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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 4, pp. 2130–2134, January 26, 2007
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Direct Regulation of an Oncogenic Micro-RNA Cluster by E2F Transcription Factors*

Received for publication, September 19, 2006, and in revised form, November 22, 2006
Published, JBC Papers in Press, November 29, 2006, DOI 10.1074/jbc.C600252200

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Micro-RNAs (miRNAs) are a class of non-coding RNAs that post-transcriptionally regulate gene expression via the RNA interference pathway. In addition to roles in normal development, miRNAs have recently been implicated in a range of human diseases, including cancer. We recently demonstrated that a polycistronic cluster of miRNAs, miR-17–92, is oncogenic in a mouse model for Burkitt's lymphoma. This is due, in part, to a reduced apoptotic program. In an effort to understand the regulation of miR-17–92, we have studied the promoter structure of this miRNA cluster. The primary transcript initiates from a consensus initiator sequence downstream of a nonconsensus TATA box. The core promoter region contains two functional E2F transcription factor binding sites. Chromatin immunoprecipitation demonstrates that E2F3 is the primary E2F family member that occupies the promoter. These data place miR-17–92 in a regulatory loop between E2F3 and the miR-17 target E2F1. We propose a model whereby miR-17–92 promotes cell proliferation by shifting the E2F transcriptional balance away from the pro-apoptotic E2F1 and toward the proliferative E2F3 transcriptional network.

It is becoming clear that non-coding RNAs play an important role in the regulation of cell function and can contribute to human disease. One group of non-coding RNAs that has generated much recent attention is miRNAs.[3] These small RNAs have widespread impact on gene expression via the RNA interference pathway. The founding miRNA, *lin-4*, was discovered over 20 years ago in the nematode *Caenorhabditis elegans* (1, 2). More recently a large number of miRNA genes have been identified in a range of organisms. There are currently 474 human miRNAs listed in the Sanger data base, and many more are predicted to exist (3).

miRNA biogenesis begins with a primary transcript that is generated by RNA polymerase II (see Ref. 4 for a review). Sequential processing events by the ribonuclease III enzymes Drosha and Dicer yield the mature miRNA species. Averaging 22 nucleotides long, the mature miRNA is loaded into the RNA-induced silencing complex where it directs translational suppression of target mRNAs. Several computational algorithms have been developed with the goal of defining the networks of genes regulated by miRNAs (see Ref. 5 for a review). While the exact list of targets is not defined, the predicted targets extend to genes involved in a myriad of cellular processes. It is estimated that 30% of the human genome is under miRNA post-transcriptional regulation.

Despite the large number of candidate targets, the exact cellular pathways that are regulated by miRNAs are only beginning to be understood, particularly in mammals. miRNAs have been implicated in muscle development, hematopoietic differentiation, insulin regulation, and cell proliferation and apoptosis (see Ref. 6 for a review).

We recently reported that a polycistronic cluster of miRNAs, miR-17–92, can cooperate with c-Myc in a mouse model for lymphomagenesis (7). This miRNA cluster has also been functionally implicated in colorectal carcinoma and lung carcinoma and has been defined as a common miRNA signature in several solid tumors (8–10). Expression of miR-17–92 is high in proliferating cells and is regulated in part by c-Myc itself (11). In an effort to understand the transcriptional regulation of miR-17–92 and its connection to cell proliferation we characterized the promoter region of this miRNA cluster. Here we describe the promoter organization of miR-17–92 and demonstrate its direct regulation by E2F transcription factors.

EXPERIMENTAL PROCEDURES

Promoter Reporter Constructs—Promoter regions were amplified from human genomic DNA using the following primer sets: pro1353 5′, TTTCAGATTTGGCCTTTTATTTT; pro230 5′, GGAGGTCGGAAGTACTTTGTTT; pro1353 3′ and pro230 3′, AGGAGAGCTTCGCGGAGGAG; thymidine kinase-1 (TK-1) 5′, AGGAACCTTGCTTGGGAAAC; TK-1 3′, ACGAACCCGAGTACTCTCCA. PCR products were cloned into the promoterless vector pGL3 Basic (Promega) using NheI/XhoI. Site directed mutagenesis was performed with the QuikChange kit (Stratagene) using the pro230/pGL3 vector as template and the following primer sets: E2F SITE SD1 5′, CGACCTGCGCCTTCGATCCACTTCGCGCCCTC; E2F SITE SD1 3′, GAGGGCGCGAAGTGGATCGAAGGCGCAGGTCG; E2F SITE SD2 5′, CTTCGCGCCACTTCGATCCCTCGGGCGTCCGG; E2F SITE SD2 3′, CCGGACGCCCGAGGGATCGAAGTGGCGCGAAG; TATA SD 5′, GGGCTTGTCCGGAATTCCGTTGAGGCGG; TATA SD 3′, CCGCCTCAACGGAATTCCGGACAAGCCC.

Promoter activity was determined by co-transfection of the pGL3 promoter reporter (400 ng/well) with pRLSV40 (*Renilla* luciferase, Promega)(100 ng/well) into HEK-293 cells using FuGENE 6 (Roche Applied Science) and measurement of luciferase activity with the dual luciferase kit (Promega). Assays were performed in triplicate and standard deviations calculated. Activity was defined as Firefly/*Renilla* ratio, normalized to control vector transfection. For E2F transactivation assays, promoter activity was measured similarly with the following exceptions: NIH-3T3 were transfected with the pGL3 promoter reporter (400

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[1] Supported by a fellowship from the American Cancer Society.

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[3] The abbreviations used are: miRNA, micro-RNA; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; TK-1, thymidine kinase-1; BD, BD Biosciences; *SC*, Santa Cruz Biotechnology.

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ng/well) and pRLSV40 (200 ng/well), with or without E2F expression plasmids (400 ng/well), using Lipofectamine 2000 (Invitrogen). Activity was defined as Firefly/*Renilla* ratio normalized to activity in the absence of transfected E2F1.

5′-RACE Mapping of Transcript—HEK-293 cells were transiently transfected with the pro1353 vector and total RNA prepared (TRIzol, Invitrogen). RNA was decapped with tobacco acid phosphatase (Epicenter) and ligated to the synthetic RNA CGGUUUAAACGGUCCUUUAAA. cDNA was prepared (Superscript) and PCR performed using the following primers: RACE 5′, CGGTTTAAACGGTCCTTTAAA and RACE 3′, GTTCCATCTTCCAGCGGATA. PCR products were cloned into TOPO TA vector pCR2.1 and sequenced.

Chromatin Immunoprecipitation—HEK-293 cells (1 × 10-cm plate/immunoprecipitation) were cross-linked/fixed in 1% formaldehyde for 10 min at room temperature. Cells were washed in PBS and the cross-linking halted with 125 mM glycine treatment for 5 min at room temperature. Cells were extracted in 300 μl of SolA for 10 min on ice. Nuclei were recovered by centrifugation and extracted with 300 μl of SolB for 20 min on ice and sonicated 5 times for 10 s each. Nuclear lysates were centrifuged, and the supernatant was diluted in 1 volume of buffer B and precleared with 60 μl of protein-A-Sepharose plus 2 μg of sheared salmon sperm DNA. Chromatin was captured with primary antibody at the following amounts: BD E2F1, 9 μg (BD #554213); SC E2F1, 3 μg (SC-193); SC E2F2, 6 μg (SC-633); SC E2F3, 3 μg (SC-878); glyceraldehyde-3-phosphate dehydrogenase, 2 μg (Chemicon MAB374). After overnight capture the chromatin was precipitated with 20 μl of protein-A-Sepharose, washed sequentially in TSE1, TSE2, TSE3, and TE. Chromatin was eluted with 250 μl of buffer E for 15 min at room temperature. Eluate was adjusted with 4 μl of 500 mM EDTA, 9 μl of 1.0 M Tris, pH 7.6, 2 μl of 10 mg/ml proteinase K, and 2 μl of 10 mg/ml RNase A and incubated at 45 °C for 1 h. Chromatin was decrosslinked at 65 °C overnight. DNA was recovered by phenol extraction and amplified with the following primer sets: TK1 5′, ATGCCTGGACACAGGCTATC; TK1 3′, CAAATCTCCCGCCAGGTC; POLA5′, CCAAATCTTTTCCCATCAGC; POLA3′, CTCGCCCCTATCTCACAGTC; 1792p1 5′, GAAGAAGGAGGTGCTCCTGA; 1792p1 3′, GCCCAGCTGCATTTAGTAAGA; 1792p2 5′, ACCAGCTAGCGGAGGTCGGAAGTACTTTGTTT; 1792p2 3′, ACCATCGAGAGGAGAGCTTCGCGGAGGAG; 1792p3 5′, ATTCACCCACATGGTCCTTC; 1792p3 3′, GCCTGCGCTTTACTACGAC.

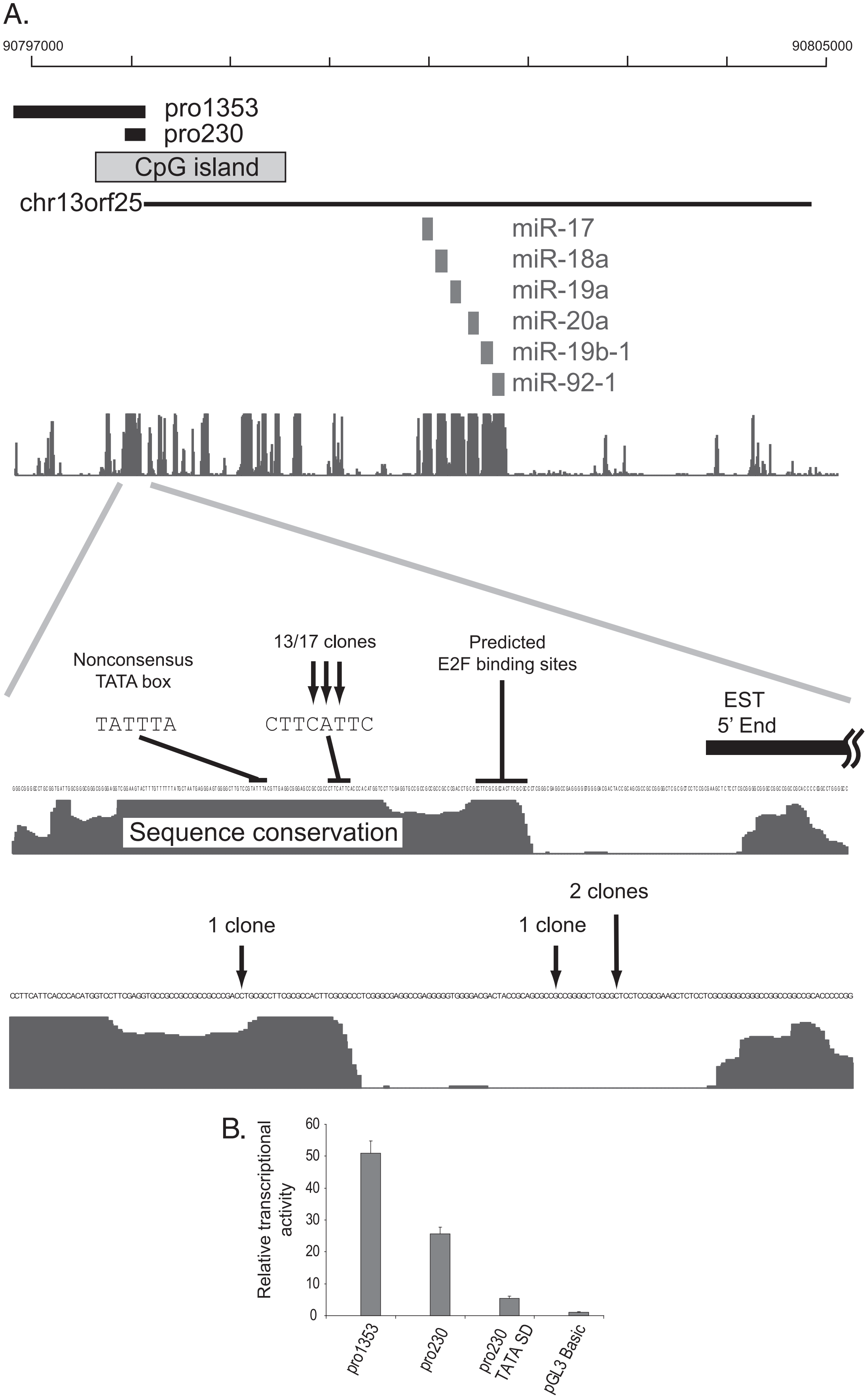
Buffers—The following buffers were used: SolA, 10 mM Hepes, pH 7.9, 0.5% Nonidet P-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, protease inhibitors; SolB, 20 mM Hepes, pH 7.9, 25% glycerol, 0.5% Nonidet P-40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, protease inhibitors; Buffer B, 1% Triton X-100, 2 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9, protease inhibitors; TSE1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-Cl, pH 8.1; TSE2, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-Cl, pH 8.1; TSE3, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.1; Buffer E, 1% SDS, 0.1 M $NaHCO_3$.

RESULTS AND DISCUSSION

The miR-17–92 cluster is comprised of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92–1, all of which are housed in the expressed sequence tag (EST) chr13orf25 (see Fig. 1*A*). The sequence upstream of the putative transcriptional start site is highly conserved across vertebrate genomes and contains an extensive CpG island, indicative of a core promoter region. In an effort to identify the true transcriptional start site we employed RACE cloning of the 5′ end. This was unsuccessful due to the extreme GC content of the 5′ end of the RNA. As an alternative strategy we cloned a 1353-nucleotide genomic region upstream of the chr13orf25 transcript, overlapping the 5′ end of the EST (construct pro1353, Fig. 1*A*). This region conferred strong transcriptional activity to a promoterless luciferase reporter vector (Fig. 1*B*). A smaller promoter region that contained the minimal conserved region had less activity, presumably due to removal of positive cis-elements (construct pro230, Fig. 1, *A* and *B*). RACE cloning of the pro1353/luciferase fusion was successful, enabling us to map the preferred start site 134 nucleotides upstream of the 5′ end of the EST (Fig. 1*A*). We believe this is the true start site, since it resides within a highly conserved region, in contrast to the terminus of the EST, which is non-conserved. The identified start site conforms to the initiator consensus sequence YYANTY, where the adenine is the start of transcription. In addition, there is a nonconsensus TATA box −32 from the start site, which is within the preferred location (−29 to −32, measured from the first thymine) (12, 13). The TATA box matches the sequence of the SV-40 early gene TATA box, which does not bind TBP but is still important for transcriptional activity at the downstream start site (14, 15). Similarly, the miR-17–92 TATA box is important for transcription, as site-directed mutation of the sequence attenuates transcriptional activity (Fig. 1*B*).

Expression of miR-17–92 has been correlated with proliferating cells. Therefore, we hypothesized that its promoter would contain binding sites for transcription factors with established roles in cell proliferation. Analysis of the promoter revealed two conserved E2F binding sites downstream of the start site (Fig. 1*A*). We tested the function of these sites by cotransfection of the pro230 and pro1353 promoter reporter constructs with an expression plasmid that directs expression of human E2F1. Both reporters were strongly transactivated by E2F1 (Fig. 2*A*). Shown for comparison is a reporter for TK-1, a promoter known to be E2F regulated. The E2F1 point mutant L132E, which lacks DNA binding activity, had a minimal effect on the reporter. E2F1 transactivates many genes, including other transcription factors such as c-Myc. To confirm that E2F-mediated regulation of miR-17–92 is direct we mutated the E2F binding sites from the pro230 reporter by site-directed mutagenesis (Fig. 2*B*). Removal of either site essentially abolished transactivation by E2F1 (Fig. 2*C*).

Nine E2F family members have been described: E2F1, E2F2, and E2F3a, which are transcriptional activators, and E2F3b to E2F8, which are transcriptional repressors (see Ref. 16 for a review). The essential role of E2Fs in the progression of the cell cycle is well known. A large number of S phase genes, including thymidine kinase, DNA polymerase α, and Cyclins E and A, are transactivated by E2Fs. Loss of all three activating E2Fs leads to a failure of S phase entry (17). Loss of a single activating E2F does not lead to cell cycle defects due to partial redundancy; however, there are specialized roles for individual family mem-


A.
90797000
90805000
pro1353
pro230
CpG island
chr13orf25
miR-17
miR-18a
miR-19a
miR-20a
miR-19b-1
miR-92-1
Nonconsensus
TATA box
TATTTA
13/17 clones
CTTCATTC
Predicted
E2F binding sites
EST
5' End
Sequence conservation
1 clone
1 clone
2 clones
CCTTCATTCACCCACATGGTCCTTCGAGGTGCCGCCGCCGCCGCCCGACCTGCGCCTTCGCGCCACTTCGCGCCCTCGGGCGAGGCCGAGGGGGTGGGGACGACTACCGCAGCGCCGCCGGGGCTCGCGCTCCTCCGCGAAGCTCTCCTCGCGGGGCGGGCCGGCCGGCCGCACCCCCGG
B.
Relative transcriptional activity
0
10
20
30
40
50
60
pro1353
pro230
pro230 TATA SD
pGL3 Basic

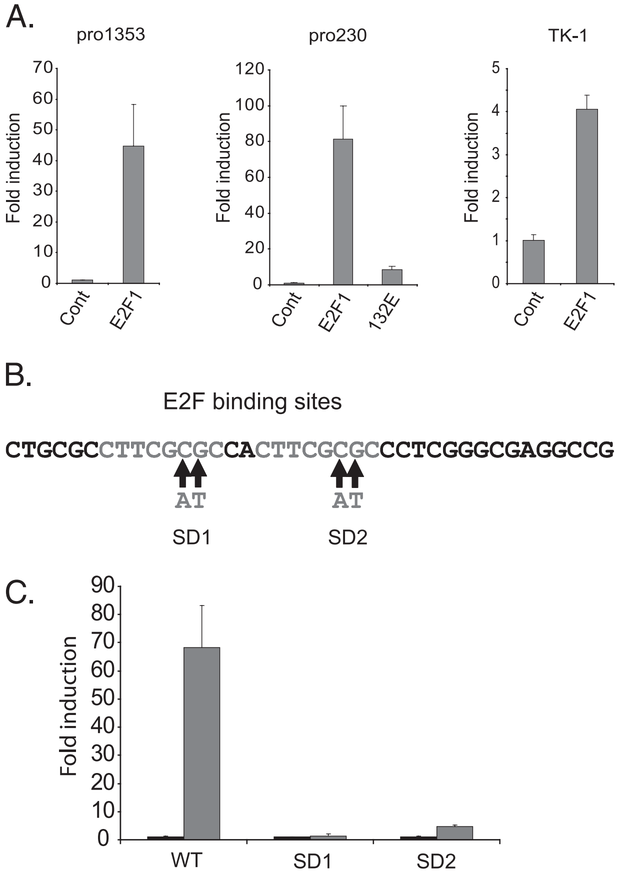


FIGURE 2. **Regulation of miR-17–92 by E2F.** *A*, the reporter constructs pro1353 (*left panel*), pro230 (*center panel*), and TK-1 (*right panel*) were co-transfected with a control vector, a vector directing expression of human E2F1, or a vector directing expression of the DNA binding domain mutant allele of E2F1 L132E, into NIH-3T3 cells. Firefly/Renilla luciferase ratios were used to calculate fold induction. *Cont.*, control. *B*, the sequence of the dual E2F sites are shown in gray. Site-directed mutants are indicated. *C*, fold induction of pro230 site-directed mutant reporters by wild type (*WT*) E2F1 is shown.

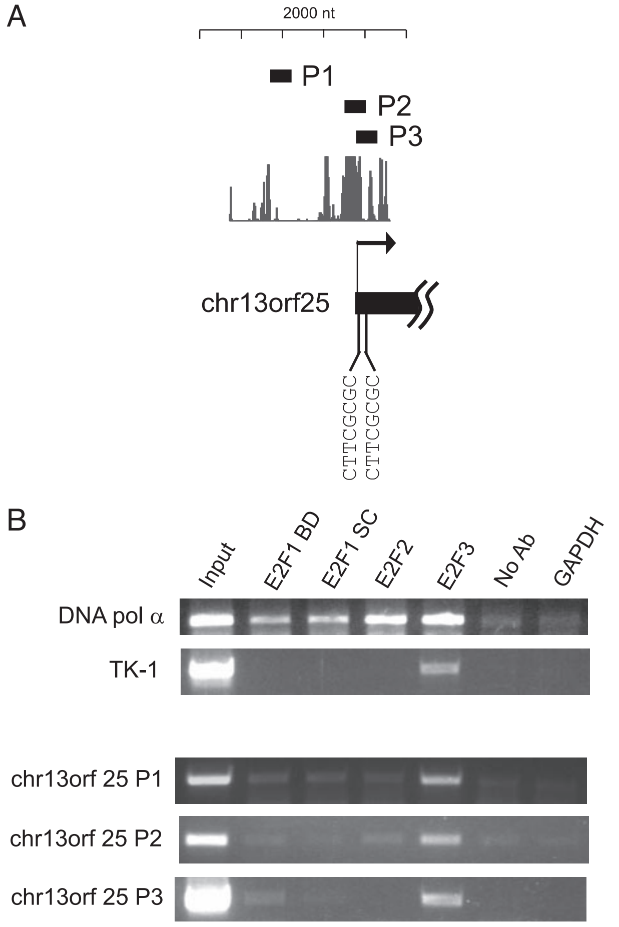


FIGURE 3. **The miR-17–92 promoter is occupied by E2F3.** *A*, the locations of chromatin immunoprecipitation primer sets are shown. The transcriptional start site and E2F sites are indicated. *B*, HEK-293 chromatin was immunoprecipitated with the indicated antibodies and associated DNA was detected by the indicated primer sets. Two different E2F1 antibodies were used, as indicated in the experimental section. Note: the P1 primer set is upstream of the indicated E2F sites but is near another predicted, but uncharacterized, E2F site. *nt*, nucleotides.

bers (18). In particular, E2Fs promote apoptosis in some cellular contexts, and this is dependent on E2F1 activity. Mice lacking E2F1 are tumor prone as a consequence, while mice lacking E2F3 are not (19–21). Similarly, mouse embryo fibroblasts that are deficient for E2F1 are resistant to apoptosis, even in the presence of elevated E2F3 (22).

While our data implicates E2F1 in the regulation of miR-17–92, it is probable that isoform specificity is lost as a consequence of overexpression. To identify the endogenous family member that occupies the miR-17–92 promoter we employed chromatin immunoprecipitation (Fig. 3). As controls we analyzed the DNA polymerase alpha promoter, which is occupied by all three activating E2Fs, and TK-1, which is occupied predominantly by E2F3 (23). Three independent primer sets flanking the miR-17–92 start site were used to detect E2F occupancy. All three primer sets detected E2F3 as the isoform predominantly associated with the promoter region. While this data does not rule out E2F1 and E2F2 association with other regions of the promoter, it confirms the role of E2F3 in the regulation of miR-17–92.

These data allow us to develop a model for miR-17–92 function (Fig. 4). Proliferative signals that converge on the Retinoblastoma gene product lead to activity of E2F transcription factors (24). In addition to induction of genes essential for S phase entry, E2Fs transactivate the oncogenic miRNAs miR-17–92. The exact cellular role of miR-17–92 is unknown, but lymphomas that express these miRNAs at a high level have reduced apoptosis (7). E2F1 is a validated target of miR-17 and miR-20 (11). Therefore, elevation of miR-17–92 would lead to decreased E2F1 protein and would thus attenuate E2F induced apoptosis. Since E2F3 is the predominant isoform that regulates miR-17–92 in cells, the targeting of E2F1 would not produce negative feedback but would rather promote E2F3 proliferative signal rather than E2F1 apoptotic signal.

FIGURE 1. **Organization of the miR-17–92 gene.** *A*, the gene structure of the primary transcript for miR-17–92 is shown. The positions of the mature miRNAs in the polycistronic cluster are shown by boxes. Vertebrate sequence conservation is indicated in the plot. The origin of the promoter reporter constructs, pro1343 and pro230, are shown. Both contain the same 3′ end, which is 10 nucleotides downstream of the 5′ terminus of the chr13orf25 EST. The expanded region indicates salient features around the start site. *B*, transcriptional activity of reporter constructs in HEK-293 cells, as measured by Firefly/*Renilla* luciferase activity. The pro230 TATA SD construct has the following TATA box mutation: TATTTA to GAATTC. pGL3 basic is the parental promoterless reporter vector.

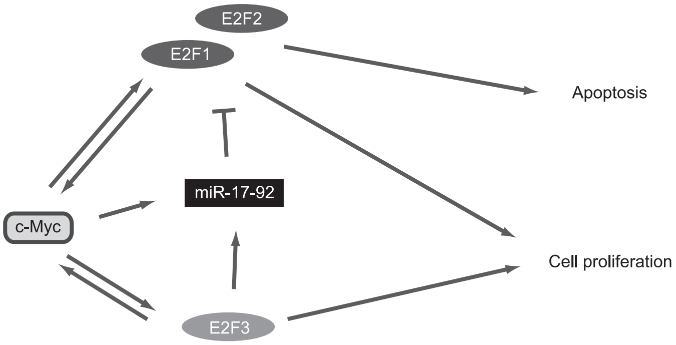


FIGURE 4. **Location of miR-17–92 in E2F signaling pathways.** Details are described under "Results and Discussion."

Our data also provide a mechanism for elevated miR-17–92 in proliferating cells. E2F activity is periodic with the cell cycle, reaching a peak during S phase (for E2F1–3) (24). We do not expect miR-17–92 to follow this periodicity, since the half-life of mature miRNAs is much longer than the typical cell cycle.[4] Rather, cycling cells would have elevated steady state levels of miR-17–92 due to the periodic burst of E2F activity during S phase, while quiescent cells would have reduced miR-17–92 levels. In a parallel pathway, miR-17–92 is regulated by c-Myc (11). E2F and Myc transactivate each other, providing a complex regulatory signal for miR-17–92 expression.

Acknowledgments—We acknowledge Yi Zhang, Ru Cao, and Dazhi Wang for advice on chromatin immunoprecipitation. We thank Dazhi Wang and members of the Hammond laboratory for advice on the manuscript. E2F1 expression plasmids were kindly provided by Azad Bonni. Chromatin immunoprecipitation control primer sequences were kindly provided by Joseph Nevins.

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