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The Leucine Zipper of c-Myc Is Required for Full Inhibition of Erythroleukemia Differentiation

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The leucine zipper motif has been observed in a number of proteins thought to function as eucaryotic transcription factors. Mutation of the leucine zipper interferes with protein dimerization and DNA binding. We examined the effect of point mutations in the leucine zipper of c-Myc on its ability to dimerize in vitro and to inhibit Friend murine erythroleukemia (F-MEL) differentiation. Glutaraldehyde cross-linking studies failed to provide evidence for homodimerization of in vitro-synthesized c-Myc protein, although it was readily demonstrated for c-Jun. Nevertheless, whereas transfected wild-type c-*myc* sequences strongly inhibited F-MEL differentiation, those with single or multiple mutations in the leucine zipper were only partially effective in this regard. Since the leucine zipper domain of c-Myc is essential for its cooperative effect in *ras* oncogene-mediated transformation, this study emphasizes the close relationship that exists between transformation and hematopoietic commitment and differentiation. c-Myc may produce its effects on F-MEL differentiation through leucine zipper-mediated heterodimeric associations rather than homodimeric ones.

Several functions have been attributed to the c-*myc* proto-oncogene. For example, its enforced expression from an inducible promoter in mitotically quiescent cells partially mimics some of the features of growth factor stimulation (3). High levels of exogenous c-*myc* potentiate the effects of several types of growth factors (33). c-*myc* can cooperate with c-*ras* to transform some types of primary cells in vitro (19) and in certain instances can act alone to produce such changes (15). Finally, alterations in the levels of c-*myc* in the Friend erythroleukemia and HL-60 promyelocytic leukemia cell lines exert profound effects on differentiation (8, 10, 14, 18, 29, 40).

Whereas the biological effects imparted by c-*myc* are manifold, the mechanisms through which these effects occur are less well understood. Although it is widely believed that c-*myc* is a transcriptional activator, the evidence to support this notion, while compelling, is largely indirect and, in some cases, incompatible with this notion (27). Although v-Myc and c-Myc proteins are capable of binding both single-stranded and double-stranded DNA (1, 2, 6), no specific target sequence has been identified as in the case of other nuclear proto-oncogene products such as c-Fos, c-Jun, and c-Myb (2, 4, 5). When fused at its N terminus to the DNA-binding region of the LexA repressor in yeast cells, c-Myc can activate transcription of genes spaced downstream of the LexA operator (21). However, this effect is much less pronounced than with c-Fos, and its relevance to transcriptional activation in higher cells is uncertain.

c-Myc is also one of a number of proteins that contain a so-called leucine zipper (20). This structural motif consists of a C-terminal region of high alpha-helical content with four to five leucine residues evenly spaced at seven-amino-acid intervals. When displayed on an idealized helix wheel, the hydrophobic leucine side chains project from the alpha helix in a common plane. It was originally predicted that this would allow for the interdigitation of the zipper regions of opposing molecules and provide a means of dimerization (20). Point mutagenesis of the leucine zipper of Fos and Jun family proteins has borne out this prediction and established the importance of the dimer structure in DNA binding (12, 13, 16, 24, 31, 32).

A functional role for the c-Myc leucine zipper has been suggested by Dang et al., who demonstrated that C-terminal deletions of c-Myc, which abolished the leucine zipper domain, also eliminated cooperativity with the EJ *ras* oncogene in the transformation of primary rat embryo fibroblasts (9). They also showed that bacterially expressed c-Myc protein could form homodimers as well as higher-order oligomers.

In this report, we have addressed the role of the c-Myc leucine zipper in the inhibition of Friend murine erythroleukemia (F-MEL) differentiation. In our initial studies, we attempted to duplicate the findings of Dang et al. by showing that in vitro-translated c-Myc protein could form homodimers. Despite the ease with which this was accomplished for c-Jun protein, we were unsuccessful in demonstrating it in the case of c-Myc. Nevertheless, when expression plasmids containing point mutations in the c-Myc leucine zipper were introduced into F-MEL cells, all mutations showed a severe impairment in their ability to inhibit dimethyl sulfoxide (DMSO)-induced differentiation. These reports suggest that any leucine zipper-mediated interactions of c-Myc which do occur are probably hetrodimeric in nature.

MATERIALS AND METHODS

Cell culture and transfections. A subclone of the original F-MEL 745 line was used in all experiments. In the absence of chemical agents that induce erythroid differentiation, <1% of these cells routinely stained positive with the benzidine reagent (28). After a 5-day exposure to 1.5% DMSO, however, >70% of the cells became benzidine positive. The cells were grown as previously described in Dulbecco modified Eagle minimal essential medium containing 10% supplemented calf serum (Hyclone, Logan, Utah), 2 mM glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml in a humidified 5% CO_2 atmosphere

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(28). Care was taken to maintain cells at a sufficiently low density (generally $<5 \times 10^5$/ml) to ensure logarithmic growth at all times. Transfected cells were maintained under identical conditions except that the culture medium contained 10% dialyzed supplemented calf serum and 0.25 μM methotrexate (MTX) (28, 29). Benzidine staining was performed as previously described (28, 29). Transfections were performed by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) with settings of 1.0 kV and 25 μF and a resultant time constant of 0.5 to 0.6 ms. A total of 2×10^7 cells in 0.5 ml of phosphate-buffered saline were subjected to transfection, using 20 μg of linearized c-*myc* expression vector plasmid DNA and 2 μg of linearized pSV_2neo DNA (34). Successfully transfected clones were selected in medium containing G418 (0.48 mg/ml, active concentration; GIBCO Laboratories, Grand Island, N.Y.). Pooled G418-resistant clones were then subsequently selected in medium containing 0.25 μM MTX and maintained under these conditions. Individual clones were obtained by limited dilution of the MTX-resistant cell population in 96-well microtiter plates.

Mutagenesis of murine c-*myc* cDNA. The starting template in all cases was single-stranded M13mp18 bacteriophage DNA containing a 2.0-kilobase *Hin*dIII fragment or murine c-*myc* cDNA (35). The single-stranded DNA was prepared from phage grown in *Escherichia coli* CJ236 (*dut ung thi relA*) as described by Kunkel (17), using the Bio-Rad Muta-Gene in vitro mutagenesis kit. Specific 20-nucleotide (nt)-long oligomers containing single nucleotide mismatches to each of the leucine zipper codons were used to generate point mutations. The identity of each mutant was confirmed by direct DNA sequencing, using a primer complementary to a region of cDNA just upstream from that encoding amino acid 410. A quadruple mutant containing the substitutions Leu-413→Pro, Leu-420→Trp, Leu-427→Ser, and Leu-434→Pro was obtained, as were three of the four individual point mutations (see Fig. 2). Leu-434→Pro could not be obtained as a single mutation because of persistent rearrangement and deletion of adjacent sequences. Double-stranded phage DNAs were digested with *Xho*I to excise the 1.4-kilobase c-*myc* coding region. The fragment was then cloned into the *Xho*I-digested pSV_2myc-dhfr expression vector (28) from which wild-type c-*myc* coding sequences had been removed.

Preparation and characterization of anti-c-*myc* antibody. A 1.16-kilobase *Pst*I c-*myc* cDNA fragment encoding the C-terminal 260 amino acids of the murine c-Myc protein was cloned into the procaryotic expression vector pATH23 and expressed as a TrpE–c-Myc fusion protein in *E. coli* DH5-α. Insoluble material from lysed indoleacrylic acid-induced cells was solubilized with sodium dodecyl sulfate (SDS) and dithiothreitol and electrophoresed through a 7.5% linear polyacrylamide gel. c-Myc fusion protein constituted approximately 50% of the total protein in such preparations. The TrpE-Myc fusion protein band was excised from the gels after visualization with fluorescamine (Sigma Chemical Co., St. Louis, Mo.) solubilized in complete Freund adjuvant and used for immunization of rabbits. The production of specific antibody was monitored by immunoprecipitation of ^{35}S-labeled c-Myc protein from rabbit reticulocyte lysates containing SP64 in vitro-synthesized c-*myc* RNA (Promega Biotec, Madison, Wis.). Affinity purification of the anti-c-*myc* antibody was accomplished by passing the immunoglobulin G fraction of immune rabbit serum twice through a CNBr-linked TrpE–c-Myc fusion protein-Sepharose column. TrpE–c-Myc fusion protein competed completely for authentic c-Myc protein in Western immunoblot assays.

Detection of c-Myc protein in F-MEL cells. F-MEL cells were either untreated or treated with 1.8% DMSO for 5 h. Cells were pelleted by low-speed centrifugation, washed three times in phosphate-buffered saline, and lysed in standard RIPA buffer containing 100 μg of phenylmethysulfonyl fluoride per ml and 1 μg each of aprotinin, leupeptin, and pepstatin per ml. Approximately 100 μg of total protein per ml was electrophoresed through a 7.5% polyacrylamide-SDS gel and then transferred to nitrocellulose by electroblotting. The filter was then blocked for 4 to 6 h in a 5% suspension of nonfat dried milk prepared in TBS buffer (0.15 M; 20 mM Tris hydrochloride [pH 7.6]). Undiluted antibody was then added to a final dilution of 1:1,000, and incubation was continued for 2 h at 25°C. After extensive washing of the blot in TBS plus 0.5% Tween 20, bound anti-c-*myc* antibody was detected with biotinylated goat anti-rabbit antibody, followed by immunodetections with an avidin-alkaline phosphatase-based detection kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Glutaraldehyde cross-linking studies. Full-length murine c-*jun* cDNA was a kind gift from R. Bravo (EMBL Laboratories, Heidelberg, Federal Republic of Germany). The cDNA and full-length c-*myc* cDNA were cloned into SP6 expression vectors, transcribed in vitro, and capped, using a Promega mRNA 5′ capping kit according to the directions of the supplier. After DNase digestion and phenol-chloroform extraction, 1 μg of each RNA was translated in vitro in 50 μl of rabbit reticulocyte lysate containing 50 μCi of [^{35}S] methionine (specific activity, approximately 1,200 μCi/mmol; Amersham Corp., Arlington Heights, Ill.). Cross-linking was carried out essentially as described by Nakabeppu et al. (24) for varying periods of time and was terminated by the addition of glycine to 100 mM final concentration. After dialysis against 1 liter of 10 mM potassium phosphate (pH 8.0) overnight, total in vitro-synthesized proteins were resolved on 7.5% SDS-polyacrylamide gels, fixed, treated with En^{3}Hance (Amersham), and subjected to autoradiography at −80°C for 24 to 72 h, using a single intensifying screen.

S1 nuclease protection assays. Total RNA was extracted from F-MEL cells by the guanidine hydrochloride method. A 10-μg sample of each RNA was hybridized overnight with 10^5 dpm of S1 probe. The probe used was a 940-nt-long *Nde*I-*Xho*I fragment from the pSV_2myc-dhfr vector, end labeled at the *Xho*I site (28). The conditions for hybridization and S1 nuclease digestion were as previously described (6, 28, 29). Reaction products were resolved in 2% agarose gels, which were subsequently dried and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.), using a single intensifying screen.

RESULTS

Absence of c-Myc dimerization. The leucine zipper motif has been shown to be important for the dimerization of a number of eucaryotic transcription factors (12, 13, 16, 24, 31, 32). Furthermore, Dang et al. have previously shown that bacterially expressed c-Myc protein can form dimers and higher-order oligomers (9). We therefore first asked whether it was possible to demonstrate c-Myc dimerization by using in vitro-translated protein. A full-length murine c-*myc* cDNA was transcribed in vitro, using SP6 polymerase. As a positive control, we also expressed a full-length murine c-*jun* cDNA. Equal amounts of each capped RNA were translated

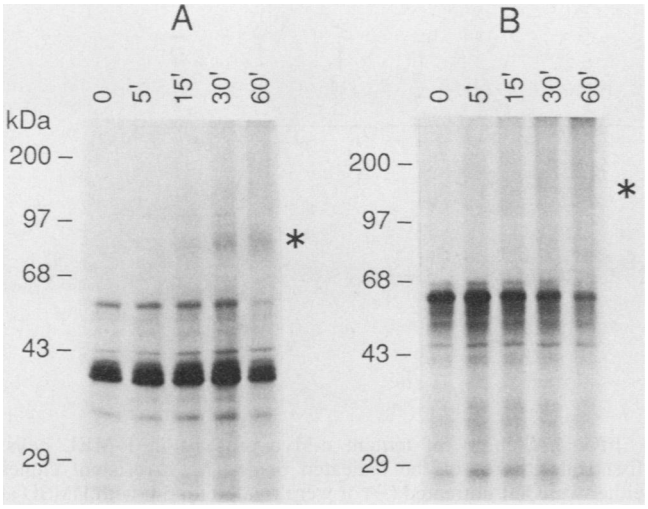

FIG. 1. Glutaraldehyde cross-linking of in vitro-synthesized c-Jun (A) and c-Myc (B) proteins. Approximately 1 μg of each in vitro-transcribed capped RNA was translated in 50 μl of a rabbit reticulocyte lysate containing 50 μCi of [^{35}S]methionine. Glutaraldehyde cross-linking was performed as described by Nakabeppu et al. (24) for the times indicated (in minutes). Total in vitro-synthesized proteins were resolved on linear 7.5% polyacrylamide-SDS gels, fixed, dried, and autoradiographed. Asterisks indicate the expected positions for dimerized c-Jun (ca. 80 kDa) and dimerized c-Myc (ca. 130 kDa).

in separate rabbit reticulocyte lysates. The products were then exposed to 1/10 volume of 0.1% glutaraldehyde for varying periods of time before terminating the reactions with glycine. After dialysis, equivalent numbers of trichloroacetic acid-precipitable counts were electrophoresed through 7.5% polyacrylamide-SDS gels, which were then processed for autoradiography. As seen in Fig. 1A and as previously reported (12, 13, 16, 24, 31, 32), in vitro-translated c-Jun protein migrated as two closely spaced bands of approximately 40 kilodaltons (kDa). With increasing lengths of exposure to glutaraldehyde, a new band of approximately 80 kDa in size was observed. That this new band corresponded to c-Jun dimers was demonstrated by showing that it, along with the two 40-kDa bands, could be precipitated with an affinity-purified anti-c-*jun* polyclonal antibody (not shown). These results confirmed those of many other laboratories and indicated that our procedure was capable of demonstrating leucine zipper-mediated dimerization.

When in vitro-translated c-Myc protein was examined in parallel after an identical exposure to glutaraldehyde, no cross-linking was observed (Fig. 1B). Higher concentrations of glutaraldehyde or longer periods of treatment also failed to reveal any evidence for oligomerization, as did gross overexposures of autoradiograms (not shown). Thus, at least under conditions that allow for dimerization of other leucine zipper-containing proteins, we were unable to obtain any evidence for such interactions of c-Myc.

Characterization of c-*myc* mutations. Despite the lack of evidence for c-Myc dimerization, we were nevertheless interested in determining whether the leucine zipper domain was important for the full biological activity of c-Myc. We investigated this by introducing point mutations into the leucine zipper region. Figure 2 shows the mutations that we introduced into a murine c-*myc* cDNA (35) by using oligonucleotide-directed mutagenesis. Individual mutants with mutations at positions 413, 420, and 427 were obtained, as was the quadruple mutant that included the Leu-434 residue. The single mutation of Leu-434 was not obtained because of a persistent rearrangement and deletion of the adjacent sequence.

Each mutant cDNA was excised from its respective phage vector with *Xho*I and used to replace the wild-type murine c-*myc* sequence in the plasmid vector pSV$_2$myc-dhfr (28). As controls, we used pSV$_2$myc-dhfr containing wild-type c-*myc* sequence (28) as well as the parental pSV$_2$dhfr vector lacking c-*myc* sequences. Linearized plasmid DNAs were coelectroporated along with pSV$_2$neo into F-MEL cells, selected sequentially in G418 and MTX, and then cloned by limited dilution in 96-well microtiter plates. Each clone was subsequently expanded so as to allow for benzidine staining in response to DMSO and, in selected cases, for molecular analysis.

We first determined how efficiently exogenous c-*myc* sequences were incorporated and at what levels they expressed. We randomly selected several individual clones from each group, purified total RNAs, and used an S1 nuclease protection assay to simultaneously detect endogenous and transfected mRNA transcripts (28). We have previously established that the sequential selection method we use ensures that virtually every clone will express transfected sequences, usually at high levels (6, 28, 29). Every clone examined contained high levels of exogenous c-*myc* mRNA (Fig. 3). As previously reported, expression of the transfected c-*myc* sequences had no effect on endogenous c-*myc* transcript levels (8, 10, 18, 28). We thus conclude that virtually every clone expresses high levels of transfected c-*myc* sequences.

We next asked whether the introduction of point mutations into the c-Myc protein might affect its level of expression. We and others have observed that changes in endogenous c-Myc protein levels in differentiating F-MEL cells parallel changes in transcript levels (34a; our unpublished observations). To confirm this, we studied a pooled, uncloned population of MTX-resistant F-MEL cells that had been transfected with the pSV$_2$dhfr vector alone. Cells were either untreated or treated with 1.8% DMSO for 5 h. We then examined cell lysates for c-Myc protein by Western blotting,

FIG. 2. Point mutations of the murine c-Myc leucine zipper region. The C-terminal 30 amino acids are shown, with the leucine moieties thought to be involved in the leucine zipper interaction depicted in bold letters. Point mutations designed to substitute the amino acids shown above the arrows were introduced by using specific 20-base oligonucleotide primers. Single mutants corresponding to Leu-413→Pro, Leu-420→Trp, and Leu-427→Ser, as well as the quadruple mutant containing all four substitutions, were obtained. The fragment was then cloned into the vector pSV$_2$myc-dhfr (28) from which the wild-type c-*myc* sequences had been removed.

using a polyclonal, affinity-purified anti-c-*myc* antibody. DMSO treatment resulted in substantial reduction in the amount of c-Myc protein, consistent with the finding of low or undetectable levels of c-*myc* mRNA at this time (28) (Fig. 4). We then examined a pooled population of MTX-resistant clones that had been transfected with the pSV_2dhfr expression vector containing unmutated, wild-type c-*myc* sequences. Treatment of these cells for 5 h with DMSO did not alter the level of c-Myc protein. Since the levels of c-*myc* transcripts originating from the pSV_2dhfr vector did not change in response to DMSO, the simplest interpretation of our results is that the c-Myc protein in the DMSO-treated sample originated from translation of vector-derived c-*myc* transcripts.

We then examined the levels of c-Myc protein in pooled populations of clones that had been transfected with each of the four leucine zipper mutants. As was the case for cells transfected with wild-type sequences, DMSO treatment did not affect c-Myc protein levels. From these as well as our previous results, we conclude that the persistence of plasmid-derived c-*myc* transcripts after DMSO treatment is accompanied by a concurrent persistence in protein levels as well and that the introduction of point mutations into the leucine zipper region does not affect the overall expression of the c-Myc protein.

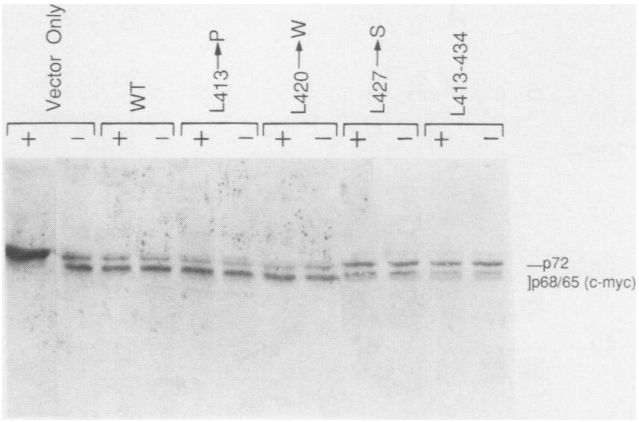

FIG. 4. Stability of mutant c-Myc proteins in F-MEL cells. Identical cultures of the indicated pooled MTX-resistant clones either were left untreated (−) or were treated for 5 h with DMSO at a final concentration of 1.8% (+). After extensive washing, the cells were lysed in RIPA buffer and particulate matter was removed by centrifugation. Then 40 μg of total protein was electrophoresed through 7.5% polyacrylamide-SDS gels, transferred to nitrocellulose by electroblotting, and treated with a polyclonal rabbit anti-*trpE*–c-*myc* antibody. Specifically reacting bands were detected with a secondary goat anti-rabbit immunoglobulin G coupled with an alkaline phosphatase-based detection system. In all cases, a protein cross-reactive with c-Myc but of slightly greater molecular size (ca. 70 kDa) was observed (p72). That this was not c-Myc was evidenced not only by its larger size but by its persistence after DMSO treatment or density arrest and by its long half-life (>4 h) compared with authentic c-Myc protein (<30 min). WT, wild type; P, proline; W, tryptophan; S, serine.

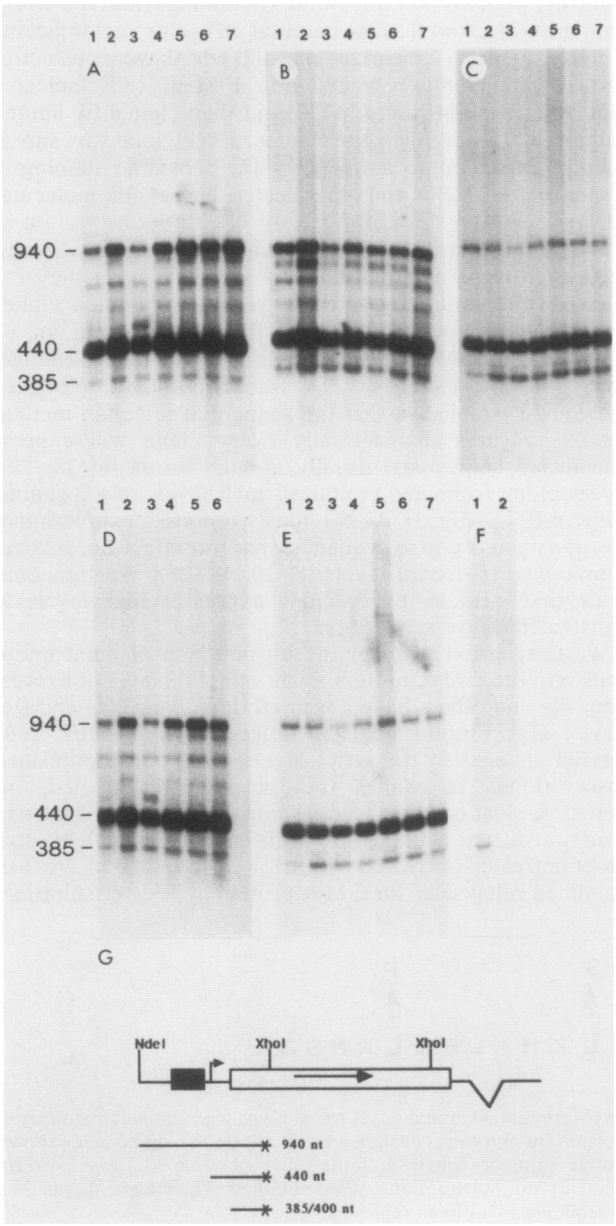

FIG. 3. Expression of transfected c-*myc* plasmids in individual F-MEL clones. The expression plasmids containing mutant and wild-type c-*myc* cDNAs as well as vector sequences only were linearized and electroporated into F-MEL cells along with linearized pSV_2neo. Cells were sequentially selected in G418 and MTX. Individual clones were then obtained by limited dilution in 96-well microtiter plates. Five to six randomly selected clones from each group were examined for the expression of endogenous and transfected c-*myc* mRNA sequences in an S1 nuclease protection assay (28). The endogenous c-*myc* transcripts protected fragments of 385 and 400 nt, whereas the transfected c-*myc* sequences protected a fragment of 440 nt. (A) Analysis of clones transfected with the wild-type expression plasmid pSV_2myc-dhfr; (B) clones transfected with the Leu-413→Pro mutant; (C) clones transfected with the Leu-420→Trp mutant; (D) clones transfected with the Leu-427→Ser mutant; (E) clones transfected with the Leu-413–434 quadruple mutant. Lane 1 in each group represents the exogenous c-*myc* RNA levels from the uncloned population of MTX-resistant F-MEL cells; lane 2 to 7 represent the individual transfected clones. (F) Uncloned F-MEL cells transfected with vector sequences only, untreated (lane 1) or 4 h after the addition of DMSO to a final concentration of 1.5% (lane 2); (G) schematic representation of the input S1 nuclease probe (940 nt) and the fragments expected from protection by transfected c-*myc* sequences (440 nt) and endogenous c-*myc* sequences (385 and 400 nt).

Differentiation of individual F-MEL clones. Individual clones of transfected F-MEL cells were tested for the ability

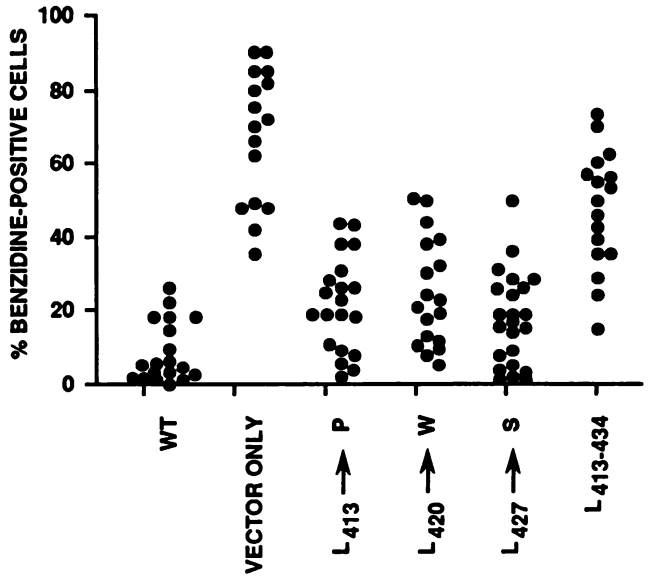

FIG. 5. Differentiation of individual F-MEL clones. A total of 16 to 23 individual clones transfected with the indicated plasmids were cultured for 5 days in medium containing 1.5% DMSO. At the end of that time, cells were stained with the benzidine reagent to determine the percentage of terminally differentiated cells (6, 28, 29). At least 200 cells were counted for each clone. See Table 1 for the *P* values between individual groups. WT, Wild-type pSV_2myc-dhfr (28); P, proline; W, tryptophan; S, serine.

to differentiate after treatment with 1.5% DMSO. Cells in the logarithmic phase of growth were treated with DMSO, and the percentage of benzidine-positive cells was scored 5 days later (Fig. 5). As previously reported, expression of the wild-type c-*myc* sequence was highly effective in suppressing DMSO-induced differentiation of F-MEL cells (8, 10, 18, 28). In contrast, clones transfected with vector sequences alone retained a high degree of responsiveness to DMSO. Mutation of any one of the first three leucines was associated with a partial but significant loss in the ability of that mutant to suppress F-MEL differentiation. Leu-427→Ser was the least disruptive of the single mutations in comparison with wild type ($P = 0.04$; Table 1), whereas Leu-413→Pro was the most disruptive ($P < 10^{-4}$). This finding may be a result of the proline substitution in the latter case, which would be more likely to affect the overall alpha-helical structure of the leucine zipper domain.

DISCUSSION

In this report, we have shown that point mutations in the leucine zipper of the murine c-Myc protein are associated with a drastic, although incomplete, loss of the ability of the protein to inhibit F-MEL differentiation. This is not the result of increased protein instability, since mutant c-Myc proteins are expressed at levels comparable to that of wild-type Myc and are equally insensitive to the effects of DMSO (Fig. 3). The finding that the c-Myc protein retained at least some of its functional capacity in each of the point mutants tested is in keeping with observations of others that single mutations in the leucine zipper domain of c-Fos or c-Jun are less disruptive to their ability to dimerize and bind their target DNA sequences than are multiple mutations (12, 16, 38). However, the finding that the quadruple mutant still retained a residual ability to inhibit differentiation was unexpected in light of the completely disabling effect of such mutations in Jun and Fos family members (12, 16, 38). There are at least two explanations for this observation. First, it is conceivable that some of the effects on F-MEL differentiation can be mediated in the absence of a functional leucine zipper. Second, other regions of the c-Myc protein might contribute to dimerization. For example, Murre et al., in a recent study of several probable eucaryotic transcriptional activators, including N-Myc, MyoD1, the immunoglobulin kappa-chain gene binding proteins E12 and E47, and the proteins encoded by the *Drosophila* genes daughterless, achaete-scute, and twist, have noted additional homologies to c-Myc in regions N terminal to the leucine zipper domain (22). Each of these proteins contains a putative amphipathic helix-loop-helix motif that might mediate dimerization through the interaction of hydrophobic side chains. This domain is necessary for specific DNA binding by these proteins (22, 23) and for the myogenic activity of MyoD1 (37). This region is notably absent from members of the Fos and Jun protein families, which might explain their complete loss of dimerization potential and function in the absence of the leucine zipper domain.

Despite numerous attempts under a variety of conditions, we were unable to demonstrate dimerization of c-Myc protein produced in reticulocyte lysates. Other methods of analysis that we have used to detect c-Myc dimerization include electrophoresis in nondenaturing gels and high-performance liquid chromatography (not shown). These results stand in contrast to those of Dang et al., who demonstrated dimers as well as higher-order oligomers of staphylococcal protein A–c-Myc fusion proteins produced in *E. coli* (9). One reason for these differences may relate to the relative concentrations of proteins used. Our reticulocyte lysates generally contain 0.1 to 0.2 pmol of in vitro-translated c-Myc protein (ca. 2 to 4 nM). In contrast, the protein concentrations used by Dang et al. were in the range of 0.75 to 1 μM (9). It is thus possible that c-Myc dimerization occurs only at concentrations of protein not attainable in

TABLE 1. Pairwise comparisons of mean levels of benzidine staining between groups of clones[a]

Group	*P* value				
	Vector only	Leu-413→Pro	Leu-420→Trp	Leu-427→Ser	Leu-413–434
Wild type (8.4)[b]	$<10^{-4}$	$<10^{-4}$	0.0003	0.04	$<10^{-4}$
Vector only (67.4)		$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
Leu-413→Pro (21.8)			0.45	0.28	$<10^{-4}$
Leu-420→Trp (25.2)				0.07	$<10^{-4}$
Leu-427→Ser (17.2)					$<10^{-4}$
Leu-413–434 (47.1)					

[a] Levels of benzidine staining between individual groups of clones were compared by single-factor analysis of variance, using the Scheffe method of multiple comparisons (significance level = 0.95) (26).

[b] Numbers in parentheses are mean levels of benzidine staining for each group of clones shown in Fig. 3.

reticulocyte lysate systems. Another possibility is that the staphylococcal A fusion proteins produced by Dang et al. were lacking N-terminal c-Myc sequences that are inhibitory to dimerization. Whatever the reasons for the differences, it is quite clear from our results that c-Myc protein is unable to dimerize under conditions that allow for leucine zipper-mediated dimerization of several other well-studied proteins. It is not at all clear what functional significance in vitro homodimerization of c-Myc or a lack thereof has in terms of in vivo effects. For example, it is now well established that c-Fos, unlike c-Jun, is incapable of forming homodimers (12, 13, 16, 24, 31, 32, 38). Nevertheless, it seems likely that the functionally relevant complex in vivo is a Fos-Jun heterodimer. A comprehensive assessment of the functional protein interactions of c-Myc must await the identification of other proteins with which it can complex via the leucine zipper or other domains as well as the DNA sequences to which such conjugates bind.

At least two highly conserved regions of the c-Myc protein are indispensable for the cooperative transformation of primary fibroblasts by *ras* proto-oncogenes (30, 36). One of these regions includes the leucine zipper. We have shown here that this region is also important for the inhibition of F-MEL differentiation. This study therefore emphasizes the close relationship that exists between the pathways leading to malignant transformation and hematopoietic commitment and differentiation.

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