MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 954–962
0270-7306/91/020954-09$02.00/0

Vol. 11, No. 2

Intracellular Leucine Zipper Interactions Suggest c-Myc Hetero-Oligomerization

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Received 6 November 1990/Accepted 21 November 1990

The physiological significance of in vitro leucine zipper interactions was studied by the use of two strategies which detect specific protein-protein interactions in mammalian cells. Fusion genes were constructed which produce chimeric proteins containing leucine zipper domains from several proteins fused either to the DNA-binding domain of the *Saccharomyces cerevisiae* GAL4 protein or to the transcriptional activation domain of the herpes simplex virus VP16 protein. Previous studies in mammalian cells have demonstrated that a single chimeric polypeptide containing these two domains will activate transcription of a reporter gene present downstream of the GAL4 DNA-binding site. Similarly, if the GAL4 DNA-binding domain of a chimeric protein could be complexed through leucine zipper interactions with the VP16 activation domain of another chimeric protein, then transcriptional activation of the reporter gene would be detected. Using this strategy for detecting leucine zipper interactions, we observed homo-oligomerization between leucine zipper domains of the yeast protein GCN4 and hetero-oligomerization between leucine zipper regions from the mammalian transcriptional regulating proteins c-Jun and c-Fos. In contrast, homo-oligomerization of the leucine zipper domain from c-Myc was not detectable in cells. The inability of the c-Myc leucine zipper to homo-oligomerize strongly in cells was confirmed independently. The second strategy to detect leucine zipper interactions takes advantage of the observation that the addition of nuclear localization sequences to a cytoplasmic protein will allow the cytoplasmic protein to be transported to and retained in the nucleus. Chimeric genes encoding proteins with sequences from a cytoplasmic protein fused either to the GCN4 or c-Myc leucine zipper domains were constructed. Experiments with the c-Myc chimeric protein failed to demonstrate transport of the cytoplasmic marker protein to the nucleus in cells expressing the wild-type c-Myc protein. In contrast, the cytoplasmic marker was translocated into the nucleus when the GCN4 leucine zippers were present on both the cytoplasmic marker and a nuclear protein, presumably as a result of leucine zipper interaction. These results suggest that c-Myc function requires hetero-oligomerization to an as yet undefined factor.

A motif in which leucines are spaced at every seventh amino acid residue in helical protein regions (leucine zipper) has been proposed to mediate dimerization (30). This hypothesis has been well substantiated by in vitro studies (2, 14, 19, 21, 29, 31, 38–40, 42, 43, 46, 52). Although the leucine zipper motif may not serve as a dimerization interface in some proteins (5), many transcription factors with this motif appear to dimerize via leucine zipper interactions to juxtapose basic regions that interact directly with DNA (25, 36, 53). The mechanism of leucine zipper interactions appears similar to that of coiled-coil helical interactions previously described in other proteins in which there are helical regions with alternating third and fourth hydrophobic residues (7, 11, 18).

Accumulating evidence suggests that leucine zippers do not interact with each other in a random fashion but that there are inherent structural features in these domains which allow specific interactions (25, 40). The structural basis for the specificity of leucine zipper interactions is still not completely understood. Hence, predictions on whether a particular leucine zipper can dimerize with itself (homodimerize) or dimerize with other leucine zippers (heterodimerize) are not yet reliable. For example, the c-Myc oncoprotein is a nuclear phosphoprotein whose normal cellular function has not yet been clearly defined (10, 16). c-Myc has a number of biochemical properties, however, that suggest it may function as a regulator of gene transcription (28, 32). Specifically, it is a DNA-binding protein with a short half-life, a high proline content, segments that are rich in glutamine and acidic residues, and a carboxyl-terminal domain containing the helix-loop-helix and leucine zipper motifs that may function in oligomerization. We have previously observed that a c-Myc mutant protein with a deletion in the amino-terminal region (amino acids 106 to 143), but not mutant proteins with deletions in the helix-loop-helix region or the leucine zipper region, behaved as a *trans*-dominant negative mutant which inhibited the ability of wild-type c-*myc* to transform rat embryo fibroblasts in cooperation with a mutated *ras* gene (14). This observation suggests that c-Myc either oligomerizes with itself or oligomerizes via its carboxyl sequences with an as yet undefined factor to transform rat embryo fibroblasts.

In the studies described below, we employed two novel techniques to determine whether c-Myc can homo-oligomerize strongly in mammalian cells. The first strategy measures the transcriptional activation of a reporter gene. Using this assay, we detected intracellular homo-dimerization of the leucine zipper domains from the *Saccharomyces cerevisiae* transcriptional activator GCN4 and hetero-oligomerization of the Fos and Jun leucine zippers, but not homo-oligomerization of c-Myc. In the second strategy, a cytoplasmic

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marker protein was shown to translocate into the nuclear compartment by binding to a nuclear protein via compatible leucine zippers. This assay also failed to detect intracellular homo-oligomerization of the c-Myc leucine zipper domains but did detect homo-oligomerization of GCN4 leucine zipper domains. Thus, data from the present studies suggest that c-Myc may function as a potential regulator of gene expression by forming hetero-oligomers with another factor(s).

MATERIALS AND METHODS

Plasmid constructions. GAL4(1-147) plasmid (gift from I. Sadowski; 44) was modified in the polylinker region (creating the pGALO plasmid) in order to fuse various c-*myc* fragments to produce in-frame GAL4–c-Myc fusion proteins (28). Reporter plasmid G5E1bCAT (gift from J. Lillie; 33) has been previously described. c-*myc* *Nde*I-*Cla*I and *Cla*I-*Nsi*I DNA fragments from pOTS*myc* (49) and MLV-*myc* (48) plasmids were used to generate GM(1-262) and GM(262-439) (or DMycLZ) GAL4–c-Myc chimeras. VP16 transcriptional activation domain (amino acids 411 to 490) *Sal*I-*Bam*HI gene fragment from C490 mutant (gift from S. Triezenberg; 51) was subcloned into MLV-*myc* In6 mutant (48) *Xho*I-*Cla*I sites with linkers to generate AMycLZ. The GCN4 leucine zipper *Xho*I-*Hin*dIII gene fragment (gift from S. McKnight) was inserted into the *Xho*I-*Hin*dIII sites of the pOTS-*myc* In413 mutant (48). The *Cla*I-*Hin*dIII fragment from this pOTS*myc-GCN4LZ* hybrid was subcloned into other *myc* vectors to replace the c-Myc leucine zipper with the GCN4 leucine zipper. Dideoxy sequencing was performed to confirm the integrity of this swap, which results in the replacement of the c-Myc leucine zipper (amino acids 413 [Leu] to 439) with the GCN4 leucine zipper (amino acids 253 [Leu] to 281).

A eucaryotic expression vector, pNLVP, encoding the N-terminal 11 amino acids of GAL4 fused to the simian virus 40 (SV40) large T nuclear targeting sequence PKKKRKVD (27) followed by the VP16 transcriptional activation domain (amino acids 411 to 455) and a multiple cloning site was constructed. This was achieved by three-piece ligation of a 60-bp *Hin*dIII-*Sph*I fragment from pGALO containing amino-terminal GAL4 sequences, a synthetic oligonucleotide encoding the SV40 T nuclear targeting signal, and a 2.9-kb *Hin*dIII-*Eco*RI fragment from pGALO containing eucaryotic promoter, enhancer, polyadenylation site, and plasmid sequences to create a new plasmid pNL. pNLVP was created by insertion of a 160-bp *Eco*RI-*Sal*I fragment from pSVGVP(Δ456) plasmid (gift from I. Sadowski) containing VP16 sequences into the *Eco*RI and *Sal*I sites of pNL. AFosLZ and AJunLZ were constructed by inserting *Nde*I-*Cla*I and *Nde*I-*Pst*I bZIP fragments from Jun250 (murine Jun amino acids 250 to 334) and Fos137-216 plasmids (human Fos amino acids 137 to 216) (gift from L. Ransone and I. Verma) into pNLVP *Nde*I-*Cla*I and *Nde*I-*Nsi*I sites, respectively. The same bZIP gene fragments were inserted into pGALO to produce DFosLZ and DJunLZ.

The c-Myc leucine zipper region (amino acids 380 to 439) *Xho*II-*Hin*dIII gene fragment from SP65*myc* (48) was inserted in-frame into the eucaryotic chicken pyruvate kinase (PK) expression vector (12) *Kpn*I and *Bgl*II sites with adapters to produce PKMycLZ. Similarly, the same Myc region with a GCN4 leucine zipper from pOTS*myc-GCN4LZ* was fused to PK, creating PK-MGCN4LZ.

Cell culture, transfection, and immunofluorescence microscopy. DUKXBII Chinese hamster ovary (CHO) cells were passaged in α minimum essential medium supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. CHO cells at 50% confluence were transfected with activator and reporter plasmids, using DEAE-dextran as previously described (33). Unless otherwise indicated, for the specific protein-protein interaction assay, 2 μg of plasmids encoding GAL4 chimeric proteins per plate, 4 μg of plasmids encoding VP16 chimeric proteins per plate, and 2 μg of reporter plasmid G5E1bCAT per plate were cotransfected, using 100-mm-diameter plates of CHO cells. Forty-eight hours after dimethylsulfoxide shock and chloroquine treatment, cells were harvested and cell lysates were prepared for assays by three cycles of freezing and thawing (33). COS7 cells were grown on coverslips in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS and transfected by using DEAE-dextran as previously described (13). Cells were fixed in 3.5% paraformaldehyde and processed for immunofluorescence microscopy as previously described (13). To stain simultaneously for both chicken PK and c-Myc, a rabbit anti-chicken PK (gift from B. Roberts) antibody and a mouse monoclonal anti-Myc peptide antibody (amino acids 171 to 188; OM-11-906; Cambridge Research Biochemicals) were used as the primary antibodies (both at 1:100 dilution). Secondary antibodies were rhodamine-conjugated goat anti-rabbit (1:200 dilution) and fluorescein-conjugated goat anti-mouse (1:200 dilution) antibodies. Transfection efficiency with COS7 cells varied from 5 to 10%. About 1 in 30 transfected COS7 cells expressed both PK and Myc hybrid proteins.

Rat embryo cells were harvested from 13-day-old pregnant Fisher rats and grown in DMEM with 10% FCS as previously described (48). For transformation assays, 2×10^5 rat embryo cells in 100-mm-diameter plates were transfected with 10 μg of *myc* plasmid or 5 μg of EJ*ras* plasmid (48), using Lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.) according to manufacturer's instructions. Transformed foci were counted 14 to 20 days after transfection.

Immunoprecipitation and CAT assays. Cells were incubated in methionine-free DMEM without FCS for 30 min followed by incubation in the same medium with [^{35}S] methionine (100 μCi/ml) for 4.5 h. Cells were harvested, and immunoprecipitations were performed as previously described (28, 33). For quantitative comparison, identical total counts per minute were used for immunoprecipitation. Acylation of [^{14}C]chloramphenicol (chloramphenicol acetyltransferase [CAT] activity) was measured by thin-layer chromatography using acetyl coenzyme A or by the phase-extraction assay using butyryl coenzyme A as previously described (45, 47). For quantitation of CAT activity, the phase-extraction assay was performed in the linear reaction range. To detect intracellular leucine zipper interactions, 20 μl (of a total of 100 μl of cell extract from a confluent 100-mm-diameter plate of transfected CHO cells) of cell extract was incubated with 0.3 mM *n*-butyryl coenzyme A, 125 mM Tris hydrochloride (pH 8.0), and 22 μM [^{14}C] chloramphenicol (57 mCi/mmol) in a total volume of 80 μl for 1 h at 37°C. The reaction mixtures were processed as previously described (47).

RESULTS

Detection of intracellular hetero-oligomerization of Jun and Fos leucine zippers. The strategy underlying the development of our first assay to detect protein-protein interactions was outlined by Fields and Song (17) for a yeast system, and the assay is illustrated in Fig. 1 with specific reference to the

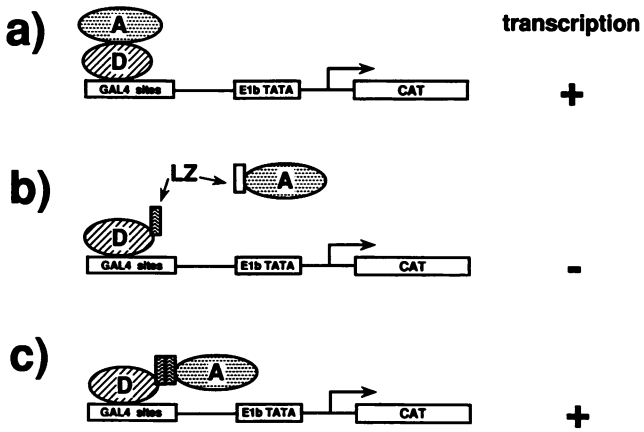

FIG. 1. Detection of leucine zipper interaction in cells by transcriptional activation. (a) The chimeric GAL4-VP16 activator potently activates transcription through GAL4 binding sites. (b) Leucine zipper (LZ) hybrids containing either the GAL4 DNA-binding (D) domain or the VP16 activation (A) domain are incapable of inducing transcription. Likewise, the presence of both D-LZ and A-LZ′ with incompatible leucine zippers is unable to initiate transcription. (c) Interaction between compatible leucine zippers brings the activation (A) domain into close proximity to the DNA-binding (D) domain and results in activation of transcription.

detection of leucine zipper interactions in mammalian cells. The assay detects transcriptional activation of a reporter gene encoding CAT located immediately downstream of five tandemly arranged GAL4 DNA-binding sites (in plasmid G5E1bCAT; 33). Previous studies have established that amino acids 1 to 147 of the yeast GAL4 protein are sufficient for specific binding to the GAL4 DNA site but that additional protein sequences are necessary for activating transcription (35, 41). The herpes simplex virus VP16 protein functions as a transcriptional activator in mammalian cells, and this transcriptional activation activity can be localized to amino acids 411 to 490 (51). A fusion gene encoding a chimeric polypeptide containing both the GAL4 DNA-binding domain and the VP16 activation domain will strongly activate transcription in mammalian cells of reporter genes downstream of the GAL4 sites (33, 44). Thus, we hypothesized that when a chimeric protein containing the VP16 activation domain is complexed via leucine zipper dimerization to the GAL4 DNA-binding domain of a second chimeric protein, transcriptional activation of the CAT gene would result.

To test this assay, we constructed eucaryotic expression vectors, which contain the SV40 early promoter and polyadenylation site and encode fusion proteins that contained the GAL4 DNA-binding domain fused to various protein regions with leucine zipper motifs. We also constructed expression vectors driven by the SV40 early promoter that produced hybrid proteins consisting of an initiating methionine preceding the SV40 large T nuclear targeting sequence (27) and the VP16 activation domain fused to the various leucine zipper regions. As many studies had demonstrated that Fos and Jun interact strongly in vitro (8, 9, 19, 21, 38, 42, 43, 46, 52) and that this interaction is likely to be mediated by the leucine zipper regions of these proteins, we sought to determine whether these regions would mediate intracellular oligomerization in the transcriptional assay. Thus, hybrid genes were constructed that produced the GAL4 DNA-binding domain (D) or the VP16 activation domain (A) fused to either the c-Fos basic leucine zipper region (FosLZ) or c-Jun basic-leucine zipper region (JunLZ) (resulting in DFosLZ, DJunLZ, AFosLZ, and AJunLZ; Fig. 2A). While each of the individual fusion genes displayed relatively low background CAT activity when cotransfected with reporter plasmid G5E1bCAT into Chinese hamster ovary (CHO) cells, transfection with combinations of plasmids encoding either DFosLZ and AJunLZ proteins or DJunLZ and AFosLZ proteins resulted in strong activation of CAT gene expression (Fig. 2B). Protein levels of DFosLZ and DJunLZ were equivalent, as determined by immunoprecipitation of metabolically labeled CHO cells with anti-GAL4 antibody (data not shown). The reason for the higher activation of transcription by DFosLZ and AJunLZ than that of DJunLZ and AFosLZ is unclear, since it does not appear to be due to lower levels of DJunLZ compared with DFosLZ protein. In contrast, in this assay for intracellular oligomerization, neither Fos nor Jun leucine zippers displayed detectable homo-oligomerization in transfected cells (Fig. 2, DFosLZ and AFosLZ proteins and DJunLZ and AJunLZ proteins). These data indicate that heterodimerization, but not homodimerization, via the leucine zipper regions of c-Fos and c-Jun is readily detectable in mammalian cells.

Homo-oligomerization of the c-Myc helix-loop-helix and leucine zipper region is undetectable in cells. Through the study of chimeric proteins containing various segments of the c-Myc protein fused to the GAL4 DNA-binding domain, we have identified an amino-terminal region of c-Myc that can potently activate transcription (28). In contrast, the carboxyl-terminal domain of c-Myc containing the nuclear targeting sequences, and basic region, helix-loop-helix, and leucine zipper motifs does not activate transcription. Thus, we constructed hybrid genes with the GAL4 DNA-binding domain (D) or the VP16 activation domain (A) fused to c-Myc amino acids 262 to 439 (creating DMycLZ and AMycLZ which contain nuclear targeting sequences, basic regions, and helix-loop-helix [HLH] and leucine zipper [LZ] regions; Fig. 3A). When the plasmids encoding chimeric proteins DMycLZ and AMycLZ were cotransfected along with reporter plasmid G5E1bCAT, the combination failed to activate CAT gene expression above background levels (Fig. 3B). In fact, transfection with a range of concentrations (2 to 12 μg) of the plasmid encoding AMycLZ with a constant amount of plasmid (2 μg) encoding DMycLZ did not result in activation of CAT gene expression above background levels (data not shown). These observations suggest that the MycHLH-LZ region is unable to mediate detectable homo-oligomerization in this assay system. However, it is possible that these hybrid proteins are unstable in the cells or display conformations which are unfavorable for the MycHLH-LZ region to mediate homo-oligomerization.

As a control for the integrity of the hybrid proteins, we exchanged the c-Myc leucine zipper region in AMycLZ and DMycLZ precisely with the leucine zipper region of the yeast protein GCN4 to produce AM-GCN4LZ and DM-GCN4LZ, respectively (Fig. 3A). Since the leucine zipper of GCN4 is known to mediate effective homo-dimerization in vitro (2, 23, 50), chimeric GAL4 and VP16 proteins containing the leucine zipper of GCN4 were expected to activate CAT gene expression when they were coexpressed. Indeed, cotransfection of plasmids encoding AM-GCN4LZ and DM-GCN4LZ produced a significant degree of CAT gene activation compared with control transfection experiments with either plasmid alone (Fig. 3B). In addition, transfections with combinations of plasmids encoding AMycLZ and DM-GCN4LZ proteins or DMycLZ and AM-GCN4LZ proteins did not result in activation of CAT gene expression above

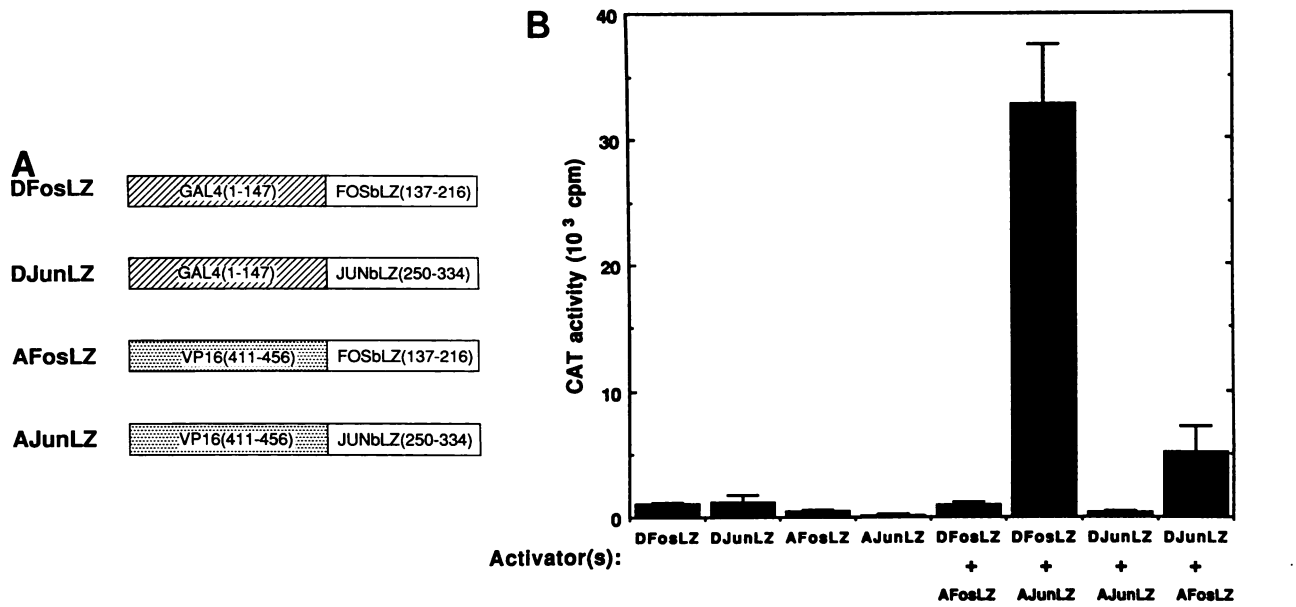

FIG. 2. (A) Schematic depiction of chimeric proteins DFosLZ, DJunLZ, AFosLZ, and AJunLZ. Amino acid residues included are indicated in parentheses. The basic regions are shown as 'b', and leucine zipper as LZ. AFosLZ and AJunLZ also contain a nuclear targeting signal at the amino terminus (see Materials and Methods). (B) Detection of c-Fos and c-Jun leucine zipper hetero-oligomerization in cells. Fusions of either Fos or Jun leucine zipper domains (LZ) to either GAL4 DNA-binding (D) or VP16 activation (A) domain yielded basal CAT activity expressed from the reporter G5E1bCAT. Combinations of FosLZ and JunLZ fusions dramatically activated CAT gene transcription. Homo-oligomerization of FosLZ or JunLZ was undetectable. CAT activity was determined by the phase-extraction assay from four separate transfection experiments and given as mean ± standard deviation in counts per minute.

background levels from control transfections with the individual plasmids (data not shown). Failure of the c-Myc leucine zipper region to mediate dimerization in cells as a result of unfavorable conformations of the hybrid proteins seems an unlikely explanation, since replacement of only the c-Myc leucine zipper in these constructs with the GCN4 leucine zipper allowed dimerization in cells. In addition, DMycLZ and DM-GCN4LZ protein levels are comparable in transfected, metabolically labeled CHO cells, as determined by immunoprecipitation and the use of anti-GAL4 antibody (Fig. 3C). When compared with the level of endogenous c-Myc (Fig. 3C, lane c), the chimeric GAL4 protein levels appear roughly the same (Fig. 3C, lanes a and b). However, since the transfection efficiency (as estimated by immunofluorescence microscopy and by the use of anti-GAL4 antiserum; 28) with CHO cells is about 10 to 20%, the chimeric GAL4 protein levels are estimated to be about 5- to 10-fold higher than endogenous CHO cell c-Myc levels (with the number of methionines in each chimeric protein taken into account).

Nuclear import of a cytoplasmic marker protein complexed to a nuclear protein via leucine zipper dimerization. We developed a second assay to assess the ability of the c-Myc leucine zipper to dimerize in mammalian cells. This assay takes advantage of the observation that the addition of nuclear localization sequences to a cytoplasmic protein will cause the cytoplasmic protein to be transported to and retained in the nucleus (12, 13). Eucaryotic expression constructs that contain the SV40 origin of replication were prepared in which leucine zipper regions were fused to the sequences of a cytoplasmic marker protein or to those of a nuclear protein (Fig. 4A). When compatible leucine zipper interactions are found, the chimeric protein containing both the leucine zipper region and the cytoplasmic marker protein sequences would be predicted to complex with the leucine zipper of the nuclear protein, and thereby the cytoplasmic protein might be imported into the nucleus (Fig. 4B). We constructed chimeric genes containing sequences of cytoplasmic protein chicken PK fused at its carboxyl terminus with c-Myc amino acids 380 to 439 (creating PKMycLZ); this chimeric protein is devoid of any nuclear targeting signals (Fig. 4C) (12). We also prepared a similar chimeric protein containing PK and c-Myc sequences, except that the Myc leucine zipper region was precisely replaced by the GCN4 leucine zipper (creating PK-MGCN4LZ; Fig. 4C) (2). In addition, a c-Myc eucaryotic expression vector was also constructed so that the GCN4 leucine zipper region replaced the c-Myc leucine zipper (creating MycGCN4LZ; Fig. 4C). We chose COS7 cells for these experiments, because anti-Myc and anti-chicken PK antibodies gave very low background staining of untransfected cells compared with the transfected cells which express proteins at very high levels from plasmids containing the SV40 origin of replication (12, 13).

Chimeric proteins PK-MGCN4LZ and PKMycLZ are cytoplasmic proteins in transfected mammalian cells, as determined by indirect immunofluorescence microscopy using anti-PK antibody (Fig. 5A). MycGCN4LZ is a nuclear protein as expected (Fig. 5B). Cotransfection with plasmids encoding PKMycLZ and c-Myc yielded cells in which c-Myc was localized in the nucleus and PK was located in the cytoplasm (Fig. 5C to F). Similarly, cotransfection with a plasmid encoding a nuclear protein containing the c-Myc leucine zipper and a plasmid encoding a chimeric protein with the GCN4 leucine zipper linked to cytoplasmic marker sequences did not result in the translocation of the cytoplasmic marker protein into the nucleus (data not shown). In contrast, when the GCN4 leucine zipper motif is present in

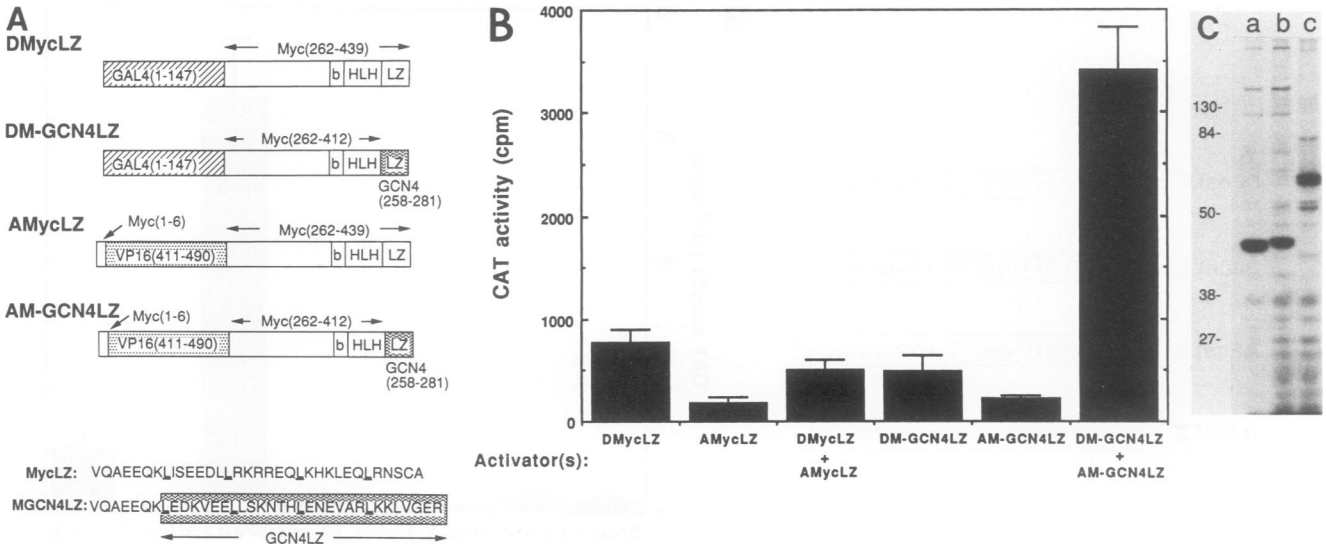

FIG. 3. (A) Schematic depiction of chimeric proteins DMycLZ, DM-GCN4LZ, AMycLZ, and AM-GCN4LZ. Amino acid residues included are shown in parentheses. b, Basic region; HLH, helix-loop-helix region; LZ, leucine zipper. The amino acid sequences for c-MycLZ and the chimeric M-GCN4LZ are given at the bottom of the panel, with the GCN4 leucine zipper domain boxed. (B) Homo-oligomerization of GCN4 leucine zipper and lack of interaction between c-Myc leucine zippers in cells. c-Myc amino acids 262 to 439 were fused to either GAL4 DNA-binding domain (DMycLZ) or VP16 activation domain (AMycLZ). Transfections with the combination of plasmids encoding AMycLZ and DMycLZ did not activate transcription of reporter plasmid G5E1bCAT more than either activator plasmids alone. GCN4 leucine zippers in the AM-GCN4LZ and DM-GCN4LZ combination (AM-GCN4LZ + DM-GCN4LZ) interacts to activate transcription. CAT activity was determined as described in the legend to Fig. 2. (C) Lack of detectable c-Myc leucine zipper domain homo-oligomerization is not due to lack of GAL4 chimeric protein production. Immunoprecipitation of DMycLZ (lane a), DM-GCN4LZ (lane b) from CHO cells (metabolically labeled with [^{35}S]methionine) with anti-GAL4 antibody, and endogenous CHO c-Myc (lane c) with anti-c-Myc antibody. Positions of prestained molecular mass markers (kilodaltons) are given on the left margin.

both the chimeric nuclear protein and the chimeric cytoplasmic protein, the cytoplasmic marker was transported into the nucleus (Fig. 5G to J). The chimeric PK protein with the GCN4 leucine zipper was completely localized in the nucleus (Fig. 5). However, there are cells in which the chimeric PK protein is partially located in the nucleus (Fig. 5I and K), presumably due a limiting amount of the chimeric nuclear protein with the GCN4 leucine zipper in the same cells. These data suggest that at the single cell level, c-MycLZ does not homo-oligomerize nor will it oligomerize with the GCN4 leucine zipper in cells. The GCN4 leucine zipper, however, readily displays homo-oligomerization in transfected cells.

A chimera of c-Myc with the GCN4 leucine zipper transforms rat embryo cells inefficiently with a mutated *ras* gene. We performed an additional experiment to test the hypothesis that wild-type c-Myc may function as a homo-oligomer in cells. In this experiment, we replaced the c-Myc leucine zipper with the GCN4 leucine zipper and then sought to determine the neoplastic transforming ability of this chimeric protein (MycGCN4LZ) encoded in a plasmid driven by the Moloney leukemia virus long terminal repeat (48). If c-Myc can function as a homo-oligomer in cells, then replacement of the c-Myc leucine zipper with the GCN4 leucine zipper (allowing the formation of homo-oligomers) should allow the chimeric protein to transform cells. In fact, the ability of chimeric protein MycGCN4LZ to transform rat embryo cells in the presence of a mutated *ras* gene is drastically reduced compared with that of wild-type c-Myc (Table 1). In addition, the number of foci of cells transformed in the presence of MycGCN4LZ were remarkably smaller then those produced in the presence of wild-type c-Myc (data not shown). Protein levels of c-Myc and c-MycGCN4LZ determined by immunoprecipitation in two randomly selected clones transformed by wild-type c-*myc* and the chimeric c-MycGCN4LZ are comparable (data not shown). These results further suggest that the majority of functional c-Myc protein within the cell is not in a homo-oligomeric form.

DISCUSSION

A recurring theme among many transcription factors characterized to date, including the yeast protein GCN4 and the mammalian proteins c-Jun, c-Fos, MyoD, E12, and E47, is the presence of dimerization or oligomerization motifs which juxtapose basic regions (26). These oligomerization motifs include regions predicted to form leucine zipper domains and regions predicted to form helix-loop-helix domains. The basic regions have been proposed to bind to specific DNA sequences when dimers are formed (25, 36, 37). Although these oligomerization motifs appear to mediate specific protein-protein interactions in vitro, the role of these oligomerization motifs in cells has not been directly studied. We report here two strategies capable of detecting oligomerization via leucine zipper domains in mammalian cells. Using these approaches, we were unable to detect homo-oligomerization of the c-Myc helix-loop-helix or leucine zipper domains. In contrast, c-Jun and c-Fos leucine zipper hetero-oligomerization and GCN4 leucine zipper homo-oligomerization were readily detectable.

One of the strategies used to detect intracellular leucine zipper interactions was based on previous work in *S. cerevisiae* (17). In this assay system, a DNA-binding domain was linked through leucine zipper interactions to a transcriptional activation domain; formation of such complexes resulted in transcription of a reporter gene. Similarly

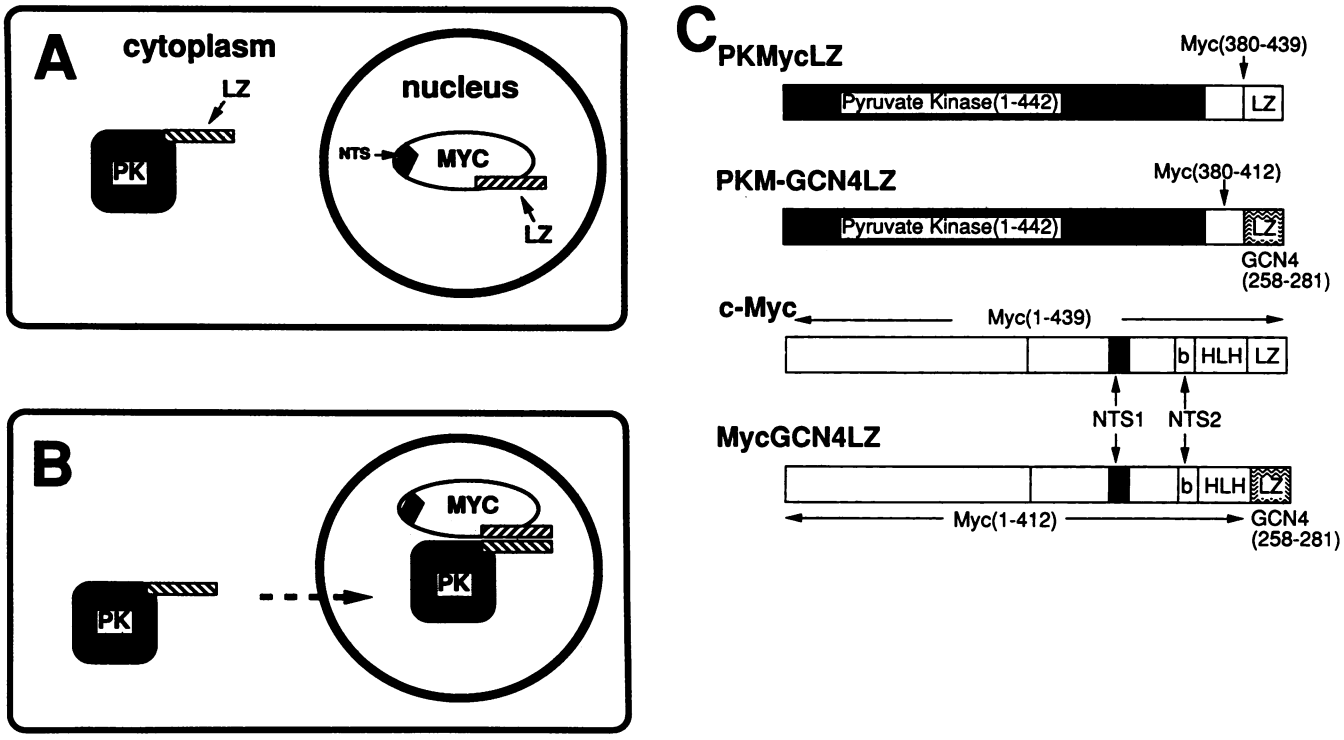

FIG. 4. Nuclear translocation of a cytoplasmic protein-leucine zipper fusion through interaction with a leucine zipper of a nuclear protein. (A) Leucine zippers (LZ) on a cytoplasmic marker protein (PK) and a nuclear protein (MYC) (NTS = nuclear targeting signal) which do not interact lead to cytoplasmic PK-LZ fusion and nuclear location of the nuclear protein. (B) The cytoplasmic PK fusion is translocated into the nuclear compartment by binding via compatible leucine zippers with the nuclear protein. (C) Schematic depiction of PKMycLZ, PKMGCN4LZ, c-Myc, and MycGCN4LZ. Amino acid residues included are shown in parentheses. The nuclear targeting signals NTS1 (amino acids 320 to 328) and NTS2 (amino acids 364 to 374) are indicated. The c-Myc b, HLH, LZ, and GCN4LZ regions are as shown in Fig. 3.

conceived experiments have recently been used to demonstrated intracellular interactions of the E1a protein with the ATF2 transcription factor and also interaction of the E1a protein with the retinoblastoma gene product (34). Our results provide further evidence for the potential of these assays for detecting specific protein-protein interactions in cells, as we were able to detect interaction between the c-Jun and c-Fos leucine zippers and also homo-oligomerization between GCN4 leucine zippers. Our inability to detect Jun-Jun homodimerization is likely to reflect the limitation of the transactivation assay, since Jun-Jun homodimers appear to exist in vivo (1, 4, 15, 22) and definitely exist in vitro (8, 21, 38, 40). Another caveat with the use of this assay is the potential for both Jun-Fos and Jun-Jun chimeric dimers to interact with cellular AP1 sites and thereby deplete the GAL4 sites of the GAL4 chimeras. The sensitivity of the transactivation assay is also likely to be affected by the conformation of the chimeric proteins.

Homo-oligomerization of the c-Myc protein region containing the helix-loop-helix and leucine zipper motifs is not detectable by the transcriptional activation assay. In contrast, replacement of the c-Myc leucine zipper with the GCN4 leucine zipper allowed the chimeric proteins to oligomerize in cells. The latter observation suggests that the inability of the c-Myc leucine zipper to mediate oligomerization was probably not a result of instability of the chimeric protein nor was it likely to have resulted from steric interference. Data from the work presented here suggests that homo-oligomerization of this c-Myc region does not occur intracellularly. As with the Jun leucine zipper, which homodimerizes in vitro in the absence of the Fos leucine zipper (40), bacterially synthesized and purified c-Myc appears to homo-oligomerize, but only at high protein concentrations (14). Likewise, MyoD appears to dimerize weakly in vitro, although it primarily heterodimerizes with E12 in vivo (37).

In order to substantiate the failure of the helix-loop-helix and leucine zipper domains of c-Myc to mediate strong homo-oligomerization in cells, we adapted a novel approach to detect protein-protein interactions. With this strategy, COS7 cells were cotransfected with a combination of fusion genes each of which produced either a chimeric protein containing both cytoplasmic marker sequences and leucine zipper sequences or a nuclear protein containing a leucine zipper. If the leucine zippers were compatible for specific interactions, the cytoplasmic protein would be transported in a piggy-back fashion into the nucleus by its complexing with the nuclear protein via the leucine zippers. In these experiments, we found that the c-Myc leucine zipper region linked to PK could not dimerize with the leucine zipper of wild-type c-Myc. In contrast, replacement of the c-Myc leucine zipper with the GCN4 leucine zipper allowed homodimerization of these proteins, resulting in the translocation of the cytoplasmic marker protein into the nucleus. In addition, this observation also suggests that leucine zipper interactions can occur in the cytoplasmic compartment prior to translocation of leucine zipper proteins into the nucleus. As in the transcriptional activation assay, the c-Myc leucine zipper failed to display homodimerization activity in this

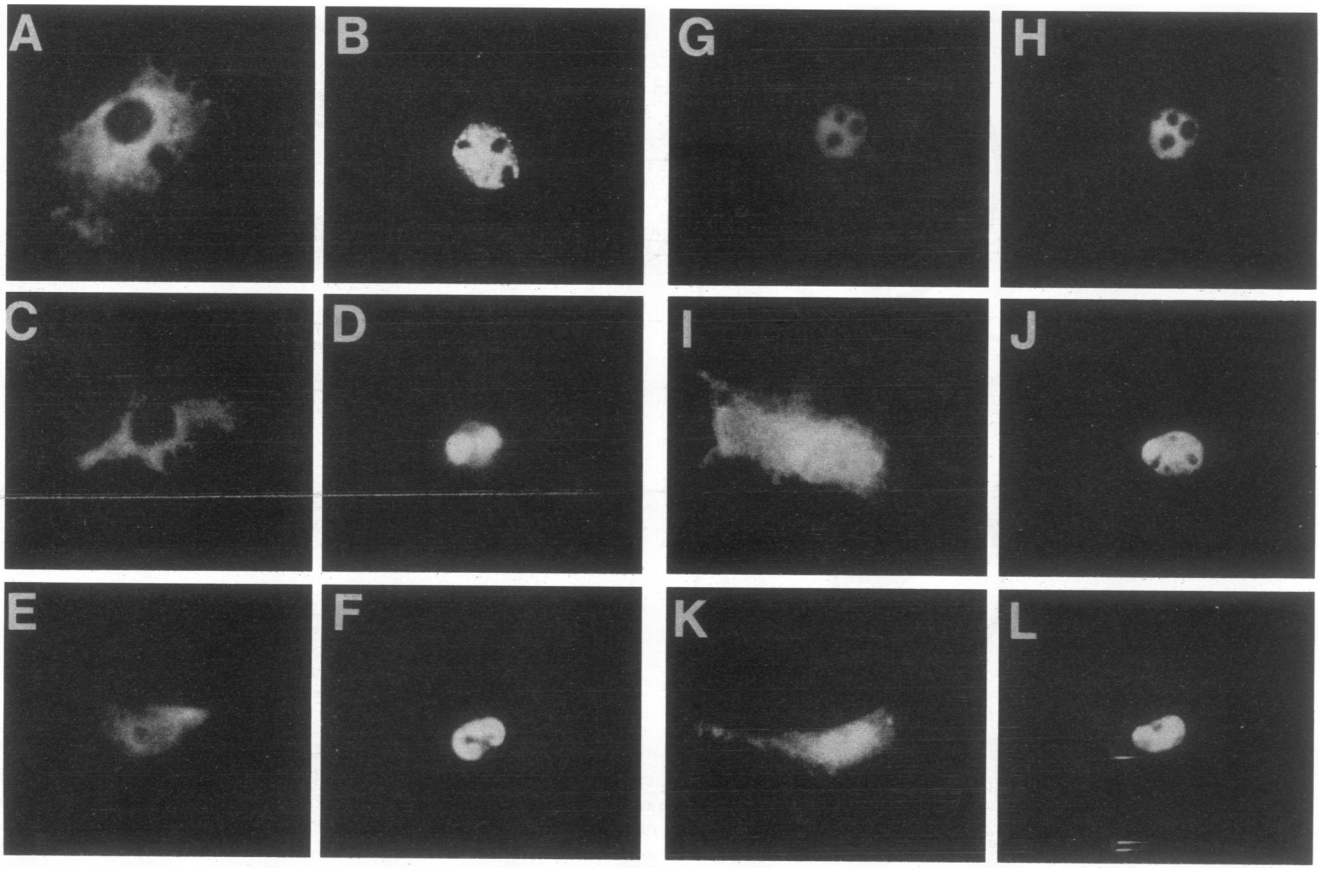

FIG. 5. Subcellular location of PK and c-Myc proteins in transfected COS7 cells. (A) Cytoplasmic location of PK-MGCN4LZ. (B) Nuclear location of MycGCN4LZ. Cytoplasmic location of PK-MycLZ (C and E) in the same cells expressing nuclear c-Myc (D and F). (G) Nuclear location of PK-MGCN4LZ in the same cell expressing MycGCN4LZ (H). Partial nuclear location of PK-MGCN4LZ (I and K) in cells expressing MycGCN4LZ (J and L). PK detection (A, C, E, G, I, and K; rhodamine fluorescence) and c-Myc detection (B, D, F, H, J, and L; fluorescein fluorescence).

assay, which does detect GCN4 leucine zipper homodimerization in cells.

In order to examine further the biological significance of the oligomerization state of c-Myc, we replaced the c-Myc leucine zipper with the GCN4 leucine zipper and examined the transforming potential of this chimeric protein. If c-Myc functions in cell transformation as a homo-oligomer, then replacement of the c-Myc leucine zipper with a homodimerizing GCN4 leucine zipper should not alter the transforming potential. On the other hand, if c-Myc functions as a hetero-oligomer, then the leucine zipper swap is likely to result in a less active or inactive chimera. Indeed, we observed that the c-Myc GCN4 leucine zipper chimeric protein is reproducibly less active than wild-type c-Myc in cotransformation assays with the mutated *ras* gene, suggesting that c-Myc does not function as a homo-oligomer in mediating neoplastic transformation.

TABLE 1. Transforming activity of chimeric protein MycGCN4LZ with a GCN4 leucine zipper replacing the c-Myc leucine zipper region compared with wild-type c-Myc[a]

Expt	No. of foci for plasmid encoding the following protein:	
	Wild-type c-Myc	MycGCN4LZ
1	59	9
2	63	15
3	65	5
4	58	7

[a] Rat embryo fibroblasts were transfected with plasmids (10 μg) encoding wild-type c-Myc or MycGCN4LZ and a plasmid (5 μg) expressing a mutated *ras* (EJ-*ras*) gene (see Materials and Methods). Transformed foci were counted 14 to 21 days posttransfection. Transfections with individual plasmids encoding c-Myc, MycGCN4LZ, or EJ-Ras resulted in no focus formation. The numbers of foci (mean ± standard deviation) for all four experiments were 61 ± 3 for wild-type c-Myc and 9 ± 4 for MycGCN4LZ.

Although methods to predict the specificity of leucine zipper interactions are not yet available, it appears that the residue 5 positions distal from each leucine (fifth residue) in the zipper plays an important role in the coiled-coil interaction (39). This fifth residue tends to be hydrophobic or uncharged in proteins that are known to homo-oligomerize in vitro. In contrast, Fos, which does not homodimerize through leucine zippers, contains several fifth residues which are charged (42). Similarly, several of the fifth residues of the c-Myc leucine zipper are charged residues which are likely to interfere with homodimerization potential. All of the above observations suggest that c-Myc function is likely to require hetero-oligomerization with another protein, although it is possible that c-Myc homo-oligomers might exist in vivo under conditions not tested in this report. Since both the helix-loop-helix and leucine zipper regions of

c-Myc are required for transforming activity (48) and since c-Myc does not hetero-oligomerize with E12, E47, or MyoD (37), the protein(s) that associate with functional c-Myc is likely to be a protein that contains both helix-loop-helix and leucine zipper motifs, such as those present in AP4 (24), TFE3 (3), TFEB (6), and USF (20). In fact, Eisenman and coworkers (16a) have recently identified a partial cDNA clone that encodes a protein with both helix-loop-helix and leucine zipper domains capable of interacting with the c-Myc oligomerization domain.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant CA51497, American Cancer Society-Maryland Chapter, W. M. Keck Foundation, Henry M. and Lillian Stratton Foundation, Inc., and Hubert and Anne E. Rogers Scholarship. C.V.D. holds an American Cancer Society Junior Faculty Research Award (JFRA-281). G.J.K., L.M.S.R., and E.R.F. are supported in part by an NIH hematology training grant.

We thank P. Agre, R. Ambinder, R. Eisenman, B. Lee, J. Lillie, S. McKnight, L. Ransonne, B. Roberts, I. Sadowski, K. Struhl, S. Triezenberg, I. Verma, and B. Vogelstein for reagents or discussions.

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