

# Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and *trans*-acting dominant mutants

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The *myc* family of cellular oncogenes encodes three highly related nuclear phosphoproteins (c-Myc, N-Myc, and L-Myc) that are believed to function as sequence-specific transcription factors capable of regulating genes important in cellular growth and differentiation. Current evidence indicates that Myc family proteins exist as biologically active heterodimeric complexes in association with another helix-loop-helix leucine zipper phosphoprotein, Max. We have investigated the common and unique properties among the Myc family, as well as the physiological role of Max in the regulation of Myc family function. We demonstrate that *trans*-activation-incompetent mutants of one Myc family member can act in *trans* to dominantly suppress the cotransformation activities of all three Myc oncoproteins, indicating that the Myc family functions through common genetic elements in its cellular transformation pathways. Employing coimmunoprecipitation with either anti-Myc or anti-Max antibodies, we show that the transfected normal c-Myc, N-Myc, and L-Myc oncoproteins associate with the endogenous Max protein in REF transformants, indicating that the Max interaction represents at least one component common to Myc family function. In addition, we observed a striking reduction in Myc cotransformation activity when a Max expression construct was added to *myc/ras* cotransfections. We discuss these biological findings in the context of a proposed model for Myc/Max function and regulation in which Max serves as either an obligate partner in the Myc/Max transcriptional complex or as a repressor in the form of a transcriptionally inert Max/Max homodimer capable of occupying Myc/Max-responsive gene targets.

[Key Words: Myc family; Max; cotransformation; dominant interference; Myc/Max complexes]

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Three highly related nuclear phosphoproteins, c-Myc, N-Myc, and L-Myc, are encoded by the *myc* family of cellular oncogenes. Although their exact function has not been determined, current evidence suggests that Myc oncoproteins act as sequence-specific transcription factors that serve to regulate genes important in normal cellular growth and differentiation processes (for review, see Lüscher and Eisenman 1990; DePinho et al. 1991). Deregulated expression of all three *myc* family genes has been implicated in the genesis or progression of naturally occurring tumors and has contributed to transformation of cultured cells as well as tissues in transgenic mice (for review, see Moroy et al. 1991). In the rat embryo fibroblast (REF) cotransformation assay, *c-myc*, *N-myc*, or *L-myc* expression constructs have been shown to cooperate with the activated *H-ras* (val12) gene to transform

early-passage REFs (Land et al. 1983; Schwab et al. 1985; Yancopoulos et al. 1985; DePinho et al. 1987a,b; Legouy et al. 1987; Birrer et al. 1988). In this assay, *c-myc* appears to be most oncogenically active, whereas *N-myc* and *L-myc* exhibit moderate and weak transforming activity, respectively (DePinho et al. 1987a,b). The tumor distribution of each *myc* family member is also different and appears to correlate strongly with their tissue-specific expression during normal development (Nisen et al. 1986; Zimmermann et al. 1986).

Structural studies have led to a better understanding of the function of Myc oncoproteins in normal cellular growth and malignant transformation. Domains essential for transcriptional *trans*-activation (Kato et al. 1990), sequence-specific DNA binding (Blackwell et al. 1990; Halazonetis and Kandil 1991; Kerckhoff et al. 1991; Prendergast and Ziff 1991), protein oligomerization (Blackwood and Eisenman 1991; Prendergast et al. 1991; Kato et al. 1992), and nuclear localization (Stone et al. 1987;

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Dang and Lee 1988) have been found to be highly conserved among c-Myc, N-Myc, and L-Myc proteins (DePinho et al. 1986, 1987a,b). The carboxy-terminal regions of Myc proteins contain two structural motifs identified previously in known transcription and differentiation factors, namely, the leucine zipper (LZ; Landschulz et al. 1988) and the basic region/helix-loop-helix (BR/HLH; Murre et al. 1989). The two motifs are found in a contiguous arrangement, with the LZ located at the carboxyl terminus and the HLH just amino-terminal to the LZ. A region rich in basic amino acid residues that overlaps the amino-terminal end of the HLH motif appears to confer sequence-specific DNA-binding activities to the hexameric core sequence CACGTG in vitro (Blackwell et al. 1990; Blackwood and Eisenman 1991; Prendergast et al. 1991). The BR/HLH and LZ structures have been found to be indispensable for polypeptide dimerization with the Myc-associated human protein Max (Blackwood and Eisenman 1991) and its murine homolog, Myn (Prendergast et al. 1991). In vitro affinity chromatography studies have demonstrated that Max can homodimerize, or heterodimerize with c-Myc, N-Myc, or L-Myc (Blackwood and Eisenman 1991). The formation of a heterodimeric complex between either c-Myc (Blackwood et al. 1992; Kato et al. 1992) or N-Myc (Wenzel et al. 1991) and Max has also been substantiated in vivo. Max appears to have the capacity to form Max/Max homodimers in vitro (Blackwood and Eisenman 1991) and possibly in vivo (Kato et al. 1992). Myc/Max heterodimers and Max/Max homodimers appear to have similar DNA sequence-binding specificities in vitro (Prendergast et al. 1991; Kato et al. 1992). Although c-Myc homodimers do not appear to exist in vivo (Smith et al. 1990), homodimer formation can be forced at high protein concentrations in vitro (Dang et al. 1989; Smith et al. 1990).

The amino-terminal region of the c-Myc protein has been shown previously to be essential for neoplastic transformation (Stone et al. 1987) and appears to be involved in *trans*-activation (Kato et al. 1990). This region has been found to be highly conserved among all members of the Myc family from lower vertebrates to humans (N. Schreiber-Agus and R.A. DePinho, in prep.). In contrast, Max does not exhibit *trans*-activation activity (Kato et al. 1992).

Activities of LZ/HLH-containing proteins have been found to be strongly dependent on dimerization (Lassar et al. 1989). This requirement forms the basis for dominant interference, a stratagem first proposed by Herskowitz (1987), which is designed to generate a null phenotype for proteins whose function relies on oligomerization. In this respect, the overexpression of a mutant Myc oncoprotein that lacks its *trans*-activation domain but still retains its ability to oligomerize would be expected to behave as a dominant-negative mutant of the normal Myc oncoprotein regardless of the specific oligomerization partner of that protein. Recent experiments with c-Myc appear to have demonstrated the effectiveness of this approach (Dang et al. 1989).

Although all three Myc family proteins exhibit oncogenic activity, it is not clear whether differences in their

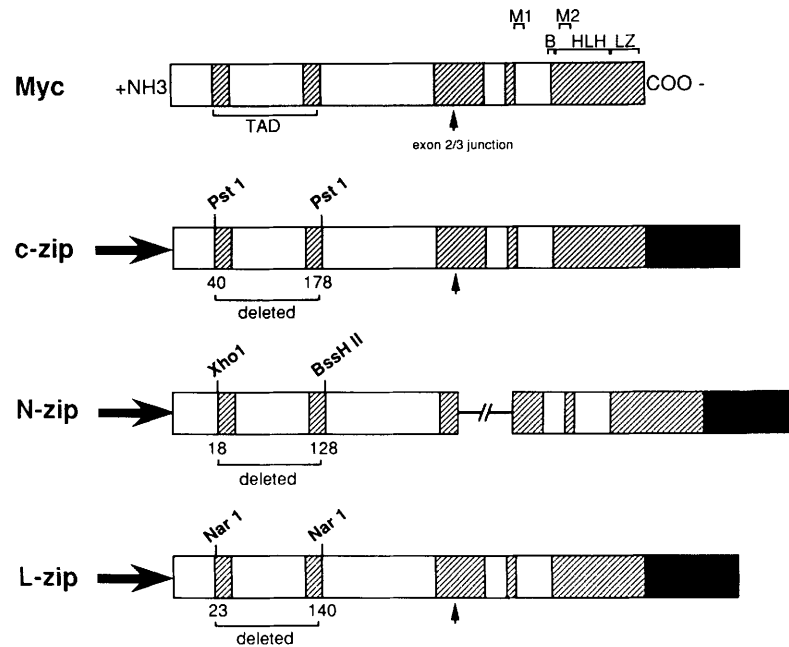
oncogenic potency and tumor distribution result from differences in their *trans*-activation activities or their affinities for Max or specific gene targets. In this study we show that dimerization-competent/*trans*-activation-incompetent c-Myc, N-Myc, and L-Myc mutants can all interfere equally with the transformation activities of either c-Myc or N-Myc in the REF cotransformation assay, indicating that Myc family oncoproteins share common genetic elements in their transformation pathways. Consistent with previous in vivo studies for c-Myc (Blackwood et al. 1992; Kato et al. 1992) and N-myc (Wenzel et al. 1991), we demonstrate that the transfected c-Myc, N-Myc, and L-Myc oncoproteins associate with the endogenous Max protein in REF transformants, indicating that the Max interaction represents at least one component common to Myc family function. In addition, the neutralizing activity for each mutant *myc* expression construct was equivalent, suggesting that differences in oncogenic potency among Myc oncoproteins may localize to regions important for *trans*-activation rather than those involved in sequence recognition and dimerization. Finally, the finding that a *max* expression vector functioned to suppress the transformation activity of all three *myc* family genes has led to a model for the regulation of Myc activity.

## Results

### *Cross-interference among the Myc family of cellular oncoproteins by trans-acting dominant mutants*

To determine whether c-Myc, N-Myc, and L-Myc function through common or distinct cellular transformation pathways, mutant c-Myc, N-Myc, and L-Myc oncoproteins were assayed for their ability to influence the cotransformation activities of oncogenically active c-Myc and N-Myc. All three mutant proteins, termed c-Zip, N-Zip, and L-Zip, encode BR/HLH and LZ sequences responsible for sequence-specific DNA-binding activity and protein oligomerization but contain large deletions in the highly conserved *trans*-activation domain as defined previously in the c-Myc protein (Kato et al. 1990) (Fig. 1). These *zip* expression constructs were not oncogenically active (data not shown). Employing the REF cotransformation assay, the oncogenic potency of each Myc + Zip combination was measured by parameters standardly used to establish the degree of malignant transformation, including the rate of foci formation, the subcloning efficiency of harvested foci, and the ability of established cell lines to exhibit anchorage-independent growth.

*The zip-induced phenotype* For each transfection point, a Rous sarcoma virus/long terminal repeat (RSV/LTR)-driven *c-myc* or *N-myc* gene and an activated *H-ras* (*val12*) gene were cotransfected onto a monolayer of early-passage REFs in the presence or absence of *c-zip*, *N-zip*, or *L-zip* expression constructs. In several experiments, the number of foci generated by *c-myc/ras* alone or by *N-myc/ras* alone was markedly reduced when a *c-zip*, *N-zip*, or *L-zip* expression construct was added to



**Figure 1.** Schematic of *c-myc*, *N-myc*, and *L-myc* dominant-negative mutant (*zip*) expression constructs. (Top) A linear representation of a Myc family protein. Highly conserved regions among the Myc family proteins are indicated by the cross-hatched regions, and the major structural-functional regions are bracketed with the abbreviations, which indicate the following: (TAD) trans-activation domain; (M1, M2) nuclear localization signals; (B) basic region; (HLH) helix-loop-helix; (LZ) leucine zipper. In the diagrams of the three dominant-negative mutant constructs, the highly conserved regions are crosshatched, and the boundaries of the deleted domains are indicated by restriction enzyme sites used and corresponding amino acid residues united in the ligation. Solid arrows and rectangles represent the RSV/LTR and SV40 polyadenylation sequences, respectively.

the cotransfection (Fig. 2, A and B, *c-myc* and *N-myc*, respectively). Most notably, all three *zip* expression constructs were found to be nearly equivalent in their capacity to attenuate *c-myc/ras* and *N-myc/ras* transformation activity. Specifically, relative to *c-myc/ras* alone, an average reduction in foci generated was 18% for *c-zip*, 13% for *N-zip*, and 20% for *L-zip*. Similar results were obtained in *N-myc/ras* assays, wherein a 14% reduction was observed with *c-zip*, 20% with *N-zip*, and 23% with *L-zip*. The suppressive activity of each *zip* expression construct was readily evident with other parameters used to assess the degree of malignant transformation, including a decreased potential for anchorage-independent growth and a decreased efficiency of subcloning, that is, the ability of harvested foci to become established as permanent cell lines (Fig. 2A,B). Because *L-myc* transforms weakly, we did not assay the impact of *zip* expression constructs on *L-myc* cotransformation activity because differences would be more difficult to detect.

**Zip does not affect E1A cotransformation activity** To determine whether the actions of Zip are restricted to Myc transformation activity, we tested the ability of the *zip* expression constructs to influence the oncogenic activities of the adenovirus E1A viral oncoprotein. In two experiments the addition of either *c-zip* or *L-zip* to E1A/*ras* cotransfections was found to have no effect on the degree of foci formation (Fig. 2C). These results suggest that *zip*-induced suppression operates through elements that are essential and specific for Myc family activities and that E1A functions through a pathway that is not dependent on Myc function.

**L-Myc repression of c-Myc and N-Myc** To validate further the experimental approach and the findings of the *zip* experiments, we took advantage of the oncogenic po-

tency differences among the *myc* family. The weakly transforming *L-myc* gene was tested for its ability to influence the oncogenic activities of the more potent *c-myc* or *N-myc* genes. The addition of *L-myc* would be expected to induce a phenotype similar to the *zip*-induced phenotype, albeit less pronounced because unlike the *zip* constructs *L-myc* can cooperate to some extent with *ras* to transform REFs. In four experiments, the addition of an equimolar amount of the *L-myc* construct to *c-myc/ras* or *N-myc/ras* cotransfections resulted in a level of cooperation activity that was intermediate between *c-myc* or *N-myc*, and *L-myc* activities (Fig. 2D). Northern analysis demonstrated that all transformants from the various combination expressed high levels of the introduced *c-myc*, *N-myc*, *L-myc*, and *ras* genes (data not shown).

**Expression analysis in the transformed REF cell lines** Steady-state mRNA levels for the various transfected constructs were examined in a large number of *c-myc/ras*-transformed cell lines that were generated in the presence or absence of the various *zip* expression vectors; a representative panel of samples is presented in Figure 3. All permanently established cell lines were found to express high levels of the 2.0-kb transcript encoded by the transfected *c-myc* gene (Fig. 3A,B, *c-myc* panels, lanes 1–32) relative to levels of *c-myc* gene expression found in the P19 mouse embryonal carcinoma cell line, a cell line that serves as a control for the levels of *myc* family gene expression typically observed in developing mouse tissues (Fig. 3A, B, P19 lanes). All transformed cell lines were also found to express high steady-state mRNA levels of the introduced *H-ras* gene (Fig. 3A, *H-ras* panel, lanes 1–15; similar results were observed for samples 16–32; data not shown). Significantly, when the

**A**

Transfected DNA	Total # of Foci		Subcloning Efficiency = # of established cell lines/ # of cell lines tested (%)	Anchorage-independent cells / cell lines tested (%)
	Exp. 1	Exp. 2		
c-myc + ras	260	290	10/12 (83%)	8/9 (89%)
c-myc + ras + c-zip	44	56	10/17 (59%)	9/15 (60%)
c-myc + ras + N-zip	38	35	6/11 (55%)	6/10 (60%)
c-myc + ras + L-zip	48	62	5/10 (50%)	4/9 (44%)

**B**

Transfected DNA	Total # of Foci		Subcloning Efficiency = # of established cell lines/ # of cell lines tested (%)
	Exp. 1	Exp. 2	
N-myc + ras	126	130	9/13 (69%)
N-myc + ras + c-zip	20	16	6/11 (55%)
N-myc + ras + N-zip	24	28	6/10 (60%)
N-myc + ras + L-zip	30	28	5/14 (36%)

**C**

Transfected DNA	Total # of Foci	
	Exp. 1	Exp. 2
E1a + ras	910	920
E1a + ras + c-zip	840	870
E1a + ras + N-zip	ND	ND
E1a + ras + L-zip	760	801

**D**

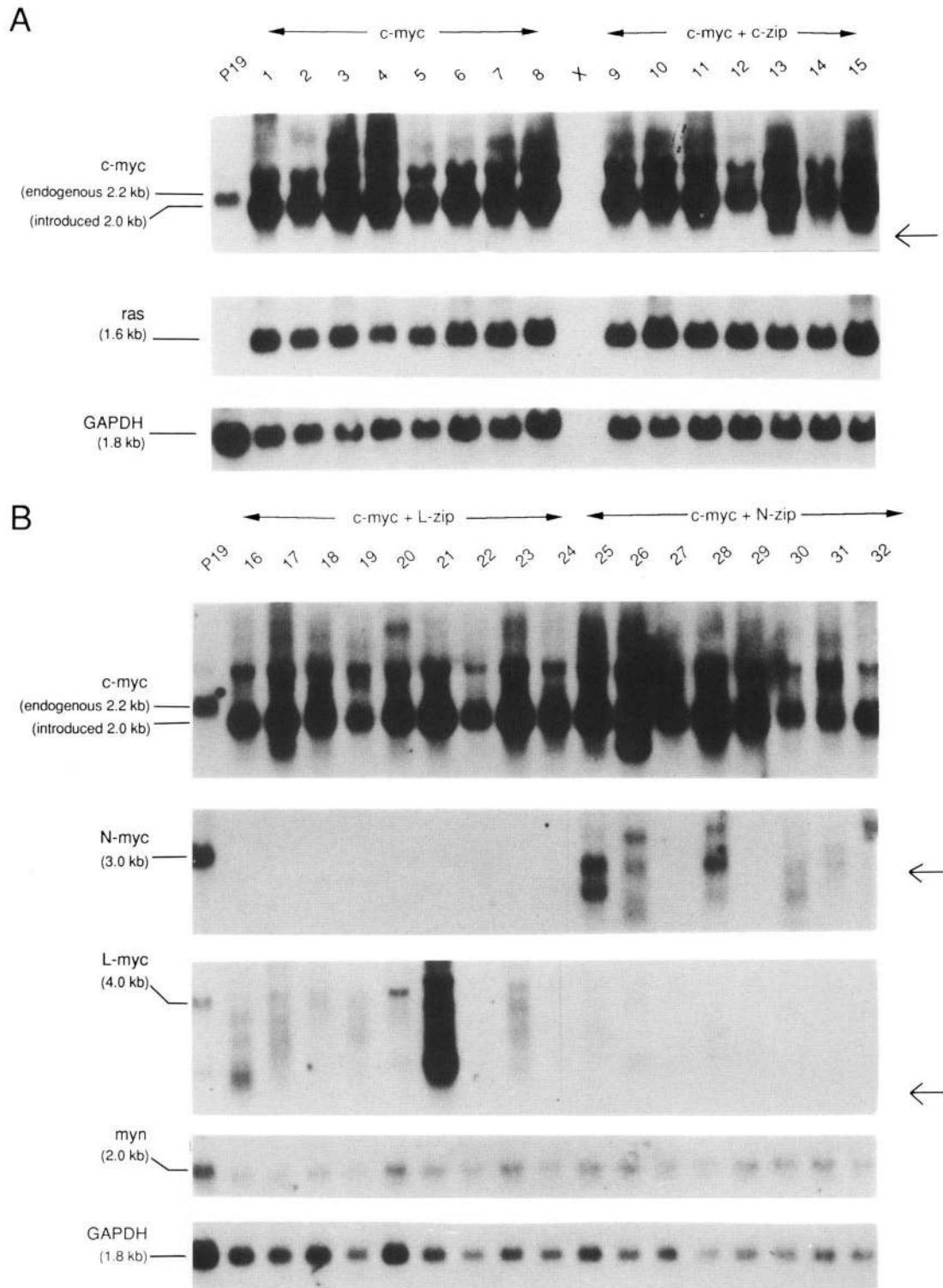
Transfected DNA	Total # of Foci				Subcloning efficiency = # of established cell lines / # of cell lines tested (%)	Anchorage-independent cells / cell lines tested (%)
	Exp. 1	Exp. 2	Exp. 3	Exp. 4		
c-myc + ras	288	304	310	325	13/16 (81%)	12/14 (86%)
c-myc + ras + L-myc	170	200	165	163	8/16 (50%)	9/14 (64%)
N-myc + ras	150	141	155	140	11/16 (69%)	ND
N-myc + ras + L-myc	112	115	111	107	7/16 (44%)	ND
L-myc + ras	12	20	16	18	0/10 (0%)	ND

**Figure 2.** Transformation of REFs with *c-myc* or *N-myc/ras* with or without *zip* constructs and with or without *L-myc*. (A) Each plate of REFs was transfected with 2  $\mu$ g of *c-myc* and 2  $\mu$ g of *ras* with or without 2  $\mu$ g of the *zip* constructs, as indicated in the transfected DNA column, and the total number of foci on 12 plates derived from 4 transfected plates was counted 11 days after transfection in two separate experiments. Foci were harvested and established as permanent cell lines with the indicated subcloning efficiency and assayed for anchorage-independent growth as described in Materials and methods. (B) Same as in A, except REFs were transfected with *N-myc/ras* with or without the *zip* constructs. (C) Same as in A, except REFs were transfected with 10  $\mu$ g of *E1a* and 2  $\mu$ g of *ras* with or without the *zip* constructs, and foci were counted 7 days after transfection in two separate experiments. (ND) Not determined. (D) Same as in A, except REFs were transfected with *c-myc/ras* or *N-myc/ras* with or without *L-myc* or with *L-myc/ras* alone.

same Northern blot was successively stripped and assayed for expression of the shorter *zip* transcripts, most of the permanently established *zip*-transfected cell lines were found to express lower levels of the *zip*-encoded transcripts relative to those produced by the transfected *myc* expression construct (Fig. 3B, *N-myc* panel, lanes 25–32; *L-myc* panel, lanes 16–24). In addition, despite linearization of the *zip* constructs, significant variability in the *zip*-encoded transcript sizes was observed. Because REFs do not express the endogenous *N-myc* and *L-myc* genes, the variable pattern of *zip*-encoded transcripts was more readily discerned in the *c-myc* + *N-zip* and *c-myc* + *L-zip* samples (Fig. 3B, *N-myc* panel, lanes 25–32; *L-myc* panel, lanes 16–24). In addition, cell lines that expressed *zip* transcripts of proper size were found to express somewhat higher levels of the wild-type *c-myc* mRNA relative to cell lines with aberrant *zip* transcript

forms (e.g., Fig. 3B, *N-myc* panel, lanes 25, 26, and 28 contain proper size *zip* transcripts). Finally, expression of the endogenous rat *max* gene was found to be similar in all REF transformants assayed (Fig. 3B, *myn* panel, lanes 16–32; similar results were obtained for samples 1–15; data not shown). Together, these results were consistent with the existence of strong selective forces operating against the high-level expression of normal *c-zip*, *N-zip*, or *L-zip* transcripts.

*Comparable expression of introduced myc and zip genes in transiently transfected REFs* Selection against *zip* gene expression was supported further by a series of transient transfection assays that were designed to compare the efficiency of *zip* versus *myc* construct expression before selection at the level of transformation. In these experiments early-passage REF monolayers were trans-



**Figure 3.** Northern analysis of stably transformed REF cell lines. (A) Twenty micrograms of total RNAs derived from P19 cells, from a panel of several *c-myc/ras*-transfected REF-transformed cell lines (lanes 1–8), and from a panel of several *c-myc/ras/c-zip*-transfected REF-transformed cell lines was probed with radiolabeled fragments specific to probes indicated at left. For the *myc* hybridizations, the same Northern blot was successively dehybridized and hybridized with radiolabeled probes of similar specific activities, followed by exposures for similar lengths of time. The transcript size is shown below each gene for both endogenous and introduced genes. The arrows indicate the positions where the *zip* transcript is expected to run. (B) Same as in A, except total RNAs derived from several *c-myc/ras/L-zip* (lanes 16–24) and *c-myc/ras/N-zip* (lanes 25–32) cell lines were assayed.

fectured with equimolar amounts of the various *myc* and *zip* expression constructs. Total RNAs were isolated 48 hr after transfection and assayed for expression of the introduced constructs. In contrast to the stably transfected REF cell lines described above, each transiently transfected *zip* expression construct generated steady-state mRNA levels that were comparable to levels produced by their respective *myc* expression constructs. Moreover, each *zip* construct was found to encode a single prominent transcript species of expected size rather than the irregular transcript forms detected in stably transfected lines. For example, in the N-*zip* and N-*myc* cotransfections, the N-*zip* construct generated a distinct 2.4-kb transcript (Fig. 4, N-*zip* lanes) and yielded levels of expression that were comparable to levels of the 2.5-kb transcript encoded by the N-*myc* construct (Fig. 4, N-*myc* lanes). Similar results were obtained for c-*zip* and L-*zip* expression constructs (data not shown). Transfection efficiencies were found to be similar for each transfection point, as determined by expression levels of a cotransfected human growth hormone gene (data not shown). Overall, the comparable levels of expression and the production of a distinct transcript for all constructs clearly indicate that selection against *zip* gene expression takes place at the level of cellular transformation in the stably transfected lines.

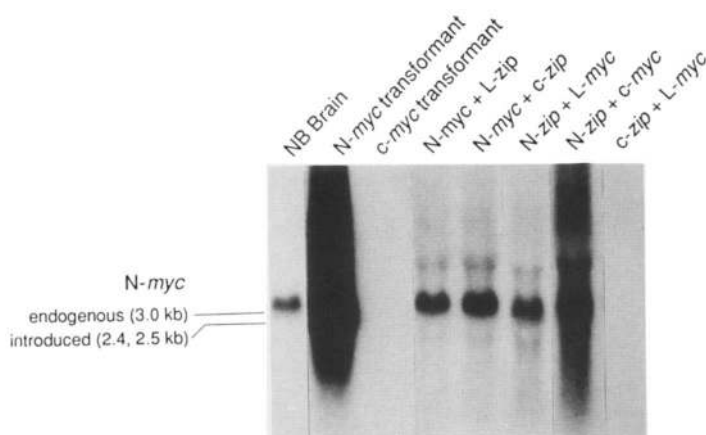
**Intracellular L-Myc/Max heterodimers exist in L-*myc*/ras transformants** The results of the previous experiments demonstrated that L-*myc* and various *zip* expression constructs can diminish c-*myc* and N-*myc* cotransformation activity. This effect may occur through competition for Max or common gene targets, or both. Although heterodimer complex formation between Max and all three Myc proteins has been shown to occur in vitro (Blackwood and Eisenman 1991), intracellular Myc/Max heterodimers have thus far been substantiated only for c-Myc (Blackwood et al. 1992; Kato et al. 1992) and N-Myc (Wenzel et al. 1991) proteins.

We attempted to document similar intracellular interactions between L-Myc and Max to determine whether L-Myc/Max interactions do represent a possible mechanism by which L-Myc or Zip can function as *trans*-dom-

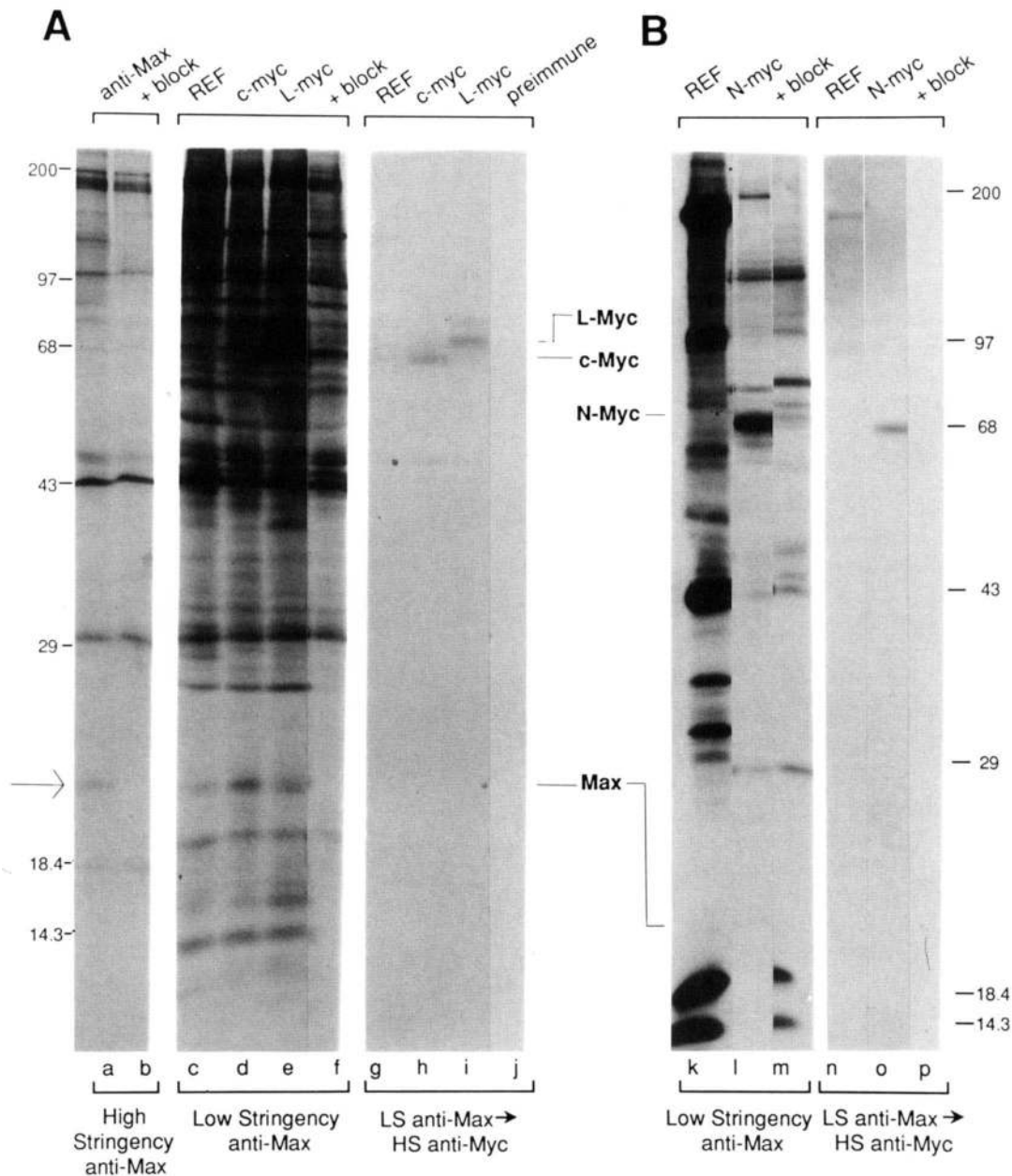
inant repressors. c-*myc*/ras- and L-*myc*/ras-transformed REFs were depleted of intracellular methionine and subsequently metabolically labeled with radioactive [<sup>35</sup>S]methionine. Cell lysates were precipitated under high-stringency conditions with an anti-Max antibody to confirm that REFs expressed Max protein (Fig. 5A, lane a). A 21- to 22-kD band, detected in all REF lines, was verified to be Max protein as it disappeared specifically when an unlabeled glutathione S-transferase (GST)-Max fusion protein was included in the immunoprecipitation (Fig. 5A, lane b). The same antibody was used in immunoprecipitations under low-stringency conditions that permitted Max-associated proteins to remain complexed with Max and be coimmunoprecipitated (Fig. 5A, lanes c-f). The immunoprecipitated fractions from the c-*myc*/ras and L-*myc*/ras transformants appeared to possess unique bands in the size range of 62–64 and 66–68 kD, respectively, which were not detected in nontransformed REFs, or *myc*/ras-transformed REFs when the anti-Max antibody was blocked in the immunoprecipitation (Fig. 5A, lanes d, e, c, and f, respectively). To confirm that these were the c-Myc and L-Myc proteins, proteins immunoprecipitated under low-stringency conditions with anti-Max antisera were dissociated and reimmunoprecipitated under high-stringency conditions with an anti-human-c-Myc antibody that cross-reacts with all members of the Myc family (Fig. 5A, lanes h,i). Because the immunoprecipitated bands migrated at mobilities observed previously for Myc family proteins and were not readily detected in the nontransformed REF control or when preimmune serum was used (Fig. 5A, lanes g,j), we conclude that these are the c-Myc and L-Myc proteins. Similar results were obtained with N-Myc and Max in the N-*myc*/ras transformants (Fig. 5B, lanes k-p). These data confirm that c-Myc/Max and N-Myc/Max heterodimers do form intracellularly and, for the first time, demonstrate that L-Myc and Max associate in vivo.

*Elevated levels of myn can profoundly inhibit the transforming activity of all three myc family genes*

A close biochemical relationship between Myc and Max has been established at the level of direct protein inter-



**Figure 4.** Northern analysis of transiently transfected REF cells. Twenty micrograms of total RNAs derived from REF monolayers transiently transfected with the indicated expression constructs was probed with a radiolabeled probe specific for mouse N-*myc*. The transcript sizes for the endogenous (3.0 kb), introduced (2.5 kb), and mutant (2.4 kb) N-*myc* genes are indicated. Newborn brain RNA serves as a control for the endogenous levels of *myc* normally expressed in a developing tissue. N-*myc* and c-*myc* transformants are stably transfected REF cell lines.



**Figure 5.** Identification of Myc-Max complexes in *myc/tas*-transformed REF cell lines. (A) Identification of c-Myc/Max and L-Myc/Max complexes. [<sup>35</sup>S]Methionine-labeled cell lysates were immunoprecipitated under high-stringency conditions with an anti-human Max antibody to identify the Max protein, which is indicated. The blocked sample included an excess of cognate immunogen to block specific immunoprecipitation (lane b), whereas the anti-Max lane represents an unblocked sample (lane a). To identify Max and Max-associated proteins, [<sup>35</sup>S]methionine-labeled cell lysates from the indicated samples were immunoprecipitated with an anti-Max antibody under low-stringency conditions that preserve Max and associated protein interactions (lanes c-f). Lanes d and e are immunoprecipitated protein from c-Myc- and L-Myc-transformed REFs, respectively; lane f represents one sample in which anti-Max was blocked. Nontransfected REFs express low levels of the endogenous c-Myc protein (lane c). The samples in lanes c-e were dissociated with ionic detergents and reprecipitated under high-stringency (HS) conditions with an anti-Myc antibody to verify the presence of Myc proteins in the Max-associated protein pool (lanes g-i). The preimmune sample was immunoprecipitated with the preimmune serum instead of the anti-Myc antibody (lane j). Sizes of molecular weight markers are indicated at left. The positions of Max and Myc proteins are indicated. (B) Identification of N-Myc/Max complexes. (Lanes k-m) Same as in A, lanes c-f, except low-stringency anti-Max immunoprecipitation was carried out with a N-Myc-transformed REF cell line without or with blocking the antibody (lanes l,m). (Lanes n-p) Same as in A, lanes g-j, except subsequent high-stringency immunoprecipitation with the anti-Myc antibody was carried out with samples in k-m.

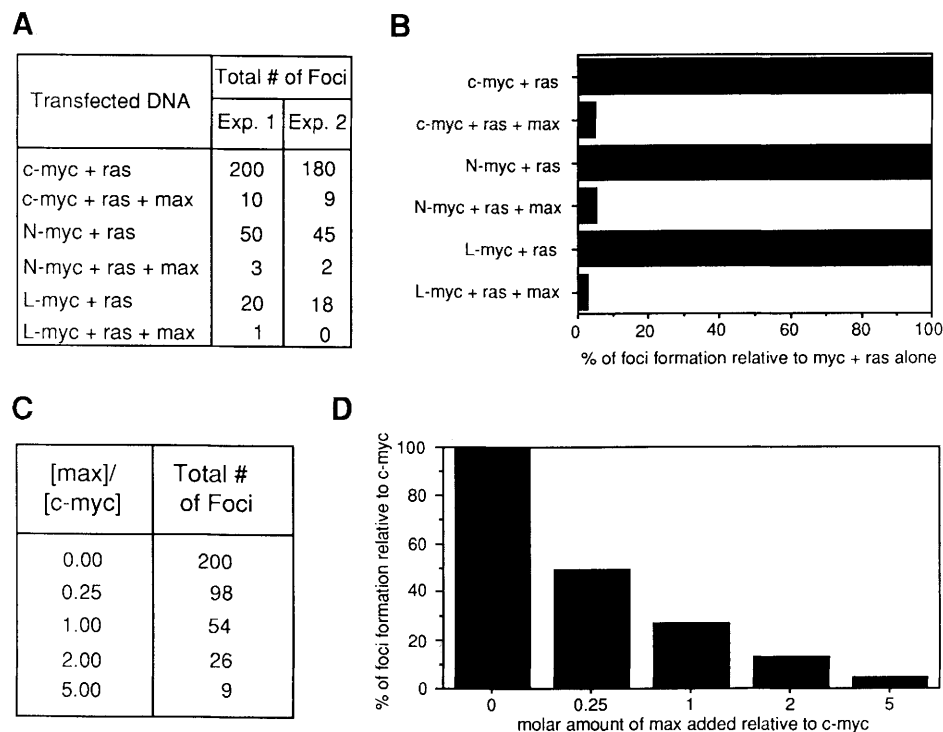
action and common sequence recognition specificity for Myc/Max and Max/Max complexes. Mutations in the HLH domain of c-Myc that inhibit dimerization with Max have been shown to eliminate c-Myc biological activities, including transformation, inhibition of differentiation, and autoregulation (for review, see Lüscher and Eisenman 1990; Penn et al. 1990). To better understand the biological role of Max with respect to c-Myc, N-Myc, and L-Myc cotransformation activity *in vivo*, an expression construct encoding the mouse Max protein was tested for its ability to influence the transforming activity of mouse c-Myc, N-Myc, or L-Myc in the REF cooperation assay. For each point, *c-myc*, *N-myc*, or *L-myc* and the activated *H-ras* expression constructs were cotransfected into early-passage REF alone or along with the *max* expression construct. As shown in Figure 6A, the addition of a 5 molar excess of *max* relative to *myc* was associated with a striking reduction in the number of foci produced for all three *myc* family genes. The *max*-induced suppression resulted in an overall reduction of 95% in *c-myc/ras*-generated foci, 94% for *N-myc/ras*, and 95% for *L-myc/ras* (Fig. 6B). To determine whether the relative molar ratio of transfected *myc* and *max* was an important parameter, a fixed amount of *myc* and *ras*

was cotransfected along with an increasing molar amount of *max*, ranging from a 0 to 5 molar ratio. An increase in the amount of added *max* was found to correlate closely with a progressive reduction in the number of foci generated (Fig. 6C). The reduction appeared to be linear over the range examined (Fig. 6D).

## Discussion

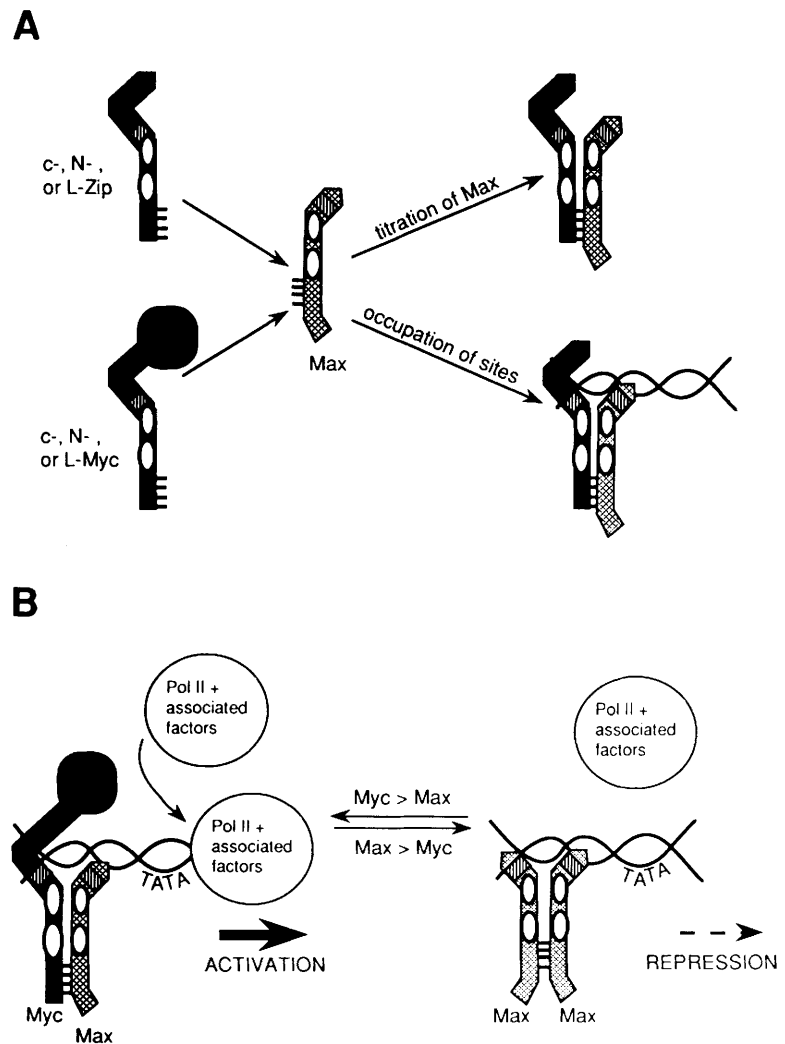
### Cross-interference among the Myc family

In this study we demonstrated that the three Myc mutants (termed c-Zip, N-Zip, and L-Zip), all lacking their putative *trans*-activation domain, significantly impaired the cotransformation activities of wild-type c-Myc and N-Myc, indicating that c-Myc, N-Myc, and L-Myc oncoproteins utilize common genetic elements for neoplastic transformation. As shown schematically in Figure 7A, coexpression of c-Zip, N-Zip, or L-Zip and Myc family proteins presumably leads to a competition for these genetic elements through the formation of Zip/Max complexes, resulting in a titration of Max away from Myc and/or an occupation of Myc/Max-binding sites by the nonfunctional Zip/Max complex. The identical neutral-



**Figure 6.** Transformation of REFs with *c-myc/ras*, *N-myc/ras*, or *L-myc/ras* with or without *max*. (A) See legend to Fig. 2, except that REFs were transfected with *c-myc*, *N-myc*, or *L-myc* with or without *max* and foci were counted 9 days after transfection. (B) Relative foci formation numbers from samples in A were derived by averaging the two experiments and expressing the total number of foci counted in each transfection that included the *max* expression construct as a percentage of the number of foci counted in the corresponding *myc* transfection, that excluded the *max* expression construct, which were each taken to be 100%. (C) Each plate of REFs was transfected with 2  $\mu$ g of *c-myc*, 2  $\mu$ g of *ras*, and the indicated molar amount of *max* DNA relative to *c-myc*, as indicated in the left column. The total number of foci from 12 plates derived from 4 transfected plates was counted 10 days after transfection. (D) Relative foci formation numbers of samples in A were derived by expressing the total number of foci counted in the *max* transfections as a percentage of the total number of foci counted with *c-myc/ras* only transfection, which is taken to be 100%.





**Figure 7.** Proposed models of Zip and Max inhibition of Myc-transforming activity. (A) Zip-induced inhibition of Myc activity may occur through (1) titration of the Max pool by formation of Zip/Max heterodimers, thereby decreasing the formation of Myc/Max heterodimers, and/or (2) occupation of target sequences by *trans*-activation-incompetent Zip/Max complexes. (B) Alteration in the expression of Myc and Max can influence the formation of active vs. inactive complexes. High-level expression of Max protein can lead to the formation of Max/Max homodimers, which may compete with Myc/Max heterodimers for binding a common gene target.

izing activity of each *zip* construct indicates that domains contained within the three Zip proteins are similar, if not identical, in function. These domains include those involved in nuclear localization, nonspecific and sequence-specific DNA binding, and protein oligomerization, all of which may allow for interaction with common genetic elements. We have provided evidence that the L-Myc protein is associated with the endogenous Max protein intracellularly in *L-myc/ras* REF transformants. These observations, taken together with the documented intracellular associations of Max with c-Myc (Blackwood et al. 1992; Kato et al. 1992) and N-Myc proteins (Wenzel et al. 1991), support the hypothesis that interaction with Max represents at least one component common to Myc family function. Although the formation of Zip/Max heterodimers has not been documented in this study, several recent studies have demonstrated clearly that deletions or substitutions in the amino-terminal end of Myc proteins do not affect the dimerization or sequence-specific binding activities of the Myc protein (Blackwood and Eisenman 1991; Berberich and Cole 1992).

The finding that c-Zip, N-Zip, and L-Zip proteins are functionally similar suggests that the differences in oncogenic potential among the Myc family are governed by biological properties encoded within the deleted amino-terminal region and are not the result of factors such as differential affinities for the Max protein or target sequences, among other possibilities. The amino-terminal sequences, deleted in the c-Myc mutant, have been shown to encode *trans*-activation potential (Kato et al. 1990) and appear to be indispensable for cotransformation activity (Stone et al. 1987). Deletion of the equivalent region in N-Myc and L-Myc also rendered them oncogenically inactive. These functional data, coupled with the high degree of amino acid sequence conservation, suggest that the amino-terminal region of N-Myc and L-Myc may also encode *trans*-activation potential. If this is the case, it would be of interest to measure the *trans*-activation potential for each Myc family member and correlate this with oncogenic potency. One would anticipate that the potent c-Myc oncoprotein would possess a superior ability, relative to N-Myc and L-Myc, in *trans*-activating the expression of growth-promoting

gene targets involved in cellular transformation. Support for such a concept awaits clear evidence of transcriptional regulation of specific growth-related genes by the Myc family. Nevertheless, this notion compares with that established experimentally for the structurally (Ralston and Bishop 1983) and functionally (Ralston 1991) related E1A viral oncoproteins, of which the 13S E1A oncoprotein has been shown to be both a potent activator of early viral transcription and a strong transforming agent, and the weakly transforming 12S form possesses minimal *trans*-activation potential.

The dominant interference approach also provided some insight into the relationship between E1a and Myc family function in cellular transformation. The finding that E1a cotransformation activities were for the most part unaffected by dominant-negative mutants of the Myc family clearly indicates that unimpaired Myc function is not an essential requirement for E1a cotransformation activities. This suggests that E1a may function to transform cells through pathways that are independent or downstream of Myc-related activities.

#### *Physiological consequences of coordinate Myc family expression*

We also sought to better understand the physiological significance of the relatively weak biological activity of the L-Myc protein in relation to that of c-Myc and N-Myc. We confirmed that the mouse L-Myc protein was capable of transforming REFs to a limited extent and have demonstrated that this reduced activity is not the result of an inability to interact with Max. We studied the physiological effect of L-Myc on c-Myc or N-Myc in the same cell by transforming REFs with *c-myc* or *N-myc* plus *ras* with or without *L-myc*. L-Myc clearly diminished the transforming potential of c-Myc and N-Myc to a level intermediate of that of c-Myc or N-Myc and L-Myc by themselves. We speculate that this effect results from the titration of Max into a complex of weak transforming ability (L-Myc/Max), which may impact on the oncogenic potency of c-Myc and N-Myc by reducing the chances of forming active c-Myc/Max and N-Myc/Max heterodimers. Alternatively, if Max is not limiting in the cell, the competition for and occupation of a common target sequence in growth control genes by the L-Myc/Max complex may decrease accessibility for c-Myc/Max or N-Myc/Max complexes and consequently reduce the overall level of activation of these common gene targets.

The theory that a normal function of L-Myc may be to regulate the formation of c-Myc/Max complexes or their access to target sequences and thereby modulate c-Myc activity is of particular significance given the observation that *L-myc* can be expressed coordinately with *c-myc* and *N-myc* in many normal developing cells (Mugrauer and Ekblom 1991; K.A. Mahon and R.A. DePinho, unpubl.) Support for this concept derives from recent experiments in transgenic mice showing that forced cell type-specific expression of *L-myc*, *N-zip*, or *L-zip* constructs in differentiating lens fiber cells, which normally

express N-*myc* only, may have induced a phenotype in which expression of specific late-stage differentiation markers has been delayed (S.D. Morgenbesser, B. Mukherjee, and R.A. DePinho, unpubl.). As such, the activity of N-Myc in these cells may have been reduced owing to titration of Max and/or occupation of common gene targets by the introduced L-Myc or Zip proteins.

#### *Role of Max in the regulation of Myc activity*

We were also interested in studying the biological impact of Max on the cotransformation activity of each Myc family member. We demonstrated that addition of a mouse *max* expression construct to the *myc/ras* cotransfections resulted in a striking decrease in the cotransformation activity of all three Myc family proteins. Suppression was found to be linear over a wide range of *max* concentrations. Our biological results are consistent with several recently described biochemical features of the Max protein, and a model depicting Max regulation of Myc activity is presented in Figure 7B. Most notably, Max can form homodimeric complexes that appear to lack *trans*-activation potential but have the capacity to bind the same hexameric sequence (CACGTG) recognized by the Myc/Max heterodimeric complex. Together, these findings are consistent with a model proposed previously (Kato et al. 1992), which states that because Max homodimers occupy the CACGTG sequence as transcriptionally inert complexes, they may deny access by the Myc/Max *trans*-activation complex, thus implicating a role for Max homodimers as negative regulators of Myc activity. It has been theorized that when Myc protein levels are limiting *in vivo*, Max homodimers form and bind in a repressive fashion to genes whose promoters/enhancers bear the CACGTG motif. With the induction of high-level Myc expression during active cellular proliferation, the balance would be shifted toward the formation of Myc/Max heterodimers. On the basis of this model, suppression of Myc cotransformation ability by Max in our experiments could have resulted from the preferential formation of *trans*-activation-incompetent Max homodimers over *trans*-activation-competent Myc/Max heterodimers. Our results clearly support the current view of Myc/Max activity (for review, see Torres et al. 1992) and are in contrast to previous studies in which addition of the same *max* expression construct caused a moderate enhancement in c-Myc cotransformation activity, even with high *max/myc* molar ratios (Prendergast et al. 1991). Although the basis for this difference is not clear, it is possible that differences in *myc* construct design may have achieved a more favorable intracellular ratio of Myc/Max in the previous study, resulting in an overall increase in Myc/Max heterodimers rather than Max homodimers.

Our studies have demonstrated that dominant interference represents a potentially useful strategy for elucidation of the role of *myc* family genes in normal physiology and malignant transformation. Although the effective use of this biological approach will require a more complete understanding of the range of potential dimer-

ization specificities of the Myc protein, dominant-negative mutants provide a means of abrogating Myc activity in a number of experimental systems. For instance, the targeting of dominant mutant proteins to specific cell lineages may prove useful in assessing *myc* family function during normal cell growth and differentiation. Similarly, we would anticipate that the conditional overexpression of Max would yield similar insights. An important and obvious application of this genetic approach would be the use of Zip or Max expression vectors as a therapeutic modality in malignant disease.

## Materials and methods

### *myc* and *zip* expression vectors and their construction

The various *myc* and *ras* plasmids used in the REF transfections are as follows: pT24-*ras* contains the mutant H-*ras* oncogene (*val12*) (Fasano et al. 1983); pKO-*myc* is a *c-myc* expression construct in which transcription of the second and third exons of the mouse *c-myc* gene is driven from an SV40 promoter; pRN2,3 is an N-*myc* expression construct in which transcription of the second and third exons of the mouse N-*myc* gene is driven from the RSV/LTR; and pRL2,3 is an L-*myc* expression construct in which transcription of the second and third exons of the mouse L-*myc* gene is driven from the RSV/LTR.

The various *zip* constructs are as follows: *c-zip* encodes a mutant *c-myc* gene derived from the mouse *c-myc* cDNA, graciously provided by Dr. Ken Marcu (pMc-*myc*54; Stanton et al. 1983); the *XhoI* fragment utilized contains a 414-bp in-frame deletion that removes amino acids 40–178; transcription of *c-zip* is driven from the RSV/LTR, and the coding domain is followed by an 847-bp *BglII*–*BamHI* fragment encoding the SV40 splice and polyadenylation signal (Gorman et al. 1982). N-*zip* consists of an N-*myc* genomic fragment containing the second and third coding regions (DePinho et al. 1986) with a deletion that removed amino acid residues 18–128; the open reading frame was maintained in-frame by a bridging oligomer. L-*zip* encodes a mutant L-Myc protein with a deletion of amino acid residues 23–140. This construct was derived from the mouse L-*myc* cDNA (Legouy et al. 1987). Both N-*zip* and L-*zip* are driven by the RSV/LTR and contain the SV40 polyadenylation signal described in *c-zip*. All *zip* expression constructs are illustrated in Figure 1. CMV*myn*, a *max* expression construct, kindly provided by Ed Ziff (New York University), consists of a full-length cDNA encoding the complete mouse *max* gene driven by the cytomegalovirus (CMV) promoter/enhancer; this construct was described previously (Prendergast et al. 1991). p1A encodes the E1A viral oncoprotein described previously (Prendergast et al. 1991).

### Analysis of RNA and DNA

DNA preparation, restriction endonuclease digestions, probe preparation by the nick-translation method, blotting procedures, and hybridization conditions were performed as described previously (DePinho et al. 1986). Total RNA was isolated by the LiCl/urea method as described previously (Auffray and Rougeon 1980) from P19 embryonal carcinoma cells (McBurney et al. 1982), from mouse newborn brain or from exponentially growing cultures of the various transfected REF cell lines. Twenty micrograms of total RNA was fractionated by electrophoresis on a 1% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized as described (Xu et al. 1991). RNAs were judged to be intact and evenly loaded by ethidium

staining, as well as by hybridization to the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Tso et al. 1985). *myc*-specific probes included (1) *c-myc*, a 780-bp *PstI*–*XhoI* fragment consisting of the coding domain derived from the *c-myc* cDNA plasmid pMc-*myc*54 (Stanton et al. 1983; a generous gift from K. Marcu); (2) L-*myc*, a 480-bp *BamHI*–*HindIII* fragment containing the third exon derived from the genomic clone B1 (Legouy et al. 1987); and (3) N-*myc*, a 635-bp *ClaI*–*HincII* fragment containing the coding portion of third exon derived from the genomic clone pN7.7 (DePinho et al. 1986). To ascertain the relative levels of *myc* versus *zip* gene expression, Northern blots were hybridized with probes of similar specific activities and exposed for the same length of time. In this manner, the various Northern blot hybridizations were found to yield comparable levels of steady-state *myc* family gene expression in the P19 control sample in all experiments.

### Preparation and transfection of REFs

Early-passage cultures of REFs were prepared as described elsewhere (Yancopoulos et al. 1985) from 12- to 14-day Fischer rat embryos. Freshly harvested REFs were initially plated at near-confluent density, allowed to grow to confluency, expanded 1 : 5, and frozen on reaching confluency. To prepare for transfection, REFs were thawed and replated at  $1 \times 10^6$  cells/10-cm plate in Dulbecco's modified Eagle (DME) medium supplemented with 10% fetal bovine serum (FBS). DNAs were introduced into REFs by the calcium phosphate precipitation method as described previously (Andersson et al. 1979) by use of 30  $\mu$ g of mouse liver carrier DNA with 2  $\mu$ g of each of the appropriate plasmid DNAs per plate, except for 10  $\mu$ g in the case of CMV*myn* (mouse *max* expression construct). For the *max* dose response experiments, each plate received 2  $\mu$ g of the *myc* and H-*ras* plasmids along with the indicated amount of CMV-*myn* (0.5, 2, 4, or 10  $\mu$ g). In the E1a cotransformations, 10  $\mu$ g of p1A was used. The cultures were refed 6–9 hr after transfection and split 1 : 3, ~20 hr after the transfection. Foci were counted 9–12 days after transfection. The transient transfection was performed in the same manner as described above, except that 25  $\mu$ g instead of 2  $\mu$ g of the indicated plasmids was added per plate and no genomic DNA carrier was used. At 48 hr, the transiently transfected plates were harvested for total RNA. Controls for cotransfection efficiency included a human growth hormone gene that demonstrated comparable expression levels in all stably and transiently transfected cells (data not shown).

### Assay for colony formation in soft agar

A single cell suspension of  $\sim 1 \times 10^4$  cells in 5 ml of 0.28% agar was overlaid onto a 60-mm culture dish containing a 0.7% agar base. All agar suspensions were made in DME media supplemented with 10% FBS. Duplicate plates were prepared for each line tested.

### Immunoprecipitation

The polyclonal rabbit antisera against human *c-myc* and human *max* have been characterized previously (Blackwood and Eisenman 1991). Metabolic labeling of REF cells and immunoprecipitation under low-stringency conditions to recover Myc/Max heterodimers and high-stringency conditions to recover uncomplexed proteins was essentially as described (Blackwood et al. 1992). All samples were analyzed by SDS-PAGE on 15% polyacrylamide gels under reducing conditions and exposed to autoradiography.

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## **Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and trans-acting dominant mutants.**

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