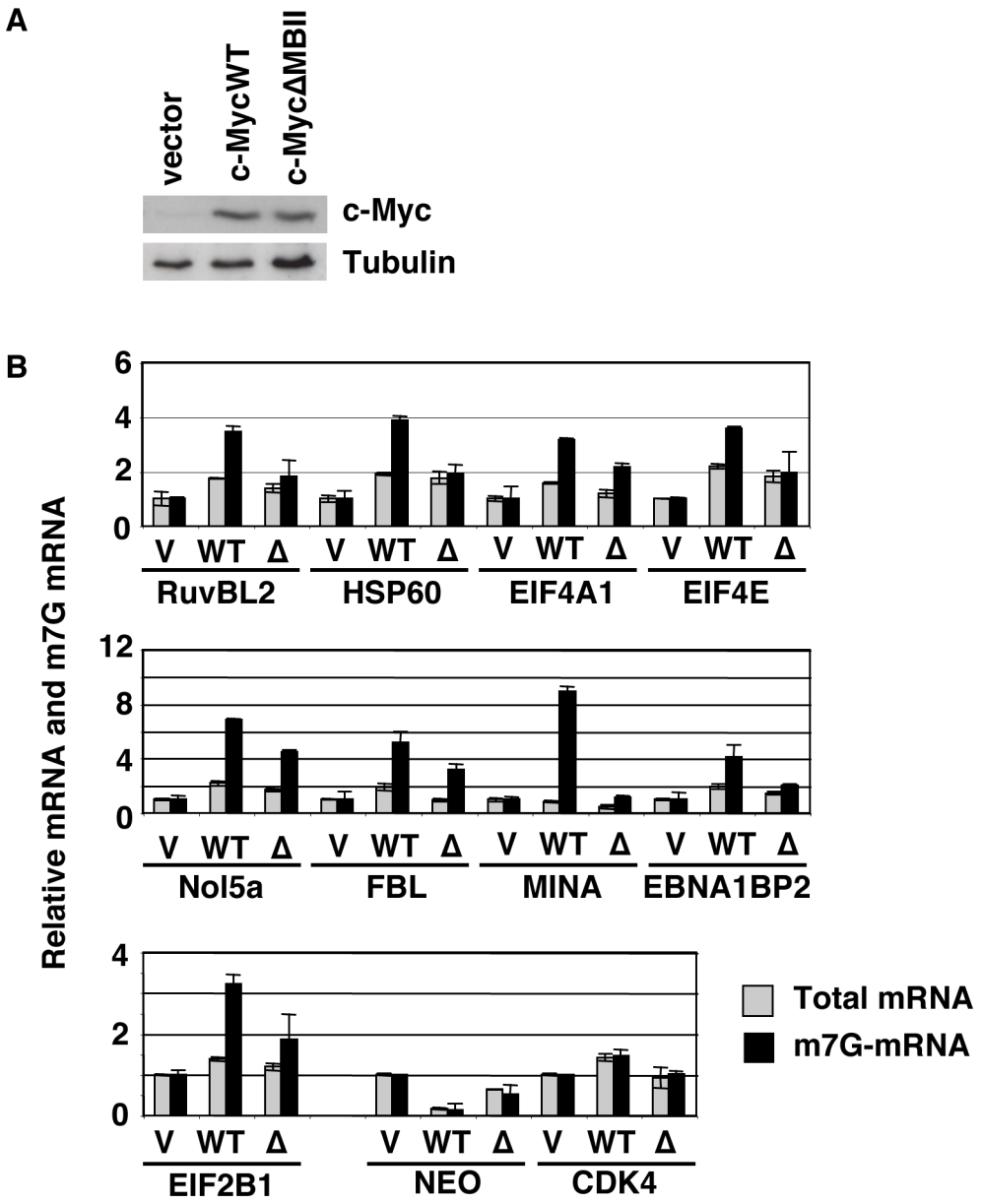

Figure 2. c-Myc promotes transcription and mRNA cap methylation

A) c-Myc and Tubulin expression was detected by Western Blot in extracts from fibroblasts expressing c-MycWT (WT), c-MycΔMBII (Δ) or vector control (V). B) RNA was extracted and an anti-m7G immunoprecipitation was carried out. Input mRNA levels (grey bars), and m7G-mRNA levels (black bars) for the genes indicated were determined by RTPCR and normalised to vector control input mRNA and vector control m7G-mRNA, respectively. The immunoprecipitations were performed in duplicate and error bars indicate within experiment variation. The m7G-immunoprecipitations were performed at least twice on each of two independently isolated RNA samples with similar results.

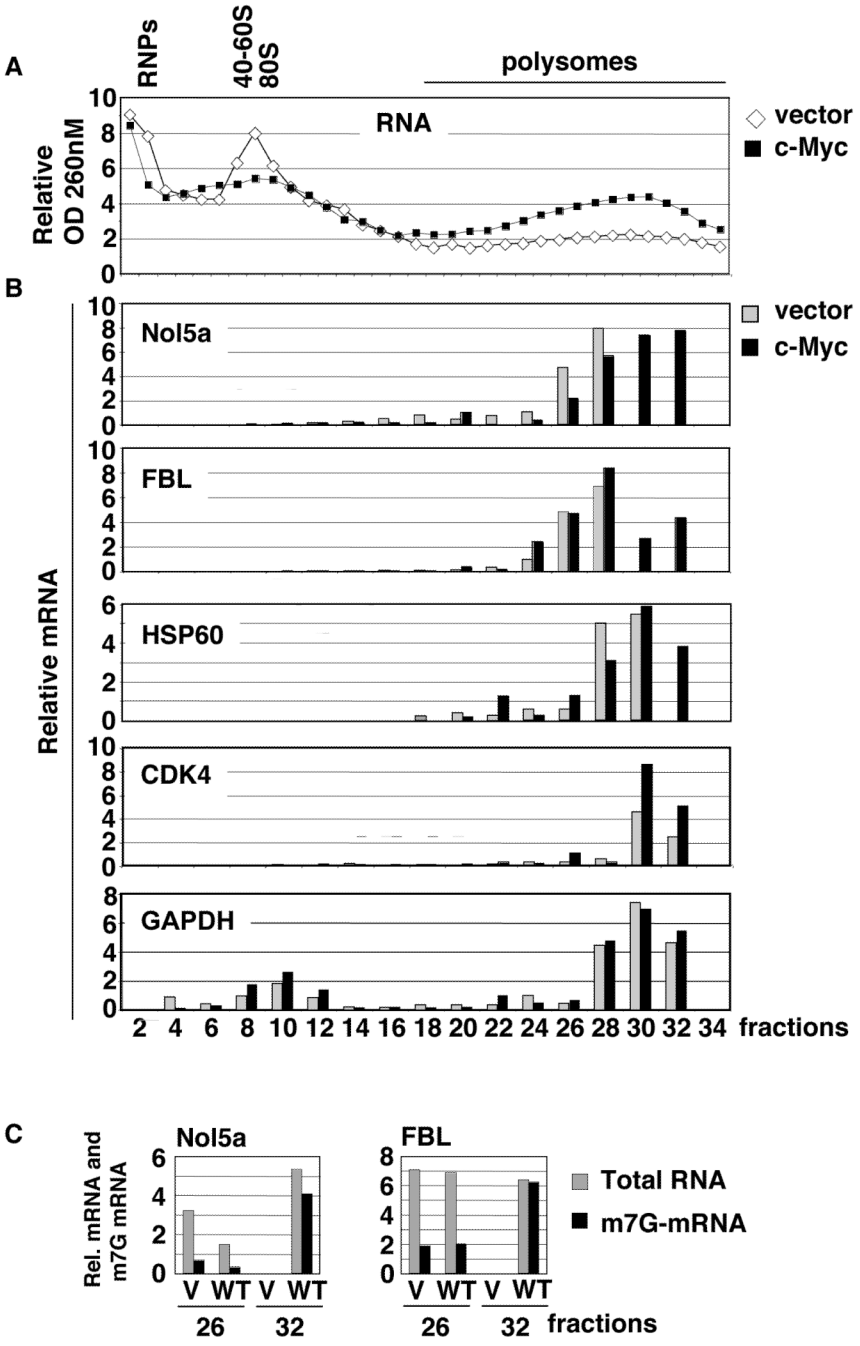

Figure 3. c-Myc promotes increased polysome loading

A) Cell extracts from fibroblasts expressing c-Myc (black boxes) or vector control (white diamonds) were separated on sucrose gradients, fractions were collected, and the absorbance at 260 nM was measured for every other fraction and reported relative to input values. Experiment was performed five times with similar results. B) mRNA levels for the genes indicated were quantified by RTPCR for c-Myc-expressing (black bars) and vector control cells (gray bars), and were reported relative to input material. Experiment was performed on three sets of independently purified polysome preparations with similar results, and a representative set of data is shown in the figure. C) FBL and Nol5a mRNA (grey bars) and

anti-m7G-mRNA (black bars) from fractions 26 and 32 was quantified by RTPCR. Experiment was performed three times with similar results.

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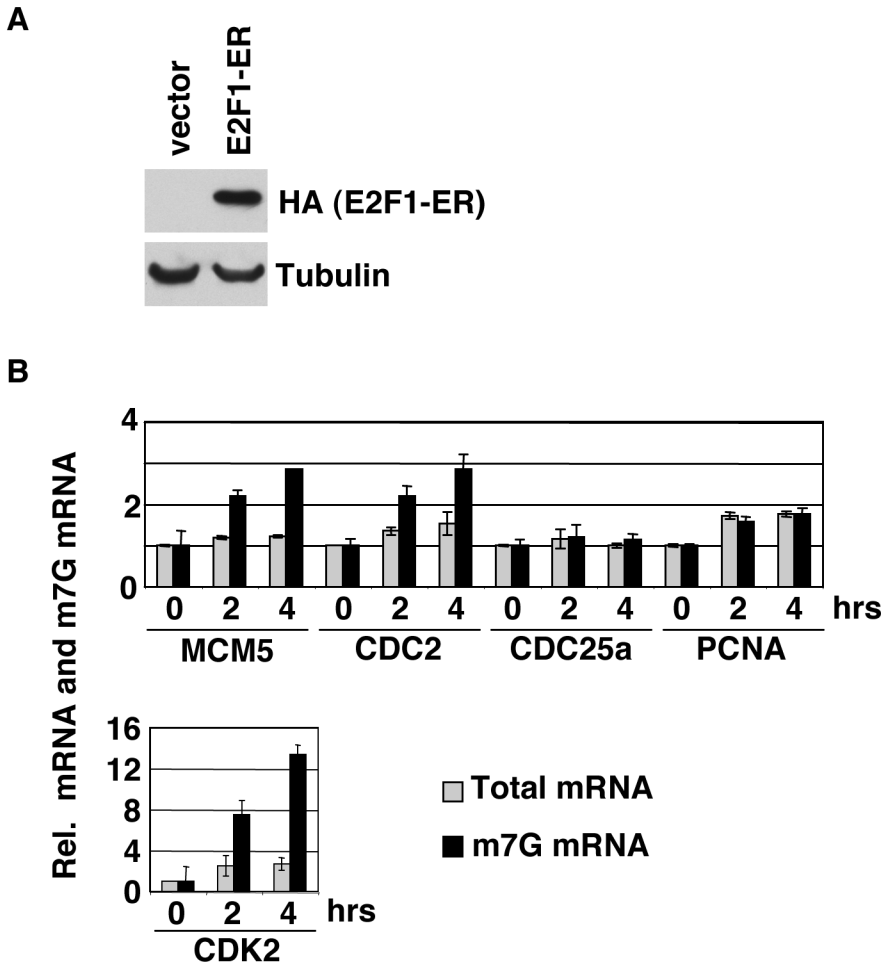

Figure 4. E2F1 induces transcription and cap methylation

A) HA(E2F1) and Tubulin expression was detected by Western Blot in extracts from fibroblasts expressing E2F1-ER or vector control. B) E2F1-ER was activated with 4-hydroxytamoxifen for 0, 2 and 4 hrs. For the genes indicated, mRNA levels (grey bars) and m7G-mRNA levels (black bars) were determined by RTPCR and normalised to the 0 hr time point mRNA and m7G-mRNA, respectively. The immunoprecipitations were performed in duplicate and error bars indicate within experiment variation. The m7G-immunoprecipitation were performed at least twice on each of two independently isolated RNA samples with similar results.