A novel factor distinct from E2F mediates C-MYC promoter activation through its E2F element during exit from quiescence

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Although C-MYC is overexpressed in a number of tumors, the mechanisms governing its expression in normal or tumor cells are not completely understood. Recruitment of the Retinoblastoma protein family members to gene promoters by E2F factors has a dominant negative effect on their activity during the G0 and G1 phases of the cell cycle. Despite the presence of an E2F-binding site on the C-MYC promoter, it escapes the repressive effect of E2F–Retinoblastoma complexes through unknown mechanisms during exit from quiescence. We hypothesized that occupancy of E2F elements by factors distinct from E2F might account for this escape. To test this hypothesis, we investigated whether the E2F element in the C-MYC promoter is regulated differently than E2F elements in promoters that are activated at the G1–S transition. Employing gel shift analysis, the E2F element from the C-MYC promoter was found to form a unique non-E2F complex, referred to as E2F C-MYC Specific (EMYCS), which is not observed with E2F elements from several other promoters. The DNA contact residues for EMYCS are distinct but overlapping with residues required for binding of E2F proteins. Finally, the approximate estimated molecular weight of the DNA-binding component of EMYCS is 105 kDa. Functional studies indicate that EMYCS has transcriptional transactivation capacity and suggest that it is required to activate the C-MYC promoter during exit from quiescence.

Introduction

The C-MYC proto-oncogene has been implicated in the development of a large number of human tumors (1). Tumoral transformation by C-MYC can take place as a consequence of its overexpression, even in the absence of mutations in its coding region (2). Chromosomal translocations or genomic amplification account for deregulated C-MYC expression in a number of tumors (3), but other studies indicate that the increase of C-MYC expression in colon and breast carcinomas is rarely due to genomic amplification or translocation (4). These studies are consistent with others showing that deregulated C-MYC expression precedes its own amplification and genomic destabilization (5,6). Moreover, it has been proposed that genomic destabilization is one of the mechanisms involved in C-MYC induction of many tumors (6,7,8,9,10).

The mechanisms governing C-MYC expression are yet poorly understood because regulation of its promoter is extremely complex with a lot of redundancy, many feedback loops and involvement of several cross-regulatory circuits (11). Its expression is barely detectable in quiescent cells, but it rapidly increases following the initial signaling mediated by stimuli that induce cell proliferation (12). Notably, C-MYC induction by growth factors is mediated, at least partly, by its E2F element (13).

The known regulatory elements in the C-MYC promoters are not sufficient for C-MYC expression after stable transfection (14) or in transgenic mice (15). Proper regulation of C-MYC expression requires additional control elements located outside a 50 kb of contiguous DNA sequences covering the human C-MYC coding region and ~20 kb of flanking upstream and downstream sequences (16). Insertion of the immunoglobulin k-intron and 3’ enhancers downstream from the coding region or insertion of the immunoglobulin heavy chain enhancer E κ upstream from the P1 and P2 C-MYC promoters activates C-MYC transcription (15,17).

The E2F family of transcription factors plays a pivotal role in cell cycle regulation of gene expression. E2F factors are generally composed of two structurally related subunits, termed E2F and DP. To date, eight E2F subunits (E2F1 through E2F8) and three DP subunits have been identified in mammalian cells (18). The members of the Retinoblastoma family (pRb, p107 and p130) bind tightly to the C-terminal transactivation domains of E2F1–E2F5, and this interaction probably blocks their association with transcriptional co-activators (19,20). Retinoblastoma family members, through their association with E2Fs, also exert dominant-negative effects on promoter activity, in part through their concomitant interaction with histone deacetylase molecules, histone methylases and nucleosome-modifying protein complexes (21).

The cell proliferation inhibitory properties of pRb family proteins are regulated by phosphorylation (22). In quiescent or differentiated cells, pRb family members are hypophosphorylated and the majority of nuclear E2F factors are bound to pRb family proteins. When cells are stimulated to proliferate, pRb family members become phosphorylated at the G1–S transition and release free, presumably active E2F (22). As a consequence of these events, most E2F-regulated genes are activated at this stage of the cell cycle. However, although the C-MYC gene contains an E2F element, it becomes promptly activated during the exit from quiescence (12) and therefore much earlier than pRb family proteins become phosphorylated and release free E2F. Moreover, C-MYC expression remains constitutive throughout the cell cycle in subsequent cycles (23,24). Hence, C-MYC is able to largely escape the dominant repressive action of pRb–E2F complexes during the exit from quiescence through unknown mechanisms.

We hypothesized that occupancy of E2F elements by factors distinct from E2F might account for this escape and we uncovered a nuclear factor referred to as EMYCS (from E2F C-MYC-Specific complex) that seems to be different from any known E2F family member. Our functional results provide evidence that this factor is a transcriptional activator that may be required to avoid the recruitment of pRb family members to the C-MYC E2F site at early phases of G1 and to elicit C-MYC promoter activation during exit from quiescence.

Materials and methods

Cell culture and preparation of nuclear extracts

X50-7, DG75, Mutu-1, Bjab, Jurkat, Molt-4, U937, HL-60 and K562 cells were grown in RPMI 1640 medium, whereas U2OS, Saos-2, HeLa and NIH-3T3 cells were grown in Dulbecco’s modified Eagle’s medium. Both media were supplemented with 10% fetal bovine serum, penicillin, streptomycin and glutamine. All cells were maintained at 37°C in a humidified 5% CO2-containing atmosphere. Peripheral blood mononuclear cells (PBMC) were obtained as described previously (25). Nuclear extracts were prepared as previously reported (26).
Electrophoretic mobility shift analysis

Gel shifts were performed with labeled double-stranded oligonucleotides encompassing the E2F elements from the DHFR, E2F1, C-MYC, C-MYB and CDC2 promoters (see supplementary data available at Carcinogenesis Online). Binding reactions were performed as described previously (26). Antibodies (Abs) against E2F1–E2F6 and p107 were purchased from Santa Cruz Biotechnology (Sant Cruz, California). The anti-E2F7 polyclonal Abs BCN4774, BCN4775 and BCN4776 recognize E2F7-A, E2F7-B or both isoforms, respectively, and were obtained from AntibodyBcn (Barcelona, Spain). The anti-DP1 and anti-pRb N9 polyclonal rabbit Abs were described previously (26).

Ultraviolet cross-linking experiments, methylation interference and chromatin immunoprecipitation analysis

These assays were carried out essentially as described in refs (26,27,28), respectively. Rabbit anti-p130 (Santa Cruz Biotechnology) was used for chromatin immunoprecipitation.

Construction of vectors

pBG-LUC has been described previously (29) and was generated by cloning the β-globin TATA box upstream from the luciferase gene in the pGL2-Basic plasmid. Details on the construction of the wild-type (wt) version of 2xcmyc-BG-LUC and its mutant derivatives are provided in supplementary data (available at Carcinogenesis Online). The generation of pEnh-Ep-CMYC, pGL2-CMYCwt, a DNA insert encompassing the C-MYC P1 and P2 promoters (–274 to +66 relative to the P2 transcription start site), pEnh-Ep-CMYC and their mutant derivatives is described in the supplementary data (available at Carcinogenesis Online).

Transfections and reporter gene assays

U2OS, Saos2 and 293-T cells were transfected employing the calcium phosphate method (30) whereas DG75, Jurkat, K562 and Mutu-1 cells were transfected by electroporation (30). Transfections included 5 µg of appropriate luciferase reporter plasmid plus 1 µg of pCMV-βgal and 24 µg carrier plasmid [Bluescript (Stratagene, La Jolla, California)].

PBMC cells were harvested and resuspended at a density of 1 × 10⁷ cells/ml in RPMI 1640 and, then, electroporated as described previously (31). Transfected PBMC were split in two 35 mm diameter dishes, incubated in RPMI with 10% FCS at 37°C and activated or not with 5 µg/ml leucoagglutinin plus 50 units/ml interleukin-2 for 24 h. PBMC transfection included 40 µg pEnh-Ep-CMYC reporter vectors and 5 µg pRL-SV40 (Promega, Madison, California).

Results

The E2F element in the C-MYC promoter binds a unique factor that does not interact with E2F elements from several other promoters

To address the possibility that the C-MYC E2F element is regulated differently than E2F elements in promoters that are activated later in the cell cycle, electrophoretic mobility shift analysis (EMSA) was employed to compare the binding of nuclear factors to the E2F element in the C-MYC promoter relative to that from genes that are induced during S phase or the G1–S phase boundary (Figure 1). Employing nuclear extract from the non-transformed human lymphoblastoid cell line, X50-7, four major protein complexes are observed (I–IV) using labeled E2F elements from the C-MYC (Figure 1C and D, respectively).
These four complexes are apparently common to the E2F probes derived from the DHFR, C-MYB and CDC2 promoters ([26] and data not shown). Competition experiments were performed to assess the specificity of the protein–DNA interactions observed with the E2F site from the C-MYC promoter and the E2F1-d element. Assays employing a labeled C-MYC E2F element as a probe revealed that complexes I, II and IV are competed by each of the E2F elements tested (Figure 1C). However, complex III was only efficiently competed by a self-oligomeric site (Figure 1C, C-MYC competed lane), whereas it was only partially competed by wt E2F elements from the other promoters (Figure 1C). This result suggests that the C-MYC E2F site forms a specific complex (referred to here as EMYCS) that is unique to the C-MYC E2F element and that migrates at the same position in EMSA assays than other E2F-containing complexes also present in band III.

As a control for E2F binding in our assays, we could observe the formation of complexes I–IV using labeled E2F1-d as a site in a probe. In this case, the four complexes were completely and efficiently competed by all other E2F elements tested, except when a mutant DHFR element (DHFRmut) was used, indicating the presence of E2F or E2F-related factors in complexes I–IV (Figure 1D).

EMYCS is not recognized by Abs against E2F and pRb family members

It is well established that DP1 is a major component of the vast majority of E2F–DNA complexes ([22] and that E2F elements can form complexes such as DP1–E2F4–p107 (complex I), DP1–E2F2–pRb (complex II) and one or more free DP1–E2F (complexes III–IV) with nuclear extracts from asynchronous non-transformed cell cultures ([22,26,32]). Accordingly, a DP1 Ab inhibits the formation of most of these complexes when E2F-specific probes are used ([26]).

Employing X50-7 nuclear extracts and the C-MYC or the E2F1-d probes, complexes I, II and IV were affected by an anti-DP1 Ab (Figure 2A–D), whereas complexes I and II were inhibited by anti-p107 and anti-pRb Abs, respectively (Figure 2A and C). Additionally, formation of complexes I and IV was inhibited by anti-E2F4 and anti-E2F5 Abs, respectively (Figure 2B and D). These data suggest that complexes I, II and IV formed with the C-MYC probe are similar to those obtained with other E2F elements contained in other promoters.

In these experiments, complex III exhibited again a unique behavior when the C-MYC probe was used. Thus, an anti-E2F4 Ab barely affected its formation with the C-MYC probe (Figure 2B), but completely inhibited its appearance with the E2F1-d (Figure 2D), or with other probes such as DHFR, C-MYB or E2F1-p ([26] and data not shown).

It is clear that the E2F-1, -2, -3, -6 and -7 Abs did not significantly affect formation of complexes I–IV with the E2Fmyc or E2F1-d probes (Figure 2B and D). These Abs inhibit formation of complexes I and IV, and partially inhibit formation of complex III (Figure 2A). However, formation of complex II is completely inhibited by anti-E2F4 Ab. This result suggests that the C-MYC E2F element revealed a strong interaction with a specific 105 kDa band that was not observed in the complex III formed with the DHFR and C-MYB elements (Figure 2F and data not shown). Additional proteins with estimated molecular weights close to 55 kDa were also observed associated to complex III on the E2Fmyc probe, but proteins with similar apparent masses were

Relative molecular weight estimation of EMYCS DNA-binding components

Because EMYCS binds the E2Fmyc probe but not the other E2F probes tested, we hypothesized that, by using ultraviolet (UV) cross-linking, we might uncover a protein as a constitutive component of E2Fmyc probe-associated complex III that would not be present in the complex III formed with the other E2F probes. UV cross-linking of complexes III on the C-MYC E2F element revealed a strong and specific 105 kDa band that was not observed in the complex III formed with the DHFR and C-MYB elements (Figure 2F and data not shown). Additional proteins with estimated molecular weights close to 55 kDa were also observed associated to complex III on the E2Fmyc probe, but proteins with similar apparent masses were
also found with complex III on the other E2F probes (Figure 2F and data not shown). These data strongly suggest that the 105 kDa band probably represents a major DNA-binding component of EMYCS. We also performed UV cross-linking experiments using the Burkitt’s lymphoma cell line, DG75, and like the results obtained with X50-7 extracts, a 105 kDa band was detected that was probably attributable to a factor in the EMYCS complex (data not shown). Several other bands were observed with complexes I–IV, most of which were in the range of the molecular weights for known E2F species (~45–65 kDa) employing the E2Fmyb probe (Figure 2F). A 25 kDa band was observed that appeared to be common to every probe and each of the other complexes tested (Figure 2F, pointed with a short arrow).

EMYCS factors recognize a distinct nucleotide sequence than E2F-containing complexes

To further analyze EMYCS, we used methylation interference analysis to compare the nucleotide-binding specificity of this complex with that of complexes known to contain only E2F. Our results showed that methylation of the G residue at position 8 did not affect binding of EMYCS but it interfered with the binding of E2F-containing complexes (Figure 3A). No differences in the methylation interference patterns were observed between any complex employing the antisense strand, probably due to a dearth of G and A residues. A summary of the nucleotides required for binding of the different complexes is shown in Figure 3B.

We used this information to design mutated oligonucleotides that interact with EMYCS, but not with E2F (E2Fmyc-sp) by specifically replacing nucleotide at position 8 (G–T), as indicated in Figure 3C (myc-sp). Oligonucleotides that could bind E2F, but not EMYCS (E2Fmyc-E2F), or oligonucleotides unable to interact with any factor (E2Fmyc-null) were also designed. The sequence of the mutated oligonucleotides is indicated in Figure 3C.

Complex formation employing X50-7 nuclear extracts and the wild-type E2Fmyc probe (E2Fmyc-wt) was competed with a

Fig. 3. EMYCS and E2F complexes interact with overlapping DNA sequences. (A) Methylation interference analysis of complexes I–IV was performed employing X50-7 nuclear extracts and the E2Fmyc probe. The sequence of the sense and antisense strands is indicated adjacent to the gel. Triangles, diamonds and squares indicate bases whose methylation completely (filled) or partially (empty) blocked formation of complexes I and II (triangles), III (diamonds) and IV (squares). The cleavage pattern observed with an unbound probe (F) is also shown. (B) Summary of methylation interference analysis shown in A. (C) Sequence of wt and mutant E2Fmyc probes. For each mutant, alterations relative to the wt sequences are indicated by dots. (D–F) Complexes formed between X50-7 nuclear factors and radiolabeled E2Fmyc-wt (D), E2Fmyc-sp (E) and E2Fmyc-E2F (F) probes were analyzed by EMSA. Reaction mixtures were incubated in the absence (indicated as None) or in the presence of a 100-fold excess of the indicated unlabeled competitor oligonucleotides. The position of complexes I–IV is denoted.
Forty hours later, cell extracts were prepared and luciferase and b-galactosidase activities were performed. Luciferase values were normalized for b-galactosidase activity and represent the means of at least four different experiments. The luciferase activity is shown relative to that of the null construct. (D) The indicated 2xmyc-BG-LUC plasmids were cotransfected with pCMV-bgal in asynchronously growing Saos-2 cells in the presence (+) or the absence (−) of plasmids encoding DP1 and E2F1. Forty hours later, cell extracts were prepared and luciferase and b-galactosidase assays were performed. Luciferase values were normalized for b-galactosidase activity and represent the means of at least four different experiments. The luciferase activity is shown relative to that in the absence of DP1 + E2F1.

Fig. 4. EMYCS is expressed in a broad range of cell lines and elicits transactivation of a minimal luciferase reporter plasmid. (A) Complex formation employing the radiolabeled E2Fmyc-sp probe and nuclear extracts from the lymphoblastoid cell line, X50-7, the Burkitt’s lymphoma DG75 and Mutu-1, the B-cell lymphoma Bjab, the T-cell leukemia Jurkat and Molt-4, the myeloid U937 and HL60, the erythromyeloid K562, the osteosarcoma U2OS and Saos-2, the cervix carcinoma HeLa and the mouse fibroblast NIH-3T3 cell lines was analyzed by EMSA. Nuclear extracts were prepared from asynchronously growing cells. The arrow indicates the position of EMYCS.

(B) Schematic representation of the reporter plasmids used. Two copies of the wt E2F site from the C-MYC promoter or its mutant derivatives that interact only with E2F (E2F), only with EMYCS (sp) or with none of them (null) were cloned immediately upstream from the b-globin TATA box in pBG-LUC. (C) The indicated 2xmyc-BG-LUC plasmids were cotransfected with pCMV-bgal in asynchronously growing DG75, U2OS, Jurkat, K562, Mutu-1 and 293-T cells. Forty hours later, cell extracts were prepared and luciferase and b-galactosidase assays were performed. Luciferase values were normalized for b-galactosidase activity and represent the means of at least four different experiments. The luciferase activity is shown relative to that of the null construct. (D) The indicated 2xmyc-BG-LUC plasmids were cotransfected with pCMV-bgal in asynchronously growing Saos-2 cells in the presence (+) or the absence (−) of plasmids encoding DP1 and E2F1. Forty hours later, cell extracts were prepared and luciferase and b-galactosidase assays were performed. Luciferase values were normalized for b-galactosidase activity and represent the means of at least four different experiments. The luciferase activity is shown relative to that in the absence of DP1 + E2F1.
Luciferase reporter plasmids were transfected in different cell lines, and the luciferase activity of cell extracts was measured to assess the respective transactivation capacity of each factor. As shown in Figure 4C, the wt sites and the mutant elements that could only interact with EMYCS (sp) both elicited 6-14 times more luciferase activity than the mutant that could bind no factors (null) in every cell line. Luciferase activities using the mutant reporter driven only by E2F (E2F) and the null mutant (null) were almost identical (Figure 4C). These results suggest that EMYCS binds in vivo to the C-MYC E2F site, that it has transcriptional transactivation activity and that it might be a C-MYC promoter transactivator.

To assess that E2F factors only bind in vivo to the wt E2Fmyc promoter and the site mutated to interact only with E2F (E2F), but not with the mutated elements that interacted in vitro with no factor (null) or only with EMYCS (sp), we cotransfected the above mentioned luciferase reporters with or without plasmids encoding E2F1 and DP1. Our results clearly indicate that E2F1 activated the Myc-wt and Myc-E2F reporters, but not the Myc-sp or Myc-null reporters (Figure 4D). These data strongly support that E2F factors are unable to interact in vivo with the E2Fmyc site mutated to interact only with EMYCS.

Regulation of C-MYC expression requires additional unidentified control elements located outside the known regulatory sequences (16). Because insertion of immunoglobulin enhancers downstream from the coding region and upstream from the P1 and P2 C-MYC promoters mimicked endogenous C-MYC transcription regulation (15,17), we constructed a vector with the E4 enhancer cloned directly upstream from these promoters in the pGL2-Enhancer vector. It is important to note that this vector contains the SV40 enhancer downstream from the luciferase gene (see Figure 5B and experimental procedures).

The wt (Myc-wt) and mutant derivative pEnh-E4-CMYC plasmids whose E2F element could interact only with E2F (Myc-E2F), only with EMYCS (Myc-sp) or with none of these factors (Myc-null) were transfected in peripheral blood T cells. Most of these cells are quiescent and do not express C-MYC, but they readily activate C-MYC messenger RNA and protein expression and enter the cell cycle following stimulation [Figure 5A and (12)]. The luciferase activity from resting and stimulated cells was measured to assess the respective transactivation capacity of each factor following T-cell activation. As shown in Figure 5C, the reporter plasmids that contained a wt E2F myc site (Myc-wt) or a mutant site that interacted only with EMYCS (Myc-sp) were activated 5- to 6-fold following T-cell stimulation, whereas reporter plasmids that contained mutant E2F sites that interact only with E2F (Myc-E2F) or with no factor (Myc-null) were barely activated. The vector that contained both indicated pEnh-E4 luciferase reporter plasmids-containing wt (Myc-wt) or mutant E2F elements that interact only with E2F (Myc-E2F), only with EMYCS (Myc-sp) or with none of them (Myc-null) were cotransfected with pRL-SV40 into PBMC. A reporter plasmid that contained both enhancers but Myc-null) were transfected in peripheral blood T cells activated for the indicated periods of time with interleukin-2 to exit from quiescence (þ) or left untreated (−). Firefly and Renilla luciferase activities were measured in cell extracts from both activated and untreated cells. Luciferase values for each indicated reporter plasmid are shown relative to untreated cells. The mean and SD of three independent experiments is shown. (C) The

Fig. 5. Activation of the C-MYC promoter by EMYCS through the E2F element during exit from quiescence. (A) Whole-cell extracts were prepared from peripheral blood T cells activated for the indicated periods of time with leucogglutinin plus interleukin-2 to exit from quiescence. These extracts were fractionated on an 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with either the 9E10 anti-C-MYC (top panel) or the DM 1A anti-tubulin (bottom panel) monoclonal Abs. (B) Schematic representation of the reporter plasmids used (pEnh-E4-CMYC). A DNA fragment comprising the E4 enhancer followed by a DNA insert encompassing the C-MYC P1 and P2 promoters, but excluding the purine-rich strand and the pausing-related sequences, was cloned into a pGL2 vector that carries the SV40 enhancer downstream from the luciferase gene. Reporter vectors with a wt E2F element as well as vectors with a mutated E2F site that interacted only with E2F, only with EMYCS or with none of them were also generated. (C) The

These luciferase reporter plasmids were transfected in different cell lines, and the luciferase activity of cell extracts was measured to assess the respective transactivation capacity of each factor. As shown in Figure 4C, the wt sites and the mutant elements that could only interact with EMYCS (sp) both elicited 6-14 times more luciferase activity than the mutant that could bind no factors (null) in every cell line. Luciferase activities using the mutant reporter driven only by E2F (E2F) and the null mutant (null) were almost identical (Figure 4C). These results suggest that EMYCS binds in vivo to the C-MYC E2F site, that it has transcriptional transactivation activity and that it might be a C-MYC promoter transactivator.

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The wt (Myc-wt) and mutant derivative pEnh-E4-CMYC plasmids whose E2F element could interact only with E2F (Myc-E2F), only with EMYCS (Myc-sp) or with none of these factors (Myc-null) were transfected in peripheral blood T cells. Most of these cells are quiescent and do not express C-MYC, but they readily activate C-MYC messenger RNA and protein expression and enter the cell cycle following stimulation [Figure 5A and (12)]. The luciferase activity from resting and stimulated cells was measured to assess the respective transactivation capacity of each factor following T-cell activation. As shown in Figure 5C, the reporter plasmids that contained a wt E2F myc site (Myc-wt) or a mutant site that interacted only with EMYCS (Myc-sp) were activated 5- to 6-fold following T-cell stimulation, whereas reporter plasmids that contained mutant E2F sites that interact only with E2F (Myc-E2F) or with no factor (Myc-null) were barely activated. The vector that contained both
with the E2F elements from the Abs to other DP or E2F family members affected formation of the activation capacity, as we show herein. In addition, neither anti-E2F7 nor although it is similar to that of E2F7. However, E2F7 is a transcrip-
ing, indicating that nucleotide recognition sequences are different for during exit from quiescence.

C-MYC enhancers, but no C-MYC promoter, was not activated following T-cell stimulation (Figure 5C). Hence, only the C-MYC promoter constructs that contained the wt E2F site or the mutated site that interacted with EMYCS, but not with E2F, were activated in vivo during exit from quiescence.

Nuclear extracts were prepared from peripheral blood T cells stimulated for different periods of time and the interaction of these extracts with the E2Fmyc-sp probe was assessed by EMSA. As shown in Figure 5D, EMYCS was almost undetectable in resting cells, but its binding significantly increased 12 h after cell activation and maximal binding was observed 18–24 h following exit from quiescence. The pRb family member p130 is a transcriptional repressor that is usually associated to E2F elements during quiescence. Chromatin immunoprecipitation assays clearly show that binding of p130 to the E2Fmyc site decreased 16 h after lymphocytes stimulation (Figure 5E). Together, our results strongly suggest that the E2F element plays a crucial role during activation of the C-MYC promoter in cells coming out of quiescence and that interaction of EMYCS with this element is required to achieve full activation of the promoter in these cells.

Discussion

C-MYC expression activation by growth factors is mediated, at least partly, by its E2F element (13) through unknown mechanisms. Because induction of C-MYC expression takes place much earlier than p130 is phosphorylated and free E2F is released, we hypothesized that occupancy of the E2F site by factors distinct from E2F might allow this element to escape from the repressor activity of the E2F–p130 complexes. We have discovered a novel factor (EMYCS) that interacts with the E2F site from the C-MYC promoter that is unique and distinct from known E2F family members. We also provide evidence that this novel factor is a transcriptional activator that might be required to avoid the recruitment of pRb family members to the C-MYC E2F site at early phases of G1, and to elicit C-MYC promoter activation during exit from quiescence.

Although EMYCS interacts with nucleotide residues that partially overlap with those required for E2F binding, all our data indicate that this factor is different from known E2F factors. There is a G nucleotide (G8) that is required for E2F binding but not for EMYCS binding, indicating that nucleotide recognition sequences are different for EMYCS and E2F factors. Furthermore, the estimated molecular size of EMYCS (105 kDa) is different from most E2F family members, although it is similar to that of E2F7. However, E2F7 is a transcriptional repressor (33,34) while EMYCS has transcriptional transactivation capacity, as we show herein. In addition, neither anti-E2F7 nor Abs to other DP or E2F family members affected formation of the EMYCS complex. Finally, while most E2F family members interact with the E2F elements from the C-MYB, DHRF and E2F1 promoters in vitro (J. Álvaro-Blanco, L. Martínez-Gac, E. Calonge, M. Rodríguez-Martínez, I. Molina-Privado, J. M. Redondo, J. A. Elcami, E. K. Flemington and M. R. Campanero, unpublished data), EMYCS only binds to the C-MYC E2F site.

At this time, the identities of factors in the EMYCS complex remain to be determined. It was previously reported that recombinant ets-1 and ets-2 bind to the C-MYC E2F element (35). In that study, however, no experiments were shown in which ets-1- or ets-2-containing complexes were detected employing cell extracts. Moreover, the high molecular weight estimated for EMYCS suggests that this factor is not ets-1 or ets-2. Finally, employing ets-1- and ets-2-binding sites in oligonucleotide competition studies, we were unable to detect any evidence of ets factor binding to the C-MYC E2F element (supplementary Figure 3 is available at Carcinogenesis Online). Therefore, any binding of ets-1 or ets-2 from nuclear extracts must be significantly lower than the level of binding observed for EMYCS or E2F family members.

The C-MYC E2F element does have some homology to previously reported Stat DNA-binding sites and treatment of HepG2 cells with interleukin 6 leads to Stat3 binding and activation of C-MYC through its E2F site (36). However, EMYCS binding to this element was not affected by Abs to Stat3 or Stat5 or by oligonucleotides known to interact with Stat3 and Stat5 (supplementary Figure 4 is available at Carcinogenesis Online). Moreover, binding of Stat to their DNA-binding sites usually requires cell activation by cytokines or chemo-
kines. Stat3 or Stat5 is constitutively activated in some, but not every tumor cell lines (37,38). Specifically, the Burkitt’s lymphoma cell line DG75 shows no basal activation of Stat-3 or Stat-5 DNA-binding activity (39). We have shown, however, that EMYCS is present in a number of cell lines from different tissues, including DG75 cells, and that it readily binds the C-MYC E2F element, even in the absence of any cell treatment. Furthermore, we have observed that EMYCS has transcriptional transactivation capacity in untreated cell lines such as DG75, U2OS, Mutu-1, Jurkat and 293-T. Our results therefore suggest that EMYCS is constitutively active in these cell lines. Hence, EMYCS plays a role in non-stimulated cells and is not probably to be a known member of the Stat family of transcription factors.

NFATc1 has also been shown to bind to and activate the C-MYC promoter through a site that overlaps with the E2F element (40). To assess whether EMYCS was a NFAT family member, we used an antiserum directed against a common epitope of the DNA-binding domain of all NFAT members (all-NFAT Ab) that completely abol-
ished formation of NFAT complexes in EMSA assays (41). This Ab did not affect binding of EMYCS to the E2F element from the C-MYC promoter, but inhibited formation of specific complexes with the distal NFAT site of the human IL-2 promoter (supplementar-
tary Figure 5A is available at Carcinogenesis Online). Furthermore, the oligonucleotide corresponding to the NFAT-binding site did not compete the binding of EMYCS to the E2Fmyc probe (supplementary Figure 5B is available at Carcinogenesis Online). Finally, NFATc1 binding to DNA requires activation in response to an increase in the intracellular calcium concentration whereas EMYCS binding to the C-MYC E2F element is constitutive in a number of cell lines from different tissues. These data therefore suggest that EMYCS is not a NFAT family member.

To identify the protein or proteins present in EMYCS, we have carried out pull-down experiments using beads conjugated with the oligonucleotide that binds specifically to complex III. Unfortunately, EMYCS non-specifically bound the different beads that were employed, even in the absence of oligonucleotides (data not shown). Other experimental approaches will be therefore required to know the makeup of this complex.

Since the DNA recognition sequences of EMYCS overlaps with the E2F element, EMYCS complex formation is likely mutually exclusive with the binding of E2F proteins, suggesting that compe-
tition between these factors for binding to the C-MYC E2F element might dictate the mode of regulation governing C-MYC expression. Thus, this element might be a potent C-MYC expression repressor in quiescent cells by recruiting pRb family members through E2F (Figure 6A). Following cell growth stimulation and entry into the cell cycle, most E2F-regulated genes are induced near the G1–S phase transition, once pRb family members have become hyperphosphory-
lated (22). C-MYC, however, is turned on quickly after the induction of cell proliferation, much earlier than kinases responsible for pRb family proteins phosphorylation become active. We propose that C-MYC induction during exit from quiescence might depend on the binding of factors such as EMYCS to its E2F site and exclusion of the E2F–pRb repressor complex from the promoter, rather than on the release of pRb family members from E2F (Figure 6B). In accordance with our model, we have shown that, C-MYC expression, binding of EMYCS to the C-MYC E2F site, and C-MYC promoter activity all are readily induced following exit from quiescence in T lymphocytes with a similar kinetics. EMYCS interaction with the E2F element not only would prevent C-MYC promoter repression but might also elicit its activation since we have observed that this factor has transcriptional transactivation capacity and is required for C-MYC promoter activation during exit from quiescence.
Finally, our findings may be relevant to the possible role of C-MYC overexpression in oncogenesis. Since EMYCS is required for C-MYC gene activation during exit from quiescence, it is tempting to speculate that deregulated expression of this factor may, in some cases, contribute to the overexpression of C-MYC that has been observed in multiple tumors (1). It could be therefore of great importance to identify the factors present in EMYCS in the next future and determine its role in tumor formation and C-MYC expression.

**Supplementary material**

Supplementary Data and Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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**References**


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