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The biological activities of the v-Myc oncoprotein, including cellular transformation, transcription regulation and apoptosis, depend on the integrity of the v-Myc amino terminus. Analysis of this region using deletion mutagenesis has yielded interesting results. In addition to further defining regions of the amino terminus required for transcriptional activation, a potential inhibitory region of v-Myc transactivation has been identified. New data suggest that the ability of individual deletions to function in transcription assays is not due to different levels of protein expression, supporting an actual difference in the transcriptional abilities of the novel proteins generated by these deletions. The identification and characterization of a protein that specifically interacts with a region of the v-Myc amino terminus has suggested another form of regulation of the v-Myc oncoprotein. This protein, termed OS-9, interacts with a highly conserved and functionally important region of v-Myc called Myc Homology Region II. While no effect of OS-9 has been observed on cellular transformation mediated by v-Myc, OS-9 does impact v-Myc transcriptional activation. Transcriptional activity of the v-Myc activation domain is reduced by nearly 75% when OS-9 is overexpressed in cells. This repression does not extend to other activators -- for example, the activation domain of MyoD is unaffected by OS-9. These data point to the interaction of OS-9 with Myc as being important in regulating the acivities of v-Myc as a transcription factor.

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FOREWORD

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Elizabeth J. Japanowsky 6/2/97

PI - Signature Date

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INTRODUCTION

The Myc oncoprotein is a nuclear transcription factor containing both an amino-terminal transcription activation domain (TAD) and a carboxy-terminal basic helix-loop-helix/ leucine zipper (bHLH/LZ) motif (Henriksson and Luscher, 1996). Work by many others has demonstrated that Myc heterodimerizes with another nuclear protein Max via their HLH/LZ regions (Blackwood and Eisenman, 1991) and that these heterodimers bind specifically to a DNA sequence know as an E-box (-CACGTG-, Blackwell *et al.,* 1990).

The amino-terminal TAD is important for Myc's ability to activate transcription, and it is also necessary for many of Myc's other activities, including cellular transformation (Stone *et al.,* 1987), transcriptional repression (Li *et al.,* 1994) and apoptosis (Evan *et al.,* 1992). There is a region of high conservation across all Myc family members known as Myc Homology Region II (MHR II) that is located within the amino terminus of the Myc proteins. Because of the many biological activities associated with the amino terminus of Myc and the presence of such a well-conserved domain in the region, much effort by our lab and others has been expended to identify factors that could interact with this small region of the v-Myc TAD. It is believed that such factors could mediate the functions of the Myc oncoprotein under various cellular conditions.

A former postdoctoral fellow in the lab, Dr. Michael Dorsey, screened a human cDNA library using the yeast two-hybrid system to identify cellular factors that specifically associate with v-Myc MHR II. It was shown that OS-9, a protein encoded by a gene amplified in several human osteosarcomas (Su *et al.*, 1996), interacts with MHR II but not with a mutant form of MHR II, indicating the specificity of the interaction. Since this v-Myc-interacting protein is implicated in tumorigenesis, and because of Myc's known involvement in many malignancies, characterization of the OS-9/ Myc association was begun. It has been my primary goal to investigate the effects of OS-9 overexpression in cells and what effect this has on Myc-mediated activities.

BODY

As outlined in the Revised Statement of Work (October 28, 1996), quantification of protein expression of the previously reported GAL4-v-Myc amino terminal deletions will determine if the differences noted in transcriptional activity among the deletions reflects a true difference in transactivation potential or merely is the result of unequal expression of the fusions. Figure 1 reviews some of the GAL4-v-Myc deletions presented in earlier reports. Fig. 1A depicts residues remaining in the v-Myc amino terminal transcription activation domain (TAD) after deletion mutagenesis was performed. Transcriptional activity of these v-Myc TAD deletions, when fused to the heterologous DNA binding domain of the yeast protein GAL4 and assayed on a chloramphenicol acetyl transferase (CAT) reporter gene, is presented in Figure 1B. Of particular concern are the differences in transcriptional activity between similar 5' v-Myc deletions that extend to either residue 219 or 244 of the TAD (i.e., between GAL4-v-Myc(12--244) and GAL4-v-Myc(12--219)). To examine the level of protein expression of these fusions, COS-1, a cell line established from African green monkey kidney cells using SV40 T antigen, were transiently transfected with selected GAL4-v-Myc deletions, and whole cell extracts were prepared. Figure 2 shows Western blot analysis of these extracts using a monoclonal antibody against the DNA binding domain of GAL4. Both 5' and 3' GAL4-v-Myc deletions appear to be expressed at similar levels. The different 5' GAL4-v-Myc fusions, i.e., GAL4-v-Myc(12--244) and Gal4-v-Myc(12--219), are produced equivalently. Therefore, the large variation in CAT activity observed using these two v-Myc variants is not due to protein instability or degradation.

To finish my analysis of the v-Myc TAD, the DNA binding ability of the GAL4-v-Myc deletion constructs must be assesed. Since Western blots worked well with COS-1 cells, nuclear extracts from COS-1 cells transiently transfected with the selected GAL-4-v-Myc deletions have already been prepared. These extracts will be incubated with a ^{32}P-labeled probe containing a GAL4 protein binding site and EMSA performed to assess whether the differences in CAT activity could be due to the inability of certain fusion proteins to bind to DNA. The inhibitory action of v-Myc residues 219--244 will be further analyzed (beyond any potential differences in DNA binding ability) by expressing this small region of the v-Myc TAD fused to a strong transactivator such as GAL4-VP16. If an effect of transcriptional activity is observed on GAL4-VP16, then residues 219--244 can be considered a transferable repression domain. Interestingly, this region of v-Myc contains a highly charged strectch of amino acids, and high charge is a feature common to many "repression motifs" (Hanna-Rose and Hansen, 1996). Another experiment to address the

role of residues 219--244 on the function of the Myc protein is to delete this region from full-length v-Myc. This novel Myc protein will then be tested for TAD activity and transformation ability and compared with its full-length counterpart.

Figure 3 depicts the amino acid sequence of the v-Myc amino terminus-interacting protein, OS-9 (formerly referred to as TRC). This protein was identified by our lab in a yeast two-hybrid screen designed to isolate proteins that could interact with a highly conserved region of the Myc TAD, MHR II. The gene encoding OS-9 has recently been shown to map to a region of human chromosome 12 which is amplified in several human sarcomas (Su *et al.*, 1996). Immunofluorescence studies by Dr. Michael Dorsey to determine the cellular location of OS-9 have shown that the OS-9 protein is found predominantly in the cytoplasm (Dorsey *et al.*, submitted). However, a truncated version of the protein, OS-9 (296-667), which lacks the amino terminal half of the protein (see Figure 3), is able to localize to the nucleus. This result indicates that within the carboxy terminal half of the OS-9 protein there is a functional nuclear localization signal which potentially could be used under yet to be identified cellular conditions.

To address if OS-9 has any effect on the function of v-Myc, the functions ascribed to MHR II were considered. MHR II is essential for Myc-mediated cellular transformation, for transcriptional repression through INRs and for Myc's ability to induce apoptosis. The effect of OS-9 on cellular transformation was analyzed first. Figure 4 reports the results of stable transfections in C3H10T1/2 cells. Three different OS-9 clones were utilized: full-length OS-9 (1-667) and two amino-terminal deletions of OS-9, (141-667) and (296-667) {Figure 3 indicates the sequences removed by these deletions}. Focus formation by activated human H-Ras and v-Myc was set at 100%. Very little effect on transformation was observed when any of the OS-9 clones were coexpressed with activated Ras alone or with Ras and Myc. Therefore, I conclude that OS-9 has no measurable effect on cellular transformation in this assay system.

An important property of the Myc amino terminus that I have been investigating is transcriptional activation. To examine if OS-9 is involved in this aspect of Myc function, C3H10T1/2 cells were transiently transfected with various GAL4 activators, the $(GAL4)_5$ E1B TATA CAT reporter, and either the pECE vector alone or full-length OS-9 in the pECE vector. As can be seen in Figure 5, a two-fold molar excess of OS-9 has a profound effect on transcriptional activity by v-Myc. Both the v-Myc (and c-Myc) TADs are repressed in their ability to activate transcription from the GAL4-CAT reporter by OS-9. A smaller region of the v-Myc TAD, residues 90--219 (which contains MHR II), is also repressed by OS-9, whereas v-Myc residues -32--42 are not. This is in agreement with *in vitro* binding studies performed by Dr. Michael Dorsey which indicate that OS-9 can bind

to residues 90--219 but not to residues -32--42 (Ref). An interesting observation I have made is the repression of the transcriptional activity of the VP16 TAD by OS-9. This supports previous data from the lab suggesting that the TADs of v-Myc and VP16 interact with common cellular factors necessary for the function of these two proteins (Min *et al.*, 1994). Neither the activation domains of E1A or MyoD are affected by the presence of OS-9, indicating that OS-9 does not have a repressive effect on transcription activation domains in general. In order to ensure that the repression observed was not a feature associated with GAL4-DB-fusion proteins, the LexA reporter system was tested. The v-Myc TAD was fused downstream of the LexA DNA binding domain, and transcriptional activity of this construct was measured on the $(LexA)_6$ E1B TATA CAT reporter. As can be seen in Figure 6, OS-9 represses activation of the v-Myc TAD when fused to the LexA DNA binding domain as well. I conclude from these experiments that the transcriptional activation properties of Myc (and another similar activator, VP16) are reduced by OS-9 and that this reduction is not due to a general repression of transcription or due to the reporter system used.

In order for the interaction of OS-9 with the v-Myc TAD to be physiologically revelant, it is important to demonstrate an effect of OS-9 on the function of full-length Myc. All previous activation experiments were performed with only the amino terminus of v-Myc or smaller regions of the amino terminus. Figure 7 shows the effect of OS-9 on the transcriptional properties of full-length Myc on a Myc-responsive reporter, $p(MBS)_3$ TK CAT. A high background level of activity is observed with the reporter alone, presumably due to endogenous Myc. This level of activity is reduced when OS-9 is present. The same phenomenon is seen with the pTK CAT reporter, which supports data that Myc upregulates the TK promoter (Min *et al.*, 1994). A measurable increase in CAT activity is seen when Myc is assayed with the reporter alone (however, all samples in Figure 7 tested without co-expression of OS-9 are set at 100% to indicate the strength of repression by OS-9). The activation by full-length Myc is greatly reduced when OS-9 is co-expressed with Myc. Since full-length Myc is affected by OS-9, the interaction of these two proteins could have physiological significance.

Another function of MHR II is its involvement in Myc-mediated repression of certain promoters that contains INRs. Our lab has recently identified a gene that is downregulated by v-Myc, the adrenomedullin precursor gene, or AM (X. Wang and E.J. Taparowsky, unpublished observations). Analysis of the promoter of the AM gene has yielded the presence of both a TATA box and an INR. Since Myc does have an effect on this promoter, a 2 kb 5' flanking sequence of AM was inserted upstream of the CAT gene to create the AM-CAT reporter gene. This reporter was tested in the presence of both Myc

and Myc plus OS-9 to see if OS-9 also affected Myc's ability to repress transcription. Figure 8 shows that this is indeed the case. An approximate 60% reduction in CAT activity was observed when Myc was co-transfected with AM-CAT. In the presence of Myc plus OS-9, activity of AM-CAT was reduced by 95%. OS-9 alone does have an effect on the activity of AM-CAT; however, this result is not surprising since OS-9 appears to influence endogenous Myc on other reporter tested also. Co-transfection of an unrelated protein (B-ATF) with AM-CAT and v-Myc does not enhance Myc's repressive activity, indicating that the OS-9 observation is specific. Figure 9 represents an additional control of OS-9 activity. The activity of the β-actin-CAT reporter in the presence of OS-9 and OS-9 plus v-Myc was examined. Experimentally, a slight but measurable decrease in β-actin-CAT activity is seen when v-Myc is co-expressed in cells. However, OS-9 does not enhance this Myc-mediated event, nor does OS-9 affect the β–actin-CAT reporter alone. It seems that the effect of OS-9 on v-Myc is only applicable to revelant targets of Myc, such as reporters that contain Myc protein binding sites (E-boxes). To support this hypothesis, preliminary results using other Myc-responsive promoters as reporters indicate OS-9 can repress Myc function at naturally-occurring E-boxes. Both the ornithine decarboxylase promoter (ODCΔCAT, Bello-Fernandez *et al.* 1993) and the murine p53 promoter (mu p53 CAT, Roy *et al.* 1994) are activated by Myc. This activation by Myc is repressed significantly by co-expression of OS-9 (data not shown). In our experiments, therefore, both the activation and repression functions of the Myc amino terminus are affected by OS-9.

As a further way to characterize the interaction of Myc and OS-9, an artificial reporter containing both LexA and GAL4 protein binding sites was utilized ($p(LexA)_8(GAL4)_5$ CAT, Hollenberg *et al.*, 1995). Activators fused to the LexA DNA binding domain activate this reporter (i.e., LexA-v-Myc, LexA-VP16, etc.). Figure 10 demonstrates when expressed as a GAL4-DB fusion protein, OS-9 represses all activators tested. This implies that the interaction of OS-9 with Myc is needed to target it to a promoter, where OS-9 then can influence other factors, such as the basal transctiption factor machinery.

Finally, the effect of OS-9 on cellular apoptosis is beginning to be examined. Myc neo 13A, a cell line that expresses the v-Myc protein (Davenport and Taparowsky, 1992) was stably transfected with 1 μg pECE-OS-9 and 100 ng pBABE-puro vector (which contains the gene coding for puromycin resistance). Colonies resistant to both Geneticin and puromycin were selected and further analyzed. Whole cell extracts of Myc neo 13A

cells and OS-9 clone 5 were harvested and used for Western blotting. Incubation with anti-HA antibody indicates the presence of the OS-9 protein (which is tagged with the HA epitope), and incubation with an anti-gag antibody illustrates continued production of the gag-Myc fusion protein (Figure 11, panels A and B). OS-9 clone 5 and the parental Myc neo 13A cells were then grown in both high serum (10%) and low serum (0.5%), and their growth rates determined. As shown in Figure 11 panel C, OS-9 clone 5 does not grow as well as the Myc neo 13A cells in high serum and does not reach the same saturation density at the end of the counting period. In low serum, however, OS-9 clone 5 is slightly more vigorous and grows to a higher final density. (Four independent trials of cell counts were averaged in Figure 11. The data in the low serum graph are represented as a fitted curve for ease of interpretation.) Since OS-9 does appear to affect the growth of cells in which it is expressed, further studies are underway.

C3H10T1/2, Myc neo 13A, and OS-9 clone 5 cells will be induced to undergo apoptosis by exposure to staurosporine, an inhibitor of phospholipid/calcium-dependent protein kinases. Myc neo 13A cells do undergo apoptosis under these conditions (R. Wang, unpublished observations). Apoptosis will be determined by analysis of genomic DNA prepared from the cells. In cells undergoing apoptosis, internucleosomal DNA is degraded, and this can be visualized by the appearance of a "DNA ladder" on an agarose gel. These experiments will determine if OS-9 has an effect on Myc-mediated apoptosis.

Last, an *in vitro* analysis of ternary complex formation by OS-9 will be performed. Using *in vitro* translated GAL4-v-Myc TAD plus or minus *in vitro* translated OS-9 protein (HA-tagged), EMSA will be performed with a ^{32}P-labeled GAL4 protein binding site probe. It is possible, however, that the interaction of OS-9 and Myc may not be strong enough to detect this way, or the binding of OS-9 to Myc could be stabilized by other proteins in cells. If this is the case, nuclear extracts will be prepared from cells co-transfected with full-length Myc, Max, and the pECE vector alone or OS-9 in the pECE vector. Since OS-9 affects Myc function at the E-box, Myc's heterodimerization partner Max will be included in the transfection. EMSA will then be performed using a ^{32}P-labeled probe containing the Myc binding site.

CONCLUSIONS

The examination of the impact of OS-9 on v-Myc function has yielded interesting results. Repression of transcriptional activity of v-Myc by OS-9 appears to be specific to regions that interact, at least *in vitro*, with OS-9. This repression by OS-9 is not due to repression of transcription in general or a result of the reporter system used. Indeed, both the GAL4- and LexA-v-Myc fusions are repressed by OS-9. Additionally, reporters that utilize naturally-occurring Myc-responsive elements (the E-box) are also inhibited by the overexpression of OS-9. This result suggests that there is a physiological relevance to the interaction of Myc and OS-9.

Since OS-9 is amplified in some human osteosarcomas, it was surprising that OS-9 did not impact Myc-mediated cellular transformation and that OS-9 seems to function as a negative regulator of Myc. However, it is possible that OS-9 acts to attenuate Myc's activities to avoid cellular death via apoptosis. In the artificial reporter systems used, OS-9 represses Myc transcriptional activity broadly. In the cell, however, perhaps OS-9 functions more subtly, reducing the deleterious effects of Myc activation or amplification that would, under normal circumstances, target the cell for destruction before a malignancy could develop. Preliminary evidence suggests this could be true, as seen by the slowed cellular growth in high serum of OS-9 clone 5 cells versus Myc neo 13A cells, and in low serum by the slightly enhanced survival of OS-9 clone 5 versus Myc neo 13A cells. It is very important to continue the characterization of OS-9/Myc interaction to understand the impact this protein association has on cellular growth and death.

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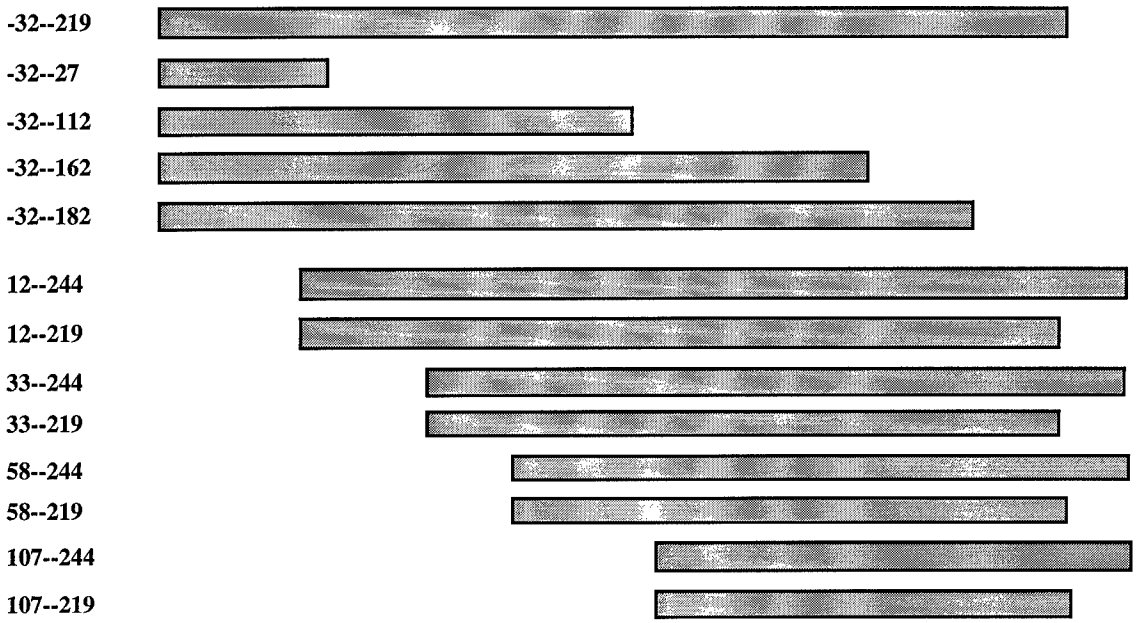
A. Deletions of the v-Myc Amino Terminus



B. Transcriptional Activity of the GAL4-v-Myc Deletions

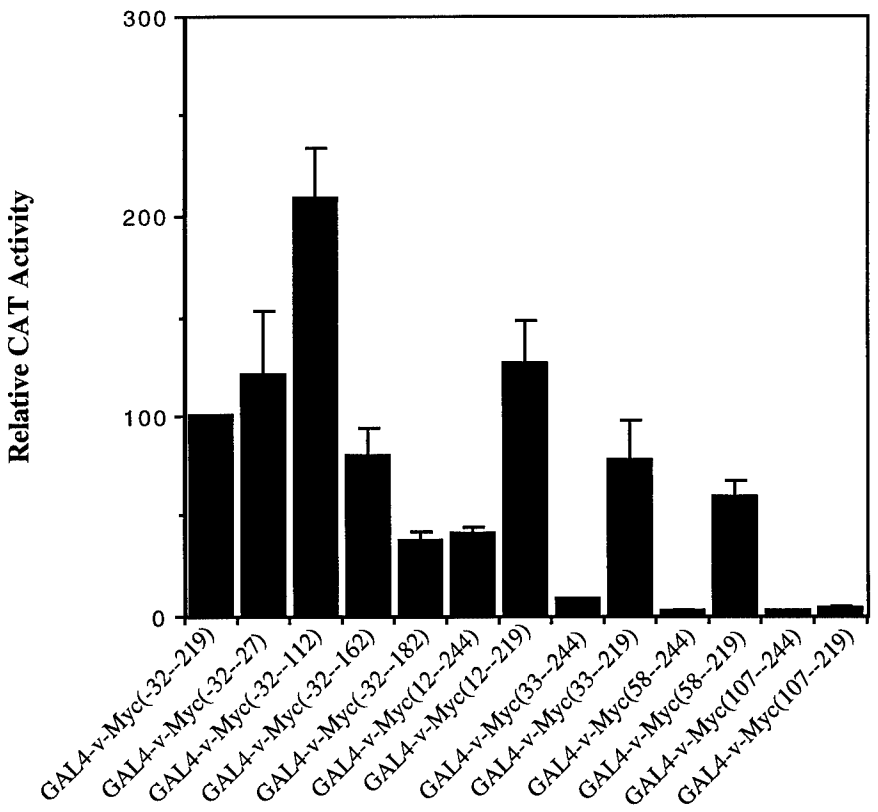


Figure 1. Selected v-Myc amino terminal deletions and their transcriptional activities as GAL4 fusion proteins.

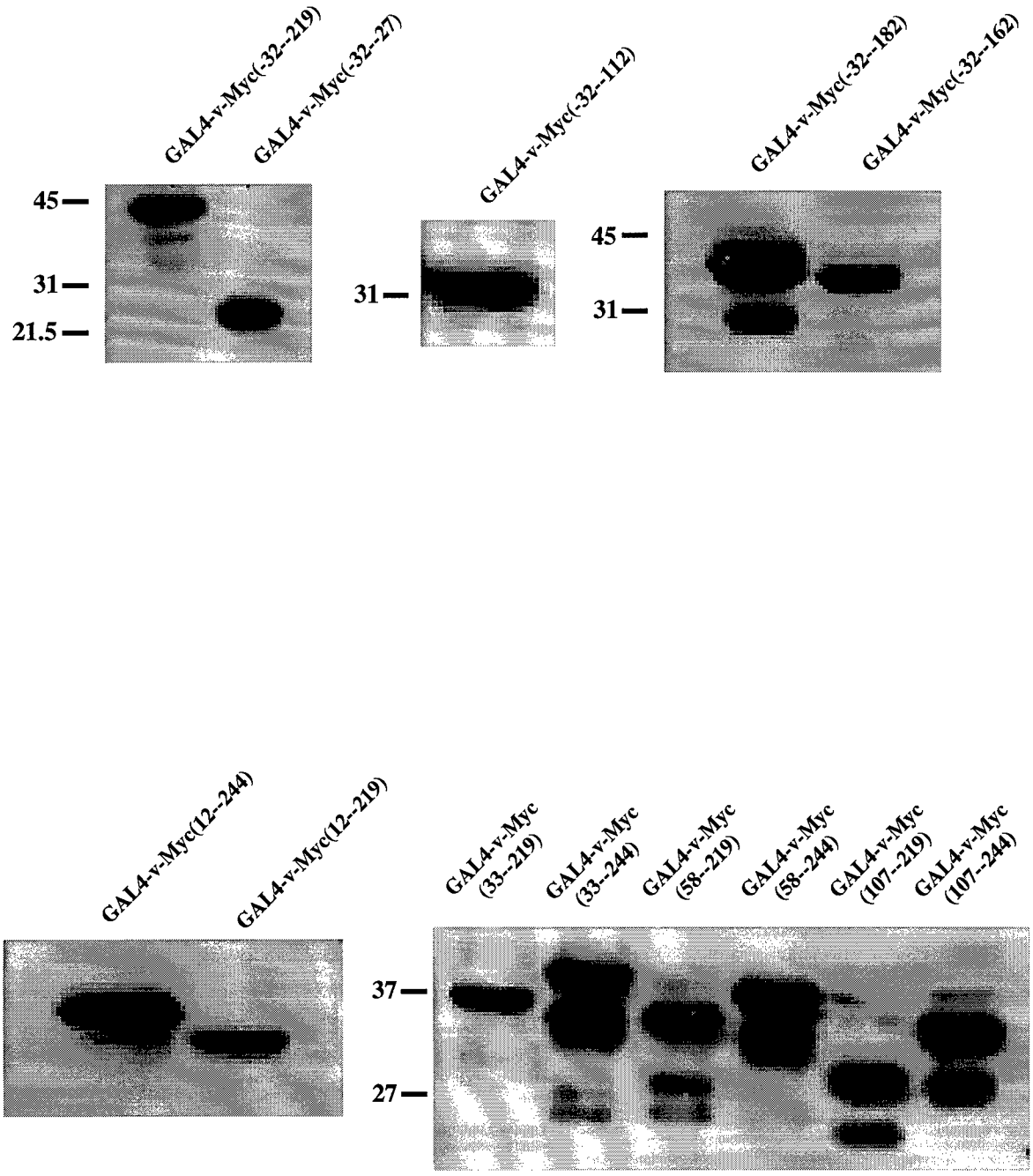


Figure 2. Western blot analysis of selected GAL4-v-Myc amino terminal deletions.

potential signal sequence

MAAETLLSSLLGLLLLGLLLPASLTGGVGSLNLEELSEMRYGIEILPLPVMGGQS 55

QSSDVVIVSSKYKQRYE**C**RLPAGAIHFQREREEETPAYQGPGIPELLSPMRDAP**C** 110

OS-9 (141-667)

LLKTKDWWTYEF**C**YGRHIQQYHMEDSEIKGEVLYLGYYQSAFDWDDETAKASKQH 165

RLKRYHSQTYGNGSK**C**DLNGRPREAEVRFL**C**DEGAGISGDYIDRVDEPLS**C**SYVL 220

TIRTPRL**C**PHPLLRPPPSAAPQAIL**C**HPSLQPEEYMAYVQRQADSKQYGDKIIEE 275

OS-9 (296-667)

LQDLGPQVWSETKSGVAPQKMAGASPTKDDSKDSDFWKMLNEPEDQAPGGEEVPA 330

EEQDPSPEAADSASGAPNDFQNNVQVKVIRSPADLIRFIEELKGGTKKGKPNIGQ 385

EQPVDDAAEVPQREPEKERGDPERQREMEEEEDEDEDEDEDEDERQLLGEFEKEL 440

EGILLPSDRDRLRSEVKAGMERELENIIQETEKELDPDGLKKESERDRAMLALTS 495

nuclear localization signal

TLNKLI**KRLEEKQSPELVKKHKKKR**VVPKKPPPSPQPTEEDPEHRVRVRVTKLRL 550

GGPNQDLTVLEMKRENPQLKQIEGLVKELLEREGLTAAGKIEIKIVRPWAEGTEE 605

GARWLTDEDTRNLKEIFFNILVPGAEEAQKERQRQKELESNYRRVWGSPGGEGTG 660

DLDEFDF 667

Figure 3. Amino acid sequence of OS-9.

GROUPS	RELATIVE FOCUS FORMATION								AVERAGE
	1	2	3	4	5	6	7	8	
Ras	17%	37%	37%	30%	62%	12%	34%	44%	34%
Myc	0	---	---	---	---	---	---	---	0
OS-9 (1-667)	0	---	---	---	---	---	---	---	0
OS-9 (296-667)	0	---	---	---	---	---	---	---	0
Ras + Myc	100%	100%	100%	100%	100%	100%	100%	100%	100%
Ras + OS-9 (1-667)	---	39%	69%	38%	---	17%	---	---	41%
Ras + OS-9 (141-667)	28%	---	---	---	---	---	---	---	28%
Ras + OS-9 (296-667)	---	---	---	53%	66%	30%	---	---	50%
Ras + Myc + OS-9 (1-667)	---	82%	---	---	---	---	63%	98%	81%
Ras + Myc + OS-9 (141-667)	127%	---	---	---	---	---	---	---	127%
Ras + Myc + OS-9 (296-667)	---	---	---	---	---	---	99%	90%	95%

Figure 4. Effect of OS-9 on Ras-mediated cellular transformation.

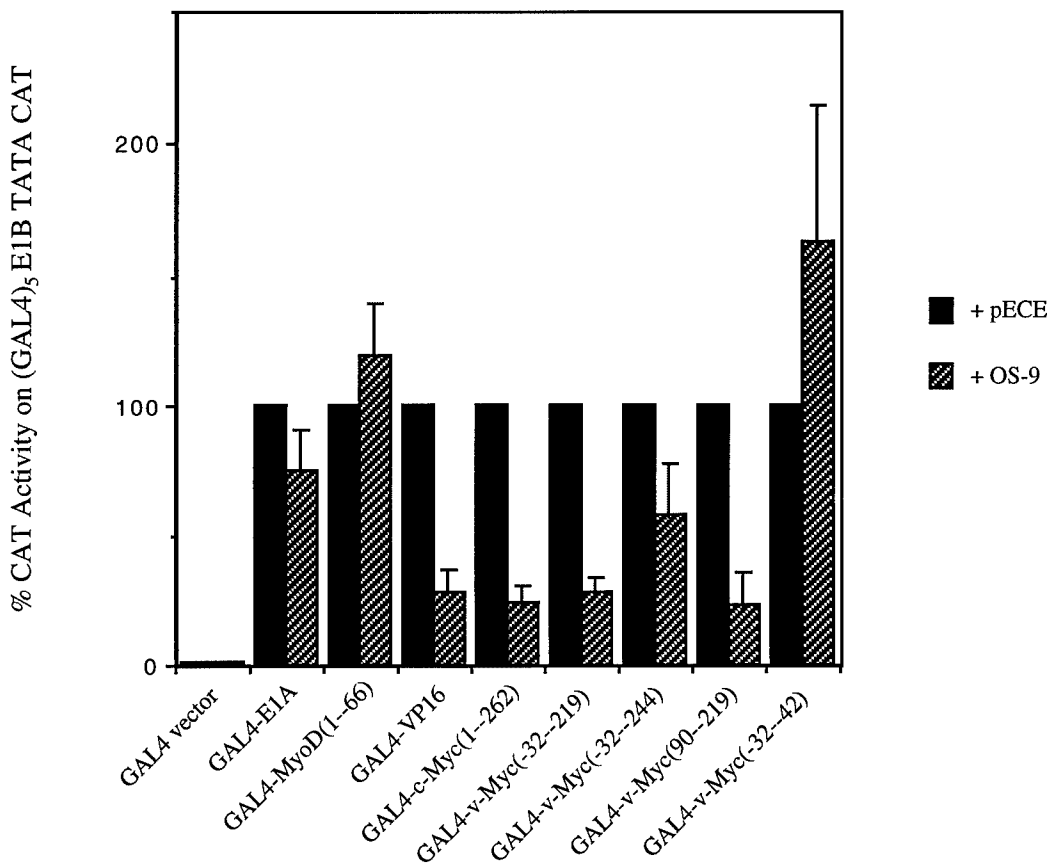


Figure 5. Effect of OS-9 on the transcriptional activity of GAL4 fusion proteins.

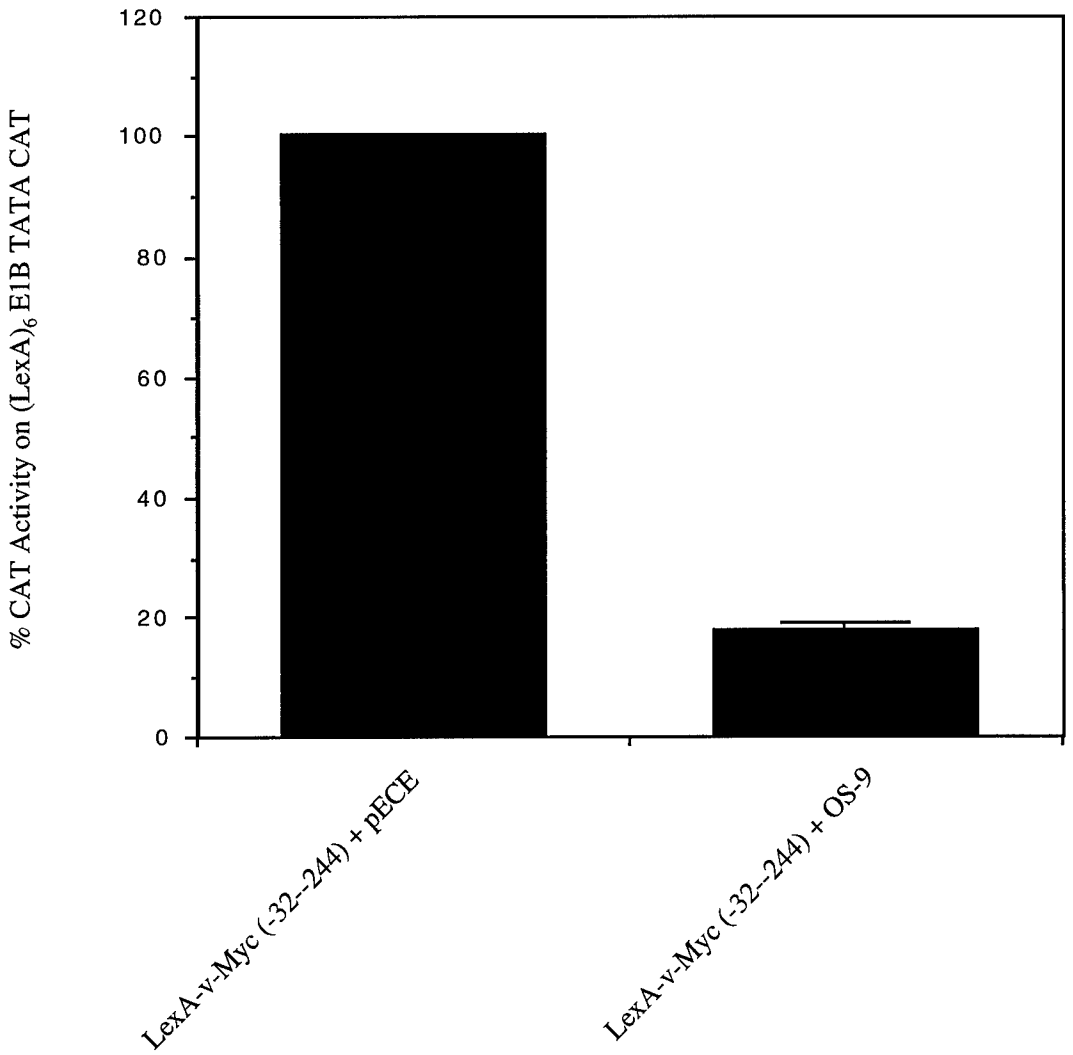


Figure 6. Effect of OS-9 on the transcriptional activity of a LexA-v-Myc fusion protein.

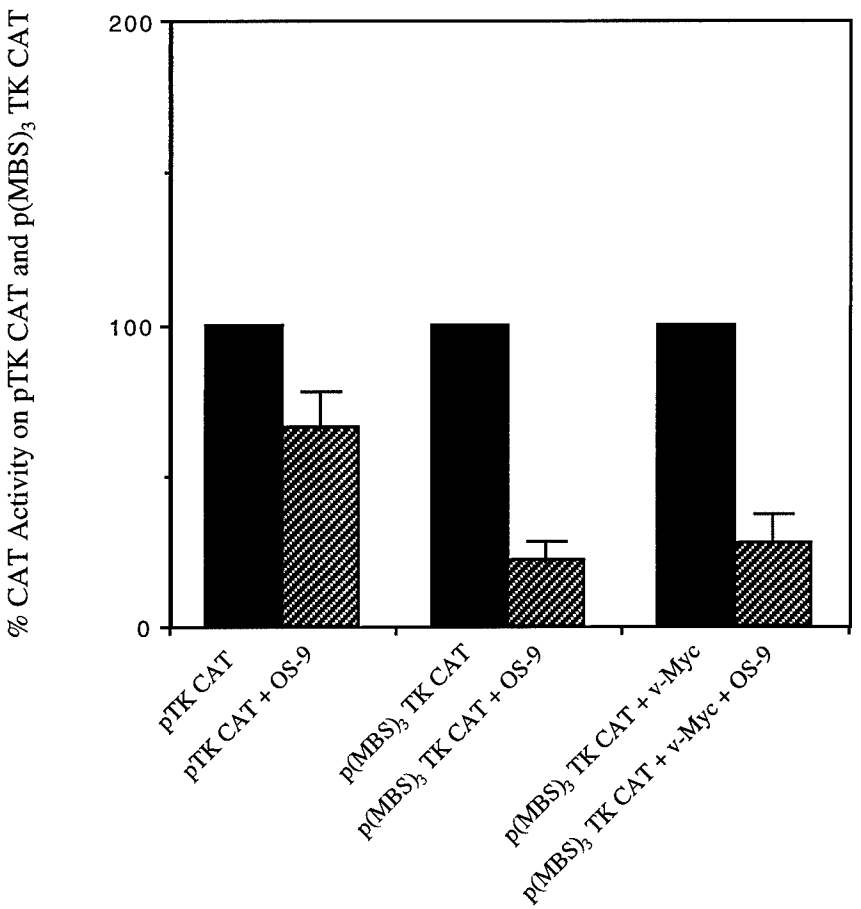


Figure 7. Effect of OS-9 on Myc-mediated activation of a synthetic Myc reporter gene, p(MBS)$_3$ TK CAT.

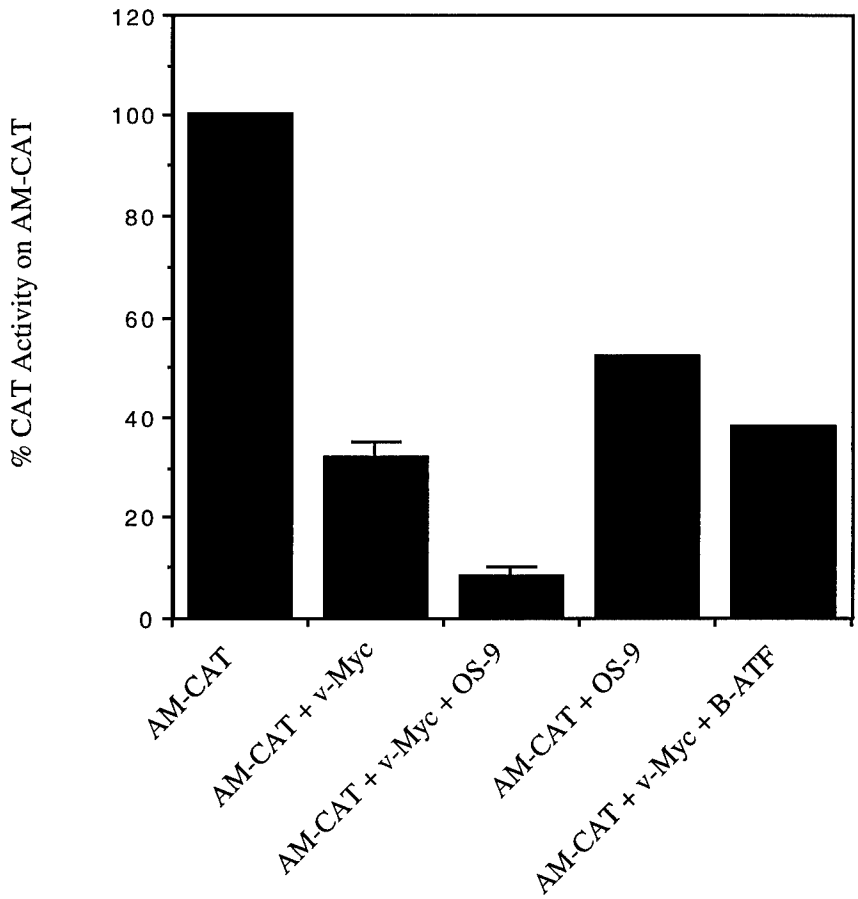


Figure 8. Effect of OS-9 on AM-CAT, a target of Myc-mediated transcriptional repression.

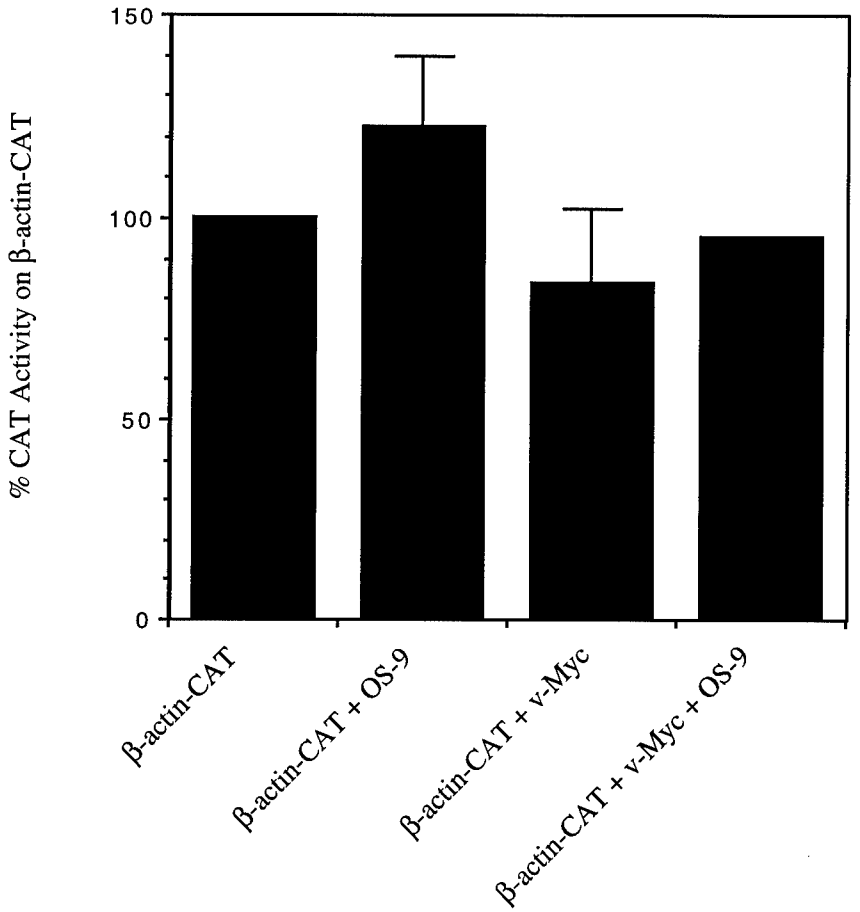


Figure 9. Effect of OS-9 on β-actin-CAT gene expression.

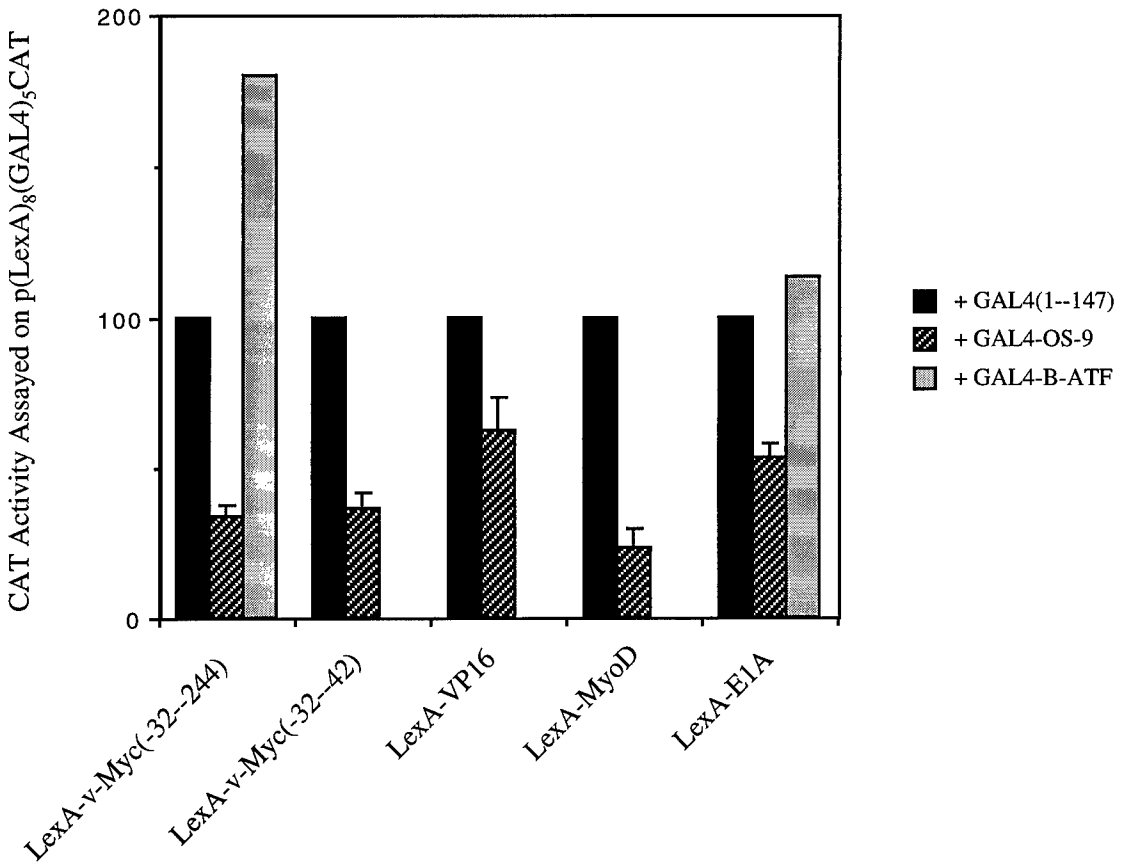
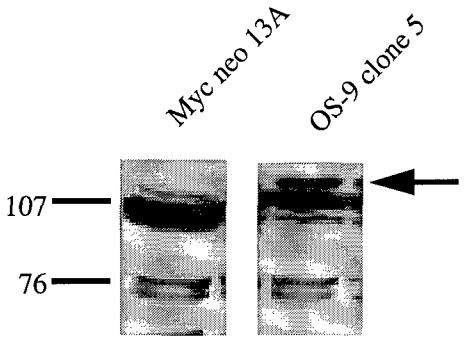
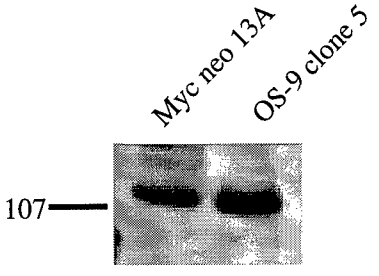


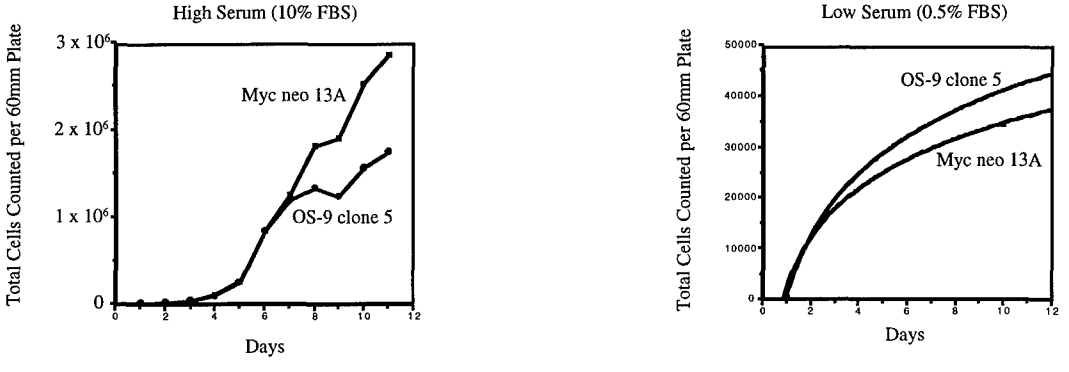
Figure 10. Effect of OS-9 on transcriptional activation by LexA fusion proteins when targeted to DNA using a GAL4 DNA binding domain.



A. Detection of OS-9 HA fusion protein in the OS-9 clone 5 cell extracts but not the parental Myc neo13A cell extracts.



B. Detection of p110 gag-Myc fusion protein in both the Myc neo 13A and OS-9 clone 5 cell extracts.



C. Analysis of the growth properties of the cell lines Myc neo 13A and OS-9 clone 5 in high versus low serum. (The growth data in low serum are presented as a fitted curve.)

Figure 11. Characterization of cell lines expressing v-Myc and/or v-Myc plus OS-9.