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Distinct Factors Bind the AP-1 Consensus Sites in Gibbon Ape Leukemia Virus and Simian Virus 40 Enhancers

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We have demonstrated that the gibbon ape leukemia virus (GALV) enhancer AP-1 element and the simian virus 40 AP-1 enhancer element bind different factors in HeLa nuclear extracts. A 39-kilodalton HeLa nuclear protein and the c-*fos* protein bind to the GALV element. Antibodies to c-*fos* abolish binding to the GALV AP-1 site. In contrast, anti-c-*fos* immunoglobulin fails to inhibit formation of the simian virus 40-specific complex from extracts of HeLa cells. Thus, AP-1-binding complexes are subject to compositional variation at different binding sites.

It was previously shown that the gibbon ape leukemia virus (GALV) Seato strain enhancer element is active in several uninfected cell lines and that the sequence responsible for this activity resides within a 155-base-pair (bp) area of the U3 region of the long terminal repeat (13). This region contains three tandem direct and perfect repeats of a 48-bp sequence (22). Within each of these repeats, a 22-bp sequence binds a cellular factor; this sequence has enhancer activity in vivo (22). The 22-bp enhancer region contains the consensus sequence TGAG/CTCA, which is found in the binding site for the transcription factor AP-1 (2, 17).

Purified AP-1 consists of a group of polypeptides ranging in size from 40 to 60 kilodaltons (kDa), with a major polypeptide of 47 kDa (2, 18); however, it is not clear whether these are different forms of the same molecule or related polypeptides. A functional relationship between AP-1 and c-*fos* has been suggested in that c-*fos* is part of a complex binding at AP-1 sites in certain cell lines (8, 24). Consistent with this, the 39-kDa protein in AP-1 preparations purified by oligonucleotide affinity chromatography is the same as p39, a 39-kDa protein previously shown to coprecipitate with antibodies to c-*fos* (23). This p39 is identical with the c-*jun* gene product (1, 3, 4). The c-*jun*–c-*fos* complex from H9 cells has been shown to bind to the GALV enhancer element (8).

Although it has been shown that the 39-kDa protein in these AP-1 preparations is functional, several lines of evidence indicate the existence of a larger AP-1 family of proteins binding to AP-1 consensus sequences. There is strong homology of the murine *jun* B cDNA clone with v- and c-*jun* (20, 25). In yeast cells, the simian virus 40 (SV40) AP-1 element is *trans*-activated by proteins distinct from GCN4, thus demonstrating that different factors can *trans*-activate AP-1 sites under different conditions (12, 15).

To study the possibility that various (possibly related) factors would bind different AP-1 sites, double-stranded oligonucleotides conforming exactly to the GALV enhancer and SV40 AP-1-binding sites (16, 17, 21, 22) were compared by using gel retardation and competition studies. We also identified directly from crude nuclear extracts the cellular protein(s) binding to the GALV enhancer element by modification of a protein-DNA-binding affinity technique for analysis of mammalian sequence-specific DNA-binding proteins (19).

MATERIALS AND METHODS

Cells and media. HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. MLA-144 and Jurkat cells were grown in RPMI 1640 medium containing 10% fetal bovine serum. Metabolic labeling was performed by growing 10^9 cells in 100 ml of methionine-free DMEM containing 5% dialyzed fetal calf serum with 10 mCi of *trans*-[^{35}S]methionine (ICN Radiochemicals, Irvine, Calif.).

Preparation of extract. Nuclear extracts were prepared by the procedure of Dignam et al. (6) with the following modifications. Buffer C contained 20% glycerol–0.42 M NaCl, and buffer D contained 20% glycerol–80 mM KCl. All buffers contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.5]). Extracts contained 30 mg of protein per ml.

Gel retardation. Gel retardation analysis was performed as described previously (10, 26, 27). Binding reactions contained a ^{32}P-labeled 3′-end double-stranded oligonucleotide probe.

Protein purification. Unless stated otherwise, each experiment contained 0.5 μg of either a multimer of the 48-bp element from the GALV long terminal repeat enhancer region cloned adjacent to a *lac* operator in pUC18 or bacteriophage λ DNA cloned adjacent to the *lac* operator in pUC8. Each fragment was bound via the *lac* operator in the vector to 1 μg of β-galactosidase–*lac* repressor fusion protein (7) linked to immunobeads via anti-β-galactosidase antibody. This complex was mixed with crude nuclear extract (2 mg of protein) and 100 μg of poly(dI)-poly(dC) and incubated for 20 min at 24°C. The immunobead complex enriched for specific DNA-binding proteins was precipitated by brief centrifugation and washed several times with 100 mM KCl buffer D. The specific DNA-binding proteins were dissociated from the DNA with 1 M KCl buffer D. This same treatment disrupts the binding of the *lac* repressor fusion protein to the *lac* operator sequence. The immunobead-*lac* repressor fusion protein complex was pelleted, leaving the DNA and the enriched DNA-binding protein preparation in

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the supernatant. This mixture was then adjusted to 100 mM KCl by the addition of 0 M KCl buffer D in the presence of either 5 μg of the 48-bp enhancer multimer, pUC18, or λ DNA linked to the *lac* repressor fusion protein-immunobead complex. This new mixture was incubated for 20 min at 24°C with or without specific oligonucleotide competitors as noted. The resulting complex was pelleted and washed extensively with 100 mM KCl buffer D. The DNA that specifically bound to the *lac* repressor, together with bound nuclear proteins, was eluted from the fusion protein by 3 mM isopropyl-β-D-thiogalactopyranoside and 5 μg of poly(dA)-poly(dT) in a final volume of 20 μl. The eluted material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of anti-*fos* antibody. A synthetic peptide corresponding to amino acids 129 through 153 of c-*fos* (11) was coupled to succinylated keyhole limpet hemocyanin with EDC and emulsified at a final concentration of 500 μg/ml in a 1:1 ratio of saline to complete Freund adjuvant. Intradermal-subcutaneous injections were made at multiple sites in the back of the male New Zealand White rabbits at 10- to 14-day intervals. After six injections, antibody was affinity purified by passage over a column made of the peptide coupled to cyanogen-activated Sepharose 4-B. After passing crude serum through the columns, the columns were washed first with 150 mM NaCl–50 mM Tris-NaCl (pH 7.4) and then with 2 M NaCl until the optical density at 280 nm was 0. Antibodies were diluted after a 15-min washing with 4 M $MgCl_2$.

Western immunoblotting of GALV-specific complexes precipitated with immunobeads. Precipitation of the protein complexes with GALV DNA immobilized on immunobeads was performed by a variation of the method described above. HeLa extract (2.5 mg of total protein) was mixed with 10 μg of poly(dI)-poly(dC), 0.8 mg of bovine serum albumin, and 0.1% Tween 20 for 4 min at 4°C. The mixture was centrifuged for 5 min (4°C, 1,000 rpm in an Eppendorf microcentrifuge), and the supernatant was used for binding to either 150 ng of GALV DNA (multimerized) previously immobilized on immunobeads or pUC19 DNA similarly immobilized as a control. The mixtures were incubated (24°C for 25 min) and washed twice with buffer D (80 mM), and the bound material was eluted with 0.2% SDS.

The eluted proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose filter (550 mA, 4°C at 90 min). The filter was blocked with Blotto (5% Carnation nonfat milk powder, 50 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [14]) for a 90-min reaction time, probed with affinity-purified anti-*fos* antibody (dilution ratio, 1/2,500) in Blotto (24°C for 24 h), and rinsed with TNE-50 (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The filter was then incubated with anti-rabbit immunoglobulin (^{125}I-labeled Fab' [24°C for 2 h] [Amersham Corp., Arlington Heights, Ill.]), washed several times with TNE-50, and dried for autoradiography.

RESULTS

The specific protein in HeLa cells that binds the GALV enhancer is distinct from the major protein which binds the SV40 AP-1 element. The 22-bp oligonucleotide that has GALV enhancer activity contains a segment homologous to

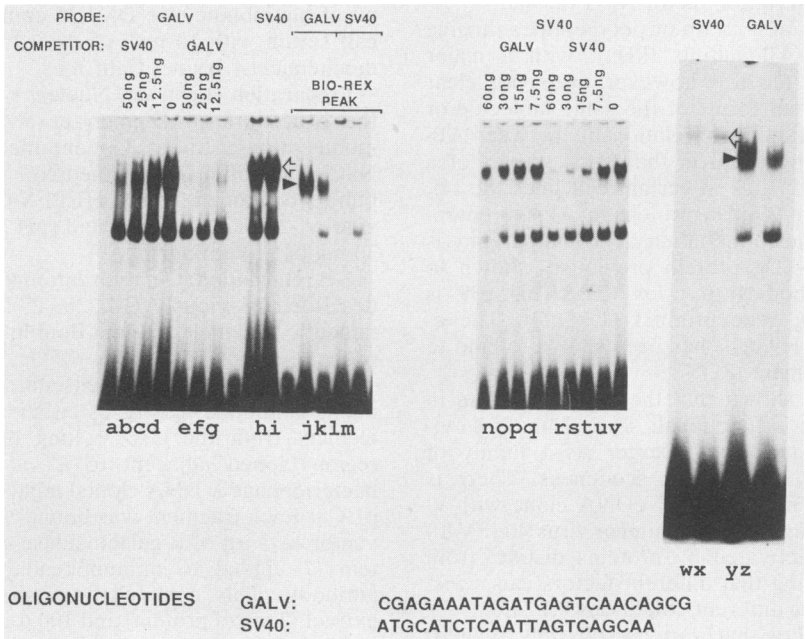

FIG. 1. Gel retardation analysis of the distinct binding specificity of GALV AP-1 and SV40 AP-1 sites. Gel retardation studies were performed with end-labeled double-stranded oligonucleotide probes comprising segments of the SV40 or GALV enhancers; the sequence of one strand of each probe is displayed at the bottom. Binding reactions included the indicated probes and competitors. All reactions included HeLa nuclear extract except those shown in lanes j through m, which contained the pooled peak of GALV-binding activity from the BioRex-70 column precipitated with ammonium sulfate (0 to 13%, lanes j and l, or 35 to 70%, lanes k and m). Note the different positions of the retarded complexes with the GALV (lanes a through g, j, and k) or SV40 (lanes h, i, and w through x) probes. The BioRex peak specifically shifts only the GALV probe. Unlabeled lanes are from binding reactions with no extract. Extracts were loaded onto a Bio-Rex column in 200 mM KCl buffer D and eluted with a 200 to 800 mM KCl gradient in buffer D, and SV40 and GALV enhancer-binding activities were monitored by gel retardation.

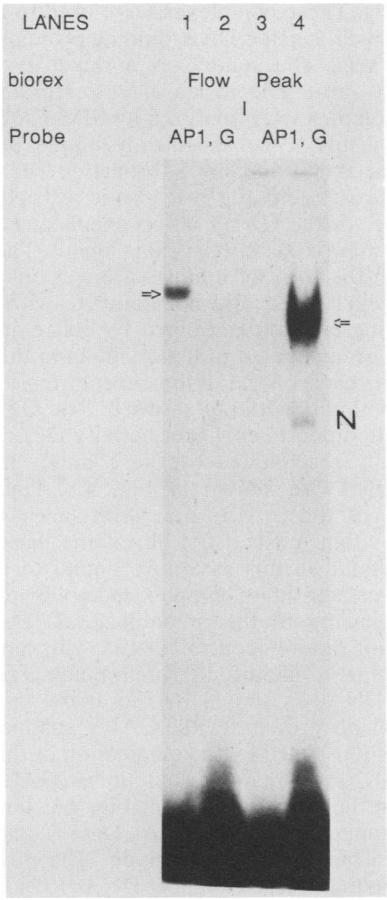

FIG. 2. The proteins binding specifically to the GALV AP-1 and SV40 AP-1 sites are separable by BioRex-70 chromatography. The flowthrough of the BioRex-70 column (Flow) will shift the SV40 AP-1 probe (AP1) but not the GALV-Seato probe (G). The BioRex-70 peak will shift the GALV probe but not the SV40 probe. N indicates the less-retarded shift that was not reduced by the addition of homologous competitor.

the binding site of the previously purified *trans*-acting factor AP-1 (2, 17, 18). AP-1 preparations contain a major peptide of 44 to 47 kDa. To explore the relationship between the factor(s) that binds to the GALV enhancer and those that interact with the SV40 enhancer, reciprocal competition studies were performed with the GALV 22-bp oligonucleotide (corresponding to the enhancer sequence) and the previously identified SV40 AP-1-binding site (17) as probes; each of these probes generated a distinctive shift with HeLa nuclear extract. Inclusion of excess unlabeled homologous or heterologous competitor indicated that the protein responsible for the GALV-specific shift binds with greater affinity to the GALV probe than the protein which binds the SV40 AP-1 site does and, conversely, that a protein specific for the SV40 AP-1 site binds more tightly to its binding site than that of the GALV sequence (Fig. 1). Each of these oligonucleotides will cross-compete only when present in vast excess. In our gel retardation studies, there is also a less retarded shift that is not reduced by the addition of a homologous oligonucleotide competitor. The shifts generated by the SV40 and GALV probes migrated differently, which allowed us to separate the GALV protein from the SV40 protein by ion exchange chromatography on BioRex-70. When HeLa nuclear extract was fractionated on BioRex-70, the SV40-binding material was found in the flowthrough from the column, whereas the GALV-binding factor was retained on the column (Fig. 2). That the GALV factor rather than the SV40 factor is the major biological effector of GALV enhancer activity is suggested by the correlation of the relative abundance (or affinities for cognate sequences) of these proteins in various cell lines with the relative enhancer activities of the GALV and SV40 enhancers in the same cells (Fig. 3). The SV40 protein, which binds the SV40 enhancer, is considerably more abundant in HeLa cells than the GALV factor, whereas a reciprocal relationship exists in MLA-144 cells, in which the GALV enhancer has higher activity (13, 22). Extracts of Jurkat cells, which support the SV40 enhancer but not the GALV enhancer, will shift the SV40 sequence but not the GALV sequence. These results suggest that the factor determining the activity of the GALV enhancer by binding to the 22-bp AP-1 sequence is distinct from the major factor binding to the SV40 AP-1-binding site.

Identification of the GALV enhancer-binding protein. The 48-bp GALV enhancer region was cloned as a multimer adjacent to a *lac* operator and bound to a *lac* repressor–β-galactosidase fusion protein which, in turn, was bound to anti-β-galactosidase immobilized on acrylamide beads. Control procaryotic DNA segments were similarly bound to

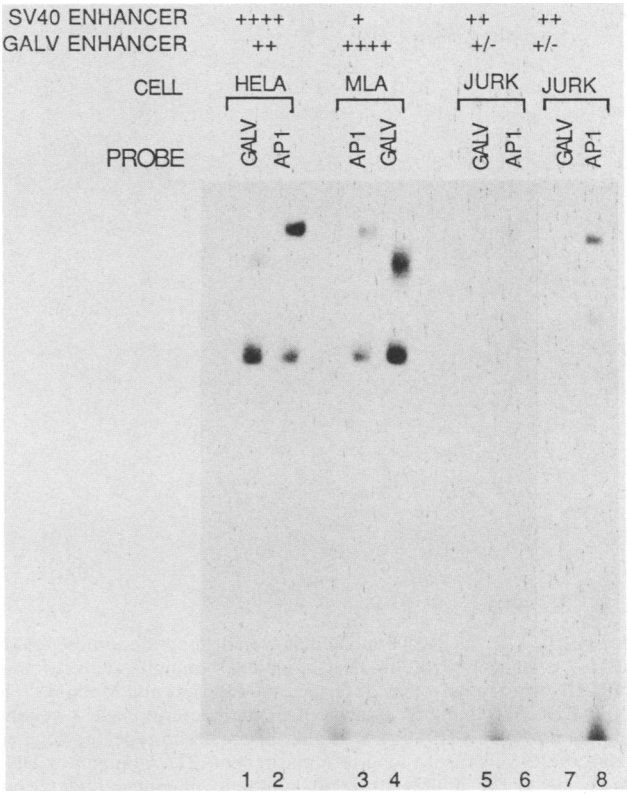

FIG. 3. Gel retardation analysis of enhancer-binding activity in HeLa, MLA-144, and Jurkat cells. The relative amounts or affinities of the GALV- and SV40-specific proteins in different cells parallel GALV-Seato and SV40 enhancer activity. Fifteen-microgram amounts of nuclear protein of HeLa and MLA-144 cells or 8 μg of Jurkat (JURK) nuclear protein was incubated with the indicated probes and adjusted to the same specific activity, and specific complexes were resolved by electrophoresis. Lanes 7 and 8 show a fourfold exposure of lanes 5 and 6. Presence of enhancer activity is indicated by a plus (+) sign; amount of activity is indicated by the number of plus signs; marginal enhancer activity is shown by +/−.

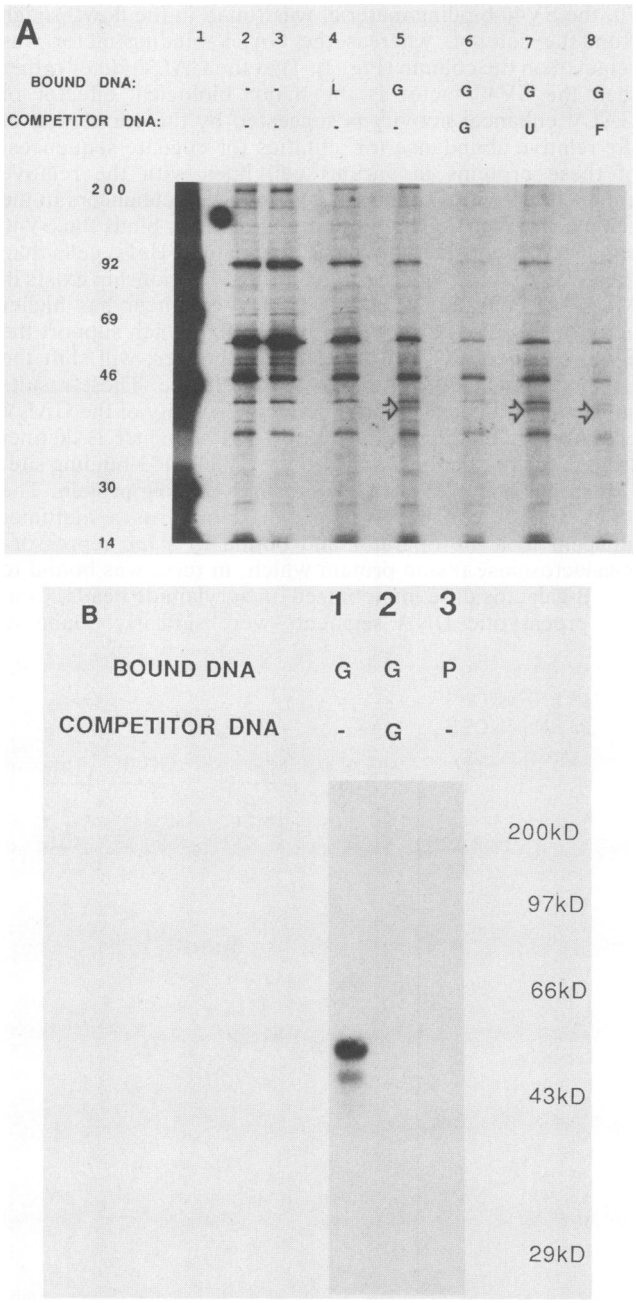

FIG. 4. (A) A 39,000-molecular-weight peptide binds to the GALV enhancer. Protein-DNA complexes immobilized on immunobeads were prepared as described in Materials and Methods. The bound DNA was either a segment of the bacteriophage λ genome cloned into pUC8 (19) or a multimer of the 48-bp repeated segment from the GALV-Seato long terminal repeat (22). Competitor DNA molecules were double-stranded synthetic oligonucleotides comprising a segment of the GALV-Seato enhancer which possesses enhancer activity (22) (G), a GALV-Seato segment immediately upstream with no enhancer activity (U), or a segment from the Friend leukemia virus enhancer (F). Lanes: 1, molecular weight standards of the indicated sizes; 2, extract proteins precipitated by immunobeads with bound anti-β-galactosidase and bound *lac* repressor–β-galactosidase fusion protein but no DNA; 3, proteins precipitated by immunobeads with bound anti-β-galactosidase but no fusion protein or DNA. Arrows indicate the presence of a protein specific for GALV DNA. L, Lambda DNA. (B) *fos* binds specifically to the GALV enhancer DNA. The proteins specifically bound to immobilized DNA on immunobeads were blotted onto nitrocellulose as described in Materials and Methods and probed with affinity-purified antibodies to c-*fos*. Lane 1 demonstrates that c-*fos* (the peptide at 55 kDa [kD]) is specifically enriched with GALV DNA (G), compared with pUC19 DNA (P) (lane 3). The specificity of c-*fos* binding to this sequence is confirmed by elimination of the signal when GALV oligonucleotide competitor is added to the initial binding reaction (lane 2). The additional bands below 55 kDa are *fos*-related antigens (9).

immunobeads. These complexes were used to directly bind GALV enhancer-specific DNA-binding proteins from crude nuclear extracts. The complexes enriched by binding the immunobead–anti-β-galactosidase–*lac* repressor fusion protein–DNA complex were analyzed by SDS-PAGE (Fig. 4A). This figure displays the specifically eluted proteins from HeLa nuclear extract in lanes 2 through 8. The DNA bound to the complex was either a procaryotic restriction fragment or a multimer of the GALV 48-bp enhancer element; both DNAs were approximately the same length. The competitor DNA was in the form of double-stranded oligonucleotides (Fig. 4A), which either did not compete with the specific GALV-binding factor (determined by gel retardation [data not shown]) or eliminated protein binding to the footprinted region within each of the 48-bp repeats (as assayed by a variety of methods [data not shown]). The GALV oligonucleotide itself supports enhancer activity (22). The proteins nonspecifically precipitated by the complex itself but not bound to DNA are shown in Fig. 4A, lanes 2 and 3. Comparison of these two lanes with lanes 4 through 8 demonstrates that most, if not all, of the background proteins precipitated in this assay are bound to the immunobead–anti-β-galactosidase complex and not by proteins binding nonspecifically to the immobilized DNA. Strikingly, comparison of lane 4 (control DNA) with lane 5 (GALV DNA) allows us to identify a protein binding specifically to the GALV DNA, as shown by the arrow in lane 5. The specificity of this protein for the GALV enhancer sequence is further demonstrated by the elimination of this protein by competitor DNA containing functional GALV enhancer sequences in the binding reaction (Fig. 4A, lane 6). Heterologous oligonucleotides (Fig. 4A, lanes 7 and 8) did not eliminate the binding of this peptide. The protein binding specifically to the GALV enhancer DNA is 39 kDa. In Jurkat cells in which the enhancer is minimally active, little or no protein of this molecular weight could be precipitated by the GALV-specific DNA (data not shown). These results are consistent with a potential role for the 39-kDa factor in enhancer-mediated transcriptional activation and with the weak or absent signal observed by gel retardation analysis of Jurkat cell nuclear extracts with the 22-bp element as a probe (Fig. 3).

***fos* is part of the complex at the GALV AP-1 site in HeLa cells.** Although we detected no specifically precipitated peptides other than this 39-kDa protein binding directly to the GALV enhancer sequence, other specifically bound proteins such as *fos*, which has previously been reported to interact with the GALV enhancer element (8), could be masked by comigration with one of the proteins precipitated nonspecifically by the immunobead–anti-β-galactosidase complex. In addition, c-*fos* migrates heterogenously due to posttranslational modification (5). To answer the question of whether c-*fos* was bound to the GALV complex, blots of the material precipitated by the specific DNAs bound to the immunobeads were probed with anti-*fos* immunoglobulins.

HeLa cell nuclear extract was incubated with GALV

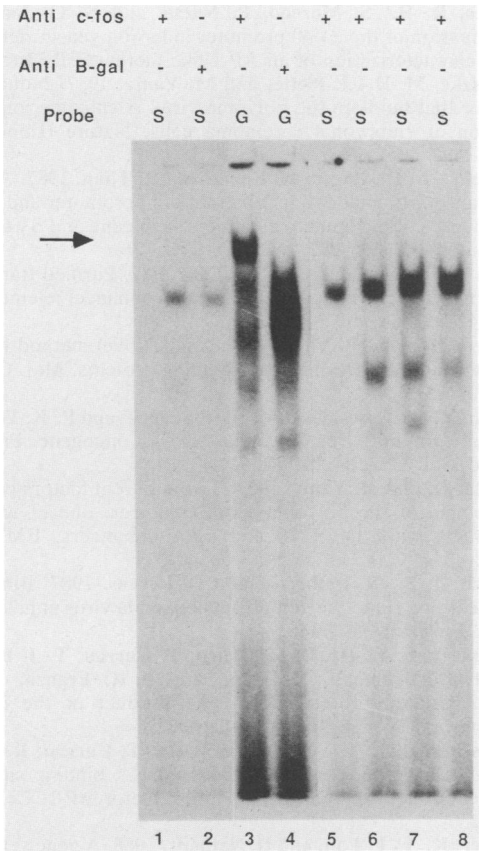

FIG. 5. Gel retardation analysis of antibodies to c-*fos* which inhibit the formation of the GALV- but not the SV40-specific shift in HeLa cells. Lanes: 1, SV40 (S) probe with *fos* antibody; 2, SV40 probe with β-galactosidase antibody; 3, GALV (G) probe with c-*fos* antibody (note the elimination of the specific shift and the appearance of a slower migrating species [indicated by the arrow] presumably from addition of antibody to the complex); 4, GALV probe with β-galactosidase antibody; 5 through 8, SV40 probe with 1, 1:10, 1:100, and 1:1,000 dilutions of the affinity-purified c-*fos* antibody. Presence or absence of antibody is indicated with a plus (+) or minus (−) sign.

enhancer DNA immobilized on beads or with pUC19 DNA immobilized on beads as a control. After the binding reaction, DNA-protein complexes on the beads were washed, and then the bound protein was eluted with 0.2% SDS. The eluted proteins were separated by SDS-PAGE and were then transferred to a nitrocellulose filter. The filter was probed with anti-*fos* antibody. The GALV DNA precipitated a major immunoreactive protein of 55 kDa, the correct molecular weight for c-*fos*, and related proteins (9) (Fig. 4B, lane 1). pUC19 DNA precipitated only a trace of *fos*-reactive material (Fig. 4B, lane 3). The binding of the 55-kDa peptide to the immobilized GALV DNA could be eliminated by inclusion of GALV DNA as a competitor in the binding reaction (Fig. 4B, lane 2). This result confirms the presence of c-*fos* and/or *fos*-related proteins that participate in specific GALV DNA-protein complexes.

Effect of *fos* antibody on AP-1 complexes. The abundance of c-*fos* protein precipitated with GALV-specific DNA bound to immunobeads suggested that c-*fos* was part of the complex with the 39-kDa protein. Addition of anti-c-*fos* immunoglobulin to gel retardation reactions inhibited the formation of the GALV-specific complex (Fig. 5). The SV40-specific complex, which is shown to be distinct from the GALV complex by both reciprocal competition and chromatography, is not affected by addition of this same antibody. This argues against the SV40-specific complex containing c-*fos* and confirms that, in contrast, the GALV enhancer specifically binds c-*fos* in HeLa cells.

DISCUSSION

We have demonstrated that a protein of 39 kDa from HeLa cells is bound by the 48-bp repeated element of the GALV long terminal repeat shown previously to have enhancer activity. The binding of this protein can be eliminated by an oligonucleotide competitor corresponding to the previously footprinted region within the GALV element and which itself has enhancer function (22) but not by heterologous oligonucleotides. The 39-kDa protein is detected in HeLa cells that support GALV enhancer-activated transcription but not in Jurkat cells that do not *trans*-activate this enhancer; thus, the abundance and affinity of this protein are correlated with enhancer activity in these cells. The size of the bound protein is compatible with the known size of the product of the c-*jun* gene.

Reciprocal competition studies indicate that the GALV and SV40 AP-1 sites bind specific proteins and that the binding of these proteins is only reduced by cross-competition with a vast excess of competitor. The distinctive electrophoretic pattern of each of these oligonucleotide-protein complexes allowed us to separate the GALV factor and SV40 factor by ion exchange chromatography. The GALV complex, in addition to binding the 39-kDa protein, is also inhibited by antibodies to c-*fos*, whereas the SV40 complex is insensitive to addition of this antibody. This result suggests that c-*fos* is not part of the complex at the SV40 AP-1 site and dictates that, in the same cell line, proteins apart from c-*jun* and c-*fos* can interact with AP-1 sites.

These results have expanded the range of proteins that are capable of binding to the AP-1 site in addition to c-*fos* and c-*jun*. What determines specificity at AP-1 sites and allows discrimination between various DNA-protein complexes? It may be that we have to expand the consensus sequence such that flanking sequences confer additional specificity or that the variation known to exist within this consensus sequence allows for a limited number of proteins that are able to distinguish one site from another. Preliminary evidence suggests that the factors which bind the GALV and SV40 AP-1 sites can be different in various cell lines (data not shown). Modulation of the presence or abundance of factors which are available may determine the physiological or pathological activities of these sequences. Further purification of the factors from HeLa and MLA-144 cells should better our understanding of the combinatorial use of factors at AP-1 sites.

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