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A novel function for Myc: Inhibition of C/EBP-dependent gene activation

(v-*myc* oncogene/C/EBP transcription factors/myelomonocytic cells/signal transduction)

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ABSTRACT **We have investigated the effect of the v-Myc oncoprotein on gene expression in myelomonocytic cells. We find that v-Myc dramatically down-regulates the expression of myelomonocytic-specific genes, such as the chicken *mim*-1 and lysozyme genes, both of which are known targets for C/EBP transcription factors. We present evidence that Myc down-regulates these genes by inhibiting the function of C/EBP transcription factors. Detailed examination of the inhibitory mechanism shows that amino-terminal sequences of v-Myc, but not its DNA-binding domain, are required for the suppression of C/EBP-dependent transactivation. Our findings identify a new function for Myc and reveal a novel mechanism by which Myc affects the expression of other genes.**

Numerous studies have implicated the c-*myc* gene in a variety of cellular processes, such as proliferation, differentiation, and apoptosis, and have shown that mutant forms of the gene, such as transduced (v-*myc*) or rearranged *myc* alleles, are involved in tumorigenesis (1–6). It is now widely believed that c-*myc* plays a central role in a switch mechanism by which normal cells decide between the alternative fates of proliferation, differentiation, and apoptosis and that deregulation of c-*myc* causes an imbalance in this switch mechanism, resulting in the development of neoplasia.

Structural and functional analyses have identified the c-*myc* protein product (c-Myc) as a transcription factor. The carboxyl terminus of c-Myc forms a basic region-helix-loop-helix-leucine zipper (B-HLH-LZ) DNA-binding domain, heterodimerizes with the Max protein (7), and recognizes the consensus Myc binding site, CACGTG (8–10), whereas the amino terminus of c-Myc functions as a transcriptional activation domain (11–14). Several genes whose expression is induced by c-Myc have been identified (15–20). In addition, c-Myc also inhibits gene expression. The C/EBPα gene (21, 22) and the adenovirus-2 major late promoter (22, 23) are down-regulated by c-Myc. Interestingly, repression of these genes does not depend on Myc-specific DNA-binding sites but is mediated by so-called initiator elements located in the promoters of these genes (22, 23). Thus, increasing evidence suggests that c-Myc affects gene expression by several different mechanisms. Nevertheless, an unambiguous functional relationship to the physiological or malignant activities of *myc* has not been established for any of the putative target genes identified so far.

We have studied the effect of v-Myc on gene expression in myelomonocytic cells. We have found that v-Myc inhibits the expression of several genes, including *mim*-1 and the lysozyme gene, both of which have been shown to be targets for C/EBP transcription factors (24, 25). We have analyzed the mechanism by which Myc down-regulates these genes and have found that amino-terminal sequences of Myc interfere with the function of C/EBP transcription factors. Our findings identify a novel function for Myc and reveal a novel mechanism by which Myc affects the expression of other genes.

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MATERIALS AND METHODS

Cells. BM2 is a line of avian myeloblastosis virus-transformed chicken myeloblasts (26). Infection of BM2 cells with MC29 virus has been described (27). BM2 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) tryptose phosphate broth, 5% (vol/vol) fetal calf serum, and 5% (vol/vol) chicken serum. The QT6 line of quail fibroblasts (28) was grown in Iscove's modified DMEM supplemented with 8% (vol/vol) fetal calf serum and 2% (vol/vol) chicken serum.

Expression Vectors. The chicken C/EBPβ expression vector pCRNC-CCR and its frameshift derivative pCRNC-fsCCR have been described (24). pCRNC-chC/EBPα encodes chicken C/EBPα and was obtained by inserting the C/EBPα-coding region as an *Eco*RI/*Cla*I restriction fragment (derived from plasmid pcC/EBPpSG5; ref. 29) into expression vector pCRNC-M (gift of A. Leutz, MDC, Berlin, Germany). pMC29(Q10–4) contains a complete proviral copy of MC29 (30) and encodes the MC29-specific Gag–Myc fusion protein. pMC29(Q10–4)Notfs is a derivative of pMC29(Q10–4) carrying a frameshift at a unique *Not*I restriction site in the v-*myc*-coding region. pSPc-myc (14) encodes human c-Myc. pRCAS-wtMyc encodes v-Myc initiated at the authentic Myc start codon and lacking *gag*-derived amino acid sequences. pRCAS-MycH336G is a point-mutated derivative of pRCAS-wtMyc with a His-336 → Ala substitution (S. Scheidler, R.W., M. Hartl, and K.B., unpublished data). pCDNA3-wtMyc encodes v-Myc initiated at the authentic Myc start codon and was constructed by cloning the v-*myc*-specific sequences of MC29 into pCDNA3 (Invitrogen). pCDNA3-B2Myc, pCDNA3-H1Myc, pCDNA3-H2Myc, pCDNA3-ΔPAMyc and pCMVMycΔVP16 are derivatives of pCDNA3-wtMyc. In mutant Myc proteins B2, H1, and H2, specific amino acid residues were substituted using site-directed mutagenesis (31). In B2, Lys-332, Arg-333, and Arg-334, located in the basic region, are all replaced by glutamine; in H1, Phe-352, located in helix 1 of the HLH domain, is replaced by proline; and in H2, Glu-378, located in helix 2 of the HLH domain, is replaced by proline. In the mutant Myc protein ΔPA, v-Myc amino acids 45–148 are missing. Numbering of v-Myc amino acids starts at the chicken c-Myc start codon. pCMVGal4Myc(Cla), pCMVGal4-Myc(Not), pCMVGal4Myc(Apa), pCMVGal4Myc(Pvu), and pCMVGal4MycN105 encode fusion proteins of the Gal4 DNA-binding domain and the first 240, 185, 148, 84, or 105 amino-terminal amino acids of v-Myc. pCMVGal4Myc(ΔPA), pCMVGal4Myc(ΔMB1), pCMVGal4Myc(Δ45–83), and pCMVGal4Myc(Δ45–97) are derivatives of pCMVGal4-

Abbreviations: B-HLH-LZ, basic region-helix-loop-helix-leucine zipper; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Myc(Not) from which v-Myc amino acids 45–148, 45–56, 45–83, or 45–97, respectively, have been deleted. pGal4VP16 encodes a Gal4/VP16 hybrid protein (32) and was obtained from P. Chambon (CNRS, Strasbourg, France). pRSVc-jun encodes human c-Jun; the pRSVc-jun stop is a frameshift derivative of pRSVc-jun (33).

Transfections and Northern Blotting. The reporter plasmids p3xTRECAT (33), p-240Luc (34), pC3Luc200 (35), pluc1000 (36), and pCH110 (Pharmacia) have been described. pG5E4–38Luc contains five Gal4-binding sites upstream of the adenovirus E4 promoter (positions −38 to +38) and the luciferase gene. QT6 cells were transfected as described (24). The amounts of DNA used are indicated in the figure legends and refer to a 10-cm cell culture dish. Luciferase, chloramphenicol acetyltransferase, and β-galactosidase assays were performed 48 hr after transfection as described (24). Detection of the chicken *mim*-1, lysozyme, chicken C/EBPα and C/EBPβ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs by Northern blotting has been described (24).

RESULTS

Inhibition of *mim*-1 and Lysozyme Gene Expression by v-Myc. To identify target genes for v-Myc in myelomonocytic cells, we searched for genes differentially expressed between the avian myeloblastosis virus-transformed chicken myeloblast cell line BM2 (26) and subclones of this line infected with the chicken retrovirus MC29 (27). To our surprise, by simply comparing the expression of known genes in these cells, we identified several genes whose expression was down-regulated by v-Myc. As shown in Fig. 1*A*, *mim*-1 and lysozyme gene mRNA levels were substantially decreased in several clones of v-Myc expressing BM2 cells compared with the parental cell line. Both genes are normally activated during myelomonocytic differentiation. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate, an inducer of differentiation in BM2 cells (37), strongly enhanced *mim*-1 and lysozyme gene expression in BM2 cells but not in the v-Myc-expressing subclones (Fig. 1*B*). Thus, v-Myc strongly inhibits the basal expression as well as the differentiation-dependent activation of at least two genes in myelomonocytic cells.

v-Myc Inhibits C/EBP-Dependent Transactivation. To examine the inhibitory effect in more detail, we focused on the *mim*-1 gene. A *mim*-1 reporter gene was at least 10-fold more active in BM2 cells than in MC29-infected BM2 cells, suggesting that the sequences responsible for the inhibition by v-Myc are located between positions −240 and +150 of the promoter (data not shown). The *mim*-1 promoter lacks canonical Myc-binding sites as well as an initiator element; however, it does contain C/EBP-binding sites and is activated by C/EBP (24, 25), raising the possibility that the v-Myc-induced down-regulation is mediated by C/EBP. Consistent with this idea, our finding was that the levels of C/EBPα and C/EBPβ mRNA were suppressed in BM2 cells expressing v-Myc (Fig. 1*C*).

To determine whether v-Myc exerts its effect only on the level of C/EBP expression, we cotransfected a *mim*-1 reporter gene with a constitutively active C/EBPβ expression vector, either in the presence or in the absence of v-Myc. Interestingly, the ability of C/EBPβ to activate the *mim*-1 promoter was strongly compromised in the presence of v-Myc (Fig. 2*A*), raising the possibility that v-Myc suppresses C/EBP-mediated transactivation by inhibiting the function of C/EBPβ and not only its expression. The inhibition of C/EBPβ-dependent transactivation by v-Myc did not depend on the particular promoter used to assay C/EBPβ activity but was also observed when reporter genes pC3Luc200 (human complement component C3 promoter from −200 to +1 bp; ref. 35) and pLuc1000 (human albumin promoter from −1000 to +1 bp; ref. 36) were used (Fig. 2*A*). We confirmed by Western blotting that the inhibitory effect of v-Myc was not due to a reduction of the amount or change of the subcellular localization of C/EBPβ (Fig. 2*B*). We concluded that v-Myc interferes with the function of C/EBPβ and not only its expression. Immunoprecipitation experiments using Myc- or C/EBP-specific antisera showed that Myc and C/EBPβ are present in roughly comparable amounts under these conditions (data not shown).

To examine the specificity of the inhibitory effect, we asked if v-Myc also inhibits other transcription factors. Transactivation by chicken C/EBPα was strongly suppressed by v-Myc (Fig. 2*C*). We confirmed by Western blotting that the amount of C/EBPα was not reduced in the presence of v-Myc (data not shown). Thus, the inhibitory effect of v-Myc affects several C/EBP family members. By contrast, transactivation by c-Jun or a Gal4–VP16 hybrid protein was not inhibited by v-Myc (Fig. 2*C*). Thus, among the transactivators analyzed, inhibition

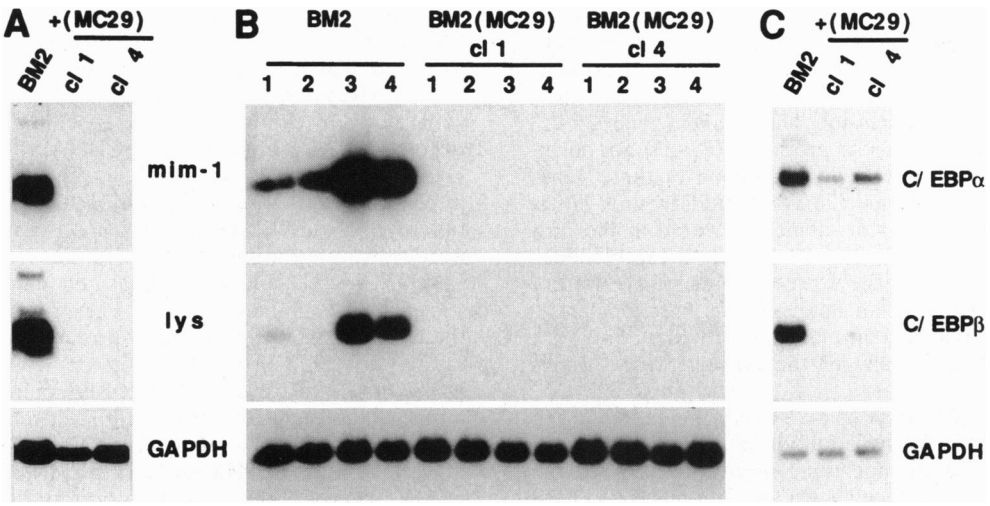

FIG. 1. Down-regulation of gene expression by v-Myc. (*A*) Polyadenylated RNA from BM2 cells or from two clones of MC29-infected BM2 cells was hybridized to probes specific for chicken *mim*-1, the chicken lysozyme gene, or GAPDH. (*B*) Northern blot analysis of the same cells as in *A*. The cells were grown in regular growth medium (lane 1), in medium supplemented with dimethyl sulfoxide to a final concentration of 0.1% (vol/vol) (lane 2), or grown for 24 hr (lane 3) or 48 hr (lane 4) in the presence of 250 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate and 0.1% (vol/vol) dimethyl sulfoxide. The blot was hybridized as in *A*. (*C*) RNA from the same cells as in *A* was hybridized to probes for chicken C/EBPα and C/EBPβ, and GAPDH. The different intensities of the signals for basal *mim*-1 and lysozyme mRNA expression in *A* and *B* are due to different exposure times of the autoradiographs.

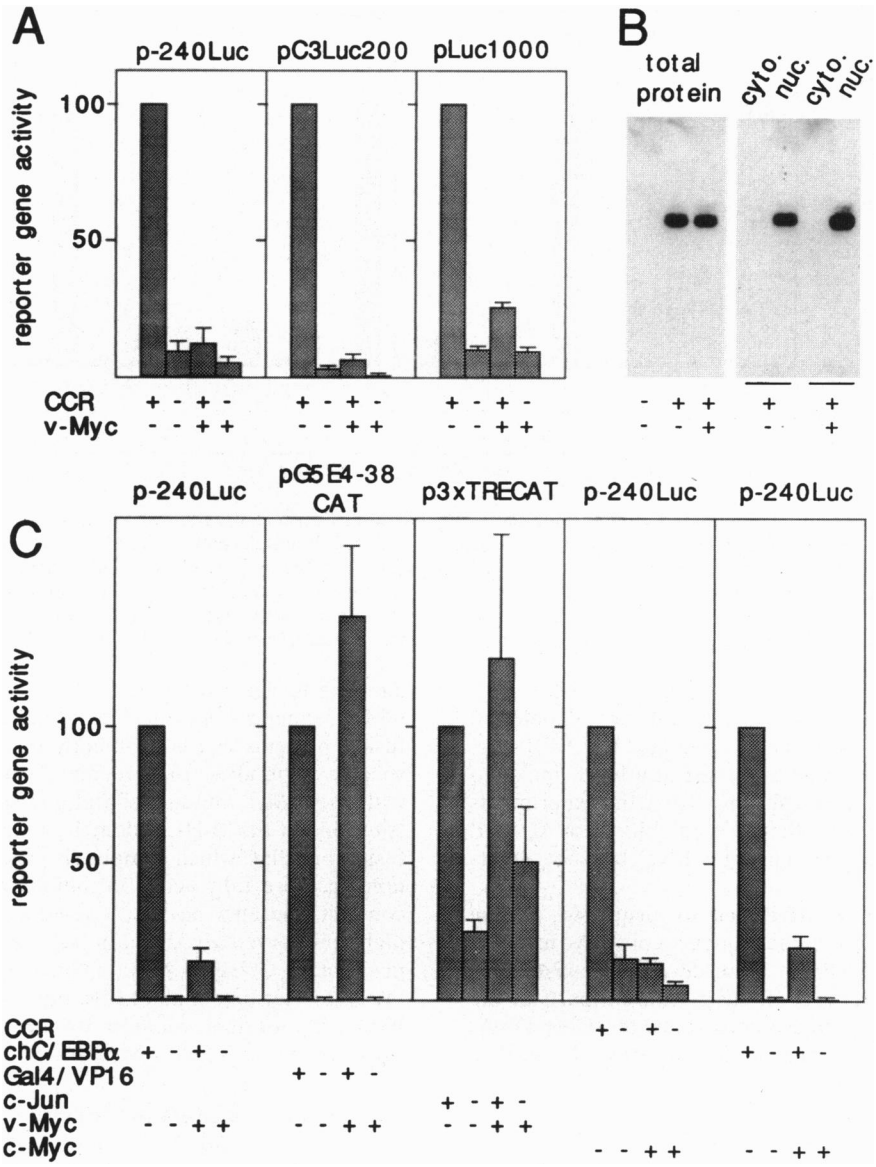

FIG. 2. Inhibition of C/EBP-dependent transactivation by Myc. (*A* and *C*) QT6 cells were transfected with 3 μg of the reporter genes shown at the top; 1 μg of the β-galactosidase reference plasmid pCH110; and expression vectors for chicken C/EBPβ (pCRNC-CCR, 1 μg), v-Myc [pMC29(Q10–4), 2 μg], chicken C/EBPα (pCRNC-chC/EBPα, 1 μg), Gal4/VP16 (pGal4-VP16, 0.2 μg), c-Jun (pRSVc-jun, 1 μg), c-Myc (pSPmyc, 2 μg), or the same amounts of the relevant control plasmids, as indicated by dashes below the columns. The columns show the average luciferase or chloramphenicol acetyltransferase activity, normalized with respect to the cotransfected pCH110 plasmid. The activity of each reporter in the presence of the appropriate exogenous transactivator was designated as 100%. Thin lines show standard deviations. (*B*) QT6 cells were transfected with the indicated expression vectors (same amounts as in *A*) and 1 μg of the luciferase reference plasmid pSV2B2. After transfection (48 hr) total cellular protein (*Left*) or nuclear and cytoplasmic fractions (*Right*) were analyzed by Western blotting with use of chicken C/EBPβ-specific antiserum. The activity of the cotransfected pSV2B2 plasmid was measured in aliquots of the transfected cells to ensure that transfection efficiencies were similar.

was only observed for C/EBP family members. Finally, we showed that human c-Myc inhibits C/EBPα and C/EBPβ to an extent similar to v-Myc. Thus, the ability to inhibit C/EBP function is not specific to v-Myc but is shared with c-Myc.

The Amino Terminus of v-Myc, but Not Its DNA-Binding Domain, Is Required for Inhibition of C/EBP. To explore the molecular basis of the suppression of C/EBP function by v-Myc, different mutants of v-Myc were tested for their ability to inhibit C/EBPα and C/EBPβ. For convenience, the mutants were generated in the context of a v-Myc protein initiated at the authentic Myc start codon. v-Myc lacking *gag* amino acid sequences inhibited C/EBPα and C/EBPβ, indicating that the *gag*-derived sequences of the Gag–Myc fusion protein of MC29 are not required to suppress C/EBP (Fig. 3*A*). In the Myc mutant His-336 → Gly, a histidine in the basic region of v-Myc, which is involved in the specific amino acid–base contacts in the Myc–DNA complex (38), is replaced by glycine. The protein encoded by the His-336 → Gly mutant exhibits strongly reduced *in vitro* DNA-binding activity and is defective in fibroblast transformation (R.W., K.B., S. Scheidler, and M. Hartl, unpublished data). The His-336 → Gly mutant inhibited C/EBPα and C/EBPβ almost as efficiently as unmutated v-Myc, suggesting that the DNA-binding function of v-Myc is not required for the inhibition of C/EBP function (Fig. 3 *A* and *B*). To substantiate this conclusion, additional mutations were generated in the basic region (B2) and the HLH region (H1, H2) of v-Myc, all of which had completely lost DNA-binding activity (31). As illustrated in Fig. 3*C*, all of the mutants inhibited C/EBPβ and C/EBPα (data not shown) with an activity similar to the unmutated v-Myc, suggesting that the

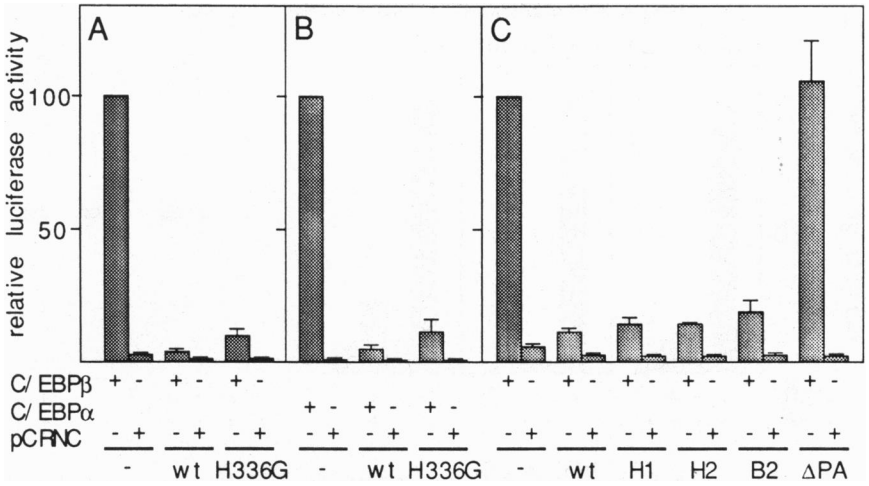

FIG. 3. Inhibition of C/EBP transactivation by v-Myc mutants. QT6 cells were transfected with 3 μg of *mim*-1 reporter plasmid p-240Luc, 1 μg of β-galactosidase reference plasmid pCH110, and 1 μg of expression vectors for full-length chicken C/EBPα or C/EBPβ. Control transfections (lacking C/EBP) were performed by use of 1 μg of the empty pCRNC expression vector instead of C/EBP expression vectors. Additionally, transfections contained 3 μg (*A* and *B*) or 0.2 μg (*C*) of expression vectors for wild-type (wt) or the indicated mutant v-Myc proteins, or an empty expression vector (minuses). The columns show the relative luciferase activity, normalized with respect to the cotransfected β-galactosidase plasmid, pCH110. The luciferase activity in the presence of C/EBP and in the absence of v-Myc was designated as 100%. (Error bars = SD.)

DNA-binding function of v-Myc is not required for the inhibition of C/EBP. By contrast, an amino-terminal deletion mutant of v-Myc (ΔPA) was unable to inhibit C/EBPβ (Fig. 3*C*), although this protein was expressed at a level similar to the wild-type protein (data not shown). Thus, the experiments shown in Fig. 3 suggested that the inhibition of C/EBP depends on the amino terminus of v-Myc, but not on its DNA-binding domain.

Inhibition of C/EBP Is Mediated by Sequences in the Vicinity of Myc Box 1. To map the sequences of v-Myc involved in the inhibition of C/EBPβ in more detail, we constructed Gal4–v-Myc fusion proteins containing different parts of the v-Myc amino terminus. In these constructs, the Gal4 DNA-binding domain provides a nuclear translocation signal, thus allowing nuclear transport of the proteins irrespective of the v-Myc sequences present. Furthermore, the expression of the fusion proteins can conveniently be monitored using Gal4-specific antibodies. In agreement with the results obtained with the v-Myc mutants containing specific amino acid substitutions in the B-HLH domain, several of the Gal4–v-Myc fusion proteins, which completely lack the Myc DNA-binding domain, were fully active in inhibiting C/EBPβ (Fig. 4). By contrast, mutants having a deletion endpoint close to the highly conserved Myc-box 1 or lacking Myc-box 1 were inactive in inhibiting C/EBPβ. Mutant Gal/v-Myc(ΔMB1), which lacks the amino-terminal half of Myc-box 1, still inhibited C/EBPβ. Expansion of this deletion toward the carboxyl terminus (deletion mutants Gal/v-MycΔ45–83 and Gal/v-MycΔ45–97),

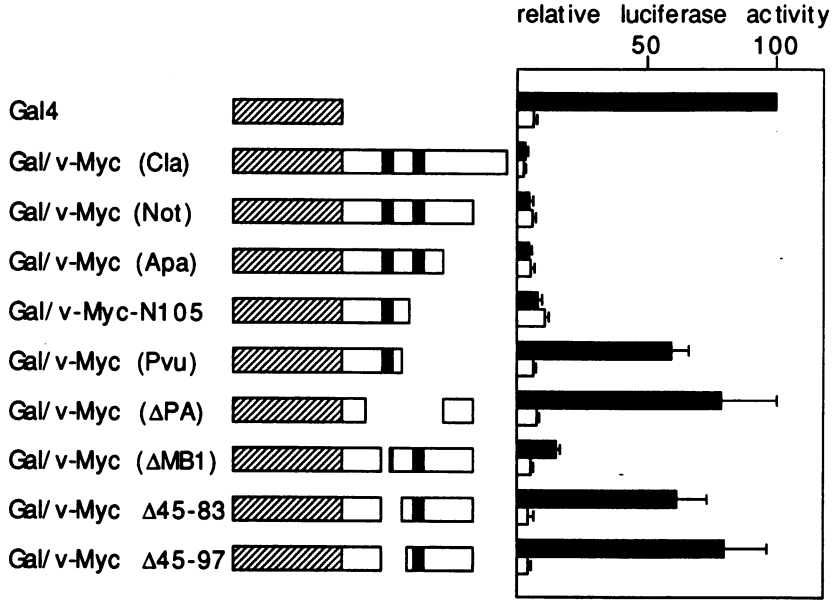

FIG. 4. Inhibition of C/EBPβ-dependent transactivation by Gal4–v-Myc proteins. QT6 cells were transfected with 3 μg of *mim*-1 reporter gene p-240Luc, 1 μg of β-galactosidase reference plasmid pCH110, 1 μg of chicken C/EBPβ expression vector pCRNC-CCR (black bars) or empty expression vector pCRNC-M (open bars), and 0.5 μg of expression vectors for the Gal–Myc fusion proteins shown at the left. Gal4 and v-Myc sequences are represented as hatched and open bars, respectively. Myc boxes 1 and 2 are shown in black. The columns on the right side show the relative luciferase activities, normalized with respect to the cotransfected pCH110 plasmid. The luciferase activity in the presence of C/EBP and in the absence of v-Myc was designated as 100%. (Error bars = SD.)

however, lead to a loss of the inhibitory activity of Myc (Fig. 4). This result suggested that the carboxyl-terminal half of Myc box 1 or sequences located between Myc boxes 1 and 2 are responsible for the inhibition.

DISCUSSION

It is widely believed that c-Myc controls a switch by which cells select between the alternative fates of proliferation, differentiation, and apoptosis. Attempts to identify target genes for Myc involved in this switch have focused so far almost exclusively on fibroblasts and on genes involved in transformation. To identify genes whose expression is affected by Myc in differentiating cells, we have studied the effect of v-Myc on a myelomonocytic chicken cell line. We have identified several genes whose expression is repressed by v-Myc in these cells. Analysis of repression of the these genes by Myc has revealed a novel function of Myc: inhibition of C/EBP-mediated gene expression. This novel inhibitory activity appears to be due to the ability of Myc to interfere with the function of C/EBP transcription factors.

It is important to distinguish between inhibition of C/EBP function and suppression of C/EBP expression. Myc inhibits C/EBPα- and C/EBPβ-mediated transactivation even when the amounts of these transcription factors remain constant. Thus, the inhibitory activity of Myc is exerted on the level of C/EBP protein function. However, Myc also down-regulates C/EBPα and C/EBPβ gene expression. The promoters of these genes contain C/EBP binding sites and are up-regulated by their own gene products (22, 39; unpublished data). Thus, by blocking C/EBP function, Myc can interfere with autoregulation and cause repression of both genes.

How does Myc block C/EBP-mediated transactivation? Although we have not yet addressed in detail the inhibitory mechanism, our results exclude several possibilities. In addition to binding to canonical Myc binding sites, Myc has been shown to recognize C/EBP-binding sites, at least *in vitro* (40). The inhibition of C/EBP cannot be explained by competition of v-Myc and C/EBP for the same binding sites, because the inhibitory effect does not require the DNA-binding function of Myc. This also excludes as a possible mechanism binding to and activation by Myc of other genes whose products then interfere with C/EBP. That the amino terminus of Myc is required for inhibition of C/EBP is somewhat reminiscent of the repression of the adenovirus-2 major late promoter and of C/EBPα genes, which also involves the amino-terminal domain of Myc (22). In these cases, however, the inhibition is mediated by initiator elements present in the promoters of the repressed genes. The inhibitory mechanism described here is distinct from this initiator-dependent repression mechanism, because the *mim*-1 and lysozyme gene promoters lack initiator elements. Furthermore, Myc box 2 is required for the initiator-dependent inhibition mechanism (22) but is dispensable for the inhibition of C/EBP-dependent transactivation. We note that the C/EBPα promoter, which contains C/EBP binding sites as well as an initiator element, can be inhibited by Myc by two different mechanisms. This suggests a complex relationship between Myc and C/EBPα expression.

What is the biological relevance of the suppression of C/EBP-dependent transactivation by Myc? A number of studies suggest important roles for C/EBP in differentiation processes. Several C/EBP isoforms are directly involved in the transcription of differentiation-specific genes in terminally differentiated liver cells, fat cells, and macrophages (24, 25, 41–46). High levels of C/EBP expression inhibit cell proliferation and, thus, appear to be responsible for the proliferation arrest of terminally differentiated cells (21, 47, 48). Finally, antisense inhibition of C/EBPα mRNA is sufficient to block fat cell differentiation (45). Several studies have shown that Myc inhibits myelomonocytic as well as fat cell differentiation (24, 27, 49). Given the relevance of C/EBP proteins for these differentiation processes, our findings raise the intriguing possibility that the suppression of C/EBP is causally related to the inhibition of C/EBP-dependent differentiation processes by Myc. Whether the novel function of Myc described here also plays a role in oncogenic transformation is unclear. Numerous studies have shown that cell transformation by Myc depends on its DNA-binding function, suggesting that transformation involves the direct activation or suppression of certain as yet unknown target genes by Myc. However, it is possible that inhibition of C/EBP, whose expression at high levels leads to growth arrest, provides an additional mechanism for locking transformed cells in a proliferative state.

Further work is required to explore these ideas and to understand, on the molecular level, how Myc suppresses C/EBP-mediated gene activation. Since cross-talk between different transcription factors often involves direct protein–protein contacts, it will be interesting to determine whether the inhibitory mechanism described here is facilitated by direct protein–protein interactions involving Myc and C/EBP. In any case, the increasing complexity and surprising diversity of mechanisms by which Myc affects the expression of other genes underlines its role as a multifunctional integration point in the transcription factor network.

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