Identification of a cDNA for SSRP1, an HMG-box protein, by interaction with the c-Myc oncoprotein in a novel bacterial expression screen

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ABSTRACT

We describe a system for screening cDNA expression libraries in *Escherichia coli* based on protein–protein interactions. The system utilizes fusion proteins containing the DNA binding domain of the lambda phage cI repressor and a heterologous dimerization domain, which is the target of the screen. Such chimeric proteins were functional as transcriptional repressors in *E.coli*; function was dependent on the presence of the heterologous dimerization domain, and function of the chimeras was disrupted by expression of excess dimerization domain. A screen was designed to identify factors that could interact with the heterologous dimerization domain and thereby inactivate the chimeric repressor. We used this screen to identify factors that could interact with the basic helix–loop–helix/leucine zipper domains of c-Myc, and isolated the cDNA for a previously characterized HMG domain protein that interacts specifically with c-Myc in this system. This screening method could be used with proteins that have the ability to homo- or heterodimerize.

INTRODUCTION

The identification and characterization of protein–protein interactions has led to significant insights into many different biological processes and their underlying mechanisms. Traditionally, protein–protein interactions have been understood only after extensive genetic or biochemical characterization of the components involved. Recently, systems have been developed for the purpose of identifying interacting factors and dissecting protein domains involved in mediating interactions (1–4). The objective of these systems is to gain insight into the function(s) of proteins through an understanding of the protein complexes in which they are found. The system that has proven most useful for this purpose takes advantage of the genetics of *Saccharomyces cerevisiae* to select or screen for protein–protein interactions (4). The ‘two-hybrid system’ identifies protein–protein interactions based on the ability of the proteins in question to form a functional transcrip-

tion factor by bringing together a DNA binding domain fused to one of the proteins and a transcriptional activation domain fused to the other protein. A second approach that has proven useful is the screening of *E.coli*-based expression libraries with a labeled protein (2). These systems have been used to screen cDNA libraries and have identified many novel genes involved in a variety of cellular processes (e.g. 2,5–9).

We describe a system that utilizes the genetics of bacteriophage lambda in *E.coli* for the identification of protein–protein interactions. The advantages of performing a screen in *E.coli* include the ability to screen larger numbers of cDNAs and the ease with which cDNAs can be recovered for further characterization. The screening system described here takes advantage of the observation that a functional transcriptional repressor can be created by fusing a heterologous dimerization domain to the DNA binding domain of the lambda cI repressor protein (1; we initially established this system independently of Hu *et al.*, who have suggested a similar screening procedure to that described here). The lambda cI repressor consists of an N-terminal helix–turn–helix DNA binding domain and a globular C-terminal dimerization domain connected by a flexible hinge region (10). cI binds a dyad symmetric DNA element (lambda operator) in a dimerization-dependent manner and represses transcription by blocking the binding of RNA polymerase to the promoter. The N-terminal DNA binding domain alone is incapable of binding DNA at physiological concentrations, such as that found in lysogenic bacteria. Therefore, the function of a chimeric repressor under similar conditions would be dependent on dimerization mediated by the heterologous domain to which the cI DNA binding domain was fused.

A chimeric repressor has been used by Hu *et al.* to perform a structural analysis of the GCN4 leucine zipper (1). A screen was performed that isolated mutations in the leucine zipper domain that would inactivate the repressor and thereby identified residues critical for dimerization of the leucine zipper. In this paper, we examine the possibility of screening for potential interacting proteins by identifying heterologous factors that will inactivate a chimeric cI repressor. In such a screen, a plasmid-based cDNA library is transformed into an *E.coli* strain expressing a cI chimera and carrying a cI-regulated reporter gene. cDNA products that can interact with the cI chimera are identified by a visible

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phenotype resulting from inactivation of the cI chimera and derepression of the reporter gene.

Specifically, our objective was to develop a screen with which to identify proteins that could interact with the C terminus of the c-Myc oncoprotein. This region of c-Myc contains a basic DNA binding domain followed by contiguous helix-loop-helix and leucine zipper motifs (B-HLH/LZ) that function as a dimerization domain (11,12). Proteins that interact with the HLH/LZ portion of c-Myc might be expected to disrupt activity of a c-Myc-cI chimeric repressor.

A DNA sequence to which Myc will bind has been determined and a partner B-HLH/LZ protein, Max, has been identified (11–14). Myc and Max can form stable heterodimers that can bind DNA with high affinity (11,15); given the fact that separate regions of the Myc protein are capable of activating transcription, it appears that Myc functions, at least in part, as a transcription factor (16–19). We were interested in trying to identify other proteins, in addition to Max, that interact with the B-HLH/LZ region of Myc, and which, in turn, would be relevant to the function of Myc.

We have screened a human cDNA library and identified a protein that interacts specifically with a cI-Myc B-HLH/LZ fusion protein. This methodology could be utilized with other chimeric repressor fusions containing distinct dimerization domains, and thus could be applied to the study of a variety of homo- and heterodimeric interactions.

MATERIALS AND METHODS

Constructs for bacterial expression

cI derivatives were constructed in pUC18 and were expressed from the weak β -lactamase promoter (20,21); the *lacO* and Shine-Delgarno sequence of *lacZ* were positioned between the promoter and the first codon of cI (Fig. 1B). The N-terminal 336 bp, encoding the first 112 amino acids, of cI was inserted as a PCR product 3' to the promoter/operator region. The human Myc basic helix-loop-helix (B-HLH) region plus and minus the contiguous leucine zipper (LZ) was inserted as a PCR product immediately 3' to amino acid 112 of cI. The B-HLH/LZ region is the C-terminal 85 amino acids from residue 355 (Lys) to 439 (Ala); the B-HLH region is 56 amino acids and contains residues 355 (Lys)–410 (Glu). PCR products for each were generated to include a termination codon after the final residue. Each of the cI expression constructs was subcloned into pACYC177 in order to lower cI fusion expression level. The resulting plasmids were referred to as p177cI, p177cI-BHLH/LZ, and p177cI-BHLH. pACYC177 is a low copy number (5–20 copies per cell) plasmid carrying a kanamycin resistance marker (the ampicillin marker was deleted) and which is compatible with pBR- and pUC-based vectors (22). The pACYC177 derivative expressing cI-TR β was kindly provided by David D. Moore. cI-TR β was constructed by inserting the C-terminal 169 residues of the rat thyroid hormone receptor β into p177cI (23). p177cI-E2/5 was kindly provided by Tom Kadesch. The region of E2/5 used in this fusion encodes the C-terminal 158 residues, which includes the B-HLH domain (24). cI-GCN4 was expressed from pACYC184, which contains the same origin as pACYC177, but carries resistance markers for tetracycline and chloramphenicol (22). The cI-GCN4 expression construct pJH370 was kindly provided by J. C. Hu and R. T. Sauer. The cI-GCN4 fusion encodes residues 1–132 of cI fused

to the leucine zipper (LZ) of GCN4 (1). The promoter region of pJH370 was replaced with that described above and then the cI-GCN4 expression cassette was subcloned into pACYC184 to create p184cI-GCN4.

Constructs expressing the Myc BHLH/LZ and BHLH domains were made by placing them in-frame with the *lacZ* coding sequence in pUC18; these domains were identical to those used in the cI fusions. The Max expression construct was made by inserting the full-length coding sequence in-frame with the *lacZ* coding sequence in pSK(+). The Max insert was generated by PCR from a cDNA library.

Over-expression constructs for cI-Myc BHLH/LZ and cI-Myc BHLH were made by inserting the corresponding coding sequences downstream of the T7 promoter in pSK(+); over-expression was achieved in BL21(DE3) (Novagen) upon induction of T7 polymerase. The GST-Max construct was made by inserting full-length Max in-frame with the GST gene in pGEX-3X. GST-Max was over-expressed and purified on glutathione-agarose.

pNNP_L387 was the reporter plasmid used in repressor assays and the cDNA library screen for interacting factors. This reporter was constructed by inserting the lambda left promoter (P_L) as a PCR product upstream of the *lacZ* gene in pNN387 (kindly provided by S. Elledge) (25). pNNP_L387 is a single copy plasmid which is compatible with pACYC177 and pBR derivatives and carries a chloramphenicol resistance (Cm^R) marker.

All DNA constructs were sequenced, either at their junction points or in their entirety, as necessary. All PCR products were sequenced in their entirety.

Dot plaque assay

The dot plaque assay (DPA) was used as a measure of cI fusion repressor activity. Typically, Y1090 derivatives expressing cI fusions were grown overnight in LB media containing appropriate drugs for plasmid selection, 10 mM MgSO₄, and 0.2% maltose. Cells were diluted ~10-fold in 2.5 ml of top agar (at 50°C) and plated. After ~5 min, 5 μ l aliquots of lambda phage KH54 (kindly provided by J. Hu) were spotted on the top agar. The phage concentration range was from 10¹ to 10⁶ plaque forming units (p.f.u.) at 10-fold intervals. A lambda phage carrying the immunity region of 434 (*imm*⁴³⁴) was used as a control for infection.

β -gal assays

Colorimetric assays were done on LB plates containing appropriate drugs plus 0.004% X-gal. Liquid β -gal assays were done according to Miller (26); assays were done in duplicate starting with single colonies. β -gal assays were done following fresh transformation with tester plasmids (pUC based) of the strains below. Colony color determinations were made following plating and overnight incubation at 37°C. Two bacterial strains were used for β -gal assays: (1) Y1090 strains containing pNNP_L387 and a p177cI fusion derivative, (2) Y1090 strains lysogenic for λ 200 and carrying a p177cI derivative or p184cI-GCN4. The λ 200 prophage has the immunity region of P21 and carries a P_R-*lacZ* reporter gene (27). Repression of *lacZ* expression by cI-GCN4 was assayed in Y200, because its expression plasmid carries the same resistance marker (Cm^R) as pNNP_L387. Repressor activity by cI fusion proteins was identical in these two strains (Fig. 5 and data not shown).

Fold repression values were calculated as the ratio of β -gal expression in a strain carrying the empty vector over that from a strain carrying the given cI repressor-expressing vector. Derepression was calculated as the ratio of β -gal activity from a strain expressing cI repressor plus a cDNA-expressing plasmid over that carrying cI repressor plus the empty vector [pSK(+)].

cDNA expression library screen

Strain 10B18 is the Y1090 derivative carrying pNNP_L387 and p177cI-BHLH/LZ. 10B18 was made competent for electroporation and transformed with a plasmid-based cDNA library made from human peripheral blood lymphocytes that had been transformed with Epstein-Barr virus (a generous gift of S. Elledge). There were 10^7 recombinants in this once-amplified library. The library was converted from a phage-base to a plasmid-base according to a published protocol (28); conversion was nearly 100%.

10B18 was electroporated on two separate occasions with 500 ng of library DNA. Cells were allowed to recover for 45 min at 37°C in SOC media and then plated on M9/0.2% mannitol with chloramphenicol (20 μ g/ml), kanamycin (30 μ g/ml), ampicillin (50 μ g/ml), IPTG (2 mM), and X-gal (0.004%). Approximately 3×10^6 transformants were obtained, of which ~500 were blue. Blue colonies were restreaked to isolate single colonies; these were then grown up and plasmid DNA was prepared and used to retransform 10B18 and plated as above.

Gel shift assay

Protein extracts were prepared from BL21(DE3) strains containing pSKcI-MycBHLH/LZ or pSKcI-MycBHLH 1 h after induction of T7 polymerase with IPTG (1 mM). Extract preparation, DNA binding, and gel shift assays were essentially those of Gaitanaris (29). The final reaction buffer contained 40 mM Tris, pH 7.5, 42 mM KCl, 1.6 mM MgCl₂, 5 mM CaCl₂, 0.5 mM DTT, 10% glycerol, 50 μ g/ml BSA, 0.2 mg/ml non-specific DNA and 0.04 mg/ml poly(dI-dC). Up to 60 μ g of extract were incubated with 1.0 ng of radiolabeled lambda O_R1 probe for 15 min at 0°C. In experiments where GST-Max was included, protein mixtures were incubated at 30°C for 20 min prior to incubation with the O_R1 probe. The O_R1 sequence is 5'TATCACCGCCAGAGG-TA3'. Competition experiments were performed with an excess of unlabeled human heat shock element (HSE) DNA (30), which is unrelated to the lambda operator or the λ P_L DNA fragment used in the pNNP_L387 reporter described above and which contains three lambda operators. The antibody used in gel shifts was 51F7G11, a monoclonal antibody to an epitope in the N-terminal domain of cI (31). This was kindly provided by R. T. Sauer. Gel shifts were performed in 5% polyacrylamide/0.5×TBE gels run at 10 V/cm at 4°C.

Mammalian tissue culture

Transient transfections of Balb/c 3T3 cells were done by calcium phosphate precipitation (32). The ratio of SV40-Myc and CMV-Max expression vectors that gave the greatest level of Myc-dependent transactivation was empirically determined (data not shown). The GAL4-VP16 expression vector was pBXG-VP16 (kindly provided by M. Ptashne). The Myc reporter contained four consensus Myc binding sites of the sequence 5'GGACCACGTGGTCT3' upstream of the human hsp70 TATA

A.

B.

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GGATCCTCTAAATACATTCAAATAAGTATCCGCTGATGACAAATAAC
Bam H I          -35       $\beta$ -lactamase promoter      -10

AACGGTAACAGAAATTGTGAGCGCTCACAATTTTGATCGATAGGAAA
Bst E II          lac operator          Cla I          S. D.

CTCGAGATG-[ $\lambda$  cI aa 1 - 112]-GGGTCTAGA
Xho I      Met1          Gly112-Xba I

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Figure 1. (A) Schematic of the c-Myc protein. The region between amino acids 355 and 439 contains the basic region and helix-loop-helix/leucine zipper (B-HLH/LZ), which correspond to the DNA binding and protein-protein interaction domains, respectively. The B-HLH/LZ region was used in fusions to the cI DNA binding domain. (B) Sequence of the expression cassette for lambda repressor cI derivatives. Shown is the sequence between the BamHI and XbaI sites in pUC18. The -35 and -10 elements of the β -lactamase promoter, the lac operator, Shine-Delgamo (S.D.) element and amino acids 1 and 112 of cI are highlighted. Myc sequences were inserted at the XbaI site.

box and CAT reporter gene; the GAL4 reporter contained five GAL4 sites and was otherwise identical. The SSRP1 C-terminal region was expressed from a CMV promoter. This expression construct was co-transfected with the above DNAs and with pXGH5, which expresses human growth hormone, as a control for transfection efficiency (32). Equivalent amounts of CMV and SV40 promoters were used in all experiments. All transfections were done in duplicate and experiments were repeated at least three times. CAT activity was determined in cell lysates by the phase extraction assay (32). Relative CAT expression corresponds to the ratio of CAT from cells transfected with activator plus empty vector divided by that of transfections in which the test construct was included.

RESULTS

We made chimeric repressors that contain the N-terminal DNA binding domain of cI fused to a heterologous dimerization domain which replaced the C-terminal dimerization domain of cI. We fused the C-terminal 85 amino acids of human c-Myc to the N-terminal 112 amino acids of cI (NcI). This region of the c-Myc protein contains the basic DNA binding region and helix-loop-helix/leucine zipper dimerization domains (B-HLH/LZ) (Fig. 1A) (33). The cI derivatives were expressed from the weak β -lactamase promoter (Fig. 1B) (20,21). Expression vectors were derivatives of pACYC177, a low copy number plasmid (~5–20) that confers kanamycin resistance (Kan^R), and which is compatible with colE1-based plasmids, such as pBR322 and pUC18 (22). By maintaining expression at a low level we avoided complications arising from the fact that NcI alone is able to bind DNA as a dimer when expressed at high levels in bacteria. Therefore, the low level of expression ensured that repressor activity was strictly dependent on dimerization mediated by an appropriate domain to which NcI was fused (Fig. 2A, see below).

Figure 2. (A) Schematic of cI repressor function. Wild-type lambda cI repressor binds as a dimer to lambda operator sequences and represses transcription. The N-terminal DNA binding domain of cI is unable to dimerize and therefore is inactive as a repressor. Fusion of the B-HLH/LZ domains of c-Myc to the N-terminus of cI creates a functional repressor protein. (B) Model of an expression screen based on specific interference with B-HLH/LZ dimerization. Using a *lacZ* reporter under the control of cI-Myc, an expression library could be screened for proteins able to interact with cI-Myc and derepress reporter expression.

Function of chimeric repressors was determined by their ability to prevent infection by phage lambda (see Materials and Methods, Dot plaque assay), and by their ability to repress expression of *lacZ* driven by the λP_L promoter. NcI alone was essentially inactive in this system (Fig. 3A, line 1); the two fold repression of β -gal activity is probably the result of a low level of NcI dimerization. In contrast, repression by cI-Myc was greater by three orders of magnitude, a level similar to that wild-type lambda cI (Fig. 3A, lines 2–3, and data not shown).

Parameters of a screen for proteins that interact with the Myc B-HLH/LZ domain of cI-Myc

Our objective was to screen a cDNA expression library for protein products that would inactivate the cI-Myc repressor through interaction with the Myc portion of the fusion. We used a *lacZ* reporter gene under the control of a lambda promoter that is repressed by cI-Myc, resulting in white or pale blue colonies on X-gal indicator plates. We anticipated that inactivation of cI-Myc following transformation with specific cDNAs would result in the appearance of blue colonies (Fig. 2B), thus allowing us to screen large numbers of cDNAs.

An essential aspect of such a screen is the ability to determine the specificity of the interaction between a cI chimera and a cDNA product. Therefore, we tested several different dimerization domains for whether they could restore repressor activity to

Regulation of Repressor Function Through Dimerization Domain Interactions.

A.

<u>Repressor Protein</u>	<u>P_L/lacZ: Rel. Exp.</u>	<u>(Repression)</u>	<u>λ Infection (pfu)</u>	
1. cI N Term.	Blue	1300	2.4 × 10⁵	Sensitive (10⁴)
2. cI-Myc (B-HLH/LZ)	White	1.0	3.0 × 10⁵	Resistant (10⁶)
3. cI-Myc (B-HLH)	White	0.4	8.0 × 10⁵	Resistant (10⁶)
4. cI-E2/5	White	0.08	4.0 × 10⁴	Resistant (10⁶)
5. cI-TRβ	White	3.0	9.0 × 10⁵	N.D.

B.

Repressor Protein	Competitor	Colony Color ($\pm P_L$ - <i>lacZ</i> Exp.)	PFU to infect ($\pm \lambda$ Resistance)
1. cI-Myc(B-HLH/LZ)	Control	Pale Blue	10^6
2.	Myc (B-HLH/LZ)	Blue	10^5
3.	Myc (B-HLH)	Blue	10^5
4.	Max	White	10^6
5. cI(I84V)-Myc(B-HLH/LZ)	Control	Blue	10^2
6.	Max	Pale Blue	10^3 - 10^4

Figure 3. (A) Heterologous dimerization domains can confer repressor function on the DNA binding domain of lambda cI. Repressor activity of the indicated fusion to cI is presented as measured by expression from the P_L -*lacZ* reporter on pNPN λ 387 and by resistance to lambda phage infection by DPA. A functional repressor protein expressed from a p177cI derivative inhibits *lacZ* expression, resulting in white colony color, and confers resistance to lambda infection to a high plaque forming unit (p.f.u.) dose of lambda phage relative to the cI N-terminus alone (line 1). The fusion proteins tested here contained the Myc B-HLH/LZ (line 2), the Myc B-HLH domain without the LZ (line 3), the B-HLH region of E2/5 (line 4), or the dimerization domain of thyroid hormone receptor β (TR β) (line 5). Fold repression was calculated as the average (three or more clones) β -gal activity of a strain carrying the empty vector divided by that of a strain expressing a given cI chimera. (B) Dimerization of cI-Myc can be altered by co-expression of interacting factors. Co-expression of cI-Myc with the Myc dimerization domains alone reduces cI-Myc repressor activity resulting in depression of *lacZ* and lower resistance to lambda infection (lines 2–3). Co-expression with Max resulted in slightly greater repression of *lacZ* (line 4). Max had the same enhancing effect on repression by cI(I84V)-Myc, which is a weaker repressor (compare lines 5 and 6). cI repressor expression was from a p177cI derivative and Myc dimerization domains and full-length Max were expressed from pUC18 and pSK(+), respectively. The controls correspond to the empty vectors in each case.

NcI in this system (Fig. 3A). Fusions containing the dimerization domains of E2/5, which is an HLH protein, and thyroid hormone receptor β (TR β) were able to confer a high level of resistance (up to 10^5 – 10^6 p.f.u.) in a dot plaque assay (DPA) and repress a P_L -*lacZ* reporter on the order of 10^3 – 10^4 -fold (Fig. 3A, lines 4–5). We tested cI fusions containing either the c-Myc B-HLH/LZ or the B-HLH. While both were highly functional, the B-HLH domain fusion was consistently a slightly better repressor than that with the B-HLH/LZ (Fig. 3A, compare lines 2 and 3).

Characterization of the screen with proteins known to interact with Myc

To address the feasibility of a screen for interacting factors, we asked whether we could inactivate the cI-Myc chimeric repressor

Figure 4. cI-Myc formed a higher order oligomer with Max, while retaining DNA binding activity. Bacterial extracts made from cells over-expressing either cI-Myc HLH/LZ or cI-Myc HLH were used in gel shift assays with a DNA probe containing the lambda O_{R1} operator sequence. Lanes 1–3 show the cI-Myc HLH/LZ shifted complex and a lower mobility complex the intensity of which increases with increasing amounts of GST-Max: 35 ng, 70 ng, or 140 ng, respectively. Addition of a monoclonal antibody against cI, in lane 4, abolishes both shifted complexes. Lane 5 shows that GST-Max alone is unable to shift the probe. Lanes 6 shows the cI-Myc HLH/LZ extract (13 μ g) plus GST-Max (0.7 μ g) complex, while lanes 7–9 show that the cI-Myc HLH extract (7.25 μ g, 29 μ g, or 58 μ g, respectively) plus GST-Max (0.7 μ g) do not form this complex. Lane 10 shows a lighter exposure of a cI-Myc HLH/LZ gel shift and lanes 11–14 show that this shifted complex is not competed by an excess of unrelated competitor DNA (HSE), but is competed by an excess of λ P_L DNA, which contains three lambda operators. The figure shows data from three separate gel shift reactions; the conditions of these reactions were identical.

by co-expressing the B-HLH or B-HLH/LZ domains of c-Myc with cI-Myc. We would expect that the B-HLH or B-HLH/LZ portions of c-Myc would form heterodimers with the same domains in cI-Myc, thus suppressing homodimerization and inactivating the repressors. We found that both Myc dimerization domain polypeptides, when expressed from pUC18 (a high copy plasmid), could interfere with cI-Myc function (Fig. 3B, lines 2–3). Interference could be seen both as an increase in β -gal expression resulting in more blue colonies on X-gal plates, and as a decrease in resistance to phage infection of ~ 3 orders of magnitude in a DPA. Both cI-Myc (B-HLH) and cI-Myc (B-HLH/LZ) could be impaired in their function by either the B-HLH or the B-HLH/LZ domains (data not shown). The activity of the cI-E2/5 repressor was not affected by expression of these domains (data not shown). These data established the feasibility of isolating peptides that impaired activity of the cI-Myc protein.

To further test the feasibility of a screen, we used Max, a B-HLH/LZ protein that has been shown to form stable complexes with c-Myc both *in vivo* and *in vitro* (11). Surprisingly, we found that co-expression of Max resulted in a modest increase in cI-Myc (B-HLH/LZ) repressor activity, instead of the expected decrease (Fig. 3B, line 4). This was not observed with the B-HLH fusion (data not shown). In order to confirm this observation, we substituted a mutation (I84V) into the cI DNA binding domain of cI-Myc that disrupts stabilizing dimeric interactions between the N-terminal domains of cI (34). This mutation reduced significantly the repressor activity of cI-Myc chimeras, as assayed by *lacZ* expression (colony color) and phage resistance (DPA). The activity of the weakened cI(I84V)-Myc was increased in these assays by the expression of Max (Fig. 3B, compare lines 5 and 6).

The observation that Max could increase repressor activity of the cI-Myc fusion proteins suggested that Max had an unexpected ability to interact with the intact cI-Myc dimer and increase its DNA binding activity. We confirmed that cI-Myc and Max could form a stable oligomer with increased DNA binding activity using the electrophoretic mobility shift assay (EMSA). We prepared extracts from bacteria expressing cI-Myc (B-HLH/LZ and B-HLH) and tested the effect of adding purified GST-Max protein on cI gel shift activity with a DNA probe containing the lambda O_{R1} operator sequence. Extracts containing cI-Myc were able to shift the mobility of the lambda operator probe (Fig. 4, lane 10). The gel shift reactions shown in lanes 10–14 of Figure 4 contained cI-Myc B-HLH/LZ extracts; cI-Myc B-HLH produced an essentially identical shifted complex (Fig. 4, lanes 7–9 and data not shown).

Incubation of cI-Myc B-HLH/LZ-containing extracts with increasing amounts of GST-Max resulted in a more dramatic shift in probe mobility (Fig. 4, lanes 1–3). This complex and the cI-Myc complex running closer to the probe were both disrupted by the addition of a monoclonal antibody against an epitope in the N-terminal domain of cI (31) (Fig. 4, lane 4), arguing that both complexes contain cI-Myc. As expected, GST-Max alone did not shift probe mobility (Fig. 4, lane 5). In contrast to the B-HLH/LZ fusion, cI-Myc B-HLH-containing extracts were unable to produce a slow migrating complex in the presence of GST-Max (Fig. 4, lanes 7–9). Figure 4, lane 9 shows that using nearly 20-fold more cI-Myc B-HLH extract (58 μ g) than that of cI-Myc B-HLH/LZ (3 μ g) used in lane 1 did not yield a complex with GST-Max. The abundance of active cI-Myc in the extract was apparent from the presence of the cI-Myc shifted complex;

Repressor Protein	P_L lacZ		De-Repression
	Clone 10 (-)	Clone 10 (+)	
1. cI-Myc (B-HLH/LZ)	White	Blue	8.5
2. cI-Myc (B-HLH)	White	Blue	7.7
3. cI-E2/5	White	White	1.7
4. cI-TR β	White	White	N.D.
5. cI-GCN4	White	White	1.2

Figure 5. The clone isolated in a screen for proteins able to interact with cI-Myc was specific for the Myc dimerization domains. Co-expression of clone 10 with either cI-Myc fusion resulted in derepression of a *lacZ* reporter. Co-expression with related or unrelated fusions had no effect on the repressor activity of those fusions. Interference with cI-Myc function by clone 10 was dependent on expression in the sense (+) orientation. Derepression was calculated as the average (three or more clones) β -gal expression in strains carrying a cI repressor and clone 10 (+) divided by that of strains carrying the cI repressor plus SK(+) vector.

this shift was more readily distinguishable from the free probe on a lighter exposure (data not shown). The specificity of the cI-Myc B-HLH/LZ complex was demonstrated by the inability of an excess of unrelated HSE DNA (30) to disrupt the labeled O_{R1} -containing complex (Fig. 4, lanes 11–12). In contrast, this complex was abolished by the inclusion of an excess of λP_L DNA, which contains three cI binding sites (Fig. 4, lanes 13–14). We have demonstrated similar specific binding of cI-Myc B-HLH extract as well (data not shown).

These *in vitro* data were consistent with our observations in bacteria that Max could increase the repressor activity of cI-Myc B-HLH/LZ, but not cI-Myc B-HLH, and suggested that Myc and Max were able to form a higher order oligomer, perhaps a tetramer, which was more stable and had greater repressor activity than the cI-Myc homodimer. The effects of Myc and Max on cI-Myc demonstrated that repressor activity could be modulated by the expression of heterologous interacting factors. Furthermore, the effects that were seen in our bacterial strains appeared to reflect direct interaction as assayed by *in vitro* means. While these data indicated that this methodology might enable us to isolate factors capable of disrupting cI-Myc dimers, clearly another class of interacting factors, exemplified by Max, would go undetected in this screen.

Isolation of a cDNA that encodes a factor that interacts with cI-Myc

We used the cI-Myc repressor in a screen to identify novel cDNAs encoding proteins that could interact with the B-HLH/LZ domains of c-Myc. We screened for cDNAs whose expression would interfere with cI-Myc function and derepress a P_L -*lacZ* reporter. We used an *E. coli* strain (Y1090) (Promega) which is mutant for the Lon protease and which, therefore, may be better for the expression of heterologous proteins (35). We introduced into this strain the single copy reporter plasmid pNNP_L387, which carries the *lacZ* gene under the control of the lambda P_L and a chloramphenicol resistance (Cm^R) marker (25), and a pACYC177 derivative which expresses cI-MycB-HLH/LZ. This strain was referred to as 10B18 and formed white to pale blue colonies on X-gal plates.

Strain 10B18 was electroporated with a pBR322-based cDNA library (kindly provided by S. Elledge) made with mRNA from Epstein-Barr virus-transformed human peripheral blood lymphocytes. A total of 3×10^6 transformants were screened for blue colonies; ~500 dark blue colonies were found and these were restreaked to isolate individual blue colonies. 97 blue clonal colonies were isolated and plasmid DNA was isolated from these for retransformation of 10B18. Only one clone (designated #10) reproducibly caused colonies to turn blue. We have not investigated the larger number of colonies in the screen that were moderately blue, but darker than the parental strain. The high number of false positives which appeared blue in the initial screen could have been due to an increase in copy number of the P_L -*lacZ* reporter plasmid titrating out repressor dimers or instability of the cI-Myc-expressing plasmid.

The clone 10 cDNA was subcloned into pSK(+) and retested for specificity. Transformants of 10B18 with pSK10 showed a blue phenotype on X-gal plates only when the cDNA was in the same orientation as the *lac* promoter on pSK(+); transformants with the opposite orientation plasmid remained white (Fig. 5, lines 1–2). The ability of clone 10 to turn colonies blue was specific for strains in which the *lacZ* gene was repressed by cI-Myc fusions; strains containing cI fused to either the B-HLH or B-HLH/LZ domains showed reduced repressor activity (derepression). Transformation with pSK10 into strains isogenic to those expressing cI-Myc fusions in which the only difference was that the repressor contained the dimerization domain of E2/5, TR β , or GCN4 remained white on X-gal plates (Fig. 5, lines 3–5), indicating no effect on the repressor activity of these chimeras. Therefore, clone 10 interfered specifically with cI-Myc function in a manner that required the c-Myc portion of the chimeric molecule.

The specificity of the interference by clone 10 was demonstrated further in quantitative assays of β -gal expression (Fig. 5). Assays were performed in the above strain using the pNNP_L387 reporter and in a λ 200 lysogen of Y1090 (Y200), which carries an integrated P_R -*lacZ* reporter gene. Y200 was used to test the effect of clone 10 on cI-GCN4 activity, since the cI-GCN4 expression plasmid and pNNP_L387 both carry Cm^R markers. Repression by each of the cI fusions that we have tested is similar for Y200 and Y1090 derivatives carrying pNNP_L387; the specific inhibitory effect of clone 10 on cI-Myc function was essentially the same in both strains, as well (Fig. 5 and data not shown). As measured in β -gal assays, clone 10 had only a minimal effect on cI-E2/5 and cI-GCN4 repressor activity, whereas parallel cultures expressing cI-Myc chimeras were derepressed ~8-fold (Fig. 5).

Clone 10 expresses a portion of the HMG domain protein SSRP1

We sequenced the insert in clone 10, and found that it encoded the C-terminal 304 amino acids of the human SSRP1 gene (36). The predicted protein encoded by the clone 10 cDNA (SSRP-C) contains an HMG-box DNA binding domain and is very highly charged. The full length SSRP1 gene encodes a protein of 709 amino acids, of which the only recognizable functional domain is the HMG-box. The human SSRP1 gene was isolated based on the ability of the encoded protein to bind to a cisplatin-DNA adduct; in addition, a murine clone was isolated based on binding to V(D)J recombination signal sequences (36,37).

Figure 6. The interacting clone, SSRP-C, impaired transactivation by Myc in transfected mammalian cells. SSRP-C-mediated dose-dependent repression of a CAT reporter expressed from a minimal promoter containing four Myc binding sites. The effects of SSRP-C were assayed in co-transfections with Myc and Max; the latter was included to facilitate observation of activation by transfected Myc by lowering reporter expression mediated by endogenous Myc. SSRP-C had no effect on expression mediated by GAL4-VP16 from a similar reporter containing five GAL4 sites.

Clone 10 could interact specifically with cI-Myc in bacteria; we were therefore interested in determining whether it could alter the function of intact c-Myc in mammalian cells. Myc is able to modestly activate expression from reporter constructs that contain Myc binding sites when appropriate levels of Max are also produced in mammalian cells (18,38). We established conditions in which we could observe activation by Myc (~3-fold activation), and tested the effect of co-transfecting the C-terminal portion of SSRP1. We found that the SSRP-C cDNA had a repressive effect on transactivation by c-Myc in Balb/c 3T3 tissue culture cells (Fig. 6). SSRP-C caused a 2-fold inhibition when it was co-transfected with a c-Myc-expressing plasmid, and thus could repress most of the 3-fold *trans*-activation activity that was dependent on Myc expression. SSRP-C had no effect on activation of a GAL4 reporter by GAL4-VP16 (Fig. 6). All transfections were performed with 5 µg of a mixture of the SSRP1-C expression construct and the empty vector, and repression levels were dependent on the presence of the insert (Fig. 6). Therefore, SSRP-C was able modulate c-Myc function both in our bacterial assay and in mammalian tissue culture cells.

DISCUSSION

We describe a system with which to screen cDNA expression libraries for proteins that can interact with a tester dimerization domain fused to the lambda repressor DNA binding domain. Dimerization of a chimeric lambda repressor enables the protein to bind to lambda operators and repress gene expression. The expression of a cDNA-encoded protein which is able to interfere with dimerization and thereby derepress a reporter gene provides a means of screening for cDNAs encoding interacting factors.

We found that this system could be utilized with the HLH/LZ dimerization domains of c-Myc. Our observation of a visible phenotype resulting from interference with dimerization of a chimeric repressor suggested the feasibility of a cDNA library screen based on the ability of encoded proteins to mediate such interference. The observation that Max had the opposite effect on repressor dimerization, namely to stabilize cI-Myc, indicated a limitation of this method of screening. This screen would not

detect factors that could interact with a dimerization domain but could not interfere with its function.

We used cI-Myc in a screen and isolated a clone which appeared to interact specifically with the Myc HLH/LZ domains and with none of the other dimerization domains that we tested. This clone was found to be identical to the C-terminal region of the SSRP1 gene and contains an HMG DNA binding domain and is very highly charged. We did not isolate any c-Myc-encoding cDNAs. This is most likely to be because we picked only colonies that were dark blue in color, whereas we had found colonies in which cI-Myc and Myc polypeptides were co-expressed were qualitatively less blue. Although we observed many colonies that were of an intermediate blue color, we did not investigate these due to their large number. It is possible that a Myc cDNA may have been present in that class of clones.

A shortcoming of the screen as we have used it is the high number of false positives. One likely means of generating blue colonies in this system is by loss of copy number control of the reporter plasmid resulting in relief of repression by titration of the cI chimera; a straightforward means of overcoming this problem is to use an integrated reporter such as a λ200 prophage (see above). We found that such a reporter was indistinguishable from the plasmid reporter with respect to regulation by cI chimeras (Fig. 5 and data not shown). A second possible shortcoming of the screen is that eukaryotic proteins might require post-translational modification to interact, and that modification might not occur in bacteria.

The clone that we isolated (SSRP-C) showed a phenotype in bacteria indicative of an interaction with cI-Myc. Despite this clear result, we have been unable to demonstrate a direct interaction by other means. We fused the Myc dimerization domains to LexA and SSRP-C to an activation domain (3), and did not find evidence for an interaction as measured by the yeast two-hybrid system (data not shown). We also tried to demonstrate an interaction by cross-linking and immunoprecipitation experiments with *in vitro*-translated proteins, as well as by gel shift assay. These methods also did not detect an interaction between Myc and SSRP-C (data not shown). A possible explanation for these negative results is that a weak interaction between these

proteins might not occur at the low concentration of protein produced by *in vitro* translation. However, in transfection experiments (Fig. 6), we did observe an effect of SSRP-C on c-Myc function. It is therefore possible that the nature of the interaction between SSRP and Myc is such that it is not detectable by some assays, and that some aspect of the bacterial screening system enabled the detection of a transient or weak interaction.

The interaction that we have observed between Myc and SSRP1 has precedents in that there are numerous examples of important regulatory interactions between HMG domain-containing proteins and other transcription factors (reviewed in 39). LEF-1 is a lymphoid-specific, HMG domain transcription factor that activates transcription in part by bending sharply the DNA to which it is bound; this is thought to bring the factors bound adjacently into close proximity with each other and with LEF-1, perhaps promoting the formation of a stable complex (39–41). HMG2, which does not bind DNA sequence-specifically, appears to interact with components of the pre-initiation complex and/or pol II to regulate transcription (42). The pol I transcription factor UBF interacts with SL-1 such that DNA binding by these factors is cooperative; this interaction is mediated by HMG domains 3 and 4 of UBF (43). While the functional significance of the interaction between Myc and SSRP1 is not yet apparent, it is possible that this interaction has a role in transcriptional regulation by Myc of its target genes. A test of this hypothesis requires isolation of *in vivo* targets for Myc regulation.

An interaction between Myc and SSRP1 could regulate the function of SSRP1 as well. The function of SSRP1 is not yet understood. The observation that it can bind to kinked DNA, such as that of a cisplatin–DNA adduct, does not indicate the nature of its physiological role in the cell. An intriguing observation, which would be consistent with an effect of Myc on SSRP1 function, is that there is a correlation between high levels of Myc expression and increased resistance of cells to cisplatin (44). This may be a reflection of Myc altering the function of SSRP1 to enhance or impair binding to cisplatin–DNA adducts.

The cloning system that we have described and utilized with c-Myc may be applicable to the identification of protein–protein interactions involving various dimerization domains tested as fusions to the lambda repressor DNA binding domain. The observation that different types of dimerization domain are functional as fusions to cI (see Fig. 2) suggests that this screening method may be applicable to other dimerizing proteins. Furthermore, it is possible that proteins that specifically heterodimerize could be used in such a screen by co-expressing cI fusions that could form heterodimers; such heteromeric cI fusions should be functional as repressors as well.

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