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Activation of c-*myc* promoter by c-*myc* protein in serum starved cells

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The function of the c-*myc* protein, the product of a proto oncogene, is not clearly understood although many reports, including ours, suggest that the c-*myc* protein plays several roles in the regulation of transcription and DNA replication. Here we examined the effects of c-*myc* protein on transcription from the c-*myc* promoter, and by inference its role in auto-regulation, after introducing into cultured cells a c-*myc* expression vector and a CAT reporter gene linked to the promoter and upstream region of the human c-*myc* gene. To minimize the effects of the endogenous c-*myc* protein on the exogenously added CAT reporter gene, the transfected cells were treated under serum-free conditions. The results show that CAT expression from the *myc* promoter increased in a dose-dependent manner after addition of the c-*myc* expression vector, and that it also required the presence of a c-*myc* binding sequence previously identified 2 kb upstream from c-*myc*'s first exon.Moreover, the domains of the c-*myc* protein important for transactivation were determined by use of various deletions mutants of c-*myc* cDNA. The results showed that the N-terminal portion in the c-*myc* protein was necessary for transactivation beside the C-terminal portion containing basic region, helix–loop–helix, and leucine zipper.

c-*myc*; Autoregulation; Activation

1. INTRODUCTION

The c-*myc* protein, a nuclear oncogene product, has been considered to have an important role for cell proliferation. Although many reports suggest that the c-*myc* protein is directly or indirectly involved in transcription and/or DNA replication, there is still no strong consensus about its molecular functions (for reviews see [1–4]). As for its transcriptional functions, it was reported that c-*myc* protein activated the heat shock 70(HSP70) promoter and repressed the metallothionein promoter [5,6], and that the adenovirus E4 promoter could be transactivated by the c-*myc* protein through the same promoter region required for E1a activation [7]. Recently, using a c-*myc*–steroid receptor fusion protein that permits hormone-dependent *myc* activity [8], a *myc*-inducible transcript has been identified which is related to the α-prothymosin gene [9]. On the other hand, the autoregulation of c-*myc* has been also studied, albeit with controversial results.

Down-regulation of c-*myc* [10,11], or up-regulation [12–14] after introduction of exogenous *myc* have been reported. These discrepancies are likely to be due to the system used for the assays; cells, time of transfection, amount of c-*myc*, and so on. Here we report that the expression from the c-*myc* promoter in its natural context was regulated by the c-*myc* protein itself in a dose-dependent manner, that it required the presence of a c-*myc* binding region upstream of the promoter, and also that the N-terminal portion as well as the c-terminal of the c-*myc* protein were important for transactivation.

Abbreviations: CAT, chloramphenicol acetyl transferase; MEM, minimal essential medium.

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2. MATERIALS AND METHODS

2.1. *Plasmid constructions*

The c-*myc* expression vector, pSVc-*myc* , was constructed as follows. c-*myc* cDNA, cloned in pSPT64 [15], was first digested with *Sph*I and *Bam*HI, treated with exonuclease III followed by mungbean nuclease, and then self-ligated. A resulting clone that contained the sequence from −33 to the 3′-end of the c-*myc* cDNA was digested with *Hin*dIII and *Eco*RI, and the fragment containing the c-*myc* gene was inserted into the *Hin*dIII-*Eco*RI sites of pSVO. The latter consists of the SV40 enhancer and promoter cloned in pBR322 [16]. The two reporter genes, pHX-CAT and pPX-CAT were constructed with the region upstream of the protein coding sequences of the genomic human c-*myc* DNA . A c-*myc* protein binding sequence is present between the *Hin*dIII and *Pst*I sites about 2 kbp upstream of the transcription initiation site, while c-*myc*'s promoters P1 and P2 are located within the first exon. The 610 bp long *Sma*I–*Pvu*II fragment (which contains c-*myc*'s first exon) was inserted upstream of the bacterial chloramphenicol acetyl transferase gene (CAT), yielding pEXON1-CAT. pHXCAT was constructed by joining the c-*myc* gene's *Hin*dIII–*Sma*I region upstream thereof. Analogously, pPX-CAT was constructed by junction of the relative *Pst*I–*Sma*I fragment.

2.2. *Cell culture and transfection*

Human HeLa, mouse L, Balb 3T3, and monkey CosI cells were cultured in Dulbecco modified Eagle MEM supplemented with 10% fetal calf serum. 5 μg of CAT reporter plasmid and various amounts

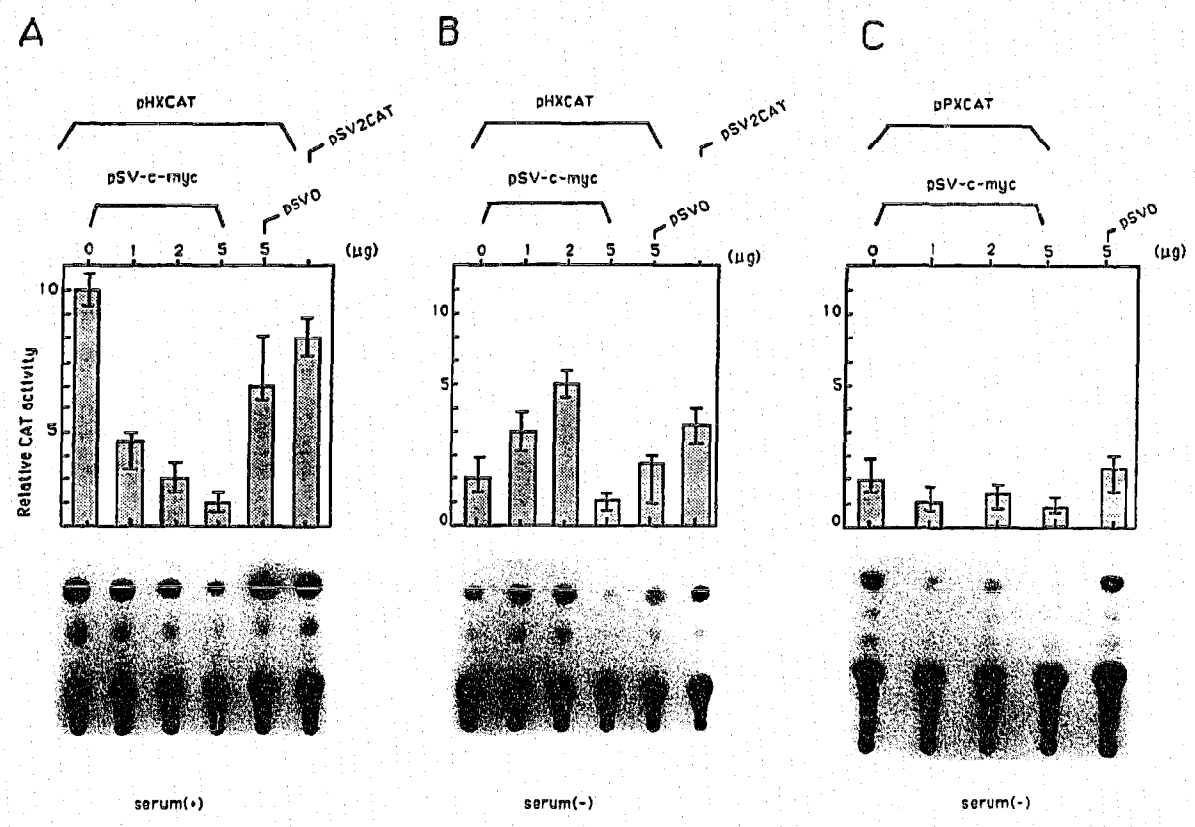

Fig. 1. Effect of c-*myc* protein on CAT expression in mouse L cells. pHXCAT, which contains the region upstream of the c-*myc* gene was co-transfected with a c-*myc* expression vector, pSVc-*myc*, or the vector pSVO, into mouse L cells. Cells were cultured with (A) or without serum (B) 16 h after transfection. pPXCAT which corresponds to pHXCAT without the *Hin*dIII-*Pst*I region of human the c-*myc* gene was also transfected in to L cells, and the cells were cultured without serum. pSV2CAT, which harbours the SV40 promoter and enhancer was used as a positive control of CAT expression. CAT assays were carried out as in section 2. The results from one of the CAT assays are shown in the photograph and the relative CAT activities of the test samples (the lowest CAT activity among the activities of the test samples was set as relative value 1) were shown above the autoradiograph. Bars in the graph represent the variations of 3 independent experiments.

of pSVc-*myc* were contransfected into 100 mm-diameter plates (50% confluent cells) by the calcium phosphate precipitation technique (17). Four hours after transfection, the cells were boosted with 25% glycerol, cultured for 15 h with 10% fetal calf serum, eventually cultured for another 24 h without serum, and then harvested. The CAT assay using cell extracts has been described previously [18]. Quantification of the relative CAT activities was performed by use of a Bioimage image analyzer.

3. RESULTS

3.1. *Establishment of a system to detect the transcriptional activity of exogenously added* c-myc

Numerous controversial results about c-*myc* transcriptional activity reported so far are likely to be due to different experimental conditions including cell type, cell condition, transfection methods, and so on. An important point is also that the effect seen on transcriptional events is likely to be derived from the combined action of endogenous and exogenous c-*myc* protein. To see the effect of the exogenously added c-*myc* on transcription, the level of the endogenous should be minimized. To this purpose, various cell types were transfected with a c-*myc* expression vector, pSVc-*myc*, and with the reporter plasmid pHXCAT which contains the region upstream of the coding sequence in the human c- *myc* gene. Nineteen hours after transfection, the cells were cultured with, respectively without serum, and CAT assays were carried out . Cells cultured without serum expressed little endogenous c-*myc* , as deduced from an S1 mapping (data not shown). The absolute CAT activities obtained varied with the cell types: as an example, the results from mouse L and monkey CosI cells are shown in Figs 1 and 2, respectively. However, the patterns of activation were similar in all cell types tested. Basically, transcriptional activation occurred with low levels of c-*myc* , while increased amounts of c-*myc* led to suppression in the cells cultured without serum. In the cells with serum, on the other hand, only suppression could be seen, in all cell types. Difference of the activation range observed is likely to be due to the strength of the SV40 promoter in two cells. In fact, while in L-cells activation occurred with 1–2 μg pSVc-*myc*, only as little as 0.2–1 μg were needed in CosI cells,

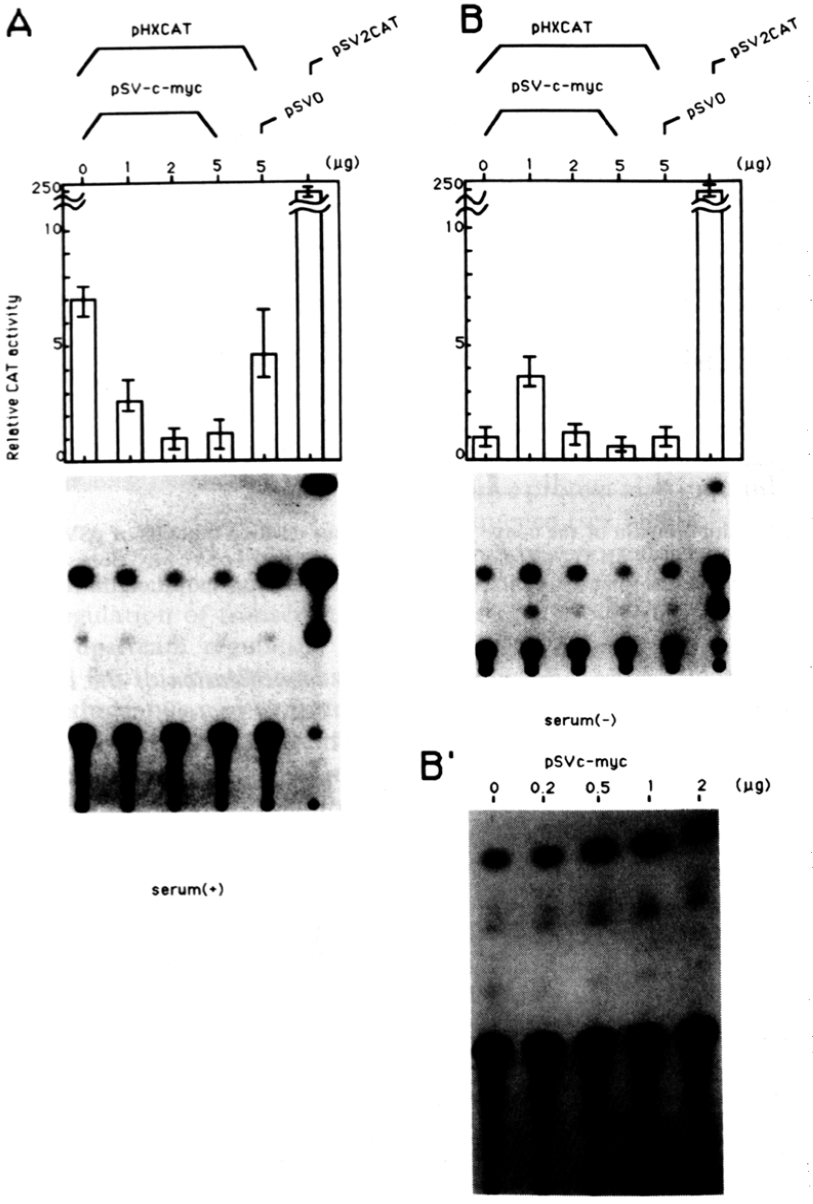

Fig. 2. Effect of the c-*myc* protein on CAT expression in monkey CosI cells. pHXCAT was transfected into monkey CosI cells. The cells were cultured with (A) or without serum (B and B'), and CAT assays were carried out as in Fig. 1. Fig. B' is a complement to Fig. B, in that it shows the transcriptional activation patterns obtained with small increases of pSVc-*myc* from 0 to 2 μg.

where amounts greater than 1 μg already inhibited transcription.

The range where activation occurs was relatively wide in L cells cultured without serum. The L cells/serum-free combination was therefore used in all subsequent experiments.

Oddly, the basal level of the reporter plasmid alone, pHXCAT, always seemed slightly higher than that of the same pHXCAT co-transfected with vector pSVO or with pSVO-derived expression vectors for inactive deletion mutants of c-*myc* (see below). We think this may happen because of the presence of SV40 enhancer/promoter sequences in pSVO and its derivatives. These sequences possibly interfere with the cellular transcription machinery, for instance by competing for transcription factors.

3.2. *Importance of the specific interaction between* c-myc *protein and DNA*

The reporter plasmid used in the CAT assays covered the region from *Hin*dIII to *Pvu*II sites of the c-*myc* gene. We have already suggested that the c-*myc* protein, or a complex including the c-*myc* protein, binds to the region from *Hin*dIII to *Pst*I ((H–P) region) of the c-*myc* gene. As a control, pPXCAT (which corresponds to pHXCAT without the ((H–P) region) was constructed, and the function of the c-*myc* promoter in this context was again tested (Fig. 1C). The results showed that in this

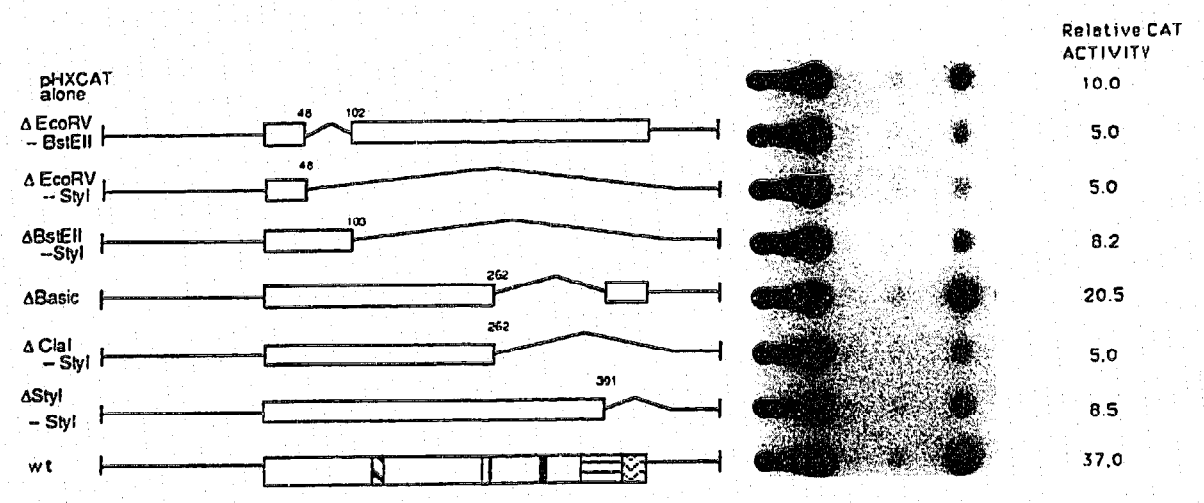

Fig. 3. Determination of the transactivation domain of the c-*myc* protein. Various deletion mutants of pSVc-*myc* were constructed; the regions deleted are shown in the figure. CAT expression of pHXCAT as affected by 1 μg of deleted pSVc-*myc* clones was tested in L cells and the relative CAT activity as compared to that of pHXCAT alone (value 10.0) was shown on the left of the figure.

case c-*myc* had little effect on CAT expression, suggesting that the target of the c-*myc* protein in pHXCAT was indeed the (H–P) region.

3.3. *Identification of the transactivation domains of the* c-myc *protein*

The c-*myc* protein possesses several interesting domains which may affect transactivating functions. Deletion mutants of c-*myc* cDNA encoding such domains

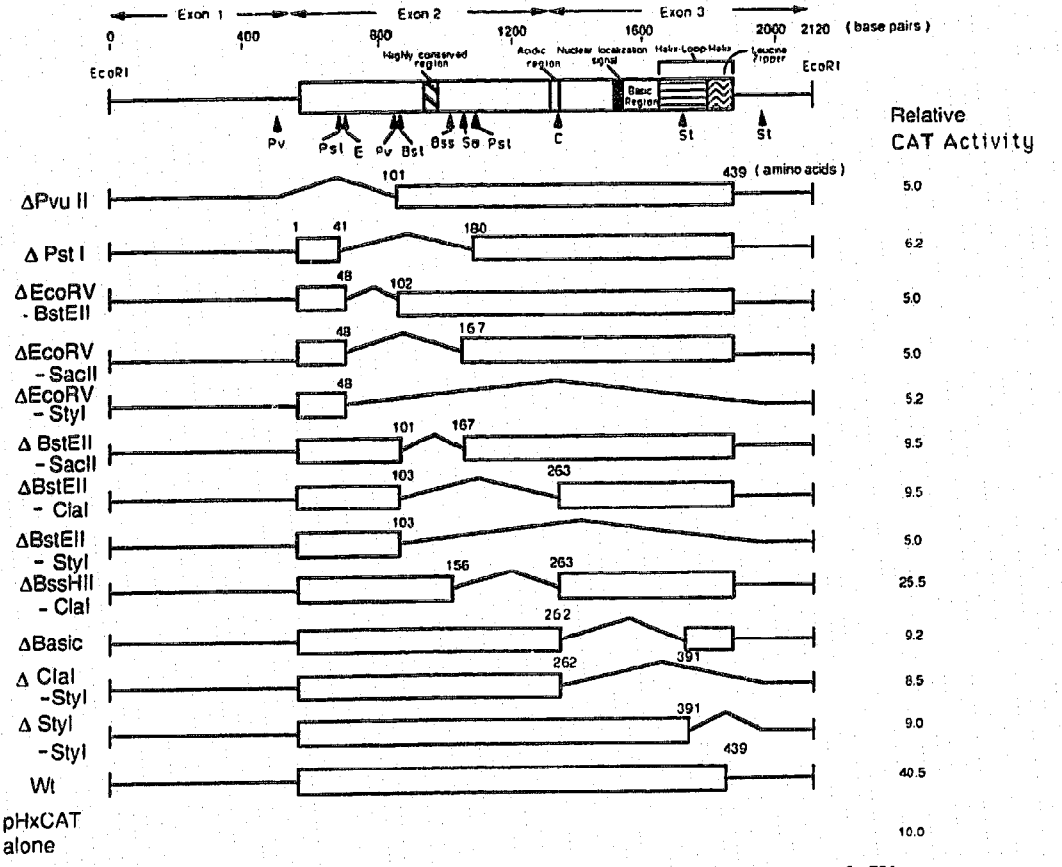

Fig. 4. Determination of the precise transactivation domain of the c-*myc* protein. Various deletion mutants of pSVc-*myc* were constructed, and their effect on CAT expression of pHXCAT was tested as in Fig. 3. The relative CAT activities of the test samples as compared to that of pHXCAT alone (value 10.0) are shown.

were constructed (Figs 3 and 4) and assayed for their transactivating function on a reporter plasmid containing the c-*myc* promoter. Experiments were repeated at least 3 times, and the relative CAT activities were shown. It is clear that deletions from amino acid No. 48 to 102 and 101 to 167 gave weak activities, indicating that the N-terminal portion is important for activation. There also exists important regions near the C-terminus of the c-*myc* protein: basic region, helix–loop–helix (HLH), and leucine zipper region. Mutants lacking these domains also lost activity, suggesting that DNA binding of the c-*myc* protein and/or protein–protein interactions are necessary. On the other hand, the portion between amino acids 156 to 263 seemed to be dispensable for transactivation.

4. DISCUSSION

In this work we have attempted to investigate the role of the c-*myc* protein in the regulation of transcription from its own promoter and upstream regulatory sequences. A condition essential for this purpose was to minimize the effects of the endogenous c-*myc* protein. We have achieved this goal by culturing the cells in serum-free conditions several hours after transfection.

The results show that transcription from the c-*myc* promoter can be induced by co-transfection of a c-*myc* protein expression vector. Transcriptional activation was found to take place with low amounts of c-*myc*, in a dose-dependent manner. Increasing amounts of c-*myc* protein led instead to inhibition of transcription, which is consistent with the fact that only inhibition effects could be seen, with any amount of c-*myc* expression vector, in the cells cultured with serum (that is, which already contain high levels of endogenous c-*myc*). In addition, transactivation seemed to depend on the presence of the (H–P) sequence, which is located about 2 kb upstream of c-*myc*'s first exon and contains a specific binding site for the c-*myc* protein (alone or in complex form). In fact, no effect on transcription could be seen with a reporter gene that lacked the (H–P) region. These results strongly suggest that c-*myc* autoregulates its own expression. It was reported that the α-prothymosin is activated by the c-*myc* protein at the entry into and progression through the cell-cycle [9]. It was also reported that the nuclear localization of the c-*myc* protein in the lower eukaryote *Physarum polycephalum* changed during the cell cycle, being transiently but specifically bound to the periphery of the nuclear matrix, where DNA replication and transcription are thought to take place, during the S-phase [19]. It is therefore possible that the trans-acting effect of c-*myc* on its own expression may somehow be related to the cell cycle: activation would occur in the period between G1 and S phases or immediately after entry of the S phase.

We have also investigated what portions of the c-*myc* protein are responsible for transactivation. One essential domain was found near the N-terminus, in particular between amino acids 41–167, where there exists a region highly conserved in the *myc* family. This is consistent with another report [20] that an activation domain spans amino acids 1 to 143. Results presented here also identified as essential the region close to the C-terminus, which contains basic region, HLH and leucine zipper structures. These are considered to be necessary for specific DNA binding and protein–protein complex formation. As a matter of fact, both N- and C-terminal regions are important for oncogenic transformation [21,22]. On the other hand, the region between amino acids 156 to 263 seemed to be dispensable. This region comprises an acidic domain.

As mentioned above, the trans-acting function of the c-*myc* protein may require its binding to a specific target sequence (see also refs [12–14]). On one side, we have already reported the existence of a c-*myc* binding sequence in the (H–P) region upstream of the c-*myc* gene itself ([13], Negishi, Iguchi-Ariga and Ariga, submitted). On the other hand, a distinct sequence was recently reported [23,24], where the c-*myc* protein can specifically bind in heterodimeric association with a novel *myc*-associated protein (MAX) [24]. Latest experiments of ours showed that the c-*myc* protein complex can indeed bind to either sequence, and that its binding to the one can be competed-out by the other (Negishi et al., submitted). These data indicate that c-*myc* plays manifold roles in cellular life. Transcriptional functions of the c-*myc* protein will be further elucidated with more biochemical approaches. For this purpose, we envisage in vitro assays with the purified, biologically active c-*myc* protein that we have enriched from human cells by use of an affinity column carrying the c-*myc* binding core sequence present in *myc* (H–P) (Negishi et al., submitted; Taira, Negishi, Kihara, Iguchi-Ariga and Ariga, submitted) and also cDNA cloning encoding c-*myc* associated proteins. These experiments will dissolve the question that c-*myc* protein directly or indirectly activates the gene expression.

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