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fos/jun and Octamer-binding Protein Interact with a Common Site in a Negative Element of the Human c-*myc* Gene*

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A negative element has previously been localized to a 57-base pair segment approximately 300 base pairs upstream of the human c-*myc* promoter P1. Within this element, a 26-base pair region was protected *in vitro* from DNase I digestion with a HeLa cell nuclear factor(s). Two specific DNA-protein complexes were identified in gel retardation assays using HeLa cell nuclear extracts and an oligonucleotide probe spanning the footprinted region. Exonuclease and chemical footprint analyses suggested that the binding sites for both complexes are almost entirely overlapping. One of the complexes was eliminated by oligonucleotide competitors possessing known AP-1 binding sites. This same complex reacted strongly with anti-*fos* immunoglobulin suggesting a role for c-*fos* in governing c-*myc* expression. Precipitation of *fos* protein bound to c-*myc* DNA that was immobilized on beads confirmed the involvement of c-*fos* in a specific complex with the c-*myc* upstream sequence. In contrast, the other complex seen by the c-*myc* probe could not be competitively inhibited by AP-1 binding sites and was not affected by anti-*fos* antibody. Instead, this complex was efficiently eliminated by unlabeled oligonucleotides containing the octamer DNA motif found in immunoglobulin gene promoters. Purified octamer-binding proteins formed stable complexes with the 26-base pair c-*myc* sequences. These results demonstrate that degeneracy in the consensus recognition sequences of these distinct factors allows each of them to bind the c-*myc* negative element. The interaction of known transcriptional activators with a negative element suggests that the same factors can mediate both transcriptional activation and repression.

Nuclear proto-oncogene products play critical roles in cell growth and differentiation, mediate signal transduction in the nucleus, and participate in the regulatory processes of transcription and DNA replication (1-11). The proto-oncogene c-*myc* has been studied extensively. Expression of the c-*myc* gene is closely associated with the growth state of cells; the deregulation of c-*myc* gene expression by various mechanisms, including chromosomal translocation, retroviral insertion, gene amplification, and point mutation, contributes to the immortalization or transformation of normal cells (12-15). The expression of c-*myc* gene is regulated by several mechanisms, including transcriptional initiation, transcriptional elongation, mRNA stability, and translation (16-19). The human c-*myc* gene is composed of three exons: the first exon is a noncoding leader sequence, and the second and third exons encode the protein product of c-*myc*. Transcriptional regulation of c-*myc* is responsive to many physiological signals, such as growth factors, and to biological agents, some of which trigger the differentiation of certain cell types (3, 5). Transcription of c-*myc* starts from at least three initiation sites, with distinct promoters (P0, P1, and P2). P1 and P2 are the major promoters located upstream of exon I and within exon I, respectively. P0 is a minor promoter, located 700 base pairs (bp)[1] upstream of P1, and accounts for less than 10% of the total c-*myc* transcription (20). The transcription of c-*myc* from P1 and P2 is regulated by a composite of positive and negative elements located both upstream and downstream of these promoters (17, 21-25). A negative transcriptional element, which acts on both P1 and P2, has been mapped between −350 to −294 bp relative to the transcriptional starting site, P1. DNase I footprint and exonuclease assays have identified a nuclear factor binding to this element (17).

In this report, we show that two biochemically distinct factors bind specifically to the 26-bp DNase I footprinted region of the c-*myc* negative element. One of the factors was identified as the *fos/jun* (AP-1) complex (40-46), and the other as the ubiquitous octamer-binding protein (27, 28, 47, 62, 63). Interestingly, the two complexes bind highly overlapping sequences. The presence of interdigitating binding sites for distinct factors may confer multiple functions on a single *cis*-element or allow cooperative interactions between *trans*-acting factors. *fos/jun* (AP-1) and the ubiquitous octamer-binding factor are known to participate in transcriptional activation and/or DNA replication (10, 26-29, 40, 45); this study suggests that repression of c-*myc* expression may employ components common to transcriptional activation and/or DNA replication.

EXPERIMENTAL PROCEDURES

Cells and Media—HeLa and WEHI 231 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. PC12 cells were grown in medium with 7% fetal calf serum and 7% horse serum. NGF (7S-NGF, Collaborative Research, Inc., Bedford, MA) treatment of PC12 cells was at a concentration of 125 ng/ml for 90 min.

Preparation of Nuclear Extracts—Nuclear extracts were prepared by the procedure of Dignam *et al.* (30) with the following modifications: buffer C contained 20% glycerol and 0.45 M NaCl; buffer D contained 20% glycerol and 80 mM KCl. All buffers contained 20 mM HEPES (pH 7.5). The protein concentration of the extracts was 30

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[1] The abbreviations used are: bp, base pairs; DTT, dithiothreitol; FRA, *fos*-related antigen; FT, flow-through; GALV, gibbon ape leukemia virus; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethylpiperazineethanesulfonic acid); $NaDodSO_4$, sodium dodecyl sulfate.

mg/ml. Whole cell extracts were made from PC12 cells; cell pellets were extracted with the addition of 3 volumes of buffer C and incubated for 40 min on ice.

Gel Retardation Assay—Gel retardation assays were performed as described previously (31, 32). Binding reactions contained a ^{32}P-3′-end-labeled double-stranded oligonucleotide probe, 0.5 μg of poly[d(I-C)], 25 μg of HeLa nuclear extract or fractionated extracts, and additional competitor DNA as specified in the figure legends. Oligonucleotides used in this study are described in Table I, except for the 40-bp oligonucleotides used in Fig. 6*A*. The sequences of the 40-bp oligonucleotides are as follows: octamer(5/6): 5′-GATCGCTTCTTAATTTGCATACCCTC-3′, NS: 5′-TCGAGACGAAAATCCACACTCATGAGTGTGCAACATATCG-3′.

Antibodies and Peptides—A *fos* peptide (amino acid from 129 to 153) (33) and affinity-purified antibodies to this peptide (*fos*-I) were kindly donated by Dr. Michael Iadarola (National Institutes of Health, Bethesda, MD). Affinity-purified antibodies to the M peptide of mouse c-*fos* (*fos*-M) were the generous gift of Dr. T. Curran (Roche Institute of Molecular Biology, Nutley, NJ). Rabbit antisera against a human c-*fos* fusion protein (*fos*-S) were prepared by Dr. Dennis Salomon (UCLA School of Medicine). Affinity-purified antibodies to β-galactosidase were prepared as described previously (34).

Fractionation of Nuclear Extracts by Bio-Rex 70 Chromatography—HeLa cell nuclear extracts were fractionated by cation chromatography on Bio-Rex 70 (Bio-Rad). The extracts were dialyzed against buffer D containing 150 mM KCl and loaded onto a Bio-Rex 70 column equilibrated with the same buffer. Approximately 1 ml of resin was used for every 10–20 mg of nuclear protein. The flow-through (FT) fraction was collected, and the column was washed with buffer D, 150 mM KCl, until the absorbance of 280 nm was less than 0.05. The column was eluted with a linear gradient from 150 to 800 mM KCl in buffer D. Gradient fractions were collected and, along with the FT, subjected to gel retardation assay using specific, double-stranded, radiolabeled probes. The FT fraction was precipitated with 70% ammonium sulfate. The peak binding fractions resulting from gradient elution were pooled and precipitated with ammonium sulfate from 0 to 35% and from 35 to 70%. The precipitated proteins were dissolved in a minimum volume of buffer D and dialyzed against the same buffer.

Immunobeads—Monoclonal mouse anti-β-galactosidase was purchased from Promega Corp. (Madison, WI) and coupled to Immunobeads (Bio-Rad), as described by the manufacturer. The *lac* repressor-galactosidase fusion protein was prepared using the procedure of Fowler and Zabin (35).

Exonuclease Assay—Exonuclease assays were performed as described (17, 36). *Pvu*II/*Sma*I or *Pvu*II/*Sca*I fragments of the human c-*myc* gene were cloned into the *Sma*I site of pUC19 or pUC18 DNA (pUCPvuII/SmaImyc or pUCPvuII/ScaImyc, respectively). The c-*myc* and *lac* operator-bearing *Bst*NI fragments of pUCPvuII/ScaImyc or pUCPvuII/SmaImyc were purified and end-labeled with [α-^{32}P] deoxyribonucleotides at both 3′ ends by *Escherichia coli* DNA polymerase (large fragment). One to three nanograms of the DNA probes were immobilized on Immunobeads as described previously (17, 34, 36). Binding reactions, with or without specific competitors, were performed in a total of 15 μl of buffer D, including extract and 5 μg of poly[d(I-C)] (Sigma). Following incubation for 25 min at 20 °C, the beads were recovered by centrifugation and resuspended in 15 μl of exonuclease buffer (10 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 0.2 mM dithiothreitol (DTT), and 5% glycerol). Twenty units of T7 gene 6 exonuclease (United States Biochemicals, Cleveland, OH) were added; the reactions were incubated for 3 min at 30 °C and stopped with the addition of an equal volume of 10 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 0.5% $NaDodSO_4$. The reactions were then extracted sequentially with phenol and chloroform. Then the DNA was recovered by ethanol precipitation, separated by gel electrophoresis (6% acrylamide/bisacrylamide (29:1), 8.3% urea), and visualized with autoradiography.

Chemical Footprint Analysis—Chemical footprint analysis was performed with the procedure described by M. Kuwabara and Sigman (37). A ^{32}P-5′-end-labeled 45-bp oligonucleotide was annealed with unlabeled complementary strand to generate probe DNA. Sequences of the 45-bp oligonucleotides are: template strand: 5′-GCAGCTGTTCCGCCTGCGATGATTTATACTCACAGGACAAGGATG-3′, nontemplate strand: 5′-CCGCATCCTTGTCCTGTGAGTATAAATCATCGCAGGCGGAACAGC-3′. A 6-fold increased binding reaction followed by gel retardation was performed as described above. After electrophoresis, the gel was soaked in 50 mM Tris-HCl, pH 8.0, 0.4 mM 1,10-phenanthroline, 0.09 mM $CuSO_4$, and 3-mercaptopropionic acid (1/2000 diluted) for 7–12 min at room temperature. The cleavage reaction was quenched by the addition of 2,9-dimethylphenanthrone to a final concentration of 2.8 mM. The gel was rinsed with distilled water and autoradiographed for 30 min at room temperature. The bands were excised from the gel, and the DNA was eluted in 10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 0.5% $NaDodSO_4$, 50 μg/ml proteinase K overnight at 37 °C. After filtration, the recovered DNA was extracted with phenol/chloroform (1:1), precipitated with ethanol, analyzed by denaturing 13% polyacrylamide gel electrophoresis (PAGE), and subjected to autoradiography.

Precipitation and Immunoblot Analysis of the c-myc DNA-Protein Complex—Eighty microliters of HeLa cell nuclear extract (2.4 mg, total protein) was mixed with 10 μg of poly[d(I-C)], 0.8 mg of bovine serum albumin, and 0.1% Tween 20 for 4 min at 4 °C. The mixture was spun (9000 × *g*, 5 min at 4 °C), and the supernatant was incubated with 150 ng of c-*myc* DNA (multimer of *Pvu*II-*Sca*I fragment) or

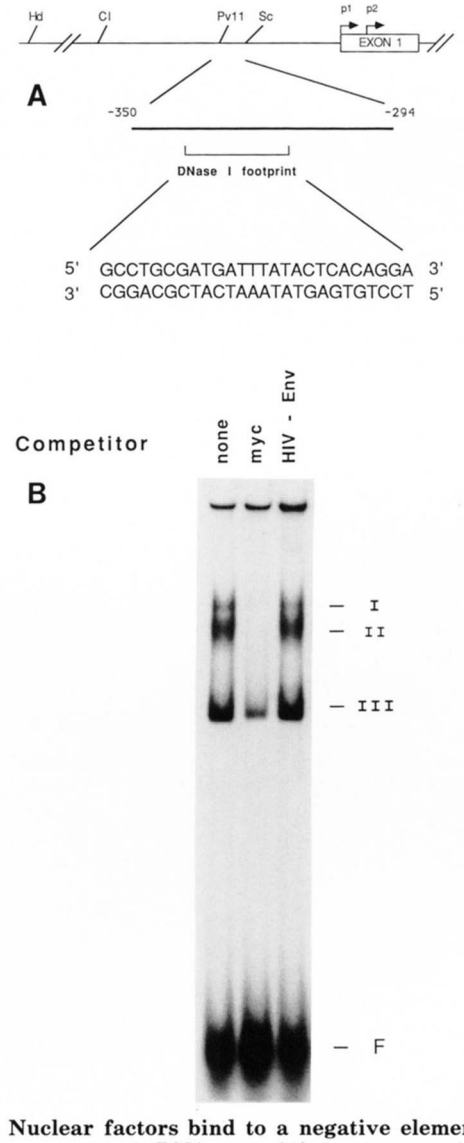

FIG. 1. **Nuclear factors bind to a negative element of the human c-*myc* gene.** *A*, DNA map of the upstream region and of exon 1 of the human c-*myc* gene. A negative element, from −350 to −294 bp relative to the transcriptional initiation site P1 of the human c-*myc* gene, is shown. A sequence within this element defined by DNase I footprint analysis is also shown. *Hd*, *Hin*dIII; *Cl*, *Cla*I; *Pv11*, *Pvu*II; *Sc*, *Sca*I. *B*, gel retardation assays identify multiple c-*myc* DNA-protein complexes. c-*myc* oligonucleotide was radiolabeled and mixed with HeLa nuclear extract and assayed by gel retardation as described under "Experimental Procedures." In each lane, 0.1 ng of the probe DNA was used. Used as competitor DNAs were 30 ng of unlabeled oligonucleotides, c-*myc*, and HIV-Env. The DNA-protein complexes are designated as *I–III*. *F* indicates free probe. Oligonucleotide sequences are shown in Table I.

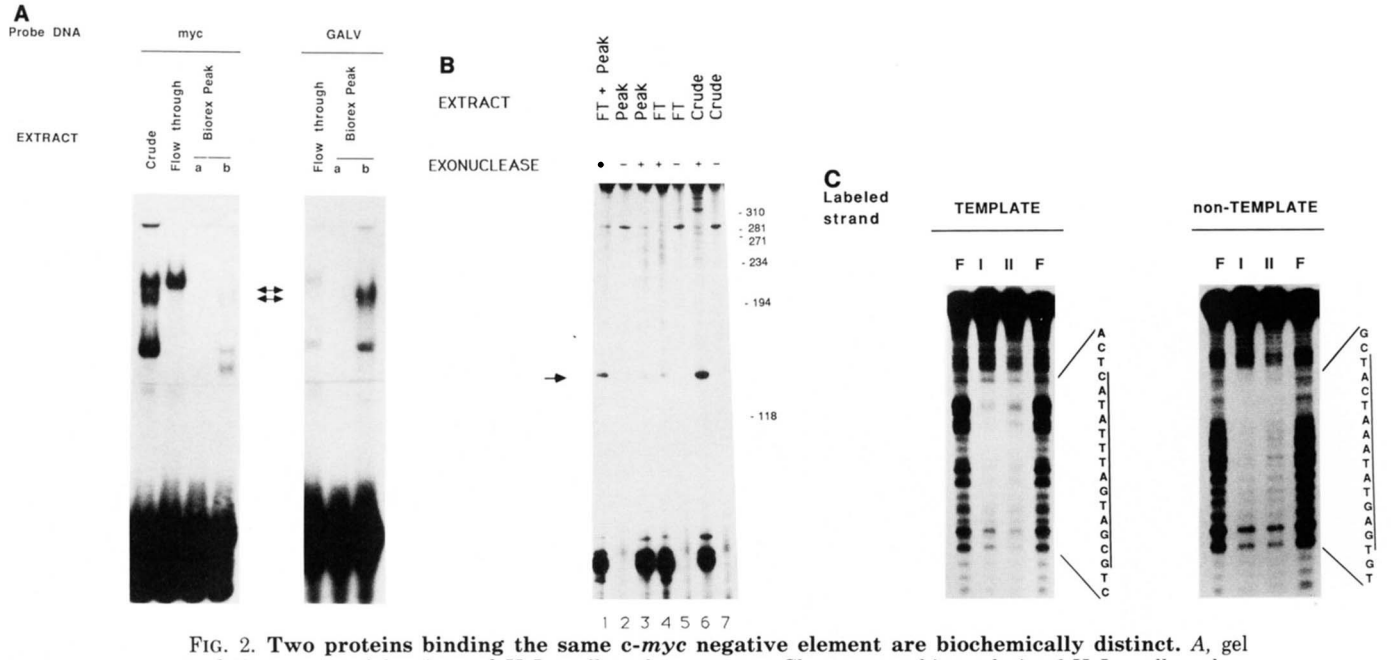

FIG. 2. **Two proteins binding the same c-*myc* negative element are biochemically distinct.** *A,* gel retardation assays of fractionated HeLa cell nuclear extracts. Chromatographic analysis of HeLa cell nuclear extracts. Chromatographic analysis of HeLa cell nuclear extracts by Bio-Rex 70, following gel retardation assays, were performed as described under "Experimental Procedures." Of each fraction, 1 μl was used in the gel retardation assays. *Arrows* indicate complexes I and II. The Bio-Rex peak fractions precipitated with 0–35% and 35–70% ammonium sulfate are indicated by *a* and *b*, respectively. *B,* exonuclease binding assay of fractionated HeLa nuclear cell extracts. The probe was the same as in an earlier study (17); the *Bst*NI fragment of pUCPvuII/SmaImyc bearing c-*myc* sequence. *Lane 1,* amounts of Bio-Rex FT fraction and pooled peak fractions precipitated with 35–70% ammonium sulfate (*Peak*) or crude nuclear extract; *lane 2,* 2.5 μl of FT plus 2.5 μl of Peak; *lane 3,* 2.5 μl of Peak; *lane 4,* 5 μl of Peak; *lane 5,* 5 μl of FT; *lane 6,* 2.5 μl of FT; *lane 7,* 3 μl of crude. The *arrow* indicates the specific bands produced by hydrolysis of probe DNAs. *C,* chemical footprint analysis of complexes I and II. Complementary 45-base oligonucleotides encompassing the c-*myc* negative element were separately labeled with [γ-^{32}P]ATP by polynucleotide kinase, and each was annealed with the appropriate unlabeled complementary strands. Gel retardation and chemical footprint analysis were performed as described under "Experimental Procedures." *I* and *II* indicate c-*myc* DNA-protein complexes I and II, respectively, and *F* indicates a free probe. DNA sequences of chemical footprints are shown, and the *lines* indicate the bases with clear protections.

control pUC19 DNA previously immobilized on Immunobeads. Competitor DNA (1 mg) was included in some experiments. The binding mixture was incubated for 25 min at room temperature and washed twice with buffer D (80 mM KCl). The bound proteins were eluted with 20 μl of 0.2% $NaDodSO_4$.

The eluted proteins were separated by 10% $NaDodSO_4$-PAGE and transferred to nitrocellulose filter (550 mAmp 4 °C for 90 min). The filter was blocked with Blotto (5% Carnation non-fat dry milk, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT for 90 min at room temperature), incubated with affinity-purified anti-*fos* antibodies (1:2500 dilution in Blotto at room temperature overnight), and rinsed with TNE-50 (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT). Anti-rabbit Ig, ^{125}I-labeled $F(ab')_2$ (Amersham), was allowed to react with the filter in Blotto for 2 h at room temperature. After several washes with TNE-50, the filter was dried for autoradiography.

Purification of Octamer-binding Factors—The ubiquitous and lymphoid octamer-binding proteins were partially purified from a nuclear extract prepared from the mouse B-cell line, WEHI 231, by affinity chromatography on an Ig octamer affinity column essentially as described (38). The sequence of the oligonucleotides used to make the affinity column was derived from the V_k 41 Ig promoter, shown in Table I. The nuclear extract was passed four times over the affinity column in the presence of poly[d(I-C)], and the column was eluted as described (38). The affinity-purified protein was subjected to electrophoresis on a 12.5% $NaDodSO_4$-polyacrylamide gel. Proteins ranging from 43 to 120 kDa were eluted from 23 contiguous gel slices and renatured as described (39). The fractions containing octamer-binding proteins were determined by gel retardation assay.

RESULTS

Identification of Multiple Distinct Factors Binding to a c-myc Negative Control Element—A 57-bp negative regulatory element has been previously identified in a region that is located −350 to −294 bp upstream of the human c-*myc* promoter P1. DNase I footprint and exonuclease analysis revealed binding of HeLa cell nuclear factor(s) to this element (Fig. 1*A*) (17).

To further characterize the nuclear factor(s), gel retardation assays were performed using an HeLa cell nuclear extract and a double-stranded oligonucleotide probe spanning the protected region from DNase I digestion. Three well-separated DNA-protein complexes were observed in the assay. Formation of complexes I and II was significantly inhibited by the inclusion of unlabeled c-*myc* oligonucleotide as competitor, confirming the specificity of these complexes for c-*myc* sequence. Formation of complex III was reduced slightly. In contrast, a double-stranded oligonucleotide containing reading frame of the HIV-1 envelope gene inhibited none of the complexes (Fig. 1*B*). In order to explore the relation of the two specific complexes, HeLa cell nuclear extract was subjected to cation exchange chromatography with Bio-Rex 70. The binding activity producing retarded complex I was present in the FT fraction, whereas complex II was retained and eluted as a single peak with a salt gradient (Fig. 2*A*). When an oligonucleotide for the gibbon ape leukemia virus (GALV) enhancer factor binding site (36) was used as a probe, formation of DNA-protein complexes was also observed in both the FT and peak fractions, and unexpectedly the mobilities of those complexes were indistinguishable from the c-*myc* DNA-protein complexes.

Two Specific Factors (Complexes) Bind to the Same c-myc

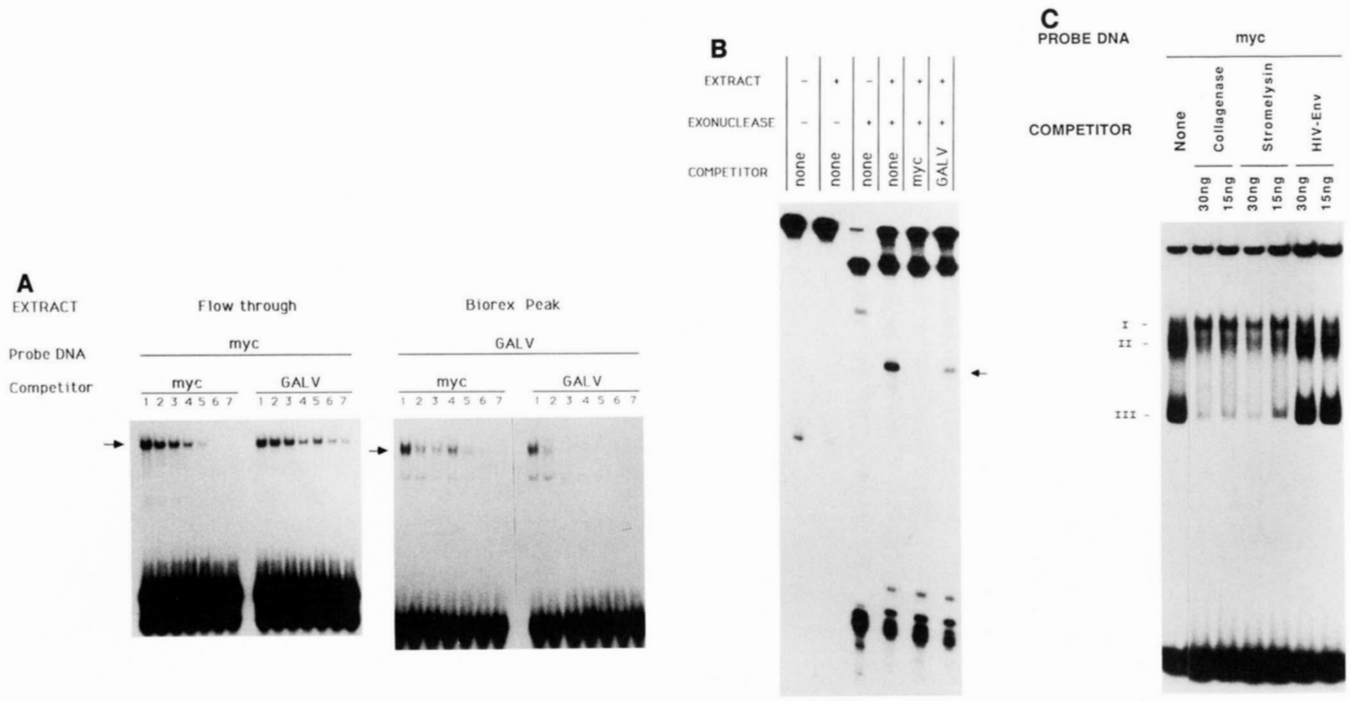

FIG. 3. **The GALV enhancer AP-1 site and c-*myc* negative element bind a common factor.** *A*, cross-competition assays comparing c-*myc* and GALV oligonucleotides by gel retardation were performed. Complex I, produced with a c-*myc* probe and FT fraction, was more efficiently reduced by c-*myc* than by GALV oligonucleotide competitor, whereas complex II, produced with GALV probe and the peak fraction (precipitated with 35–70% ammonium sulfate), was reduced more efficiently by the GALV than the c-*myc* oligonucleotide, as indicated with the *arrows*. The amounts of unlabeled oligonucleotides used as competitor DNAs were as follows: *lane 1*, 0 ng; *lane 2*, 7.5 ng; *lane 3*, 15 ng; *lane 4*, 30 ng; *lane 5*, 60 ng; *lane 6*, 120 ng; *lane 7*, 240 ng. *B*, competition exonuclease assay. Exonuclease binding assays using HeLa cell nuclear extract were performed as described under "Experimental Procedures," with or without the addition of competitor oligonucleotides. 10 μl of HeLa cell nuclear extracts were mixed with a radiolabeled BstNI fragment of pUCPvuII/ScaImyc-bearing *myc* sequence in this experiment. 600 ng each of c-*myc* and GALV oligonucleotides were used as competitor DNAs. The *arrow* indicates the bands produced by the hydrolysis of probe DNAs. *C*, effects of oligonucleotides bearing consensus AP-1 binding site on c-*myc* DNA-protein complexes in the gel retardation assays. Gel retardation assays were performed with radiolabeled c-*myc* oligonucleotides and HeLa cell nuclear extracts as described under "Experimental Procedures." Indicated amounts of unlabeled oligonucleotides were used as competitor DNAs. The sequences of the competitor oligonucleotides are shown in Table I.

TABLE I

Oligonucleotide sequences used in this study

Only the template strand is shown. Sequences underlined indicate the AP-1 consensus. Sequence overlined indicates an octamer core motif. Sequences of 40- and 45-bp oligonucleotides are shown under "Experimental Procedures."

Gene	Sequence
myc	GCCTGCGATGATTTATACTCACAGGA
HIV-Env	CTAGAGAATTCGCAATG
GALV	CGAGAAATAGATGAGTCAACAG
Collagenase	AAGCATGAGTCAGACACCT
Stromelysin	AAGGCAATGAGTCAAGGCTGFCT
Octamer	GATCGCTTCTTAATTTGCATACCCTC

Sequence—The Bio-Rex 70 FT and peak fractions were also assayed for sequence-specific DNA-binding proteins using exonuclease binding assay.

Both fractions yielded barriers to exonuclease hydrolysis at the same position as unfractionated crude extract, indicated with an *arrow* in Fig. 2*B*. A mixture of FT and peak fractions yielded coincident exonuclease-resistant bands, suggesting that both factors bound to the same, or highly overlapping, elements (Fig. 2*B*).

Chemical footprint analysis was performed to confirm the overlapping binding motif of the two complexes. Following nondenaturing electrophoresis, the gel was soaked in 1,10-phenanthroline and copper sulfate; complexes I and II as well as the free probe were then excised from the gel, and the digested DNA was recovered and analyzed as described under "Experimental Procedures." The complexes I and II showed indistinguishable footprints (Fig. 2*C*). Thus, by gel retardation, exonuclease digestion, and chemical footprint analysis, these two factors (complexes) were shown to share a common binding site.

fos/jun (AP-1) Binds to the c-myc Negative Element—As shown in Fig. 2*A*, the GALV probe interacted more strongly with the peak fraction than with the FT fraction, whereas the binding pattern was reversed with the c-*myc* probe (Fig. 2*A*). A cross-competition test was performed to determine if the GALV and c-*myc* DNA-protein complexes were related. Complex I (formed with the FT fraction) was more efficiently reduced by the c-*myc* competitor than by the GALV oligonucleotide, whereas complex II (formed with the peak fraction) was more efficiently reduced by GALV than by the c-*myc* competitor (Fig. 3*A*). Together with the direct binding data shown in Fig. 2*A*, the results suggest that the protein responsible for c-*myc* DNA-protein complex II, which fractionates in the Bio-Rex peak fraction, was closely related to GALV-enhancer binding factor (36).

Exonuclease binding assays were performed to confirm that

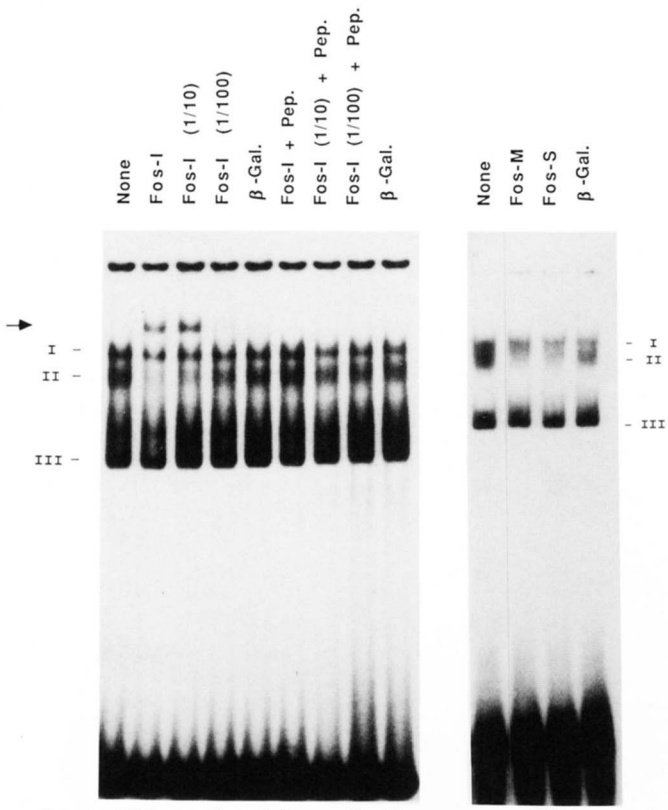

FIG. 4. **Effects of anti-*fos* antibodies on c-*myc* DNA-protein complexes in gel retardation assays.** *Left,* anti-*fos*-I antibodies were preincubated with HeLa cell nuclear extracts on ice for 60 min, and then radiolabeled c-*myc* probe and poly d(I-C) were added and incubated at room temperature for 20 min. Anti-*fos*-I antibodies selectively diminished complex II and produced new bands as indicated by the *arrow.* The *numbers* in *parentheses* indicate the dilution of the antibody. Anti-β-galactosidase antibody showed no effect. A *fos* peptide (1 μg), described as *Pep.*, abolished the inhibition by anti-*fos*-I antibody. *Right,* other preparations of anti-*fos* antibodies, *fos*-M and *fos*-S, also inhibited complex II.

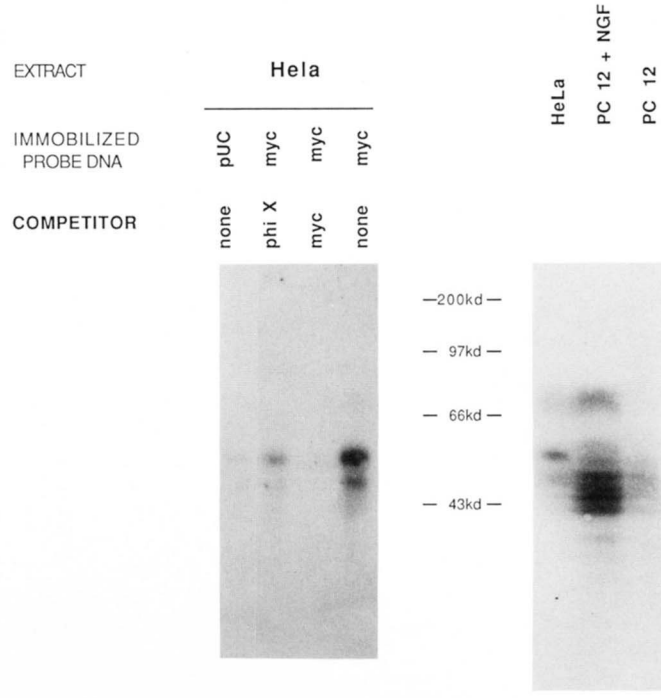

FIG. 5. **Precipitation of *fos* or FRAs with c-*myc* DNA immobilized on Immunobeads.** *Left,* precipitation of c-*myc* DNA-protein complexes on Immunobeads and subsequent Western blotting were performed as described under "Experimental Procedures." Anti-*fos*-I antibody was used to probe the filter. c-*myc* DNA precipitated a major band of 55 kDa as well as minor bands. The c-*myc* oligonucleotide completely abolished the precipitation of *fos* and FRAs, while φX DNA showed only slight inhibition. pUC DNA precipitated only trace amounts of immunoreactive materials. *Right,* unprecipitated HeLa cell nuclear and PC12 whole cell extracts, with or without NGF treatment, contain expected amounts of *fos* and FRAs.

the GALV-enhancer binding factor participated in the c-*myc* DNA-protein complex. The exonuclease assay was performed using HeLa cell nuclear extract and c-*myc* DNA probe, with the inclusion of unlabeled oligonucleotides as competitors. The GALV oligonucleotide competitor partly reduced binding to the c-*myc* probe, whereas the complete competition was observed with c-*myc* competitor (Fig. 3*B*). This result supports the notion that one of the two c-*myc* DNA-protein complexes contained GALV-enhancer binding factor. Inclusion of unlabeled GALV oligonucleotide as competitor in gel retardation assays abolished formation of c-*myc* DNA-protein complex II (data not shown).

Because the consensus binding sequence for the transcription factor AP-1 (TGAC/GTCA) is present in the GALV oligonucleotide (Table I), we examined whether the c-*myc* DNA-protein complex II also contained AP-1. Other oligonucleotides containing AP-1 binding site (Table I) were employed as competitors in gel retardation assays. Collagenase and stromelysin AP-1 sites significantly inhibited the second c-*myc* DNA-protein complex, whereas non-Ap-1 oligonucleotide, HIV-Env, did not. Complex III was also detectably reduced by the oligonucleotides of collagenase and stromelysin (Fig. 3*C*).

The c-*fos* protein has been shown to function as a transcriptional regulator (10). Recent reports have shown that the c-*fos* protein associates with a cellular protein, p39, which is the product of the c-*jun* proto-oncogene, and that the *fos/jun* complex binds to AP-1 sites (40–46). We examined the effect of affinity-purified anti-*fos* immunoglobulin on the c-*myc* DNA-protein complexes using gel retardation assays. One antibody, anti-*fos*-I, inhibited the second complex specifically, and a new shift was observed as indicated with an *arrow* in Fig. 4. In contrast, anti-β-galactosidase antibody had no effect on the c-*myc* DNA-protein complexes. The inhibitory effect of the *fos* antibody was abolished by addition of the c-*fos* peptide that was used to generate the rabbit anti-*fos* antibody. Independent preparations of anti-*fos* immunoglobulin, anti-*fos*-M and anti-*fos*-S, also inhibited the formation of complex II, but did not produce new bands. It is noteworthy that complex III, which was inhibited by AP-1 oligonucleotides (Fig. 3*C*), was not affected by the anti-*fos* antibodies (Fig. 4).

To directly demonstrate the participation of the c-*fos* protein in a c-*myc* DNA-protein complex, HeLa cell nuclear extract was incubated with c-*myc* DNA immobilized on beads. The resulting DNA-protein complex was precipitated and washed; the bound proteins were eluted with $NaDodSO_4$ and analyzed by immunoblot. Anti-*fos* antibody reacted with a major band of 55 kDa and minor bands of lower molecular masses. The molecular weight of the major band conforms to that reported for the c-*fos* protein, and the minor bands are consistent with the presence of *fos*-related antigens (FRAs) (46). c-*myc* oligonucleotide competitor completely abolished the precipitation of c-*fos* and FRAs, while φX DNA showed only slight inhibition. pUC DNA immobilized on beads precipitated only a trace amount of immunoreactive material (Fig. 5, *left*). These results demonstrate the specific binding of *fos* and FRA to the c-*myc* negative regulatory region. Unfractionated HeLa cell nuclear extract as well as PC12

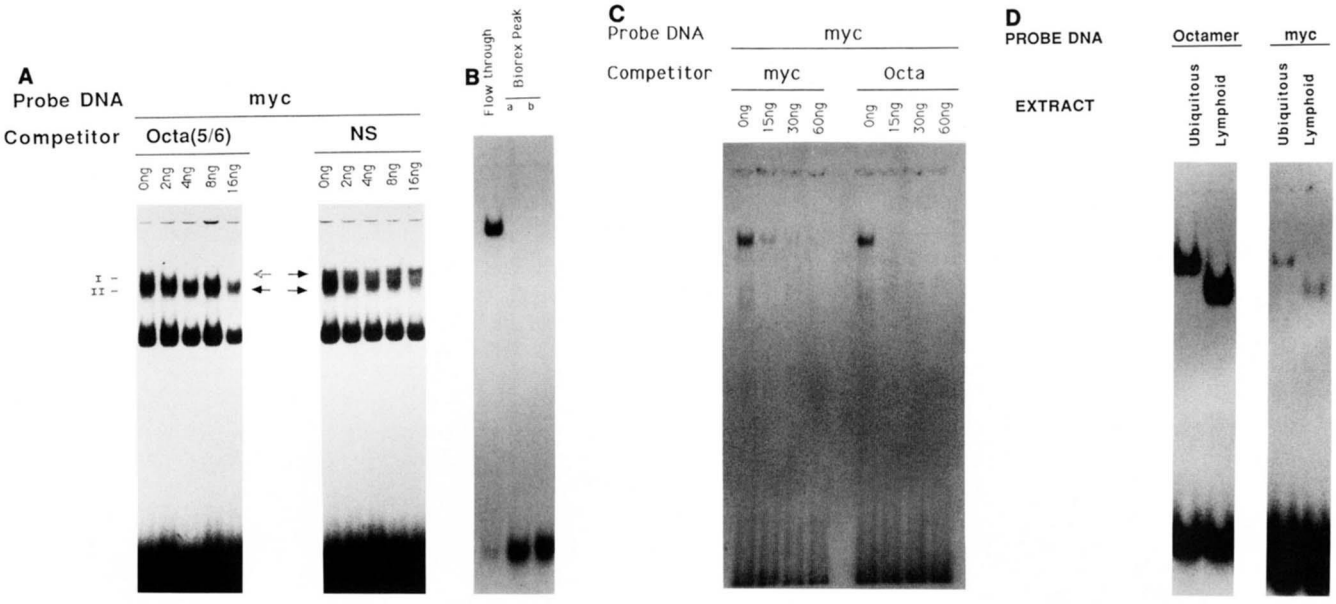

FIG. 6. **The ubiquitous octamer-binding factor participates in c-*myc* DNA-protein complex I.** *A*, oligonucleotides bearing the octamer sequence inhibit c-*myc* DNA-protein complex I in gel retardation assays. HeLa cell nuclear extracts were probed with a radiolabeled c-*myc* oligonucleotide probe, with the inclusion of oligonucleotide competitors. A 40-bp oligonucleotide bearing the octamer sequence, Octa(5/6), reduced complex I (*striped arrow*) whereas a 40-bp unrelated oligonucleotide (*NS*) showed no inhibition of complex I. Complex II was not reduced by either oligonucleotide. The complexes unaffected by competitors are indicated by the *solid arrows*. The sequences of the competitor oligonucleotides are shown under "Experimental Procedures." *B*, octamer-binding activity is observed in the FT fraction of HeLa cell nuclear extracts. Fractionated HeLa cell nuclear extracts were probed with octamer oligonucleotides. The FT fraction contained binding activity, whereas the peak fractions did not. *a* and *b* indicate the peak fractions precipitated with 0–35% and 35–70% ammonium sulfates, respectively. 1 μl of each fraction was incubated with a radiolabeled 26-bp oligonucleotide bearing the octamer motif as shown in Table I. *C*, the octamer oligonucleotide competes for the complex I more efficiently than c-*myc* oligonucleotide. The efficiency of competition for the first c-*myc* DNA-protein complex was compared using either c-*myc* or octamer oligonucleotide competitors. The FT fraction (1 μl) was incubated with probe oligonucleotide and either c-*myc* or octamer (*Octa*) oligonucleotides with the sequences shown in Table I. *D*, purified octamer-binding proteins bind to c-*myc* sequence. Octamer-binding proteins were purified from a B-cell line, WEHI 231, as described under "Experimental Procedures," and incubated with radiolabeled c-*myc* oligonucleotide. Both ubiquitous and lymphoid-specific octamer-binding proteins formed complexes with c-*myc* DNA. Radiolabeled octamer oligonucleotide was also used as a control probe, which showed stronger binding than the c-*myc* probe.

whole cell extracts, with and without NGF treatment, showed the expected pattern of *fos* and FRA immunoreactivity (Fig. 5, *right*).

An Octamer-binding Factor Recognizes the c-myc Negative Element—Gel retardation analysis in the presence of a variety of competitor sequences revealed that c-*myc* DNA-protein complex I was efficiently inhibited by sequences derived from the immunoglobulin κ promoter, which contains an octamer motif (48). Inclusion of a 40-bp double-stranded octamer oligonucleotide as competitor abolished c-*myc* DNA-protein complex I. Unrelated oligonucleotides of the same length did not reduce the signal, indicative of specific binding by octamer factor (Fig. 6*A*). When an octamer oligonucleotide of 26 bp was radiolabeled and mixed with the FT or peak fractions, specific binding was observed to be selective with the FT fraction (Fig. 6*B*): this complex comigrates with the c-*myc* DNA-protein complex (data not shown). Comparison of identical length c-*myc* and octamer oligonucleotides as competitors in gel retardation assays, with c-*myc* probe and the FT fraction, revealed that the octamer oligonucleotide reduced binding more efficiently than the c-*myc* oligonucleotide, suggesting that an octamer-binding factor constitutes the protein in c-*myc* DNA-protein complex I (Fig. 6*C*).

Two types of octamer-binding factors have been identified; one is ubiquitous (27, 28, 47, 62, 63), and the other is lymphoid specific (48, 49, 60, 61, 64). Because c-*myc* DNA complex I is formed with HeLa cell factors, it is likely to contain the ubiquitous factor. To directly demonstrate the interaction of c-*myc* sequences with octamer binding factors, both the ubiquitous and lymphoid-specific octamer protein, purified from WEHI 231 cells, were employed in gel retardation assays using the c-*myc* probe. The purified octamer proteins bonded to c-*myc* DNA, although the interaction was less than with the octamer probe. As expected, the purified lymphoid-specific factor produced a complex with greater mobility than the ubiquitous factor (Fig. 6*D*).

fos/jun(AP-1) and the Octamer-binding Factor Share a Common Binding Site in the c-myc Negative Element—From the results of the exonuclease assay and the chemical footprint analysis as well as from a comparison of the c-*myc*, GALV, and octamer DNA sequences, it is apparent that the binding sites on c-*myc* for complexes I and II overlap extensively in the central portion of the DNase I footprinted region. When comparing the c-*myc* and octamer sequence, one homologous segment was noted. Ten contiguous bp match each other at the purine-pyrimidine level with 6 identical bp (Fig. 7*A*). Between c-*myc* and GALV, there are two homologous sequences within the chemically footprinted region. In one case, 8 of 9 base pairs match at the level of purine-pyrimidine; out of the 8, 7 bp are identical (Fig. 7*A*). In the other case, 6 consecutive bp of each match at the purine-pyrimidine level; of these 6 bp, 5 are identical (Fig. 7*B*). The binding site shown

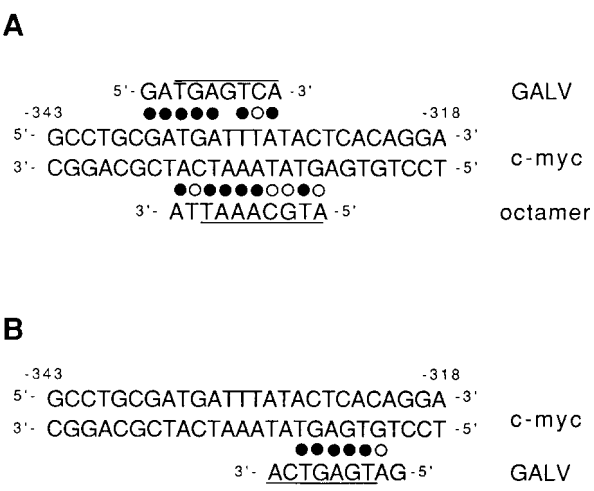

FIG. 7. **The binding sites for *fos/jun* (AP-1) and the octamer-binding factor overlap on the c-*myc* negative element.** The sequence of the c-*myc* 26-bp negative element, from −343 to −318 bp relative to the P1 promoter, is shown. *A*, the homologous sequences of the GALV and octamer oligonucleotides are aligned above and below the c-*myc* sequence, respectively. The consensus AP-1 binding site is *overlined* on the GALV sequence and the core sequence of the octamer motif is *underlined* below the octamer sequence. *B*, a second homology with the GALV sequence is aligned below the c-*myc* sequence. The consensus AP-1 binding site is *underlined* below the GALV sequence. ●, indicates an identically matched base. O, indicates a purine-pyrimidine match.

in Fig. 7 for *fos/jun* (AP-1) and the octamer binding protein overlap more extensively in case of *A* than in the case of *B*. The overlapping sequence is located in the central portion of the 26-bp c-*myc* negative element (Fig. 7).

Because purified ubiquitous octamer-binding protein produced a complex with mobility similar to complex I from crude extract and because this complex is refractory to inhibition by anti-*fos* or AP-1 oligonucleotide competition, it can be concluded that *fos/jun* is not a component of complex I. Similarly, because complex II is not altered by octamer competition and because it is unlikely that addition of *fos/jun* to complex I would increase electrophoretic mobility, it is apparent that complex II contains no octamer-binding protein. No complex migrating slower than complex I is present, which suggests that a quaternary complex of *fos*, *jun*, octamer-binding protein, and probe does not form.

DISCUSSION

We have shown that two entirely distinct factor complexes bind to a c-*myc* negative element. These two complexes bind to highly overlapping segments of the c-*myc* sequence. One of the complexes is *fos/jun* (AP-1), and the other is the ubiquitous octamer-binding factor.

There is no obvious homology between the consensus recognition sequences for these two factors. The binding assays shown above clearly demonstrate that AP-1 and octamer consensus sequences will not cross-compete binding to their respective proteins except at very high molar excess. It was unexpected, therefore, that a single element with reduced homology to each recognition sequence would bind both the *fos/jun* (AP-1) and the ubiquitous octamer protein. It is apparent that enough degeneracy exists within the sequences recognizable by AP-1 and the octamer-binding proteins to allow binding of both factors to a common site. Examination of the known binding sites for most eukaryotic transcription factors reveals considerable plasticity in the permissible deviations from the deduced consensus sequences. Such plasticity is not intrinsic to the process of sequence recognition; for example most restriction enzymes are extremely rigid with respect to sequence recognition.

By creating heterogeneity among the binding sites for a single *trans*-acting factor, some of those sites may be allowed to overlap with the binding sites for other regulatory proteins. A variety of antagonistic or synergistic interactions between factors may therefore expand the range of physiological responses mediated by a given *cis*-element. Without degeneracy in the recognition sequences the opportunities for allowing alternative binding of different factors would be reduced. For example, whereas this paper demonstrates that there exists an overlap between AP-1 and octamer-binding sites, the degeneracy of the AP-1 consensus allows overlapping binding sites for AP-1 and AP-4 in both viral and cellular genes (50).

The degeneracy of recognition sites that allows overlapping of different *cis*-elements also allows the assembly of compact, powerful regulatory regions such as enhancers. Viral enhancers, such as those found in SV40 or polyoma, possess a complicated and interdigitating array of positive and negative *cis*-elements. Without degeneracy, the constraints of rigid recognition sequences might well increase the amount of DNA needed to specify a regulatory response.

The 26-bp segment containing the *fos/jun* (AP-1) and octamer-binding sites has been shown to contain the principal determinant of a negative *cis*-element for c-*myc*.[2] At this time it is not possible to say whether the *fos/jun* (AP-1) or the octamer-binding protein is responsible for the down regulation of c-*myc* expression mediated by their common binding site. Each of these factors has already been demonstrated to play a role in more than one regulatory process. We cannot exclude a role for other proteins in decreasing c-*myc* expression from this site.

AP-1 was discovered as a transcriptional activator protein, which binds to regulatory elements of viral and cellular genes, such as SV40 and metallothionein (26). The proto-oncogene c-*jun*, a cellular homologue of the retroviral oncogene v-*jun*, has been identified as one of the proteins that binds to AP-1 sites (40, 45). Recent results indicate that the *fos* protein interacts with the product of c-*jun*, p39, and that this complex binds to AP-1 sites (41–44, 46). The role of the *fos* protein in transcriptional activation has been shown (10). However, it has been shown that c-*fos* transcription is negatively autoregulated by the *fos* protein itself and the *fos/jun* (AP-1) complex also binds to a negative regulatory element of an adipocyte-related gene, aP2 (51, 52). Identification of the *fos/jun* (AP-1) complex binding to a c-*myc* negative element comforms to these observations. It is not yet known how the *fos/jun* complex mediates both transcriptional activation and repression.

The octamer motif has been implicated in transcriptional activation of cellular and viral regulatory regions (for review see Ref. 64). Interestingly, this factor also binds to the origin of DNA replication on the adenovirus genome and mediates the initiation of replication (28, 29). Recently, one of the octamer-binding proteins was implicated as a negative regulator of immunoglobulin heavy chain gene expression.[3] There is precedent for *cis*-elements and *trans*-acting factors common to transcriptional activation, repression, and replication (53–56).

Extensive study will be required to determine the relative contributions of *fos/jun* (AP-1) and octamer-binding proteins

[2] Hay, N., Takimoto, M., and Bishop, J. M. (1989) *Gene Dev.* **3**, 293–303.

[3] Lenardo, M. J., Staudt, L., Robbins, P., Kuang, A., Mulligan, R., and Baltimore, D. (1989) *Science* **243**, 544–546.

to transcriptional repression of c-*myc*. However, this problem is complicated. First, it is difficult to mutate the binding site for a single factor, because both binding sites overlap. A mutation in the recognition sequence for one factor will be also a mutation in the other. Second, it has been shown for both the thyroid hormone and glucocorticoid receptors that some binding sites are positive elements whereas others are negative. Alteration in protein tertiary structure or conformation, caused by subtle differences in DNA-binding sequences, may alter the nature of the physiological response (56). If the deviation of the c-*myc* negative element from the consensus AP-1 and octamer sequences is critical for producing the negative effect on c-*myc* expression, then alteration of this sequence may abolish the negative response. Therefore, the direct substitution for the c-*myc* sequence by consensus octamer or AP-1 sequences cannot necessarily determine the relative contribution of either factor. Third, it is possible that both factors may act synergistically to maintain a negative effect on c-*myc* expression. Because the ubiquitous octamer-binding factor has been reported to work selectively in the S phase of the cell cycle, and *fos* and *jun* have been shown to be induced during the G_0/G_1 transition (27, 57–59), the factors may repress c-*myc* transcription at different points in the cell cycle.

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