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# An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence

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**We have used a subtraction/coexpression strategy involving two different tumors derived from *c-myc*-bearing transgenic mice to identify a gene that is a target for c-Myc regulation. The gene, expressed in certain embryonic and adult tissues and in several (but not all) *c-myc*-based tumors, bears a functional c-Myc-binding sequence located 3' to its transcription start site. This sequence is required for the binding of a nuclear protein complex which, by antibody analysis, includes c-Myc. This site is also required for expression of a reporter gene in chimeric constructs transfected into *c-myc*-overexpressing cells and, conversely, requires *c-myc* cotransfection for its enhanced expression in COS cells. Furthermore, transfection of *c-myc* blocks the normal down-regulation of this gene, which occurs in embryonic stem cells as they undergo differentiation. This target gene encodes an anonymous cDNA (ECA39) found previously to be amplified in a teratocarcinoma cell line.**

[Key Words: c-Myc; DNA-binding; transformation; transgenic mice; embryo]

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In addition to being involved in a number of malignancies, the proto-oncogene *c-myc* is thought to play a significant role in the physiology of cell division and differentiation (for review, see Cole 1986). For example, several human and animal tumors are associated with the amplification, translocation, transfection, overexpression, or abnormal regulation of this gene (Bishop 1987). In addition, studies of cultured cells have delineated a relationship between the expression of *c-myc* and the progression of cells from the resting state ( $G_0$ ) to the growth phase ( $G_1$ ) of the cell cycle (Kelly et al. 1983; Cole 1986; Kelly and Siebenlist 1986). Such progression is accompanied by a sharp induction of this proto-oncogene, an induction brought about by a number of growth factors and conditions that influence a cell to leave the resting state and enter the cell cycle.

There has also been considerable speculation, though limited evidence, to suggest that the product of the *c-myc* gene is directly involved in the regulation of gene expression or in DNA replication (Iguchi-Arigo et al. 1987; Kaddurah-Daouk et al. 1987; Prendergast and Cole 1989; Eilers et al. 1991; for review, see Lüscher and Eisenman 1990). Although convincing evidence is lacking, the structure of the c-Myc protein offers certain clues as to its possible function. c-Myc is a short-lived nuclear phosphoprotein (Hann et al. 1983; Persson and Leder 1984; Ramsay et al. 1984) that harbors structural motifs seen in a number of transcription factors: a helix-loop-helix motif (Murre et al. 1989), a leucine zipper motif (Landschulz et al. 1987), and a basic region (Prendergast and Ziff 1989).

These domains appear in several transcription factors and play a role in dimerization and DNA binding, suggesting that c-Myc might also interact with DNA as either a homo- or a heterodimer. The discovery of Max, a protein that forms a heterodimer with c-Myc and, as such, binds to a specific DNA sequence, may be evidence of just such a physiologic partnership (Blackwood and Eisenman 1991; Prendergast et al. 1991; Blackwood et al. 1992).

The recent discovery of a c-Myc-specific DNA-binding sequence, CACGTG, provides another potential clue to the eventual understanding of the molecular function of c-Myc (Blackwell et al. 1990; Halazonetis and Kandil 1991; Prendergast and Ziff 1991). Although c-Myc binds only weakly to this sequence as a homodimer, the c-Myc/Max heterodimer binds to it with high affinity (Blackwood and Eisenman 1991; Prendergast et al. 1991; Blackwood et al. 1992). Taken together, these findings strengthen the supposition that c-Myc modulates gene expression by a complex formed between c-Myc, associated proteins, and DNA.

Given these hints, it is not surprising that a number of potential genetic targets for c-Myc modulation have been identified (Prendergast and Cole 1989; Eilers et al. 1991). Nonetheless, clear evidence that any gene is the direct target for the modulating action of c-Myc is still lacking. Because understanding the potential regulatory function of c-Myc depends on the identification of its immediate targets, we set out to develop a strategy that might lead us to such a gene. The fact that c-Myc incites

the development of so many different tumors in transgenic mice (Stewart et al. 1984; Adams et al. 1985; Leder et al. 1986; Benvenisty et al. 1992) allowed us to derive two very different malignant cell lines from one of our *c-myc*-based transgenic strains. By hypothesizing that these two tumors might express common *c-Myc* target genes that are strongly up-regulated by the massive overexpression of the *c-myc* transgene and by applying a subtraction/screening strategy for cDNAs, we identified a gene that is very highly expressed in both tumors and appears, as shown below, to be directly regulated by *c-Myc*. Because a search of the data base reveals that this gene corresponds to an anonymous cDNA previously found amplified in a teratocarcinoma cell line (Niwa et al. 1990), we refer to it using its data base designation, namely *ECA39*.

## Results and discussion

### A strategy for selecting *c-Myc*-regulated genes

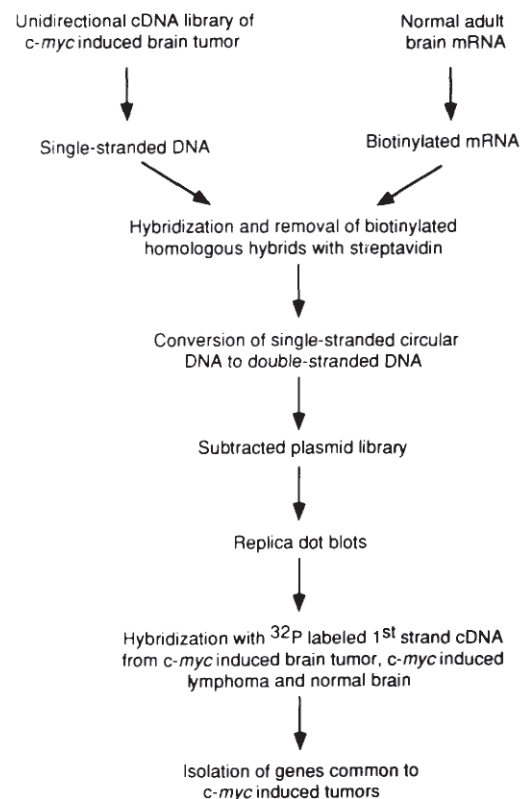
As indicated above, an assumption implicit in this work is that the product of the *c-myc* gene is involved in modulating gene expression, most likely as a positively acting transcription factor. A number of groups using cDNA subtraction/screening strategies have isolated genes from cells based on this hypothesis (Prendergast and Cole 1989; Eilers et al. 1991). The difference in our approach resides simply in our looking for up-regulated sequences that are expressed in two very different tumors derived from transgenic mice in which there is massive deregulated expression of a *c-myc* transgene.

Recently, we developed a set of transgenic mice that are particularly useful in generating *c-myc*-provoked tumors of two decidedly different cell types. One of these transgenic lines bears a *c-myc* transgene under the regulation of the human T-cell leukemia virus type I long terminal repeat (HTLV-I LTR). The other line bears and expresses the *trans*-activator (*tax*) of the HTLV-I LTR driven by an immunoglobulin promoter/enhancer (Benvenisty et al. 1992). Mice harboring the HTLV/*c-myc* transgene alone express very low levels of transgenic *c-myc* mRNA and develop T-cell lymphomas at a relatively slow rate. Mice expressing the transgenic HTLV activator alone (and thus no deregulated *c-myc* mRNA) do not develop tumors. When the two strains are bred to produce mice harboring both transgenes [the target HTLV-LTR/*c-myc* and its *trans*-activator], there is extensive up-regulation of *c-myc* expression and very rapid formation of two very different types of tumors: CD4<sup>+</sup> CD8<sup>-</sup> T-cell lymphomas and central nervous system tumors, the latter identified by morphology and by the expression of neuronal-specific markers [e.g., neurofilament proteins] (Benvenisty et al. 1992). Cell lines expressing very high levels of the *c-myc* transgene have been established from both tumor types (Benvenisty et al. 1992). For the purposes of this selection strategy, a brain tumor line (BK-1) was used as the source of the cDNA library from which normal brain cDNA sequences were subtracted before screening for genes that

are also expressed in the *c-myc*-induced T-cell lymphoma.

The specific strategy is detailed in Figure 1 and in Materials and methods. Briefly, a unidirectional cDNA phage library was made from poly(A)<sup>+</sup>-enriched RNA derived from the transgenic brain tumor cell line. To enrich for genes that are specifically expressed or up-regulated in the brain tumor, single-stranded DNA rescued from the brain tumor cDNA library was hybridized with brain mRNA from a normal animal (Duguid et al. 1988; Sive and St. John 1988; Schweinfest et al. 1990). The single-stranded cDNA that did not hybridize was used to construct a subtracted plasmid library. Individual plasmids from the subtracted library were differentially screened using labeled cDNA from brain tumor cells, T-cell lymphoma, or normal (nontransgenic) adult brain. cDNA clones representing sequences that were highly expressed in brain tumor cells and lymphoma and were expressed weakly or not at all in normal brain were studied further by RNA blot hybridization. The enrichment of the original library for brain tumor-specific cDNAs by the subtractive procedure was estimated to be ~50-fold. About 500 plasmids were screened from among ~25,000 phage in the original library; thus, we screened for relatively abundant transcripts.

From among the nine cDNA clones highly expressed in brain tumor and lymphoma and weakly expressed in normal brain, one that displayed particularly well the



**Figure 1.** Schematic representation of the construction and screening of the subtracted brain tumor cDNA library.



expression pattern that we sought was studied in detail. As noted above, we refer to the gene corresponding to this cDNA as *ECA39*. Using RNA blot analysis, we could show that *ECA39* was expressed as a 2.4-kb transcript at very high levels in cells from both the *c-myc*-induced brain tumor (BK-1) and the CD4<sup>+</sup> CD8<sup>-</sup> T-cell lymphoma (Fig. 2A). Expression was observed in all such tumors (seven) examined from several of these binary transgenic mice. This gene is also expressed in normal brain; yet, consistent with the selection strategy, the level of mRNA expression in brain is one-tenth that seen in brain tumor cells (Fig. 2A). Conversely, although there is a similarly high level of mRNA expression of this gene in T-cell lymphomas, there is no detectable expression in normal (nontransgenic) thymus or spleen. Evidently the initiation of expression of this gene in this T-cell context is either dependent on the expression of the *c-myc* transgene or it is a subsequent transformation event.

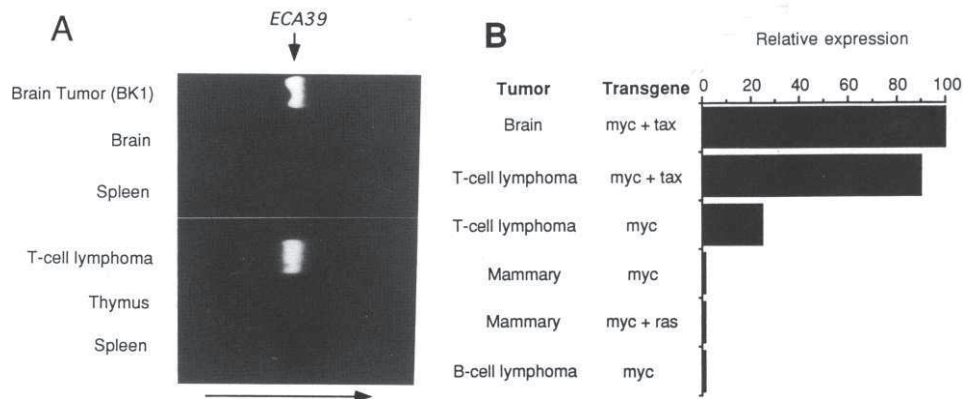
In addition to brain tumor cells and CD4<sup>+</sup> CD8<sup>-</sup> T-cell lymphomas derived from binary transgenic mice, this putative target gene is also expressed in *c-myc*-induced CD4<sup>+</sup> CD8<sup>+</sup> T-cell lymphomas derived from monogenic mice bearing the HTLV LTR/*c-myc* transgene alone (Fig. 2B). Thus, the expression of the *ECA39* gene is not dependent on the expression of the *tax* transgene but, rather, on the expression of the *c-myc* transgene. Not all *c-myc* transgene-bearing tumors, however, express this gene. In contrast, *c-myc*-induced tumors derived from other *c-myc*-bearing transgenic lines, for example, mammary carcinomas [M.TG (Stewart et al. 1984)] or B-cell lymphomas [K.TG (Leder et al. 1986)], fail to express *ECA39* (Fig. 2B). In addition, various tumors derived from transgenic mice carrying activated oncogenes other than *c-myc*, for example, *neu* [N.TG (Muller et al. 1988)] or *v-H-ras* [AC.TG (Leder et al. 1990)], were also examined for expression of this gene. Of the different tumor types screened (including mammary carcino-

mas, papillomas, bone tumors, and fibrosarcomas), only *v-H-ras*-induced fibrosarcomas expressed the *ECA39* gene, and these tumors also expressed very high levels of *c-myc* mRNA (results not shown). From this we can conclude that our cDNA subtraction/screen uncovered a gene that is expressed in several, but not all, *c-myc*-induced tumors, that its expression is not inevitable in the presence of deregulated *c-myc* expression, and that *c-myc*-induced transformation does not require *ECA39* overexpression in all cellular contexts.

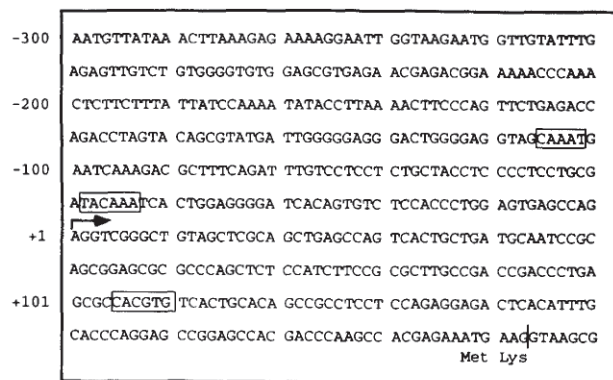
#### Identification of a potential *c-Myc*-binding motif adjacent to the *ECA39* transcription start site

To begin to determine whether the *ECA39* gene is a target for *c-Myc*-mediated regulation, it was useful to identify putative transcription regulatory structures in its genomic sequence. Using the complete structure of its cDNA (see below), we isolated the 5'-upstream genomic region of the gene, including the putative transcription start site from a mouse genomic library. Sequencing of this region revealed potential CAAT and TATA boxes upstream from the 5'-most sequence of the cDNA (Fig. 3). More interestingly, however, the sequence of the first exon revealed a putative *c-Myc* recognition site, CACGTG (Blackwell et al. 1990; Prendergast and Ziff 1991), which is located ~105 bp 3' to the putative transcription start site in an untranslated sequence in the 193-bp first exon (see below).

The recognition of these sites required us to establish the start site of transcription of the *ECA39* gene using appropriate RNase protection and primer extension assays. Thus, a 193-nucleotide RNA copy of the genomic DNA fragment corresponding to the first exon of *ECA39* (as predicted from comparing sequences of genomic DNA with those of cDNA) was protected by brain tumor RNA in an RNase protection assay (Fig. 4). In accordance



**Figure 2.** *ECA39* RNA expression analysis in tumors. RNA was isolated from tissues and cells (Chirgwin et al. 1979), blotted, and hybridized with an *ECA39* probe. (A) RNA blot of different samples (20  $\mu$ g of total RNA in each lane): Brain tumor (BK1) cells, T-cell lymphoma, normal brain, spleen, and thymus. Shown is a 2.4-kb *ECA39* transcript, as determined by 28S and 18S size markers. (B) Histogram representation of *ECA39* expression levels. The tumors are of different cell types and from different transgenic lines: Brain tumor cells (Benvenisty et al. 1992); T-cell lymphoma (Benvenisty et al. 1992); mammary tumors (Stewart et al. 1984; Sinn et al. 1987); and B-cell lymphoma (Leder et al. 1986).



**Figure 3.** Nucleotide sequence of the *ECA39* promoter and 5' region of the gene. Shown is the nucleotide sequence of 300 bp upstream from the start site of transcription, as well as the first 200 bp of the 5' region of the *ECA39* gene. Regulatory elements shown are the CAAT box, the TATA box [same sequence was reported to bind TFIID in the adenovirus E1a late promoter (Huang et al. 1988)] and the c-Myc DNA-binding site. The vertical line indicates the end of the first exon.

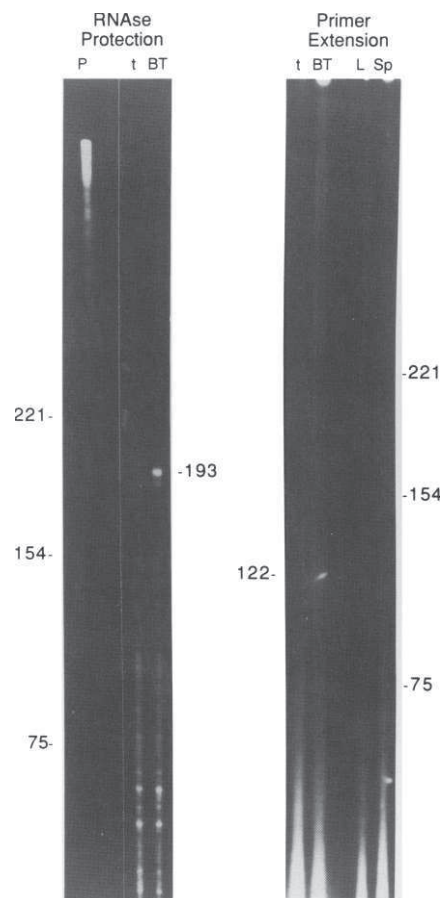
with this result, a primer located at 93–122 nucleotides downstream from the putative start site of transcription was used to generate a fragment of 122 nucleotides when extended, using RNA-dependent DNA polymerase (Fig. 4). Taken together, these results indicate that the *ECA39* transcript starts at the 5'-most base sequenced in our longest cDNA clone [and in the cDNA clone isolated by Niwa et al. (1990); see below], a position corresponding to position +1 in Figure 3.

#### *The c-Myc-binding motif is required for target gene expression and forms a complex with nuclear proteins, including c-Myc*

To evaluate the role that the putative c-Myc-binding site might play in the expression of the *ECA39* gene, various chimeric constructs containing elements of the *ECA39* 5' genomic and transcribed regions were made using the chloramphenicol acetyltransferase (CAT) gene as a reporter. These constructs were transiently transfected into c-myc-overexpressing BK1 brain tumor cells, and CAT activity was assayed. As shown in Figure 5, the chimeric gene (2–0.6-*ECA39*–CAT), containing an *ECA39* fragment covering a region 2 kb upstream and 0.6 kb downstream from the start site of transcription (including the putative c-Myc recognition site), gives maximum expression. Deleting the 0.6-kb fragment downstream from the transcription start site—the region that carries the putative c-Myc recognition site—results in a sharp loss of CAT activity (Fig. 5, 1.5-*ECA39*–CAT). Truncation of this c-Myc recognition site [Fig. 5, 2–0.1-*ECA39*–CAT] also abrogates CAT activity. Altering the putative c-Myc DNA-binding site itself by inserting an *Xba*I linker results in a fivefold reduction in CAT activity (Fig. 5, 2–0.6-*ECA39*–CAT-X). Moreover, a similar reduction in CAT activity is observed when the c-Myc

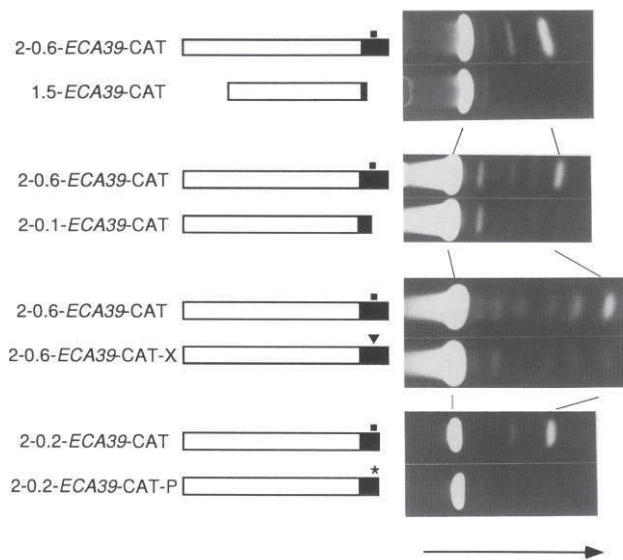
recognition site (CACGTG) is altered at a single-base position (to the sequence CACGAG) (Fig. 5, 2–0.2-*ECA39*–CAT-P). The results indicate that the c-Myc DNA-binding site is important for *ECA39* expression, as judged by this transient transfection assay.

Given the encouraging results noted above, it remained to be demonstrated that the c-Myc recognition site in the *ECA39* gene is capable of binding nuclear proteins, including c-Myc. Accordingly, gel-shift assays



**Figure 4.** Determination of the transcription start site of the *ECA39* gene. RNase protection analysis of the *ECA39* gene (left) was carried out using 20  $\mu$ g of RNA from brain tumor cells (BT) or tRNA (t). The probe (P) was a 900-bp *Sac*I–*Dra*I fragment covering 300 bp upstream and ~600 bp downstream from the putative start site of transcription of the *ECA39* gene. The protected fragment of 193 bp corresponds to the size of the first exon as predicted from the sequences of the full-length cDNA and the genomic clone (Fig. 3). Primer extension analysis of the *ECA39* gene (right) was carried out with 40  $\mu$ g of tRNA (t) or total RNA isolated from brain tumor cells (BT), T-cell lymphoma of WC.TG mouse (L), or normal spleen (Sp). A 30-bp  $^{32}$ P-labeled oligonucleotide covering positions 93–122, as designated in Fig. 3, was used as primer. Band size was determined by a DNA marker and a sequencing reaction that were run in parallel to the samples. Note a band of 122 bp in size generated from RNA of the brain tumor cells. This is consistent with the assignment of the transcriptional start site to the first nucleotide in the full-length cDNA clone.



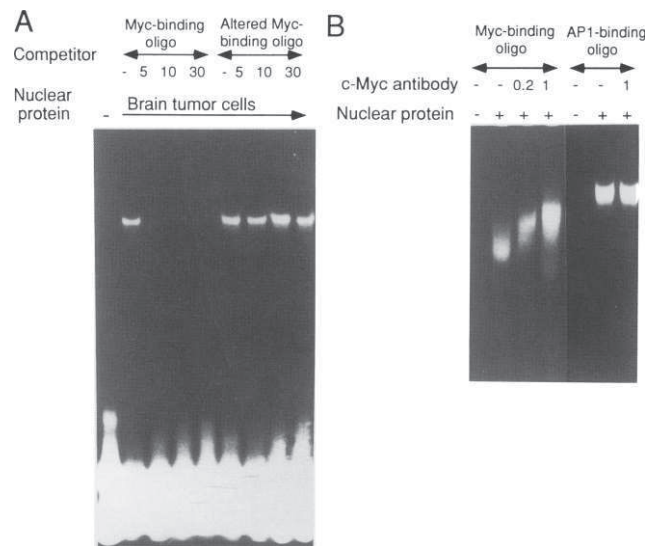


**Figure 5.** Analysis of expression of *ECA39*-CAT chimeric genes. Brain tumor (BK1) cells were electroporated with 20  $\mu$ g of different chimeric CAT reporter genes: 1.5-*ECA39*-CAT, 2-0.6-*ECA39*-CAT, 2-0.1-*ECA39*-CAT, 2-0.6-*ECA39*-CAT-X, 2-0.2-*ECA39*-CAT, and 2-0.2-*ECA39*-CAT-P (for details, see Materials and methods). CAT activity was determined 48 hr after transfection. (Open box) *ECA39* 5'-flanking region; (solid box) *ECA39* 5' region of the gene; (■) c-Myc DNA-binding site; (▼) insertion mutation in the c-Myc DNA-binding site; (•) point mutation in the c-Myc-binding site. Activity of 2-0.6-*ECA39*-CAT is  $22 \pm 3\%$  (6) that of SV2-CAT [a CAT gene driven by SV40 enhancer/promoter]. Activities of 1.5-*ECA39*-CAT, 2-0.1-*ECA39*-CAT, and 2-0.6-*ECA39*-CAT-X are  $10 \pm 1\%$  (4),  $13 \pm 2\%$  (3), and  $20 \pm 5\%$  (3) that of 2-0.6-*ECA39*-CAT, respectively. Activity of 2-0.2-*ECA39*-CAT-P is  $17 \pm 4\%$  (4) that of 2-0.2-*ECA39*-CAT. Results are expressed as mean  $\pm$  S.E. (no. of experiments in parentheses).

were carried out using the 30-nucleotide sequence harboring the putative c-Myc-binding core (CACGTG) and its surrounding sequence as derived from the cloned *ECA39* gene. *c-myc*-Overexpressing BK1 brain tumor cells were used as source of nuclear proteins which, as shown in Figure 6A, gave a clear and specific retarded band when mixed with labeled DNA. The intensity of this band was  $\sim 10$ -fold higher using nuclear extracts derived from BK1 cells than that observed using equal quantities of nuclear extracts derived from HeLa cells (results not shown). Moreover, this interaction is specific, because the addition of identical unlabeled oligomer competed and eliminated the retarded band, whereas a similar oligomer in which the core sequence was mutated (to CACCAC) did not compete for binding of the nuclear proteins (Fig. 6A).

In addition to c-Myc, other nuclear factors [e.g., TFE3 (Beckmann et al. 1990) and upstream stimulatory factor (USF) (Gregor et al. 1990)] may interact with the sequence CACGTG. It is possible that the *ECA39* sequence is a target for such interaction. However, to determine that c-Myc is part of the nuclear protein-DNA

interaction that we have observed, a monoclonal anti-body raised against a short c-Myc peptide that recognizes mouse c-Myc protein (Evan et al. 1985) was used to assess its ability to induce a supershift in the specific DNA/nuclear protein complex. The antibody used is specific to c-Myc and recognizes an amino-terminal region of the protein that is not required for DNA binding (Evan et al. 1985). The presence of c-Myc in this complex should permit the binding of anti-c-Myc antibody, altering the mobility of the complex and further retarding its migration. The complex is retarded further (supershifted) by the addition of anti-c-Myc antibody as shown in Figure 6B. The specificity of the induced retardation is illustrated by a control experiment in the which the anti-c-Myc antibody shows no effect on the mobility of the unrelated AP1-DNA nuclear protein complex (Fig. 6B). Another control using an isotype-matched irrelevant antibody does not alter the mobility of the Myc DNA-binding site complex either (not shown). These results indicate that the c-Myc DNA-binding site associated with the *ECA39* gene is capable of forming a nuclear protein-DNA complex that includes c-Myc.

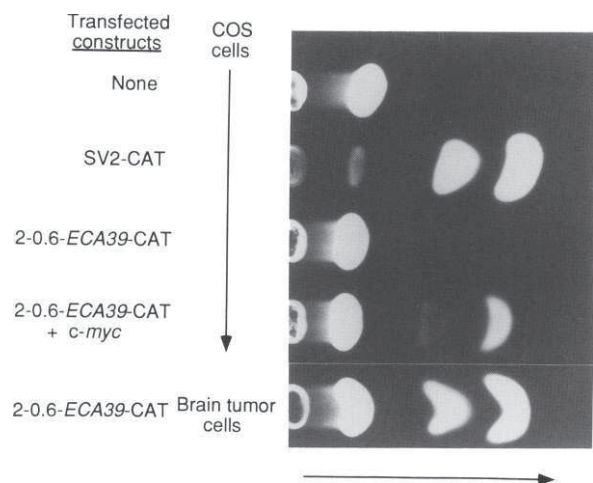


**Figure 6.** DNA-binding assay of the c-Myc-binding site in the *ECA39* gene. Nuclear extracts were isolated from brain tumor (BK1) cells and analyzed in a gel-shift assay as described (Roy et al. 1991). (A) The 30-bp Myc-binding oligomer covers the c-Myc recognition site as it appears in the *ECA39* gene (GACCCCTGAGCGCCACGTGTCACGTGACAGC). The altered Myc-binding oligomer, is a similar oligomer, in which the c-Myc recognition site was mutated (GACCCTGAGCGCCACCACTGTCACAGC). Competitor oligonucleotides were added in 5 $\times$ , 10 $\times$ , or 30 $\times$  molar excess to the labeled oligomer. (B) The  $^{32}$ P-labeled Myc DNA-binding oligomer or the 22-bp AP1 DNA-binding oligomer (Stratagene) was incubated with nuclear proteins isolated from brain tumor (BK1) cells. Thirty minutes later, either 0.2 or 1  $\mu$ g of c-Myc monoclonal antibody (Myc1-3c7) (Evan et al. 1985) was added for an additional 15 min. Note that the addition of the Myc antibody supershifted only the protein complex that binds to the Myc-binding oligomer.

*The putative c-Myc target gene requires c-Myc for its expression and is regulated by c-Myc in differentiating embryonic stem cells*

To examine in a more direct way whether c-Myc modulates expression of its putative target, a chimeric 2–0.6-*ECA39*–CAT reporter construct containing the c-Myc recognition site was transfected into COS cells in the presence or absence of a cotransfected c-myc gene driven by an SV40 promoter/enhancer (Fig. 7). In the absence of overexpression of c-myc, expression of the 2–0.6-*ECA39*–CAT reporter is relatively low, but when the reporter gene is cotransfected with the c-myc construct, its expression is enhanced three- to ninefold (Fig. 7). Similar experiments in NIH–3T3 cells also demonstrated a two- to fourfold enhancement of 2–0.6-*ECA39*–CAT expression. In addition, cotransfection of the c-myc gene did not enhance expression of the chimeric construct 2–0.6-*ECA39*–CAT-X, in which the putative c-Myc-binding site was mutated by insertion of an *Xba*I linker (results not shown). Because recent evidence has suggested that the nuclear protein Max might be a physiologic partner of c-Myc (Blackwood and Eisenman 1991; Prendergast et al. 1991; Blackwood et al. 1992), similar cotransfection experiments were carried using expression vectors for both the *max* and c-myc genes. Consistent with the fact that Max seems to be expressed constitutively at fairly high levels and is thus not likely to be a limiting factor in transcription (Blackwood et al. 1992; Wagner et al. 1992), the addition of the *max* construct did not further increase *ECA39*–CAT expression over that induced by c-myc alone (not shown).

Transient transfection assays offer an important op-



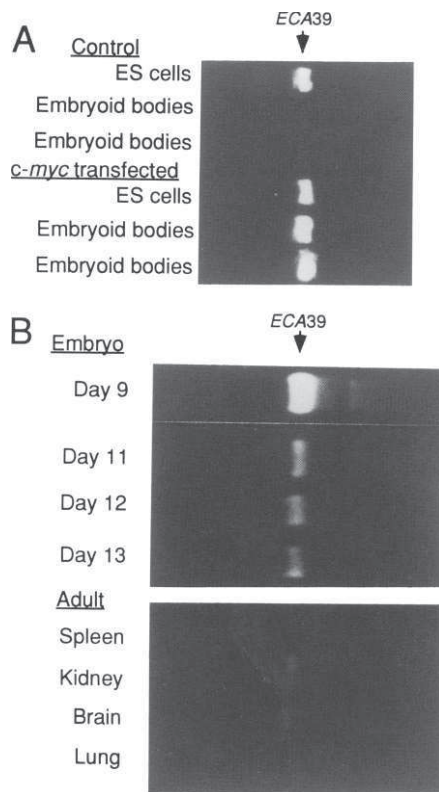
**Figure 7.** Effect of c-myc transfection on the expression of *ECA39*–CAT chimeric genes. COS or BK1 cells were electroporated with a combination of different chimeric CAT genes, and CAT activity was determined 48 hr later. (SV2–CAT) SV40 promoter enhancer driving CAT; (*ECA39*–CAT) 2–0.6-*ECA39*–CAT; (c-myc) pSV2humMycB–Bam, a vector carrying c-myc driven by the SV40 promoter enhancer. Note that cotransfection of c-myc enhanced expression of the *ECA39*–CAT construct.

portunity to evaluate the response of transfected genes in vivo, but we also wished to assess the effect of c-Myc on the endogenous *ECA39* gene in its appropriate chromosomal context. We addressed this issue first by transfecting (overexpressing) the c-myc chimeric construct in murine erythroleukemia (MEL) cells, cells that ordinarily do not express the *ECA39* target gene. Using this system, we were unable to induce de novo expression of *ECA39* (not shown). Thus, overexpression of c-myc does not, by itself, initiate expression of the target *ECA39* gene in this normally inactive cellular context. To assess the effect of overexpression of c-myc on an actively expressed endogenous *ECA39* gene, we chose a more subtle approach involving embryonic stem (ES) cells. Niwa et al. (1990) have reported that the *ECA39* gene is down-regulated in teratocarcinoma cells as they differentiate. In studies directed toward characterizing *ECA39* expression, we noted that this gene is highly expressed in ES cells and that this expression is also dramatically down-regulated as the ES cells undergo differentiation in culture (Fig. 8A; see below). Using this response as an assay, ES cell lines were established that were permanently transfected with a c-myc expression vector. Both c-myc-transfected and nontransfected ES cell lines undergo differentiation when transferred to bacteriologic dishes in the absence of the differentiation inhibiting activity factor/leukemia inhibitory factor (DIA/LIF). Differentiation was monitored by morphology of embryoid bodies and initiation of expression of  $\zeta$ -globin (A. Leder, unpubl.). In agreement with these observations, it has also been shown that transfection of ES cells with c-myc does not affect the ability of these cells to differentiate and generate normal chimeric mice (Suda et al. 1988). In our experiment, the transfected (deregulated) c-myc gene is expressed either before or after differentiation at about the same levels, whereas expression of the endogenous gene decreased by twofold upon differentiation to embryoid bodies (which consist of different cell types) (results not shown). As seen in Figure 8A, *ECA39* is highly expressed in ES cells and is sharply down-regulated as these cells differentiate. In contrast, high levels of *ECA39* expression are maintained in c-myc-transfected ES cells, even as they undergo differentiation. Thus, transfection of c-myc appears to prevent the normal down-regulation of the *ECA39* target gene.

*Structure of the ECA39 gene product and its expression in embryonic and adult tissues*

Because the *ECA39* gene appears to be a directly regulated target of c-Myc, it would be useful to know its function. Occasionally the function of a gene product can be inferred from the structure of the protein it encodes. Accordingly, we determined the sequence of a full-length cDNA from which we inferred the sequence of a protein that is 241 amino acids in length. As noted above, a search of the data base indicated that it was identical to an anonymous cDNA cloned from a teratocarcinoma cell line (Niwa et al. 1990) but was not significantly similar to any other sequence available for





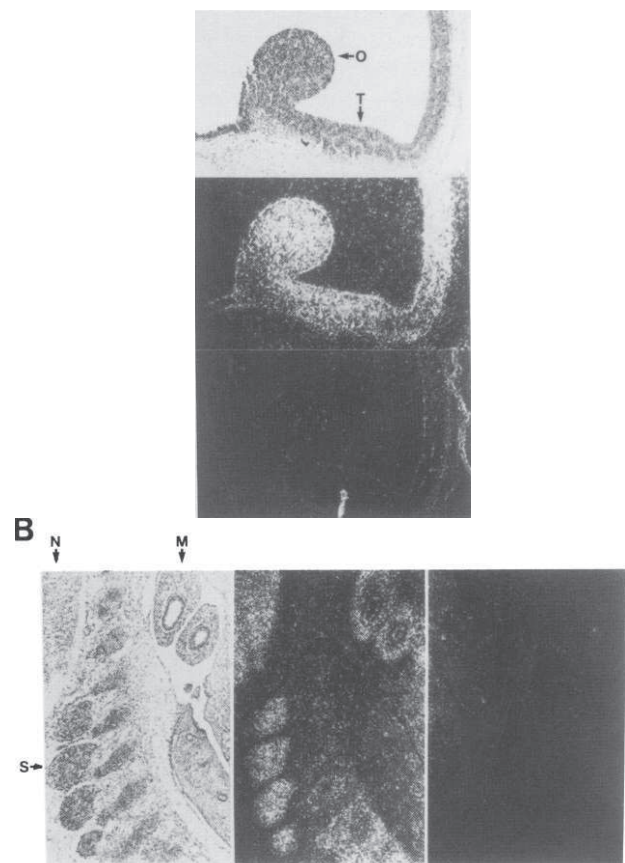
**Figure 8.** Development-specific expression of the *ECA39* gene and regulation by *c-myc*. (A) D3 ES cells (Doetschman et al. 1985) were transfected with the neomycin resistance gene alone (control) or with *c-myc* driven by the globin locus control region (Grosveld et al. 1987). The *c-myc*-transfected gene was shown to be expressed before and after differentiation. RNA was extracted from different clones of ES cells or differentiated embryoid bodies. In each lane, 20  $\mu$ g of total RNA was blotted and hybridized with an *ECA39* cDNA probe. (B) RNA was isolated from embryo and adult tissues (Chirgwin et al. 1979), blotted, and hybridized with an *ECA39* cDNA probe.

comparison. The inferred protein does not contain any obvious signal sequences or transmembrane, enzymatic, or transcription factor domains, but it does bear a putative nuclear localization signal (KKPSK, amino acids 176–180). Interestingly, Niwa et al. (1990) cloned the *ECA39* cDNA by virtue of its amplification in a teratocarcinoma cell line.

We have shown above (Fig. 2) that *ECA39* is highly expressed in brain tumors and T-cell lymphomas derived from our *c-myc*-overexpressing transgenic mice. We have also seen that the gene is highly expressed in ES cells and that it undergoes down-regulation as these cells differentiate in culture (Fig. 8A). These observations, coupled with the fact that the *myc* family of genes is also developmentally regulated (Zimmerman et al. 1986), prompted us to examine the expression of the target gene in embryonic tissue.

As shown in Figure 8B, *ECA39* is expressed at very high levels in embryos on day 9 and continues to be

expressed at moderate levels through embryonic day 13. This expression was examined in greater detail using in situ hybridization analysis. As shown in Figure 9, the antisense *ECA39* RNA probe detects specific hybridization in the brain [shown are optic chiasma and telencephalon (Fig. 9A)], the somites, and the mesonephric tubules of a day 11 embryo (Fig. 9B). Similar results were obtained using two different probes from *ECA39* cDNA (not shown). Low levels of *ECA39* expression persist in the adult brain and kidney, as demonstrated by the RNA analyses shown in Figure 8B; no expression is observed in adult lung, spleen, or thymus (Figs. 2 and 8B). Thus, it seems that *ECA39* is highly expressed in the embryonic brain and renal precursor and that expression diminishes as development proceeds. Interestingly, *c-myc* expression is also down-regulated during development in the brain (Zimmerman et al. 1986).



**Figure 9.** In situ hybridization of *ECA39* in embryos. (A) In situ hybridization analysis of *ECA39* expression in the brain of an 11-day-old embryo. Shown are the optic chiasma (O) and the telencephalon (T). (Top) Hematoxylin and eosin stain; (middle) dark field of *ECA39* antisense hybridization; (bottom) dark field of *ECA39* sense hybridization, as control. (B) In situ hybridization analysis of *ECA39* expression in somites (S), mesonephric tubules (M), and the neural tube (N) of an 11-day-old embryo. (Left) Hematoxylin and eosin stain; (middle) dark field of *ECA39* antisense hybridization. (Right) Dark field of *ECA39* sense hybridization, as control.



*Is c-Myc a factor that enhances the transcription of ECA39 through its interaction with an intragenic binding site?*

Although our data are consistent with the notion that c-Myc is a DNA-bound factor that regulates *ECA39* expression by enhancing its transcription, at this time we cannot exclude other mechanisms for its action. The c-Myc recognition site is located in the first untranslated exon of the *ECA39* gene and is therefore a part of the *ECA39* transcript. It is possible that c-Myc regulates *ECA39* by interacting with its mRNA and affecting its stability, attenuation, or transport. However, as the RNA in this region does not appear to contain a stable secondary structure and because there is ample precedent for genes being regulated by enhancers located within their transcribed sequences [e.g., the immunoglobulin heavy- and light-chain genes (Banerji et al. 1983; Gillies et al. 1983; Queen and Baltimore 1983), the thymidine kinase gene (Merrill et al. 1984), and even the *c-myc* gene itself (Sacca and Cochran 1990)], our working hypothesis holds that c-Myc enhances expression of *ECA39* through a DNA-protein interaction that affects transcription. Interestingly, it has recently been reported that p53 gene expression is up-regulated by a CACGTG sequence that is also located 3' to its promoter (Ronen et al. 1991). The factor enhancing p53 expression is believed to be a member of the MyoD family of transcriptional regulators. In addition, transcription of  $\alpha$ -prothymosin has been suggested to be activated by the Myc protein (Eilers et al. 1991); however, direct regulation of this gene by c-Myc is yet to be shown definitively.

It has also been proposed that Max is the partner of c-Myc, and we have detected high levels of *max* mRNA in both brain tumor and lymphoma cells (results not shown). Because Max expression is not cell cycle regulated, it would appear that the level of Myc, and not Max, is the limiting component in the formation of a Myc/Max complex (Blackwood et al. 1992; Wagner et al. 1992). Our failure to observe additional activation of the *ECA39* reporter construct by cotransfecting the *c-myc* and *max* genes is consistent with this view.

The *ECA39* gene appears to be regulated by c-Myc through the CACGTG sequence, which has been shown previously to bind c-Myc (Blackwell et al. 1990; Prendergast and Ziff 1991). Recently, an attempt has been made to extend the binding site of c-Myc beyond the 6-nucleotide core sequence (Halazonetis and Kandil 1991). The 12-nucleotide sequence GACCACGTGGTC, which has been identified by *in vitro* binding to a c-Myc homodimer, resembles the sequence CGCCACGTGTCA of the *ECA39* gene only partially. Thus, it seems that identification of other c-Myc target genes is needed before a longer consensus sequence for c-Myc binding *in vivo* is established.

Our ignorance as to the function of the *ECA39* gene product currently limits our ability to relate this gene to the transforming process or to cell cycle control. Nevertheless, certain conclusions can be drawn. It is clear that expression of this gene is not necessary for the transfor-

mation of all cells bearing *c-myc* as a tumor-provoking gene. The fact that we observe expression of this gene in brain tumors and T-cell lymphomas, but not in mammary carcinomas and B-cell lymphomas, indicates that it is activated in a limited range of cell types. Despite the expression of this gene in ES cells, the specificity of expression that we have observed in later stage embryos suggests that the gene product is also not required for an activity common to all cells. If the putative nuclear localization signal observed within the *ECA39* protein proves functional, the range of reactions occurring within the nucleus provides a range of candidate actions for what appears to be an entirely novel protein sequence.

Finally, the discovery of a physiologic target gene for the action of c-Myc seems to close the circle of evidence implicating c-Myc as a direct modulator of gene activity. The *ECA39* gene is likely to be only one of a number of such target genes. In addition to encouraging the further screening of appropriate libraries for additional members of the c-Myc-regulated gene family, the structure of the *ECA39* gene itself may lead to the recognition of a larger, related family of genes that, in addition to sharing common structural elements, may also be regulated by c-Myc or other members of its family.

## Materials and methods

### *Construction and screening of a subtracted cDNA library*

RNA was isolated from brain tumor BK1 cells (Chirgwin et al. 1979) and was poly(A)<sup>+</sup> selected (Aviv and Leder 1972) using oligo(dT)-cellulose (Pharmacia LKB Biotechnology, Inc., Sweden). A unidirectional cDNA library was prepared from this RNA using the  $\lambda$ ZAP II vector and cDNA cloning reagents from Stratagene (La Jolla, CA). The original library contained  $\sim 2 \times 10^6$  recombinants before amplification. Single-stranded phage of the  $\lambda$ ZAP II library were rescued using R408 helper phage and isolated in the presence of polyethylene glycol, as described previously (Sambrook et al. 1989). To enrich the library for transcripts expressed primarily in brain tumor cells, we subtracted transcripts represented in normal brain mRNA. Thus, poly(A)<sup>+</sup> enriched RNA from adult mouse brain was photobiotinylated (Sive and St. John 1988) using a 275 W sunlamp (General Electric Co. Cleveland, OH) and reagents from Invitrogen Corporation (San Diego, CA). Biotinylated brain poly(A)<sup>+</sup> enriched RNA (40  $\mu$ g) was hybridized for 48 hr with 4  $\mu$ g of single-stranded DNA from the brain tumor library using reagents obtained from Invitrogen Corporation. DNA hybridizing with the biotinylated RNA was bound to streptavidin and precipitated in the presence of phenol (Sive and St. John 1988). The unbound single-stranded DNA was then converted to double-stranded DNA with Klenow enzyme (Schweinfest et al. 1990) and transformed into *Escherichia coli*. Plasmid DNA was extracted from individual colonies, dot blotted in triplicate onto GeneScreen membranes (NEN Research Products, Boston, MA), and hybridized with <sup>32</sup>P-labeled single-stranded cDNA from brain tumor BK1 cells, normal adult brain, or lymphoma. Plasmids that hybridized more with transcripts from brain tumor and lymphoma and less with transcripts from normal brain were characterized further.

### *RNA analysis*

RNA was isolated from cells and tissues as described by Chirg-

win et al. (1979). RNA blot analysis was performed according to the procedures of Thomas (1981). RNase protection was performed by hybridizing RNA to an antisense riboprobe, as described by Melton et al. (1984). Primer extension was performed using a  $^{32}\text{P}$ -labeled oligomer as a primer, as described by Sambrook et al. (1989).

#### *In situ hybridization*

Whole embryos were fixed in 4% paraformaldehyde for 2 hr at 4°C and embedded in paraffin as described by Zeller et al. (1987).  $^{35}\text{S}$ -Labeled RNA probes were synthesized in vitro in either sense or antisense orientation. Hybridization and washing were done as described by Wilkinson et al. (1987), with some modifications (Sassoon et al. 1988).

#### *Isolation and sequencing of a genomic clone*

A genomic phage library constructed from DNA of mouse teratocarcinoma cells (catalog no. 946302, Stratagene) was screened for *ECA39* sequences according to procedures described previously (Sambrook et al. 1989). A clone covering the 5' region of the gene was isolated, and its nucleotide sequence was determined using a sequencing kit from Pharmacia LKB Biotechnology, Inc.

#### *Tissue culture and transfections*

BK1 cells (Benvenisty et al. 1992) were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 50 units/ml of penicillin G activity, 50  $\mu\text{g}/\text{ml}$  of streptomycin, 4 mM L-glutamine, and 50  $\mu\text{M}$  2-mercaptoethanol. COS cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum, 50 units/ml of penicillin G activity, 50  $\mu\text{g}/\text{ml}$  of streptomycin, and 4 mM L-glutamine. D3 mouse ES cells (Doetschman et al. 1985) were grown in DMEM supplemented with 10% fetal calf serum, 10% newborn calf serum, 4 mM L-glutamine, 50 mM 2-mercaptoethanol, nonessential amino acids, and 1000 U/ml of LIF (GIBCO BRL, Gaithersburg, MD). Differentiation of ES cells was induced by growing them in bacteriological dishes without LIF for 15–20 days, thus inducing the formation of embryoid bodies (Doetschman et al. 1985). DNA was transfected into cells by electroporation (960  $\mu\text{F}$ , 250 V, Bio-Rad Gene Pulser) (Potter et al. 1984). Transfection efficiency was verified by monitoring the amount of foreign DNA in the transfected cells.

#### *Chimeric genes and CAT assay*

To generate chimeric reporter constructs, sequences from the *ECA39* genomic clone were inserted upstream of a CAT gene. The gene 1.5-*ECA39*-CAT contains a 1.5-kb *Pst*I-*Pvu*II fragment from the promoter region of the *ECA39* gene; 2-0.6-*ECA39*-CAT contains a 2.6-kb *Sac*I fragment covering 2 kb upstream and 0.6 kb downstream from the transcription start site of the *ECA39* gene; 2-0.6-*ECA39*-CAT-X is a mutated form of 2-0.6-*ECA39*-CAT, in which an *Xba*I linker was inserted into a *Bbr*PI site (the *c-Myc* recognition site); 2-0.1-*ECA39*-CAT contains a 2.1-kb *Sac*I-*Bbr*PI fragment covering 2 kb upstream and 107 bp downstream from the transcription start site of the *ECA39* gene. The gene 2-0.2-*ECA39*-CAT contains 2 kb upstream and 166 bp downstream from the transcription start site of the *ECA39* gene. This construct has been created by insertion of a 59-bp oligomer (covering sequences 108–166 of the *ECA39* gene) into the *Bbr*PI site. The gene 2-0.2-*ECA39*-CAT-P is a construct similar to 2-0.2-*ECA39*-CAT, in which the inserted

oligomer contains a point mutation in site 109, modifying the *c-Myc* recognition site from CACGTG to CACGAG. CAT activity was determined 48 hr after transfection, as described previously (Gorman et al. 1982).

#### *DNA-binding assays*

Nuclear extracts from BK1 cells were isolated and analyzed as described by Roy et al. (1991). Briefly, double-stranded oligonucleotide probes were generated by annealing complementary oligonucleotides and end-labeling with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Nuclear extracts were incubated for 30 min at room temperature with the labeled oligonucleotide, and protein-DNA complexes were fractionated on a 6% polyacrylamide gel using reagents from Stratagene.

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