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Title	A FOS protein is present in a complex that binds a negative regulator of MYC.
Author(s)	Hay, Nissim; Takimoto, Masato; Bishop, J. Michael
Citation	Genes & Development, 3(3), 293-303 https://doi.org/10.1101/gad.3.3.293
Issue Date	1989-03
Doc URL	https://hdl.handle.net/2115/72596
Type	journal article
File Information	GenesDev1989.pdf



A *FOS* protein is present in a complex that binds a negative regulator of *MYC*

Nissim Hay,¹ Masato Takimoto,² and J. Michael Bishop¹

¹Department of Microbiology and Immunology and the G.W. Hooper Foundation, University of California, San Francisco, California 94143 USA; ²Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 USA

Regulation of the human proto-oncogene *MYC* apparently plays an important role in cellular proliferation and the genesis of diverse tumors. Transcription from *MYC* is governed principally by two promoters known as P1 and P2. Previously we have detected a negative regulator of these promoters upstream of *MYC*. We now report that this regulator comprises no more than 26 bp of DNA, with sequence that resembles the regulators of at least two other genes, and we describe nuclear factors that interact with the regulator. Nuclear extracts from human cells form three distinctive complexes with the negative regulator. One of these complexes includes the product of the proto-oncogene *FOS* or an antigenically related protein, and the *FOS* protein may, in turn, be associated with the product of the proto-oncogene *JUN*. Similarly, *FOS* and *JUN* proteins produced by translation *in vitro* bind cooperatively to the negative regulator. These results raise the possibility that *FOS* and *JUN* participate in the regulation of *MYC*.

[*Key Words*: Transcription; proto-oncogene; oncogene; *JUN*]

Received November 28, 1988; revised version accepted January 25, 1989.

The expression of the proto-oncogene *MYC* appears to be involved in maintaining the cell's capacity for proliferation. Transcription from *MYC* is increased during the response of cells to a variety of growth factors and mitogens (Greenberg and Ziff 1984; Kelly et al. 1983; Greenberg et al. 1985; Dean et al. 1986; Levine et al. 1986), whereas transcription of the gene is decreased during differentiation of the cells to a nonproliferative state (Grosso and Pitot 1985; Bentley and Groudine 1986; Eick and Bornkamm 1986; Endo and Nadal-Ginard 1986; Ramsay et al. 1986; Siebenlist et al. 1988). Regulation of transcription from *MYC* is mediated by changes in both the elongation and initiation rate of transcription (Greenberg and Ziff 1984; Greenberg et al. 1985; Bentley and Groudine 1986; Nepveu et al. 1987; Siebenlist et al. 1988). Disruption of the normal control of the gene is associated with the development of diverse types of tumors and transformation of cells in tissue culture (Cole 1986; Cory 1986; Small et al. 1987).

We have begun an examination of the mechanisms controlling *MYC* transcription by focusing on regulatory sequences within the 5'-flanking region of the human *MYC* gene. In previous studies we found that the nucleotide sequences between residues -293 and +513 are sufficient for strong and accurate transcription from the two principal promoters for the gene, P1 and P2 (+1 is the site of initiation for P1) (Hay et al. 1987). We have defined several elements that modulate the activities of the two promoters: a region that alone can sustain activity of P1 (-101/-293); two domains that exert posi-

tive effects on both P1 and P2 (-353/-1257 and -1257/-2329); and a negative regulator that acts on expression from both promoters (-293/-353).

Within the region -293/-353, we identified a binding site for nuclear protein or proteins (Hay et al. 1987). The binding site encompasses 26 bp with an AT-rich core region, overlapped and flanked by inverted repeats of eight residues. Now, we have examined in greater detail the function and the protein-binding properties of the 26-bp region. We have found that the 26-bp DNA element is sufficient to exert a negative effect on expression from both *MYC* promoters and thus have termed it the *MYC* negative regulatory element (*MYC*-NRE). *MYC*-NRE forms three complexes with proteins from nuclear extracts of HeLa cells. One of these complexes contains the product of the proto-oncogene *FOS* or a closely related protein and also may contain the protein encoded by *JUN* and/or other components of the transcription factor AP-1 (see Rauscher et al. 1988a,b; Sassone-Corsi et al. 1988a). Using translation *in vitro*, we have shown that the *FOS* protein fails to bind by itself to *MYC*-NRE, whereas cotranslated *FOS* and *JUN* bind cooperatively to *MYC*-NRE and form a specific protein-DNA complex.

Like *FOS* and *JUN*, *MYC* belongs to a group of genes whose expression is induced during the mitotic activation of quiescent cells. Expression of *FOX* and *JUN* is induced rapidly after serum stimulation of growth-arrested cells and is followed by induction of *MYC* expression. The work presented here sustains this idea by dem-

onstrating that the products of *FOS* (and perhaps *JUN*) apparently bind to a regulatory element upstream of *MYC*.

Results

A 26-bp region that inhibits transcription from MYC promoters

In previous studies we found that a 60-bp region between nucleotides -293 and -353 (where $+1$ is the transcription start site for the P1 promoter) inhibits transcription from the *MYC* promoters P1 and P2 (Hay et al. 1987). Within this region (between nucleotides -318 and -343), we identified a DNA-binding site for a nuclear protein, or proteins, from HeLa cells (Hay et al. 1987). To determine whether this DNA-binding site is sufficient to inhibit transcription from *MYC* promoters, we constructed a plasmid in which the 26-bp region between nucleotides -318 and -343 was linked to nucleotide -293 . (For a detailed description of the plasmid, see Materials and methods). This plasmid was designated as $-343\Delta-293/-318$ (Fig. 1A, construction b).

The plasmid $-343\Delta-293/-318$ and other 5' deletion mutants (Fig. 1A) were transfected into Ltk⁻ cells. Polyclonal populations of stably transfected cells that contained and expressed the various chimera were established. RNA analysis was performed with the RNase protection assay (Fig. 1B). The highest level of expression from the *MYC* promoters P1 and P2 was obtained with the 5' deletion mutant -293 (Fig. 1B, lane 4); a

level of expression of approximately twofold lower was obtained with the 5'-deletion mutant -321 (Fig. 1B, lane 3). However, a marked decrease of expression from both P1 and P2 promoters was obtained with the plasmid $-343\Delta-293/-318$. The relative inhibition of transcription obtained with the deletion mutant $-343\Delta-293/-318$ was comparable to that obtained with the 5'-deletion mutant -353 (Fig. 1B, lanes 1,2,4). In agreement with our previous studies, the level of expression by mutant -353 was ~ 10 -fold lower than that achieved with mutant -293 (Hay et al. 1987). These results indicate that the region between nucleotide -318 and -343 , which represents a binding site for nuclear protein(s) (Hay et al. 1987), is sufficient to inhibit *MYC* promoters P1 and P2.

Binding of three protein complexes to MYC-NRE

We used a 26-bp synthetic duplex that contains the sequence between nucleotides -318 and -343 (*MYC*-NRE in Fig. 2) to search for binding to protein factors. The radiolabeled 26-bp double-stranded oligomer was incubated with nuclear extract from HeLa cells in the presence of nonspecific competitor poly[d(I-C)]. Protein-DNA complexes were separated from free DNA by electrophoresis in a nondenaturing gel (Fried and Crothers 1981; Strauss and Varshavsky 1984). The *MYC*-NRE oligomer formed three complexes with nuclear protein, as demonstrated by retarded mobility, relative to that of the free DNA (Fig. 3). The three DNA-protein complexes were designated a, b, and c.

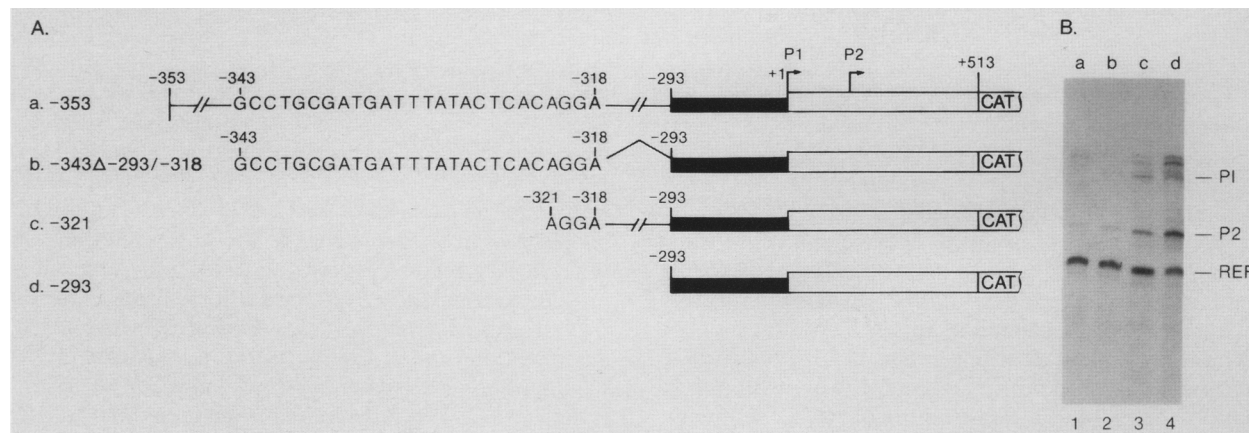


Figure 1. Control of *MYC* promoters by upstream sequences. (A). Diagram of DNAs introduced into cells. Arrowheads indicate the sites for initiation of transcription controlled by promoters P1 and P2; The coordinates for *MYC* are defined relative to the site of initiation for P1 ($+1$). (B). Quantitation of RNA by RNase protection assay. Polyclonal lines of Ltk⁻ cells bearing the DNAs described in A were established using cotransfection with hygromycin B. Transcripts derived from P1 and P2 were then detected by hybridization with RNA fragment synthesized with Sp6 polymerase and subsequent hydrolysis with RNase A and T1 (Hay et al. 1987). The protected fragments were analyzed by electrophoretic fractionation on denaturing acrylamide gels. RNA fragments representing initiation by P1 and P2 are labeled. An RNA probe for transcripts derived from the cotransfected hygromycin B gene served as an internal standard (REF). Lanes are marked according to the diagram in A.

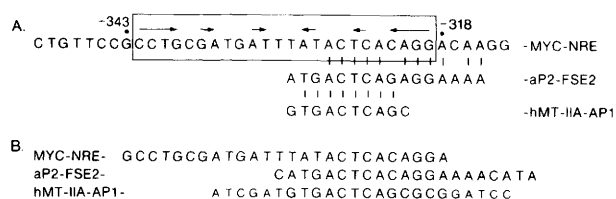


Figure 2. Similarities among MYC-NRE, FSE2, and hMT_{IIA}-AP1. (A). Comparison of MYC-NRE, FSE2 of the aP2 gene, and hMT_{IIA}-AP1. The framed sequence indicates the portion of MYC-NRE responsible for binding nuclear factors. Arrowheads indicate the dyad symmetry within MYC-NRE. Identities between different sequences are indicated by vertical lines. (B). Sequence of double-stranded synthetic oligonucleotides used for the gel retardation assays. Smaller size letters indicate nucleotides that are not part of the binding site but were added to the synthetic oligonucleotide for stability of duplexes.

A nuclear protein complex that binds to MYC-NRE and other regulators of transcription

We compared the sequence of MYC-NRE to the sequence of another negative regulator, upstream of the adipocyte-specific gene aP2 (Distel et al. 1987). This sequence, FSE2, is probably responsible for repressing expression of aP2 in preadipocyte cells. Comparison of the FSE2 and MYC-NRE sequences revealed a region of 13

bp within FSE2 that contains the recognition site for the transcription factor AP-1 and has only two mismatches with the sequence of MYC-NRE between nucleotides -315 and -327 (Fig. 2A). FSE2 also resembles the site upstream of the human metallothionein IIA gene that binds a complex of transcription factors, including the products of the proto-oncogenes *JUN* (known also as the AP-1 factor) and *FOS* (Fig. 2A) (Angel et al. 1987; Lee et al. 1987; Chiu et al. 1988; Rauscher et al. 1988a; Sassone-Corsi et al. 1988b). This site is designated hMT_{IIA}-AP1. To test whether common nuclear protein factors bind to the three different elements (MYC-NRE, FSE2, and hMT_{IIA}-AP1), we performed DNA-binding experiments and competition assays, using synthetic oligomers representing the three binding sites (Fig. 2B).

The ability of MYC-NRE to compete specifically for the binding of protein to FSE2 was determined first (Fig. 4). Incubation of the labeled FSE2 double-stranded oligomer with HeLa nuclear extract, followed by the gel retardation assay, revealed formation of two protein-DNA complexes (I and II in Fig. 4, lane 1). Complex I is equivalent to the complex b that forms with MYC-NRE DNA (Fig. 6); complex II is equivalent to complex c (Fig. 6; N. Hay, unpubl.). Formation of complex II was inhibited efficiently when the binding assay was done in the presence of increasing excess of the cognate oligomer (Fig. 4, lanes 2–5). Formation of the same protein-DNA complex also was inhibited with similar efficiency by using MYC-NRE as a competitor (Fig. 4, lanes 6–10). To demonstrate the specificity of the competition, the DNA-binding assay was performed in the presence of a

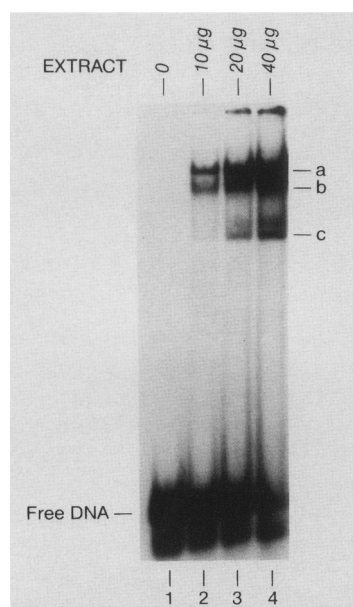


Figure 3. Binding of nuclear factors to MYC-NRE. Approximately 0.2 ng of synthetic double-stranded oligonucleotide, representing MYC-NRE and end labeled with ³²P, was incubated with increasing amounts of HeLa nuclear extract, followed by electrophoretic fractionation in an acrylamide gel (for details, see Materials and methods).

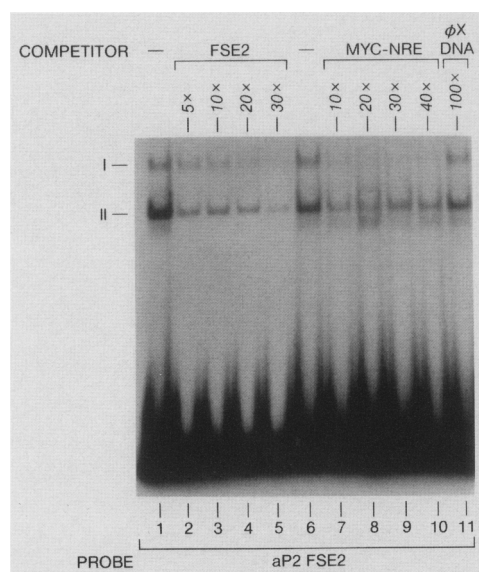


Figure 4. MYC-NRE competes with FSE2 for binding of a nuclear factor. Approximately 0.2 ng of ³²P-labeled FSE2 was incubated with HeLa nuclear extract in the absence or presence of the indicated excess of unlabeled FSE2, MYC-NRE, or ϕ X174 *Hae*III fragments.

100-fold excess of ϕ X174 DNA. No significant interference with the formation of the protein–DNA complexes was observed (Fig. 4, lane 11).

Then we performed the reciprocal competition assay to determine whether FSE2 and hMT_{II}A-AP1 could compete for binding to the MYC-NRE. As shown in Figure 5, lanes 1–3, the formation of protein–DNA complexes a and b with MYC-NRE was inhibited in the presence of excess amounts of the cognate oligomer. Protein–DNA complex c appeared to result from a nonspecific interaction, as the formation of this complex was not competed efficiently (see Figs. 4 and 5; M. Takimoto and D. Levens, unpubl.). In the presence of FSE2 or hMT_{II}A-AP1 as competitors, a marked decrease in the formation of protein–DNA complex b was observed without significant inhibition of formation of protein–DNA complex a (Fig. 5, lanes 4–9).

The results of the competition assays suggested that the same nuclear proteins that bind to FSE2 and hMT_{II}A-AP1 also participate in the formation of protein–DNA complex b with MYC-NRE. We obtained further evidence for this assumption by carrying out the DNA-binding assays with nuclear extracts from the neuroblastoma cell line Kelly. Formation of complex b could not be detected with these extracts (Fig. 6, lanes

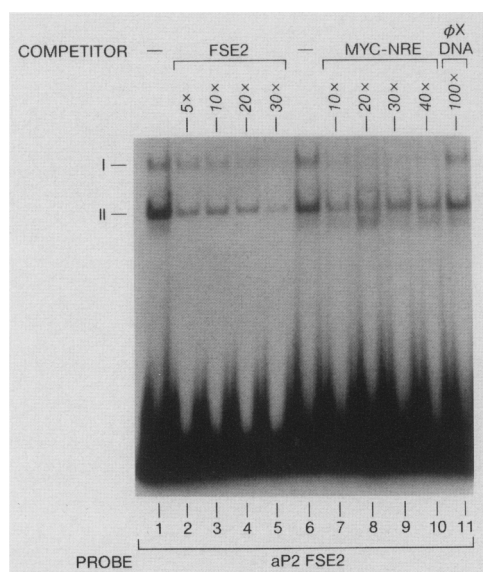


Figure 5. FSE2 and hMT_{II}A-AP1 compete with MYC-NRE for binding of a nuclear factor. Approximately 0.2 ng of ³²P-labeled MYC-NRE was incubated with HeLa nuclear extract in the absence or presence of the indicated excess of unlabeled MYC-NRE, FSE2, or hMT_{II}A-AP1.

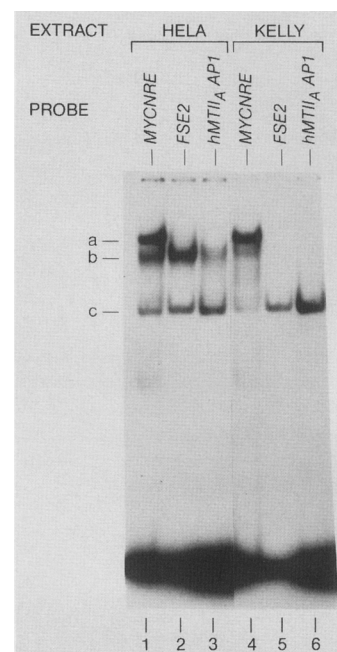


Figure 6. Gel retardation assay with HeLa and Kelly nuclear extracts. MYC-NRE, FSE2, or hMT_{II}A-AP1, labeled with ³²P, was incubated with 10 μ g of either HeLa or Kelly nuclear extract, followed by electrophoretic fractionation.

1,4). The corresponding protein–DNA complex with FSE2 and hMT_{II}A-AP1 also was not present when Kelly extract was used (Fig. 6, lanes 2,3,5,6). These results sustain the view that similar or identical proteins contribute to the formation of complex b with MYC-NRE, FSE2, and hMT_{II}A-AP1.

FOS protein or a related antigen is a component of a protein complex that binds to MYC-NRE

It was shown previously that FSE2 and hMT_{II}A-AP1 binds FOS protein specifically, in association with JUN protein—otherwise known as the nuclear factor AP-1 (Bohmann et al. 1987; Chiu et al. 1988; Rauscher et al. 1988b; Sassone-Corsi et al. 1988b). The results of the competition experiments described above led us to examine whether a FOS protein binds to MYC-NRE. We tried to inhibit the binding of nuclear factors to MYC-NRE by preincubation of the extract with antibodies directed against the FOS protein, following the strategy described previously by Spiegelman and his colleagues (Distel et al. 1987). The nuclear extract was preincubated with antibodies prior to addition of the labeled DNA. FOS-specific antibodies interfered with the binding of protein complex to MYC-NRE and specifically abolished formation of DNA protein complex b

(Fig. 7A,B, lanes 1,2). The inhibition by the antibody was specific because *MYC* antibodies or other unrelated antibodies caused no significant inhibition of the binding (Fig. 7A, lane 3; N. Hay, unpubl.). Furthermore, when the preincubation with the affinity-purified *FOS* antibodies was done in the presence of the peptide against which the antibody was made, formation of DNA protein-complex b was restored (Fig. 7B, lane 3). Similar results were obtained when the FSE2 oligomer was used as a probe in the DNA-binding assay (Fig. 7A,B, lanes 4–6), in agreement with what was shown previously (Distel et al. 1987; Rauscher et al. 1988a). The level of inhibition for the binding obtained with *FOS* antibodies varied from one experiment to the other in a range of 70–90%. However, inhibition of the binding to FSE2 was usually more efficient than that to *MYC*-NRE (Fig. 7A,B).

We used immunological means to detect *FOS* protein directly in complex b. HeLa nuclear extract was incubated with unlabeled *MYC*-NRE, followed by electrophoresis in a nondenaturing gel. The retarded complexes were transferred to nitrocellulose paper and probed with *FOS* antibodies. The immunoreactive band comigrated with protein–DNA complex b (Fig. 8).

We also were able to immunoprecipitate the complex with *FOS* antibodies. The DNA-binding assay with the labeled oligonucleotides was done as described previously. The protein–DNA complexes were then incubated with *FOS* antibodies, and the immunoprecipitate was applied to a denaturing gel. When the binding assay was done with HeLa nuclear extract and immunoprecipitation was performed with *MYC* antibodies as a control, no labeled DNA could be detected in the gel (Fig. 9A, lanes 1,6). No labeled DNA could be immunoprecipitated when Kelly extract was used in the DNA-binding assay, and *FOS* antibodies were used for the immunoprecipitation (Fig. 9A, lanes 2,5). The labeled DNA could be immunoprecipitated only when HeLa nuclear extract was used in the DNA-binding assay and immunoprecipitation was performed with *FOS* antibodies (Fig. 9A, lanes 3,4). Therefore, it appears that although preincubation of the extract with *FOS* antibodies prevents complex formation, the antibody can detect complexes once they have formed. However, the recovery of intact protein–DNA complex by immunoprecipitation with *FOS* antibodies is not very efficient, as the yield of the immunoprecipitated DNA is low relative to input DNA (Fig. 9B).

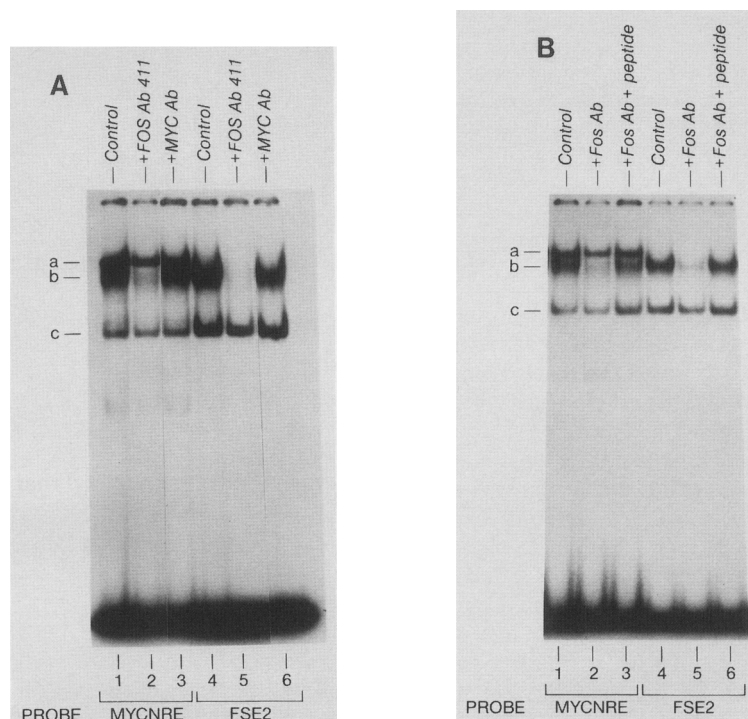


Figure 7. *FOS* antibodies inhibit binding of nuclear factors to *MYC*-NRE or FSE2. Aliquots (10 μ g) of HeLa nuclear extract were preincubated with no addition or with *FOS* antibodies. *MYC*-NRE or FSE2 oligonucleotides labeled with 32 P were added, and incubation continued for an additional 30 min (for details, see Materials and methods). Protein–DNA complexes then were fractionated by electrophoresis. (A) Preincubation of 10 μ g HeLa nuclear extract with no addition (control), or with addition of *FOS* monoclonal antibodies (+ *FOS* Ab411) or *MYC* antibodies (+ *MYC* Ab). (B) Preincubation of 10 μ g HeLa extract with no addition (control), with addition of 1 μ g of antibodies directed against the M peptide of *FOS* protein (+ *FOS* Ab), or with 1 μ g *FOS* M peptide antibodies and 5 μ g of M peptide (+ *FOS* Ab + peptide).

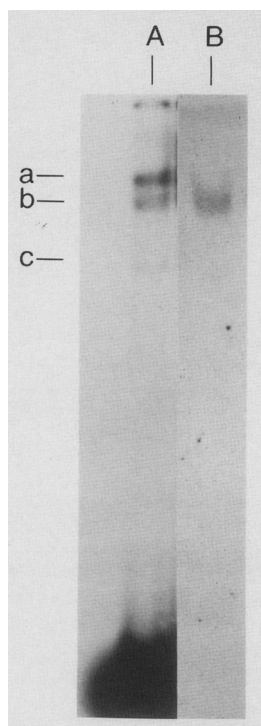


Figure 8. Presence of *FOS* protein or a related antigen in a complex formed with MYC-NRE. (Lane A) HeLa nuclear extract (10 μ g) was incubated with 32 P-labeled MYC-NRE, and protein-DNA complexes were resolved by electrophoresis. (Lane B) Protein-DNA complexes were resolved after incubation of 20 ng unlabeled MYC-NRE with 100 μ g of HeLa nuclear extract. The complexes were then transferred from the gel to nitrocellulose paper and probed with *FOS* M peptide antibodies.

Cooperative binding of *FOS* and *JUN* protein to the MYC-NRE

FOS and *JUN* proteins form a complex that binds to AP-1-like binding sites (Chiu et al. 1988; Franza et al. 1988; Rauscher et al. 1988b; Sassone-Corsi et al. 1988b). Therefore, we tried to determine whether affinity-purified AP-1 can bind to the MYC-NRE. The DNA-binding assay was performed with AP-1 that had been isolated by chromatography on an affinity column bearing TPA-responsive elements of the collagenase gene (kindly provided by Beth Allegretto and Michael Karin). As shown in Figure 10, lane 2, the preparation of AP-1 bound to MYC-NRE, forming a complex that comigrated with protein-DNA complex b (Fig. 10, lanes 1,2). When the AP-1 preparation was preincubated with *FOS* antibodies, the binding activity was reduced significantly (Fig. 10, lane 3). However, when the preincubation with antibody was done in the presence of the cognate peptide, binding activity could be restored (Fig. 10, lane 4). We attribute these findings to the copurification of AP-1 and *FOS* protein, described previously (Rauscher et al. 1988b). Because the *FOS* protein forms high-affinity complex with components of AP-1 (Chiu et al. 1988; Rauscher et al. 1988b; Sassone-Corsi et al. 1988b), we infer that this

complex binds MYC-NRE and that the binding may negatively regulate activity of the MYC promoters P1 and P2 (see Discussion).

The product of the *JUN* proto-oncogene is a major component of AP-1 preparations and generally is equated to AP-1 (Curran and Franza 1988). Therefore, we used translation *in vitro* to assess the ability of *FOS* and *JUN* proteins to bind MYC-NRE (Fig. 11). Neither gene product bound the DNA in isolation; but when combined, the products of *FOS* and *JUN* formed a complex with the oligonucleotide of MYC-NRE with similar, if not identical, electrophoretic mobility to that of the complex (b) formed with nuclear extracts (Fig. 11; data not shown). These results sustain our view that the products of *FOS* and *JUN* are principal components of the protein complex that binds MYC-NRE.

Identification of the nucleotide sequence required for binding protein complex to MYC-NRE

MYC-NRE is composed of inverted repeats of eight residues (Fig. 2A) and forms three complexes with nuclear proteins (Fig. 3). Formation of the protein-DNA complex c appears to occur with only limited specificity (see

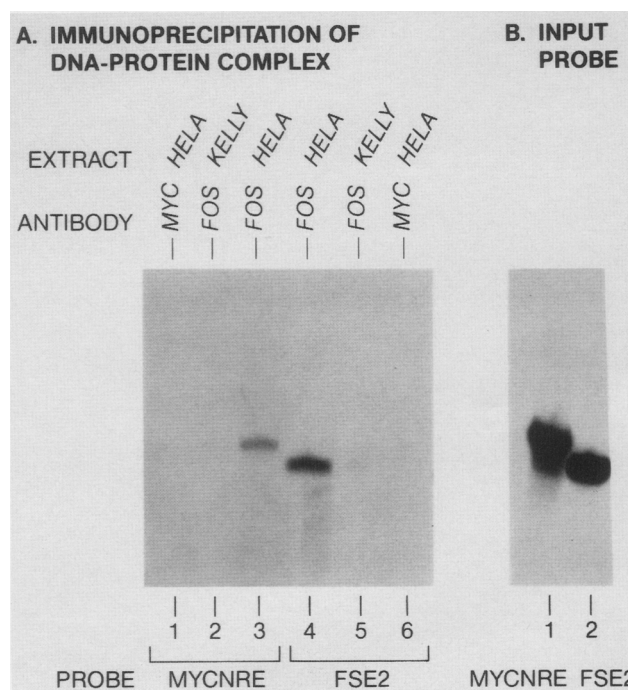


Figure 9. Immunoprecipitation of complexes containing NRE or FSE2 by *FOS* antibodies. (A) MYC-NRE or FSE2, labeled with 32 P, was incubated with either HeLa or Kelly nuclear extracts, followed by immunoprecipitation of protein-DNA complexes (for details, see Materials and methods). The immunoprecipitates were applied to a denaturing acrylamide gel. The autoradiograph was exposed for 16 hr at -70°C . (B) Quantities of 32 P-labeled MYC-NRE or FSE2 identical to those used in A were used in a binding assay without immunoprecipitation. The autoradiograph was exposed for 30 min at -70°C .

above), whereas formation of protein–DNA complexes a and b is specific. As shown in Figures 7–10, complex b contains FOS protein and may be identical to the complex that forms with FSE2 and hM1_{II}A-AP1. The portion of MYC-NRE that corresponds to the AP-1 binding site and to FSE2 is limited to residues –315 to –327 (Fig. 2A). In the DNA-binding assays described above, however, we used double-stranded oligonucleotide that contained the sequence between –318 and –343 (Fig. 2B). To better define the DNA sequence that forms complex b, we performed deletion analysis on MYC-NRE (Fig. 12).

The right hand of the dyad symmetry in MYC-NRE (between nucleotides –312 and –329, or dl1 in Fig. 12) spans the sequence shared with FSE2 (Figs. 2A and 12A). We found that this domain was not sufficient for formation of either complex a or b (which contains FOS protein), although the nonspecific complex c still formed. A double-stranded oligonucleotide that spanned the left hand and part of the right hand of the dyad symmetry also was not sufficient for formation of either complex a or b (dl2 in Fig. 12), although formation of complex c was again apparent (Fig. 12B, lane 3). Double-stranded DNA comprising the sequence between nucleotides –313 and –338, which contains the right hand of the dyad sym-

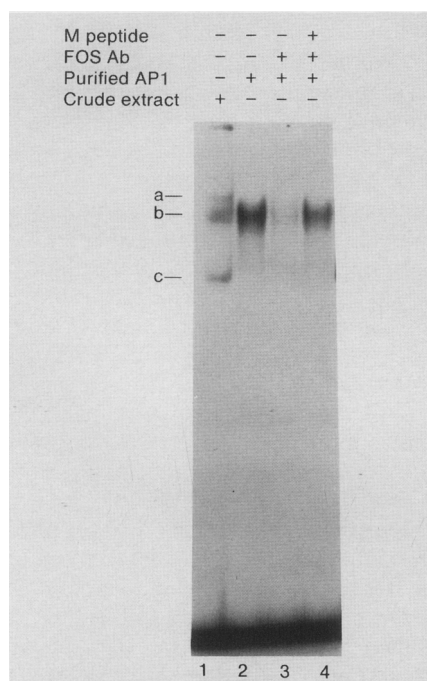


Figure 10. Binding of affinity-purified AP-1 to MYC-NRE and inhibition of the binding by FOS antibody. Binding of HeLa nuclear extract or affinity-purified AP-1 to labeled MYC-NRE was performed as described in Fig. 9. Preincubation of affinity-purified AP-1 with no addition, with addition of 1 μ g of FOS M peptide antibody, or with 1 μ g of FOS M peptide antibody and 5 μ g M peptide was performed at 4°C for 2 hr.

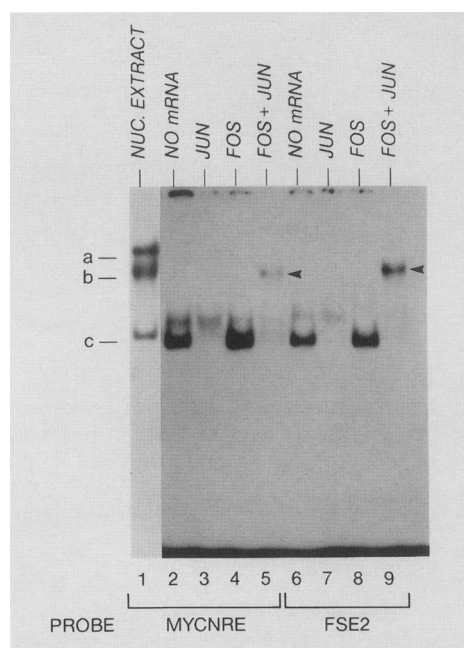


Figure 11. Binding of FOS and JUN proteins to MYC-NRE and FSE2. MYC-NRE or FSE2, labeled with 32 P, was incubated with proteins synthesized in vitro and assayed by gel retardation analysis. (Lane 1) HeLa nuclear extract; (lanes 2,6) rabbit reticulocyte lysate with no added mRNA; (lanes 3,7) JUN translated in vitro; (lanes 4,8) FOS translated in vitro; (lanes 5,9) cotranslation of FOS and JUN. The specific complex of FOS/JUN with DNA is indicated by arrows. The lower bands are related to nonspecific binding of proteins from the reticulocyte lysate.

metry and part of the left hand (dl3 in Fig. 12), was sufficient for formation of both complexes a and b (Fig. 12B, lane 4); however, the relative formation of complex b versus complex a was less efficient, in comparison with the binding to the intact dyad symmetry sequence (Fig. 12B, lanes 1,4). Thus, the DNA sequence between nucleotides –318 and –338 is necessary and sufficient for the formation of complexes a and b. Preliminary results of contact point analysis revealed that the TGA on both DNA strands (underlined in Fig. 12A) is important for the formation of complex b (M. Takimoto and D. Levens, unpubl.).

Discussion

A negative regulator upstream of the promoters for human MYC

In previous studies we showed that nucleotide sequences between residues –293 and +513 in MYC are sufficient for strong activity of the two principal promoters for the gene, P1 and P2 (Hay et al. 1987). We defined several elements that modulate the activities of the two promoters: a region that can sustain alone the activity of P1, between nucleotides –101 and –293;

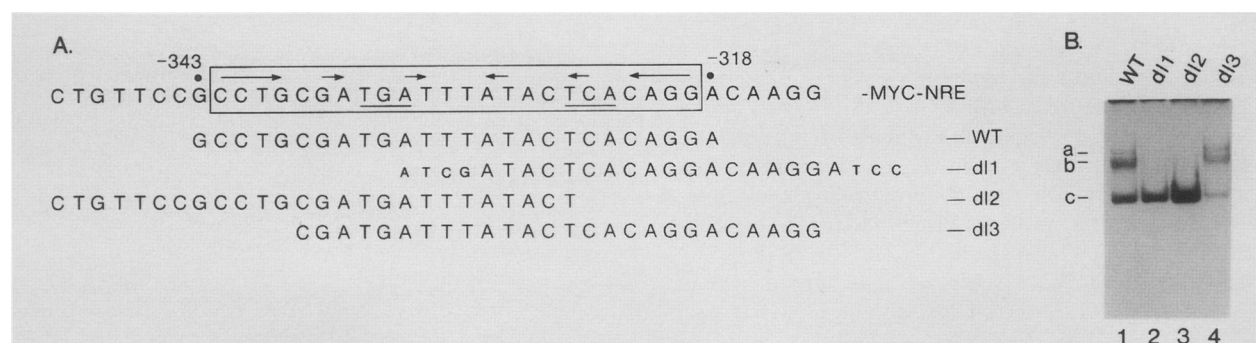


Figure 12. Characterization of MYC-NRE by deletion analysis. (A) The sequence of MYC-NRE proper between -318 and -343 (in frame) and flanking sequences. Arrowheads indicate the dyad symmetry; underlining designates the putative points of contact with protein factors. (WT) Sequence of the double-stranded synthetic oligonucleotide used in previous assays; (dl1, dl2, and dl3) sequences of oligonucleotides from different regions of the MYC-NRE used for the gel retardation assay. Small size letters indicate nucleotides that were added to the synthetic oligonucleotides and are not present in MYC-NRE. (B) Gel retardation assay with HeLa nuclear extract and labeled synthetic double-stranded oligonucleotides, described in A.

two domains that exert a positive effect on both P1 and P2 ($-353/-1257$ and $-1257/-2329$); and a negative regulator that acts on both promoters ($-293/-353$).

The negative regulator can operate as an isolated unit in proliferating cells, although the inhibitory effect can be overridden by positive regulators upstream of -353 (Hay et al. 1987), as well as by a heterologous transcriptional enhancer such as that of the early region of the SV40 genome (N. Hay and J.M. Bishop, unpubl.). Here, we have shown that the negative regulator resides within a region that was found to be protected from DNase digestion after incubation with HeLa nuclear extract (Hay et al. 1987). This 26-bp region, between nucleotides -318 and -343 , is sufficient to exert negative regulation on promoters P1 and P2 of MYC. The sequence of the protein-binding site within the MYC-NRE is comprised of AT-rich core regions, flanked and overlapped by inverted repeats of eight residues.

Previously, we reported some similarities between MYC-NRE and the negative regulator of human β interferon (Hay et al. 1987), but we have since noticed more significant homology between the MYC-NRE and FSE2—a negative regulator upstream of the adipocyte-specific gene, aP2 (see Fig. 2A). FSE2 encompasses ~ 21 nucleotides, which include a recognition site for the transcription factor AP-1 (Distel et al. 1987; Rauscher et al. 1988a). Comparison of FSE2 and MYC-NRE revealed a region of 13 bp within FSE2 that contains the AP-1 recognition site and has only two mismatches with the MYC-NRE sequence.

The negative regulator we describe here is probably not the only one that acts on transcription from MYC. Recently, another negative regulator has been reported within the first intron of human MYC (Zajac-Kaye et al. 1988); in addition, a block in elongation of transcription also contributes to repression of the gene during differentiation (Bentley and Groudine 1986; Eick and Bornkamm 1986; Siebenlist et al. 1988). It is possible that different negative regulators operate in different physiological settings. Alternatively, different regulators may interact with one another to achieve more efficient regu-

lation. For example, it would be of interest to determine whether the negative regulator described here can influence the elongation rate of transcription which, in turn, may affect the frequency of premature termination of transcription at the 3' end of the first exon of MYC (Bentley and Groudine 1988).

A complex that includes AP-1 and a FOS protein binds MYC-NRE

Previously, we have provided evidence that nuclear protein or proteins can bind specifically to MYC-NRE (Hay et al. 1987). We did not know how many factors might be involved in the binding or what their nature might be. The resemblance of MYC-NRE to FSE2, together with the finding that FSE2 can bind specifically a protein complex that includes the product of FOS associated with the transcription factor AP-1 (Distel et al. 1987; Rauscher et al. 1988a), encouraged us to examine whether a similar complex could bind to MYC-NRE.

We found that three distinct complexes form between cellular proteins and MYC-NRE. One of these complexes contains FOS protein or an antigenically related protein, and the electrophoretic mobility of the complex is identical to that of complexes formed with either FSE2 or an AP-1-binding site. Formation of this complex also is eliminated by oligonucleotide competitors possessing a known AP-1 binding site. First, there is no evidence to date that the various FOS proteins interact directly with the AP-1 binding site in DNA. Instead, the binding is mediated indirectly by other components of AP-1, such as the product of the proto-oncogene JUN (Chiu et al. 1988; Rauscher et al. 1988b; Sassone-Corsi et al. 1988b). Second, it is likely that all of the FOS proteins in preparations of AP-1 are in highly stable complexes with other proteins (Chiu et al. 1988; Rauscher et al. 1988b; Sassone-Corsi et al. 1988b). Given the heterogeneity of proteins in preparations of AP-1 (for review, see Curran and Franza 1988), we cannot specify which components of the preparations might be collaborating with FOS protein in the binding to DNA studied here. However,

we suspect that the *JUN* protein is an essential component of the complex with MYC-NRE: When produced by translation in vitro, the product of *FOS* cannot bind MYC-NRE unless the product of *JUN* is also present.

We emphasize that the portion of MYC-NRE shared with FSE2 is not sufficient for the formation of the complex containing *FOS* protein; the homology between MYC-NRE and FSE2 is limited to the sequence between nucleotides -315 and -327, but the DNA sequence between nucleotides -327 and -338 is also necessary for efficient binding of the *FOS*-protein complex.

The transcription factor AP-1 was first identified as a specific *trans*-activator of genes induced by TPA (Angel et al. 1987; Lee et al. 1987). More recently, it has been shown that in cases where AP-1 plays a role in *trans*-activation, it is associated with *FOS* protein, which also is required for efficient *trans*-activation (Chiu et al. 1988; Sassone-Corsi et al. 1988b; Schonthal et al. 1988). However, in several other cases (FSE2 in adipocytes, the long terminal repeats of HIV, and the *FOS* promoter), *FOS* protein appears to be associated with negative regulation of transcription (Distel et al. 1987; Franza et al. 1988; Sassone-Corsi et al. 1988a). Here, we have provided evidence that a complex containing *FOS* protein binds to a nucleotide sequence upstream of MYC promoters; the binding sequence mediates negative regulation of transcription from MYC.

At present, we cannot be certain whether it is *FOS* protein itself or an antigenically related protein that is associated with MYC-NRE (Cohen and Curran 1988). However, three points of evidence suggest to us that authentic *FOS* protein is involved: (1) antibodies directed against several distinct regions of the *FOS* protein blocked formation of the complex (Fig. 7); (2) *FOS* protein produced by translation in vitro bound cooperatively with the *JUN* product to MYC-NRE (Fig. 11); (3) authentic *FOS* protein has been isolated from the complex formed by nuclear extracts with MYC-NRE (M. Takimoto and D. Levens, unpubl.).

In reality, two specific complexes form with the MYC-NRE. We have focused on one of these because apparently it forms by the binding of both *FOS* and *JUN* proteins. We have not identified any components of the other complex and have no direct evidence as to the role of either of these complexes in the intact cell.

The FOS protein may regulate MYC expression

The expression of MYC, *FOS*, and *JUN* is associated with cellular proliferation. The protein products of these proto-oncogenes may function as nuclear mediators that affect the gene expression in response to signals from an extracellular environment. There is now clear evidence that *FOS* and *JUN* proteins are associated with each other and are components of transcription complexes active on several eukaryotic genes (Chiu et al. 1988; Franza et al. 1988; Rauscher et al. 1988a; Sassone-Corsi et al. 1988a,b); moreover, *FOS* and *JUN* may also regulate the expression of each other (Sassone-Corsi et al. 1988a). The data presented here raise an additional pos-

sibility: that *FOS* and *JUN* proteins may contribute to the regulation of MYC.

The induction of *FOS* in response to mitogens precedes that of MYC. It is unlikely, however, that *FOS* expression *trans*-activates MYC directly, as the induction of both genes occurs and is stimulated even when the synthesis of new protein is inhibited (Cochran et al. 1984; Muller et al. 1984; Greenberg et al. 1985). Instead, our finding that a complex containing *FOS* protein binds specifically to a negative regulator upstream of MYC suggests that the *FOS* protein may participate in the repression of MYC. Indeed, the induction of MYC by mitogens begins only after expression of *FOS* begins to decline (Muller et al. 1984; Greenberg and Ziff 1984; Greenberg et al. 1986). Reciprocal regulation of MYC and *FOS* also is observed during differentiation of at least some cell types; MYC expression ceases as those cells differentiate, whereas *FOS* is activated (Gonda and Metcalf 1984; Muller et al. 1985). Also, it is possible that *FOS* triggers MYC expression by interfering with a repressor protein that binds specifically to MYC-NRE. Whatever the role of *FOS* in the regulation of MYC might be, our observation that *FOS* protein is part of a complex that binds to a sequence upstream of the MYC promoter implies that there is an interplay of some sort between *FOS* and MYC and that further studies to explore this interplay are in order.

Materials and methods

Cells and transfection

Mouse Ltk⁻ cells and HeLa cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum. Kelly cells were grown in RPMI, supplemented with 10% fetal calf serum.

Transfection of cells was performed using calcium phosphate precipitation (Graham and van der Eb 1973). Stable transfection of Ltk⁻ cells and selection with hygromycin B was carried out as described previously (Hay et al. 1987).

Plasmids and synthetic oligonucleotides

Plasmids were constructed and prepared as described previously (Hay et al. 1987). The plasmid pBRCAT (Walker et al. 1983) was used to construct MYC-chloramphenicol acetyltransferase (CAT) hybrids. The plasmid -343Δ-293/-318 was constructed by insertion of the double-stranded oligonucleotide containing the sequence between -318 and -343, immediately upstream of nucleotide -293 in the hybrid plasmid -293 MYC-CAT. Orientation of the inserted DNA was determined by sequencing. To create the deletion with 321 bp upstream of the transcription start site, an *Sfa*NI restriction site was used. Full details of each construct are available upon request. Synthetic oligomers were purchased from Operon Technologies, Inc. (San Pablo, California). Double-stranded synthetic oligomers were end-labeled with [³²P]ATP, using T4 polynucleotide kinase (Maniatis et al. 1982).

RNA analysis

Total cellular RNA was isolated and analyzed by RNase protection as described previously (Hay et al. 1987).

Preparation of nuclear extracts and binding assays

Nuclear extracts were prepared according to the procedure of Dignam et al. (1983). The nuclear extracts were precipitated with 30% ammonium sulfate and centrifuged at 25,000g for 20 min. The pellet was resuspended in buffer D [20 mM HEPES (pH 7.9), 70 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA, and 20% glycerol]. HeLa nuclear extract (10 µg) was preincubated in 20 µl of buffer D in the presence of 1 µg poly[d(I-C)] for 10 min at 4°C. Double-stranded oligomer (0.2 ng), end-labelled with ³²P, was added and preincubation at 4°C was continued for an additional 5 min, followed by 30 min of incubation at room temperature. Binding assays with affinity-purified AP-1 were carried out in the presence of 10 µg BSA. Binding assays with in vitro translated proteins were carried out in the presence of 0.2 µg poly[d(I-C)] 0.5 µg of single-stranded DNA. Protein-DNA complexes were resolved on 5% polyacrylamide gels (30 : 1 acrylamide/bis-acrylamide) in 0.5 TBE [1 × TBE, 50 mM Tris borate (pH 8.5), 1 mM EDTA].

In vitro transcription and translation

Human *JUN* cDNA (a gift from Dr. Eric Fodor) and rat *FOS* cDNA were subcloned into pGEM3. The plasmids were linearized with appropriate restriction enzymes and were transcribed in vitro, using Sp6 RNA polymerase in the presence of m⁷GpppG. RNA was extracted by phenol/chloroform and precipitated with ethanol. Approximately 5 µg of RNA was translated in vitro, using 35 µl of micrococcal nuclease-treated rabbit reticulocyte lysate, as recommended by the supplier (Promega Biotec). One µl of the translation mixture was used in the DNA-binding assays.

Antibodies

Monoclonal antibody 411 to amino acids 4–17 of *FOS* protein was purchased from Microbiological Associates (Bethesda, Maryland). Affinity-purified antibodies to the M peptide (amino acids 127–152 of *FOS* protein; Curran et al. 1985) were a gift from Tom Curran (Roche Institute of Molecular Biology, Nutley, New Jersey). Affinity-purified antibodies to the carboxyl terminus of human *MYC* protein were a gift from Gary Ramsay (Ramsay et al. 1984). Inhibition of protein-DNA complex formation with antibodies was performed by adding antibodies as described by Distel et al. (1987).

Immunoprecipitation of labeled FSE2 and MYC-NRE

Nuclear extracts (10–20 mg/ml) were preadsorbed with an equal volume of protein A/Sepharose in buffer A for 1 hr at 4°C. The Sepharose beads were collected by centrifugation, and the supernatant was used for the binding assay. Antibodies were preadsorbed with protein A/Sepharose (1 µl of antibodies with 30 µl of beads) for 1 hr at 4°C. The beads were washed twice in 30 mM NaCl, 0.1% NP-40, 10 mM Tris (pH 8.0), and 0.5 mM EDTA. The protein-DNA complexes were formed with the preadsorbed extracts and labeled double-stranded oligonucleotides as described above. After preadsorption with protein A/Sepharose, antibodies were incubated with the protein-DNA complexes for 1 hr at room temperature. The beads then were washed three times with 1 ml of 50 mM NaCl, 0.1% NP-40, 10 mM Tris (pH 8.0), and 0.5 mM EDTA. The labeled DNA was recovered from the beads by phenol/chloroform extraction and then was loaded on a 10% acrylamide gel (19 : 1 acrylamide to bis/acrylamide) in TBE containing 7 M urea.

Immunoblotting of protein-DNA complexes

Protein-DNA complexes were formed with 2 µg of unlabeled oligonucleotides and 100 µg of nuclear extract as described above. Protein-DNA complexes were resolved on a native polyacrylamide gel and transferred to nitrocellulose in 30 min at 2.5 mA/cm² gel surface with a PolyBlot transfer apparatus (American Bionetics, Emeryville, California), using the transfer buffers described by the manufacturer. Then the filter was incubated for 1 hr at room temperature in TBS [10 mM Tris-HCl (pH 8.0), 150 mM NaCl], containing 5% calf serum, 2% nonfat milk, and 0.5% Tween 20, followed by 2 hr of incubation with *FOS* M peptide affinity-purified antibodies and ¹²⁵I-labeled protein A (Burnette 1981).

Acknowledgments

We thank David Levens for sharing information and materials and for valuable discussions, Danielle Melloul for advice on preparation of nuclear extracts, Tom Curran for *c-fos* antibody, Beth Allegretto and Michael Karin for affinity-purified AP-1, Eric Fodor for the *JUN* cDNA, and Lynn Vogel for preparing the manuscript. Work summarized here was supported by the National Institutes of Health (grants CA-12705 and CA-44338) and by funds from the G.W. Hooper Research Foundation.

Note added in proof

The unpublished results of M. Takimoto and D. Levens mentioned throughout this paper are now in press. Takimoto, M., J.P. Quinn, R.A. Farina, L.M. Staudt, and D. Levens. 1989. Fos/JUN and octamer binding protein interact with a common site in a negative element of the human *c-myc* gene. *J. Biol. Chem.* (in press).

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