MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4961–4966
0270-7306/90/094961-06$02.00/0

Vol. 10, No. 9

Domains of Human c-*myc* Protein Required for Autosuppression and Cooperation with *ras* Oncogenes Are Overlapping

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Received 5 February 1990/Accepted 4 June 1990

Amino acids 106 to 143 and 354 to 433 of the human c-*myc* protein (439 amino acids) were shown to be required for the protein to suppress c-*myc* gene transcription and were found to exactly overlap with those necessary for c-*myc* to cooperate with *ras* oncogenes in the transformation of rat embryo fibroblasts. The essential carboxyl-terminal region harbors structural motifs (a basic region, a helix-loop-helix motif, and a "leucine zipper") which, in other proteins, can mediate dimerization and sequence-specific DNA binding.

Recently it has been shown that c-*myc* down regulates the initiation of its own transcription through a putative homeostatic mechanism. The extent of this suppression is proportional to the concentration of c-*myc* protein and is observed within the concentration range of c-*myc* protein found in a normal cell. In fact, it was found that 1,000 to 2,000 molecules must be expressed in Rat-1 cells in order to approach at least half-maximal suppression of endogenous c-*myc* RNA (16). Moreover, suppression of endogenous c-*myc* transcription by v-*myc* (3, 16) and down regulation of c-*myc* expression in response to expression of exogenous N-*myc* genes have been observed (3, 6, 17; L. J. Z. Penn and M. W. Brooks, unpublished data), suggesting that the ability to repress gene expression may be conserved throughout the *myc* gene family.

In order to investigate which regions of the human c-*myc* protein are required for autosuppression, we have mapped the c-*myc* protein for this function by using a subset of the c-*myc* in-frame deletion and insertion mutants first described by Stone et al. (19) (Fig. 1A). The mutants were introduced and expressed in the Rat-1 cell line via the replication-incompetent retroviral vectors pDORneo (Fig. 1B) and pMV6 (19), which contain the neomycin resistance gene (see also Table 1). The respective c-*myc*-derived mutants were transfected into Ψ-2 cells (13), and the resultant helper-free ecotropic virus particles were used to infect early-passage Rat-1 cells. Drug-resistant Rat-1 cell colonies (150 to 250) were subsequently pooled and harvested as subconfluent proliferating cell populations. These cells were analyzed in parallel for the expression of endogenous c-*myc* RNA and exogenous c-*myc* wild-type or mutant protein (for methods, see reference 16).

To determine the capacity of each of the c-*myc* mutants to induce suppression of endogenous c-*myc* RNA expression, RNA was prepared from the Rat-1 cells (16) which had been infected with retroviruses carrying the human c-*myc* gene, an exon II and III-specific c-*myc* cDNA, mutant c-*myc* genes (Table 1), or the neomycin resistance gene alone. The level of endogenous c-*myc* expression was subsequently determined by RNase protection, using probes to detect exon I-specific sequences of endogenous rat c-*myc* RNA as well as rat glyceraldehyde-3-phosphate dehydrogenase RNA (Fig. 2; for details on methods, see reference 16). c-*myc* RNA expression from P2, the major site of transcription initiation in rat cells, is shown in Fig. 2, although c-*myc* RNA transcripts initiating from both start sites in exon I were detectable and were found to be similarly regulated (16; other data not shown). The assays also showed that wild-type (16) or mutant (data not shown) c-*myc* proteins expressed from either the genomic (exons I, II, and III) or cDNA (exons II and III) constructs suppressed endogenous c-*myc* expression with similar efficiency (Table 1).

Most of the human c-*myc* protein mutants remained competent to down regulate the expression of endogenous c-*myc* RNA. However, mutations in either of two regions of human c-*myc* rendered the protein inactive. One region was defined by the inactive deletion mutant D106-143 and the two functional insertion mutants, In 105 and In 144, which flank this critical region on either side. The second section required for autosuppression was localized to the carboxyl end of c-*myc* and included amino acids 354 to 433. All six mutations within this region of c-*myc* resulted in mutant protein which was inactive for *myc* autosuppression activity. The left-hand boundary of this domain was marked by the active deletion mutant D265-353. However, the right-hand boundary is less well defined, since the most distal insertion mutant, In 434, demonstrated only 50% wild-type levels of activity and the effect of mutations between amino acids 435 and 439 at the carboxyl terminus have not been tested.

In conclusion, we consider the regions containing amino acids 106 to 143 and 354 to at least 433 to be essential for human c-*myc* protein to suppress endogenous c-*myc* RNA expression in Rat-1 cells (Fig. 2 and Table 1).

For complete evaluation of the results described above it was critical to determine that the c-*myc* proteins expressed from the retroviral promoter corresponded to the expected mutations and that these proteins were expressed at sufficiently high levels to observe suppression of the endogenous c-*myc* gene. Therefore, the mutant c-*myc* proteins were

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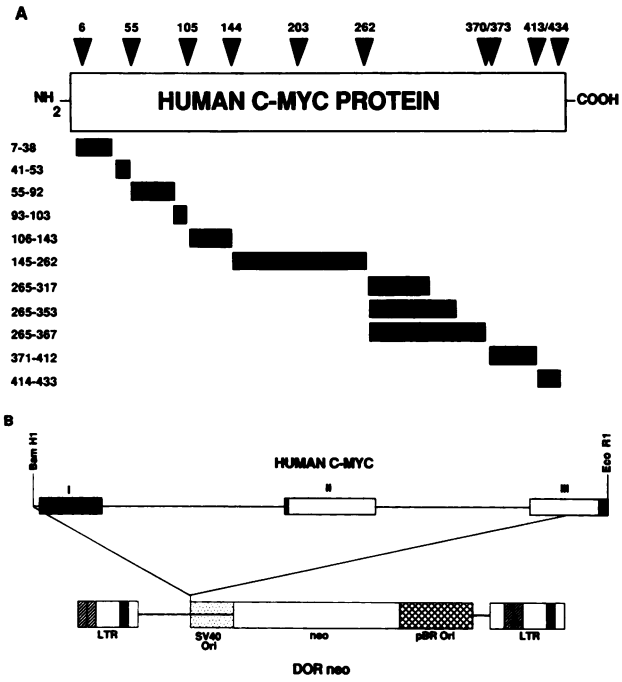

FIG. 1. Schematic representation of human c-*myc* protein mutations and DORneo recombinant retrovirus. (A) The numbers indicate amino acids of the human c-*myc* protein where in-frame deletions (■) or insertions (▼) were generated to create the c-*myc* protein mutants. (B) The human c-*myc* gene was subcloned into the pDORneo retroviral vector. LTR, Moloney murine leukemia virus long terminal repeat; SV40 Ori, simian virus 40 origin of replication; neo, neomycin acetyltransferase gene derived from transposon 5; pBR Ori, pBR322 origin of replication. Map is not to scale. The vector pDORneo is a modified form of pDOL$^-$ (10). It contains an alternative splice donor mutation (AGGT → AGCT) which supports the production of higher virus titer, an additonal *Eco*RI cloning site, a deletion of all envelope coding sequences, and a minimal pBR backbone linked to a U3-deleted 5′ long terminal repeat instead of the polyomavirus backbone. Most of the mutant human c-*myc* genes were of genomic structure (Table 1) and extended from the *Xho*I site between the promoters P1 and P2 in exon I to the *Nsi*I site downstream of the TAA stop codon in exon III. Human c-*myc* mutant-specific DNA fragments (*Bam*HI-*Nsi*I) derived from the respective pM21-based plasmids (19) (*Bam*HI cuts just upstream of the *Xho*I site of c-*myc*) were subcloned into the Bluescript plasmid KS$^+$ (Stretagene) by using the *Bam*HI and *Pst*I restriction sites in the polylinker of this vector. Subsequently, the c-*myc* genes were excised from the Bluescript–c-*myc* constructs with *Bam*HI and *Eco*RI and subcloned into the corresponding restriction sites of the retroviral vector pDORneo.

analyzed by immunoblotting (7, 16) whole-cell extracts from polyclonal Rat-1 cell populations expressing each of the mutant proteins. A rabbit pan-*myc* polyclonal antibody was used (xmyc-1; 8) (Fig. 3). All mutant proteins were detectable and migrated on sodium dodecyl sulfate-polyacrylamide gels according to the expected size for each particular mutation (19). Mutant D41-53 was the only exception, and it was undetectable because it had undergone a deletion of the epitope to which the *myc* antibody used in these experiments was raised. Together with restriction analyses of the corresponding plasmid DNAs (data not shown), these experiments confirmed that the mutant *myc* protein products did indeed correspond to the respective mutations of the c-*myc* gene. In addition, extracts of the different Rat-1 cell populations, each expressing a c-*myc* protein mutant, were analyzed quantitatively by a *myc*-specific enzyme-linked immu-

TABLE 1. Properties of human c-*myc* mutants

Mutant[a]	Presence of:		Exogenous c-*myc* protein expression in[b]:		c-*myc* activity	
	pMV6 cDNA, exons II and III	pDOR gene, exons I, II, and III	Immunoblot	ELISA (molecules/cell)	Autosuppression	Cotransformation[c] (%)
Wild type	+	+	+	5,000	+	100
In 6		+	+	7,050	+	98+/−9
D7-38		+	+	4,000	+	61+/−16
D41-53		+	ND	ND	+	53+/−29
In 55		+	+	4,050	+	74+/−22
D55-92		+	+	2,000	+	26+/−20
D93-103		+	+	5,850	+	27+/−18
In 105	+		+	8,000	+	14+/−8
D106-143	+	+	+	11,000	−	0+/−0
In 144		+	+	3,050	+	57+/−23
D145-262	+	+	+	6,000	+	80+/−35
In 203		+	+	5,500	+	74+/−23
In 262		+	+	6,700	+	58+/−33
D265-317	+		+	3,750	+	54+/−32
D265-353		+	+	2,650	+	10+/−7
D265-367		+	+	8,900	−	0+/−0
In 370		+	+	3,200	−	0+/−0
In 373		+	+	2,750	−	0+/−0
D371-412	+	+	+	7,650	−	0+/−0
In 413	+	+	+	3,550	−	1+/−1
D414-433	+	+	+	8,050	−	0+/−0
In 434		+	+	3,000	+/−	54+/−16

[a] D, In-frame deletion; In, in-frame linker insertion.
[b] ND, Not determined because of deletion of epitope to which *myc* peptide antibody was raised; ELISA, enzyme-linked immunosorbence assay.
[c] See reference 19.

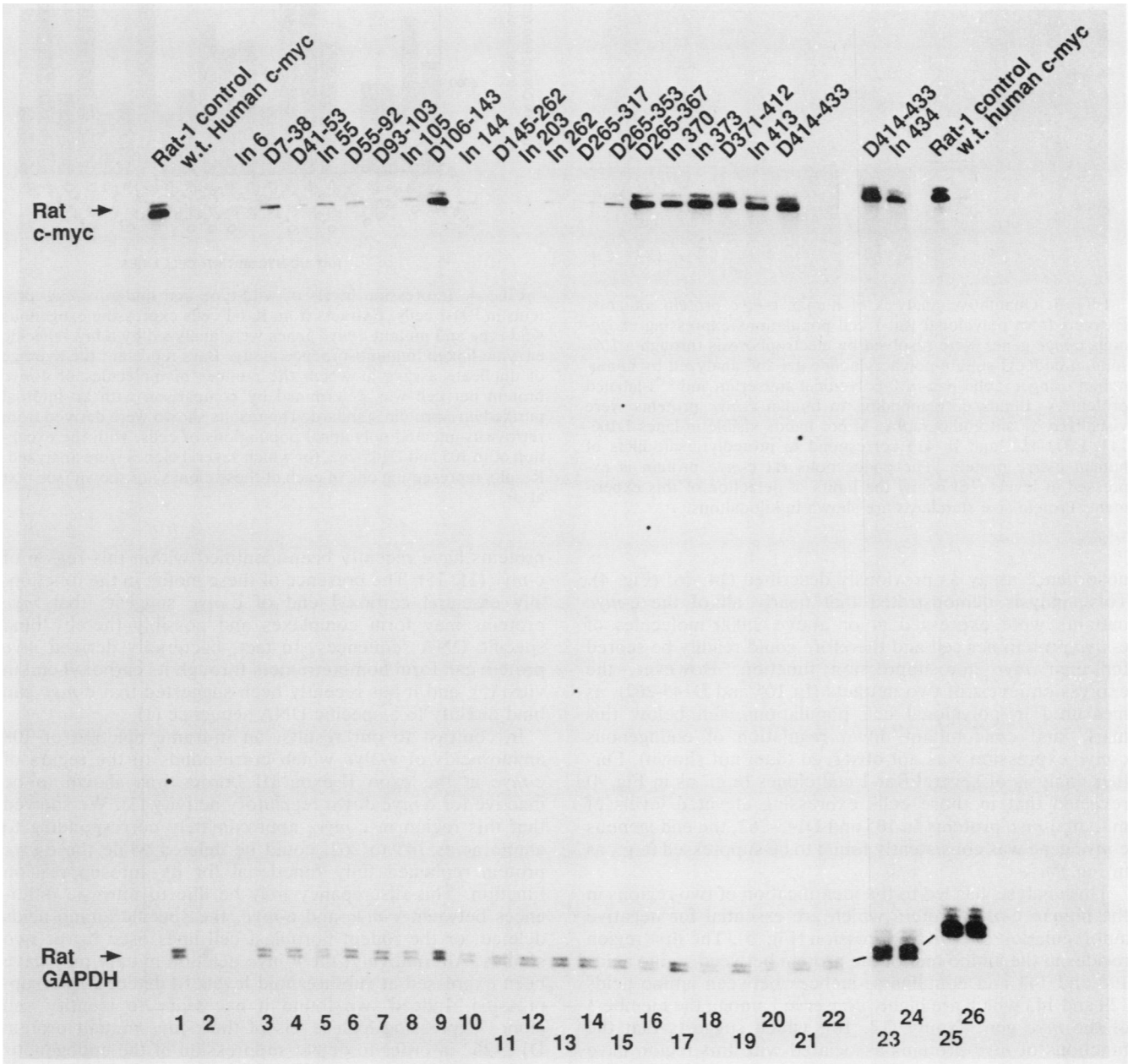

FIG. 2. Expression of endogenous rat c-*myc* RNA in response to the expression of wild-type (w.t.) and mutant human c-*myc* genes. RNAs (10 μg) from Rat-1 cells infected with control retrovirus and with retrovirus carrying wild-type or mutant human c-*myc* genes were analyzed by RNase protection using single-stranded RNA probes complementary to endogenous rat c-*myc* exon I and glyceraldehyde-3-phosphate dehydrogenase (GADPH)-specific sequences. c-*myc* RNA expression from P2, the major site of transcription initiation in rat cells, is shown. Expression of exogenous human c-*myc* RNA was not detectable under the assay conditions. The RNA analyzed was prepared from polyclonal Rat-1 cell populations, with the exception of the cells expressing mutants In 105 and D145-262, from which RNAs isolated from representative clones are shown (see text also). Following electrophoresis through a 6% denaturing polyacrylamide gel, the protected probe was visualized by autoradiography. Each of the mutant c-*myc* constructs was assayed in at least three independent experiments and consistently yielded identical results.

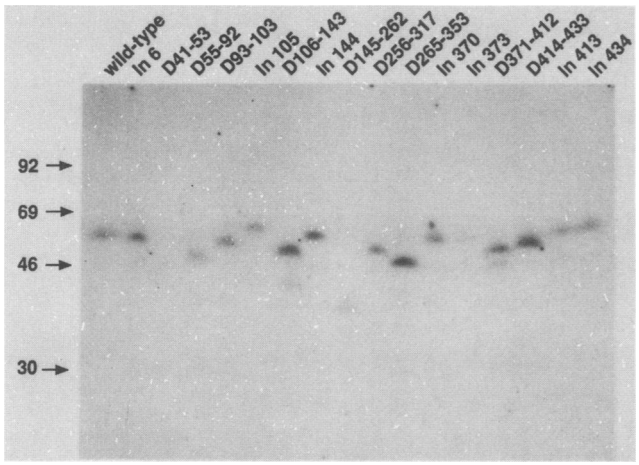

FIG. 3. Qualitative analysis of human c-*myc* protein mutants. Extracts from polyclonal Rat-1 cell populations expressing exogenous c-*myc* genes were resolved by electrophoresis through a 10% sodium dodecyl sulfate-polyacrylamide gel and analyzed by immunoblot using a rabbit pan-*myc* polyclonal antiserum and ^{125}I-labeled protein A. Bands corresponding to human c-*myc* proteins were visualized by autoradiography. Minor bands visible in lanes D106-143, D371-412, and In 413 correspond to proteolytic products of human c-*myc* protein. The endogenous rat c-*myc* protein is expressed at levels (16) below the limits of detection of this experiment. Protein size standards are shown in kilodaltons.

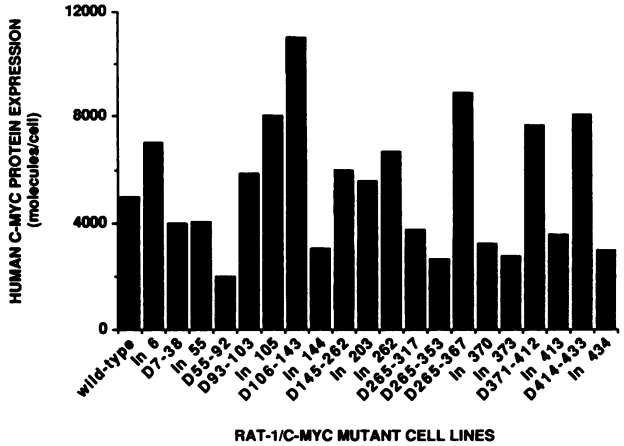

FIG. 4. Expression levels of wild-type and mutant c-*myc* proteins in Rat-1 cells. Extracts from Rat-1 cells expressing exogenous wild-type and mutant c-*myc* genes were analyzed by a *myc*-specific enzyme-linked immunosorbence assay. Bars represent the average of duplicate assays in which the number of molecules of c-*myc* protein per cell was determined by comparison with an internal purified *myc* protein standard. The results shown were derived from retrovirus-infected polyclonal populations of cells, with the exception of In 105 and D145-262, for which several clones were analyzed. Results representing one of each of these clones are shown (see text also).

nosorbence assay as previously described (14, 16) (Fig. 4). This analysis demonstrated that nearly all of the c-*myc* mutants were expressed at or above 2,000 molecules of c-*myc* protein per cell and therefore could readily be scored for their *myc* autosuppression function. However, the expression levels of two mutants (In 105 and D145-262), as measured in polyclonal cell populations, fell below this mark, and concomitant down regulation of endogenous c-*myc* expression was not observed (data not shown). Further analysis of several Rat-1 cell clones (e.g., as in Fig. 4) revealed that in those cells expressing elevated levels of mutant c-*myc* proteins In 105 and D145-262, the endogenous c-*myc* gene was consistently found to be suppressed (e.g., as in Fig. 2).

This analysis has led to the identification of two regions in the human c-*myc* protein which are essential for negative autoregulation of c-*myc* expression (Fig. 5). The first region resides in the amino half of the protein between amino acids 106 and 143 and contains sequences between amino acids 129 and 143 which are highly conserved among the members of the *myc* gene family (12, 18), which suggests that the functions of *myc* proteins associated with this region have been conserved throughout evolution. Database searches with the peptide sequence of this region did not detect significant homology with any defined structural polypeptide motif or known protein outside the *myc* family (data not shown).

The second region involved in c-*myc* autosuppression is also conserved within the *myc* gene family. It is located in the carboxy-terminal region of the c-*myc* protein between amino acids 354 and 433 and contains one of the two nuclear localization signals identified in the human c-*myc* protein (residues 364 to 374) (4). In addition, three motifs, a basic region (residues 355 to 369), a helix-loop-helix structure (residues 370 to 406), and a "leucine zipper" (residues 406 to 439 at the carboxyl terminus) associated with DNA-binding proteins have recently been identified within this region of c-*myc* (11, 15). The presence of these motifs in the functionally essential carboxyl end of c-*myc* suggests that *myc* proteins may form complexes and possibly thereby bind specific DNA sequences. In fact, bacterially derived *myc* protein can form homotetramers through its carboxyl end in vitro (5), and it has recently been suggested that c-*myc* can bind directly to a specific DNA sequence (1).

In contrast to our results, an in-frame deletion of 108 amino acids of v-*myc* which corresponds to the region of c-*myc* at the exon II-exon III border was shown to be inactive for c-*myc* down regulatory activity (3). We showed that this region in c-*myc*, approximately corresponding to amino acids 145 to 262, could be deleted while the c-*myc* protein remained fully functional for its autosuppression function. This discrepancy may be due to intrinsic differences between v-*myc* and c-*myc*, the specific amino acids deleted, or the rodent fibroblast cell lines used in the two studies. Alternatively, the v-*myc* deletion mutant may have been expressed at subthreshold levels to detect c-*myc* suppression. Indeed, we found it necessary to identify cell clones expressing high levels of the c-*myc* mutant protein D145-262 in order to detect suppression of the endogenous rat c-*myc* gene.

Regions other than amino acids 106 to 143 and 354 to 439 of the c-*myc* gene are dispensable for the *myc* autosuppression function. Nevertheless, some of these regions, such as amino acids 45 to 68 of c-*myc*, are very highly conserved among the *myc* gene family, which suggests that this domain may be important for an as yet unknown function of the *myc* protein (Fig. 5). Similarly, the highly conserved acidic region of the *myc* proteins (Fig. 5) was shown in v-*myc* to be required for chicken hematopoietic cell transformation but unnecessary for the transformation of chicken embryo fibroblasts (2, 9). These results suggest that *myc* activities can be cell-type dependent or may rely on additional and possibly tissue-specific cellular factors.

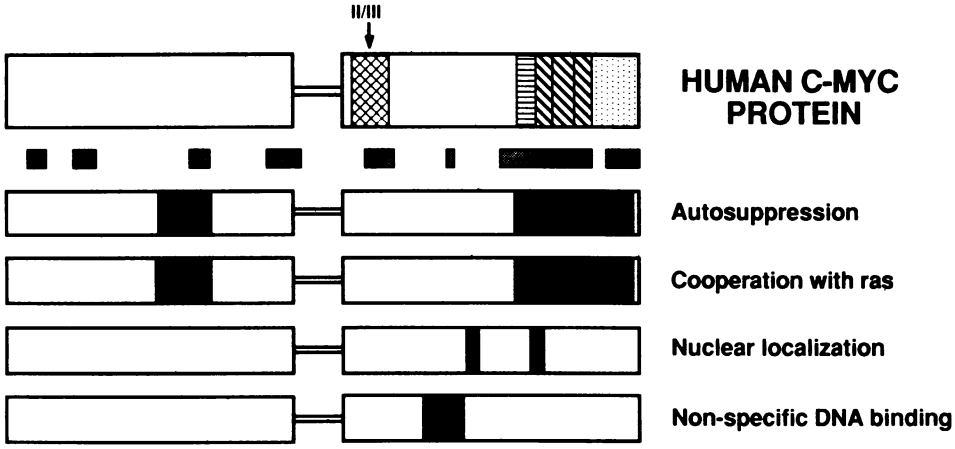

FIG. 5. Structural and functional domains of human c-*myc* protein. Secondary-structure predictions based on amino acid sequence information suggest that the 439-amino-acid nuclear phosphoprotein may be composed of an α-helix–β-sheet domain (amino acids 1 to 203) and a predominantly α-helical domain at the carboxyl end (amino acids 238 to 439) which are separated by a less-structured hinge region (amino acids 204 to 237). Additional structural motifs include a highly acidic domain ( ), a basic region ( ), a helix-loop-helix domain ( ), and a leucine zipper ( ). Regions most highly conserved among the members of the *myc* gene family ( ) are also indicated. The functionally essential domains of c-*myc* identified to date are represented by the solid black boxes in the specified schematic diagrams and map as follows: autosuppression, residues 106 to 143 and 353 to 433; cooperation with *ras* oncogenes in rat embryo fibroblasts, residues 106 to 143 and 353 to 433; nuclear localization, residues 320 to 328 and 364 to 374; and nonspecific DNA binding, residues 290 to 318. II/III, Border of exon II- and exon III-encoded sequences.

It is intriguing that the functional domains of c-*myc* required for autosuppression overlap with those essential for c-Ha-*ras* cotransformation of secondary rat embryo fibroblasts (19). Indeed, the results presented here indicate a correlation between the transforming function of c-*myc* and its ability to suppress gene transcription. Both these activities are completely lost in the mutants affecting amino acids 106 to 143 or 354 to 433 (Fig. 5). Therefore, it seems possible that cellular transformation by c-*myc* involves the repression of cellular genes which act to restrict cell proliferation.

We thank Shaun Dawkins for technical assistance.

L.J.Z.P. was supported by a postdoctoral fellowship from the Medical Research Council of Canada.

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