MOLECULAR AND CELLULAR BIOLOGY, Nov. 1989, p. 4713–4721
0270-7306/89/114713-09$02.00/0

Vol. 9, No. 11

Multiple Components Are Required for Sequence Recognition of the AP1 Site in the Gibbon Ape Leukemia Virus Enhancer

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Received 5 May 1989/Accepted 10 August 1989

At least two subunits contributed to the formation in vitro of a specific complex binding to the AP1 consensus sequence (TGAGTCA) in the gibbon ape leukemia virus (GALV) enhancer in MLA144 cells. This complex can be dissociated on a monomeric GALV oligonucleotide affinity column. One protein, termed the core protein, was retained on the oligonucleotide affinity column. The second protein flowed through the oligonucleotide affinity column and, when alone, did not bind to DNA; however, when present with the core protein, it bound strongly and very specifically to the GALV sequence. MLA144 cells contained only trace amounts of c-*fos* and c-*jun* by immunoblot analysis, suggesting that the proteins specifically binding to the GALV AP1 site were distinct from c-*fos* and c-*jun*. In addition to the major complex that recognized the GALV element, MLA144 cells contained a minor complex that is chromatographically different from and antigenically related to c-*fos*. The factor in the flowthrough complemented a human T-cell nuclear extract (Jurkat cell line), which, when alone, had no assayable complex that specifically bound to the GALV enhancer; this complementation gave rise to a specific complex similar to that seen in MLA144 cells. Together, these results suggest that the GALV enhancer can interact with multicomponent protein complexes in a cell-line-specific manner.

The enhancer activity of the gibbon ape leukemia virus (GALV) long terminal repeat is determined principally by a 22-base-pair (bp) segment of a thrice-repeated 48-bp sequence (27). A perfect AP1 consensus sequence, TGAG TCA, resides within this segment (2, 19). This consensus sequence is recognized by several distinct dimeric protein complexes composed of peptides encoded by the *fos* and *jun* gene families (1, 3, 10, 21, 29, 30). Several of these family members, distinct from c-*fos* and c-*jun*, have been isolated (e.g., *junB* [31] and *fra*1 [6]). In addition, several less well-characterized proteins are enriched by sequence recognition affinity methods when AP1 sites are used as the immobilized ligand (10). *fos*/*jun* binding sites have been shown to mediate both activation and repression (2, 9, 20). Considerable degeneracy exists in the range of binding sites showing specificity for the AP1 family of proteins (17). This degeneracy allows the overlap of a broad range of regulatory elements; for example, AP1 sites have been shown to overlap with AP4 (22) and with sites that bind the octamer-binding protein (35a). The diversity of *trans*-acting factors capable of interacting or competing at AP1 sites may expand the regulatory repertoire of this element.

The GALV enhancer, which exhibits variable activity in different cell lines, most efficiently augments transcription in a gibbon T-cell line, MLA144, whereas the human T-cell line Jurkat is the least efficient (15). The GALV enhancer element in MLA144 cells cannot be induced by the tumor promoter phorbol myristic acetate to higher levels of activity, whereas HeLa cells exhibited increased levels of activity of various AP1 elements, including the GALV element (16). The principal determinant of GALV enhancer activity was originally identified as the site of a specific protein-DNA interaction by DNase I footprint analysis, exonuclease protection, and gel retardation with extracts of MLA144 or HeLa cells; this interaction was not detected with Jurkat cell extracts (27). The physiological, developmental, and pathological roles of the various cellular factors that interact with the GALV enhancer AP1 site remain to be elucidated. As a first step toward understanding the regulation of GALV enhancer-mediated gene expression, the proteins that bind to the GALV-AP1 site from HeLa and MLA144 cells were compared. Whereas the HeLa cell binding complex is composed of c-*fos* and p39/c-*jun* (10, 28), we report the resolution of the binding activity at the GALV-AP1 site in MLA144 cells into two complexes. One complex contains a *fos*-related antigen (FRA) (10); the other, and by far the more abundant, complex appears unrelated to either c-*fos* or c-*jun*. This abundant complex could be dissociated into at least two components, which together recognize bases both within and flanking the GALV-AP1 consensus site. Evidence is also presented suggesting that the activity or abundance of each component of the most abundant complex can be independently modulated in different cells.

MATERIALS AND METHODS

Preparation of extract. Nuclear extracts were prepared by the procedure of Dignan et al. (8) with the following modifications: buffer C contained 20% glycerol and 0.42 M NaCl, and buffer D contained 20% glycerol and 80 mM KCl. All buffers contained 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5). Extracts typically contained 30 mg of protein per ml.

Gel retardation. Gel retardation analysis was performed as described previously (11, 34, 35). Binding-reaction mixtures contained a ^{32}P-3'-end-labeled double-stranded oligonucleotide probe.

Fractionation of nuclear extract by Bio-Rex 70 chromatography and oligonucleotide affinity chromatography. MLA144 nuclear extracts were fractionated by cation-exchange chromatography on Bio-Rex 70 (Bio-Rad Laboratories, Richmond, Calif.). The extracts were dialyzed against buffer D containing 200 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 to 20 mg of nuclear protein. The flowthrough fraction was collected, and the column was washed with 200 mM KCl in buffer D until the A_{280} was less

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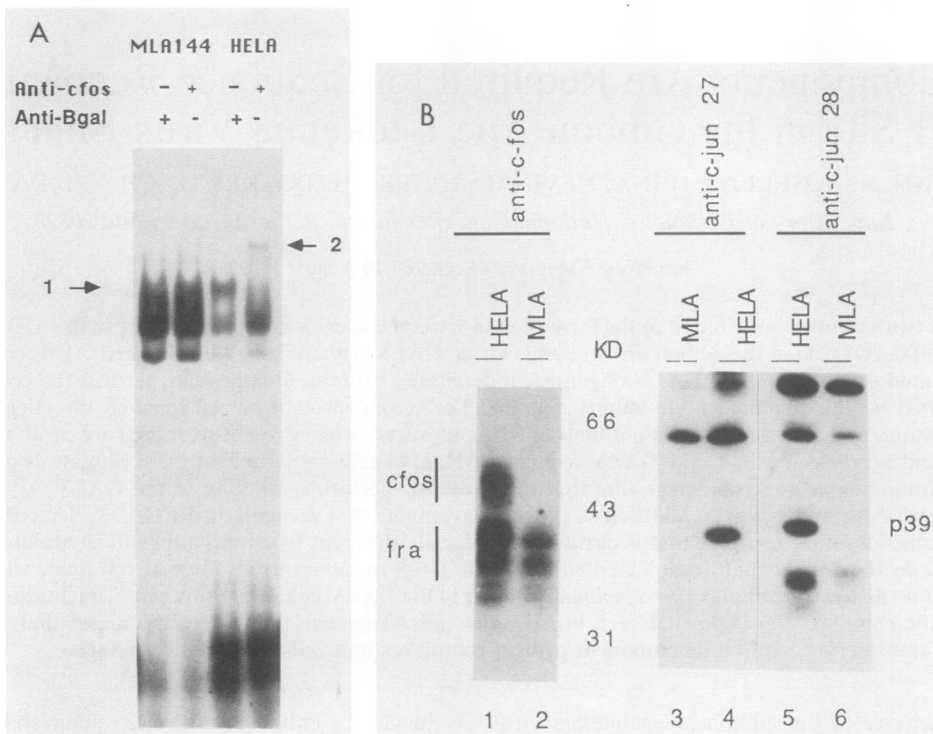

FIG. 1. c-*fos* and c-*jun* are detected in HeLa cells but not in MLA144 cells. (A) MLA144 or HeLa nuclear extract was subjected to gel retardation analysis in the presence (+) or absence (−) of anti-c-*fos* or anti-β-galactosidase immunoglobulin. The shift labeled 1 → is specifically inhibited by competition (27). The shift labeled ← 2 results from interaction of the specific complex with the antibody; note the disappearance of the specific shift with HeLa extract. (B) Nuclear extracts from HeLa and MLA144 cells were transferred to nitrocellulose after being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1 and 2 were probed with anti-c-*fos* immunoglobulin, and lanes 3 to 6 were probed with the anti-c-*jun* peptide antibodies c-*jun* 27 and c-*jun* 28, as indicated. The proteins that are identified in lanes 1 and 2 and that migrate faster than c-*fos*-specific protein are FRA (10).

than 0.05. The material that bound to the column was eluted with 0.65 M KCl in buffer D, diluted with 2 volumes of buffer D without KCl and dialyzed against 180 mM KCl in buffer D, and loaded on an oligonucleotide affinity column containing the GALV enhancer oligonucleotide, coupled as previously described by Wu et al. (38). The flowthrough was collected and stored at −70°C. The column was eluted stepwise with 0.25, 0.5, and 1.0 M KCl in buffer D. The material that eluted at 0.5 M was subsequently subjected to a second round of oligonucleotide affinity chromatography. The protein fractions obtained from this purification scheme were used in the experiments shown in Fig. 2 through 9. The material bound to the oligonucleotide column after the second pass was termed the core, and the flowthrough obtained by passing the Bio-Rex eluate over the oligonucleotide column was used to demonstrate complementation.

The flowthrough material was heat treated for 10 min at 65°C to remove 90% of the protein by centrifugation (10,000 × *g* for 5 min at 4°C) without losing significant amounts of material that would complement the core.

The proteins in the heat-treated flowthrough material were further purified by concentration on hydroxyapatite. The GALV specific activity eluted from this column at 100 mM Na_2HPO_4–NaH_2PO_4 (pH 7.4). The material from this concentration step gave rise to a specific complex with the GALV DNA. The 100 mM Na_3PO_4 eluate was subjected to affinity chromatography on a monomeric GALV oligonucleotide column as described above.

Footprinting of complexes. DNA-protein complexes were isolated by slicing the appropriate retarded band from a mobility shift gel after treatment with 1,10-phenanthroline-copper as previously described (18).

Contact point analysis. The GALV-AP1 site probe (5′-CGAGAAATAGATGAGTCAACAG-3′) was labeled with [^{32}P]ATP by using polynucleotide kinase, annealed with complementary-strand DNA, and chemically modified as previously described (4). Gel retardation was performed with this DNA, and the shifts were located by autoradiography and excised from the gel. The eluted DNA was then subjected to piperidine cleavage and analyzed by polyacrylamide gel electrophoresis on a 20% polyacrylamide gel.

Preparation of antipeptide antibodies. *jun* peptides used in the immunization procedure were prepared in house by using a Biosearch model 9600 peptide synthesizer, with standard t-Boc Merrifield resin procedures. The peptides were from the c-*jun* sequence with nomenclature used by Angel et al. (1); peptide 28 consisted of amino acids 102 to 121, and peptide 27 consisted of amino acids 227 to 247. The peptides were conjugated to bovine serum albumin by using gluteraldehyde and a published procedure (37). Two rabbits for each antigen were immunized intradermally on the back with complete Freund adjuvant in the first immunization. Serum from the fourth and subsequent immunizations was used to prepare antibody. This step was conducted by first heating the serum to 56°C for 30 min and then absorbing out pertinent antibody on the appropriate peptide Affi-Gel 10 resin (Bio-Rad) at 4°C for 18 h with gentle agitation. After being washed thoroughly, absorbed antibody was eluted from the resin with 1 M acetic acid. The eluate was neutralized to pH 7.0 with NaOH, and the resulting solution was

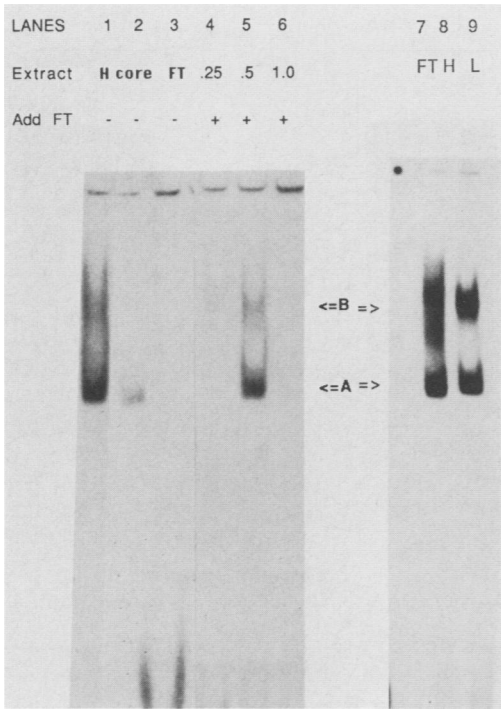

FIG. 2. Separation of the proteins that constitute the GALV enhancer complex. The probe used is 5′-CGAGAAATAGATG AGTCAACAG-3′ and is the 22-bp sequence from the GALV LTR that has enhancer function. Lanes 1, 2, and 3 are the HeLa nuclear extract, a fraction from the 0.5 M KCl elution of the oligonucleotide column, and the flowthrough of the oligonucleotide column, respectively. Lanes 4, 5, and 6 are fractions from each of the step elutions with flowthrough material added to each. Lanes 7, 8, and 9 are the flowthrough from the oligonucleotide column, the HeLa nuclear extract, and the material that was loaded onto the column, respectively. Arrows indicate shifts A and B that are present in the MLA144 nuclear extract.

concentrated and exchanged to phosphate-buffered saline (Amicon YM-10 Diaflo). c-*fos* antibody against c-*fos* residues 129 to 153 was prepared as described previously (28) and was the gift of M. Iadarola. This segment of c-*fos* overlaps extensively with the M peptide (residues 127 to 152) (7).

RESULTS

HeLa and MLA144 cell GALV-AP1 site-binding proteins are not equivalent. Previous experiments revealed the presence of factors that specifically recognize the GALV enhancer AP1 site in MLA144 gibbon T and HeLa cells (27). In extracts from each line, mobility shift analysis resolved several complexes with the GALV-AP1 probe (Fig. 1A); the most slowly migrating complex bound the GALV-AP1 site specifically, whereas the faster-migrating species were nonspecific. Several observations suggested that the major GALV-AP1 site-binding complex in MLA144 extracts might be distinct from the HeLa cell counterpart. Whereas the addition of anti-*fos* immunoglobulin to the mobility shift assay with GALV-AP1 probe resulted in virtual abolition of the major HeLa-binding complex, little or no decrease was seen with MLA144 nuclear extract (Fig. 1A). With both extracts, an additional minor shift was evident after incubation with anti-*fos* immunoglobulin. Because the MLA144 B shift was refractory to inhibition with anti-*fos* immunoglobulin, HeLa and MLA144 extracts were compared directly for anti-*fos* immunoglobulin-reactive material by using Western immunoblot analysis (Fig. 1B). HeLa nuclear extracts displayed the expected pattern of both *fos* (55 kilodaltons) and

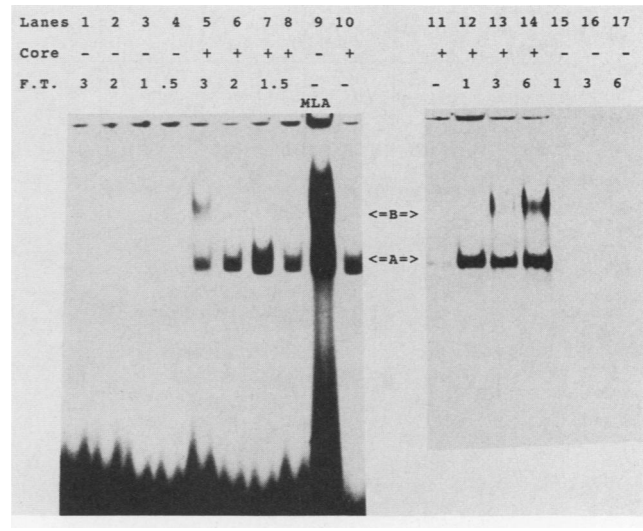

FIG. 3. Reconstitution of the specific GALV enhancer complex. Lanes 1 to 10 and 11 to 17 show two different experiments in which the probe is the GALV 22-bp enhancer sequence to which either 0.5 M core or flowthrough material, or both, are added. Lane 9 is MLA144 nuclear extract. Addition of core is indicated by +; the flowthrough was added in increasing amounts.

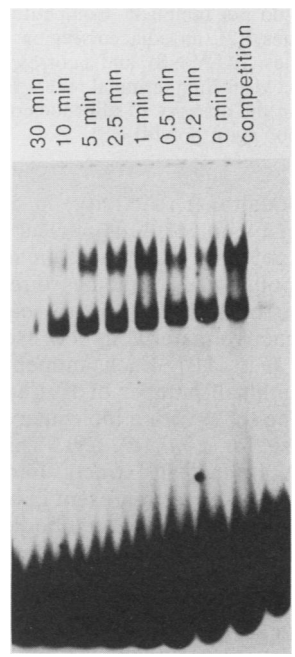

FIG. 4. The GALV-specific complex has a long half-life. Aliquots (8 μl) of a large-scale binding reaction, each containing 0.125 ng of GALV probe, 500 ng of poly(dI-dC), and 0.7 μl of second-pass core plus 2.5 μl of heat-treated flowthrough in buffer D, were withdrawn at the time indicated and mixed with 50 ng of unlabeled GALV competitor. Then, at time zero, all samples were subjected to polyacrylamide gel electrophoresis (4% polyacrylamide). In the lanes marked competition, 50 ng of unlabeled competitor was mixed with probe before addition of core and flowthrough.

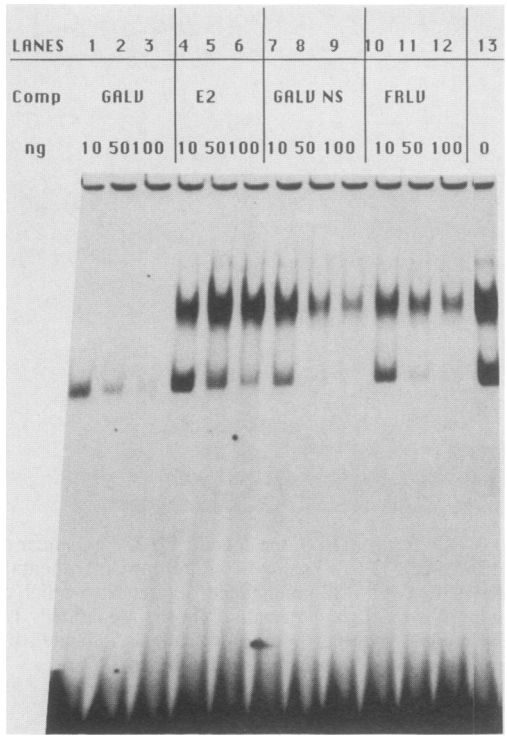

FIG. 5. The specificity of complex B is greater than that of complex A. Lanes 1 to 12 illustrate competition of the GALV enhancer oligonucleotide with various oligonucleotides when added to core (0.8 μl) plus flowthrough (3 μl). The specific shift is inhibited only by homologous competitor (lanes 1, 2, and 3), whereas heterologous competitors of approximately the same length from other regulatory regions do not inhibit it. Competitors are from bovine papillomavirus (lanes E2), an adjacent region of the GALV long terminal repeat (lanes GALV NS), and a corresponding region from the Friend leukemia virus long terminal repeat (lanes FRLV). Lane 13 shows binding in the absence of oligonucleotide competitor; all reactions contain 500 ng of poly(dI-dC).

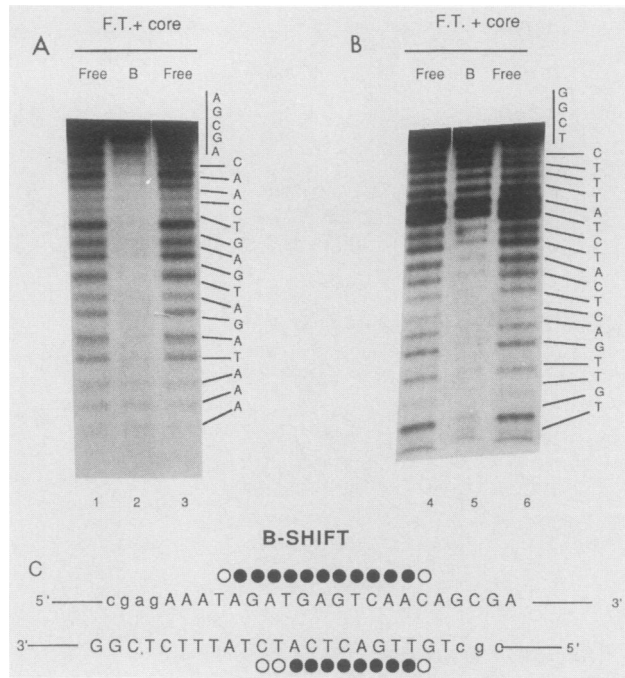

FIG. 6. Chemical footprinting of the GALV-specific binding complex. The gel retardation and chemical footprinting analyses were performed as described in Materials and Methods. (A) Lanes: 1, unbound probe; 2, B complex resulting from core and flowthrough complementation; 3, unbound probe. (B) Complementary strand. Lanes: 4, unbound probe; 5, B complex resulting from core and flowthrough complementation; 6, unbound probe. (C) Schematic summary of protection of B complex from several experiments. Symbols: ○, partial protection (50 to 60%); ●, complete protection.

fra (30 to 45 kilodaltons) reactivity; in contrast, only *fra* reactivity was seen with MLA144 nuclear extracts.

To explore a potential role for c-*jun* in MLA144 cells, affinity-purified polyclonal antibodies directed against two separate segments of the c-*jun* protein sequence were used to look for *jun*-reactive material in both HeLa and MLA144 nuclear proteins (Fig. 1B). Each antipeptide antibody reacted with only a small number of HeLa nuclear proteins, including a peptide (p39) with a molecular weight of 39,000, the expected size for c-*jun* (7, 29). This band was not detected in MLA144 nuclear extracts. In addition to p39, a small number of proteins were present in both MLA144 and HeLa cells that reacted with both antibodies. Some of these proteins may represent additional members of the *jun* family, whereas others may spuriously cross-react with both anti-*jun* antibodies. Neither anti-*jun* antibody nor two additional *jun* antibodies (a generous gift of T. J. Bos and P. K. Vogt) perturbed the binding of HeLa or MLA144 nuclear extracts with the GALV-AP1 site (data not shown).

Because MLA144 and HeLa nuclear extracts were not strictly equivalent with respect to their immunological reactions with anti-*fos* and anti-*jun* immunoglobulin and because the electrophoretic mobilities of the specific GALV-AP1 complexes with HeLa and MLA144 nuclear extracts were slightly different in numerous experiments, extracts of MLA144 cells were subjected to column chromatography to facilitate the identification and analysis of these GALV-AP1 site-binding proteins.

At least two separable components are required for formation of the GALV-AP1 site protein complex. A crude nuclear extract was prepared from MLA144 cells; in these cells, the 22-bp GALV-AP1 element exhibited strong enhancer activity (27). This extract contained a large amount of the factor(s) that interacts with the GALV-AP1 site as determined by using mobility shift analysis. This material was loaded on a Bio-Rex 70 cation-exchange column and step eluted with 0.65 M KCl in buffer D. The eluate was diluted and dialyzed against 180 mM KCl in buffer D and loaded on an affinity column coupled with monomeric, double-stranded GALV-AP1 site oligonucleotide. The flowthrough material was collected; the column was then washed and step eluted. The flowthrough and eluted fractions were assayed by gel retardation (Fig. 2). The A and B shifts, indicated by the arrows in Fig. 2, are routinely seen after incubation of the 22-bp GALV probe with MLA144 nuclear extracts. The binding activities producing the A and B shifts were abundant in the load of the oligonucleotide column (Fig. 2, lane 9). Band B contained the complex that was specifically reduced by GALV enhancer DNA as the competitor in our previous studies, whereas shift A was nonspecific (27). Singly, none of the recovered fractions produced the specific shift B. The flowthrough and the fractions from the affinity column were then analyzed to determine whether an inhibitor was present or whether a certain fraction could complement the activity of another. The specific B shift was reconstituted by the addition of the affinity column

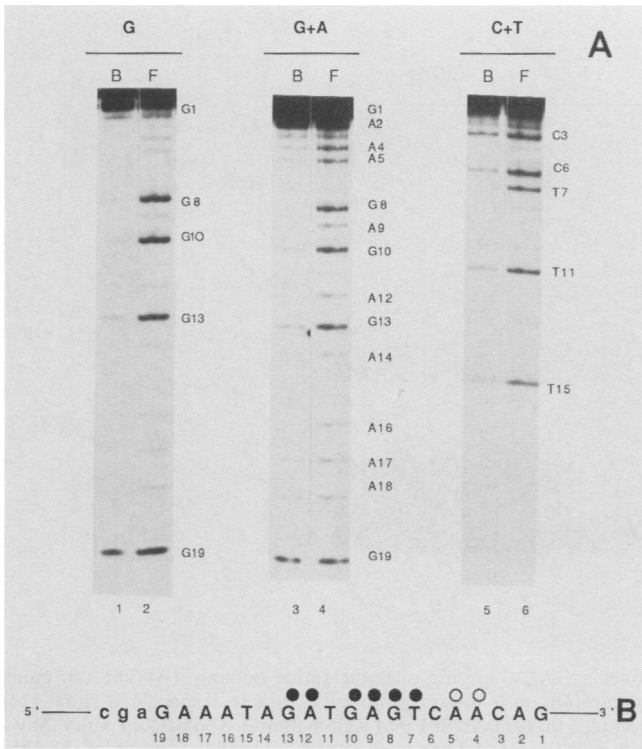

FIG. 7. Contact point analysis of the specific GALV enhancer-binding complex. (A) The gel retardation and contact point analyses were described in Materials and Methods. Methylation depurination (G only), acid depurination (G + A), and depyrimidation (C + T) are shown. Lanes: 1, 3, and 5, DNA isolated from B shift; 2, 4, and 6, free DNA. (B) Schematic summary of contact points of B complex shown above from several experiments. Symbols: ○, partial contact (50 to 60%); ●, complete contact.

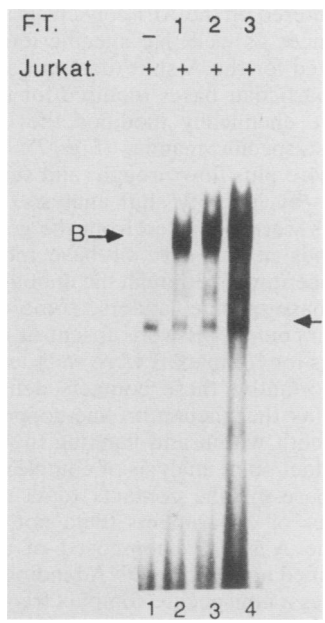

FIG. 8. Complementation of the flowthrough factor (F.T.) by addition of Jurkat extract. A constant amount of Jurkat extract (8 μg) was added to GALV probe. Increasing amounts of flowthrough material (in microliters of flowthrough added) were added. Increasing amounts of flowthrough give rise to shift B. The nonspecific shift in Jurkat extract is denoted by the bottom arrow.

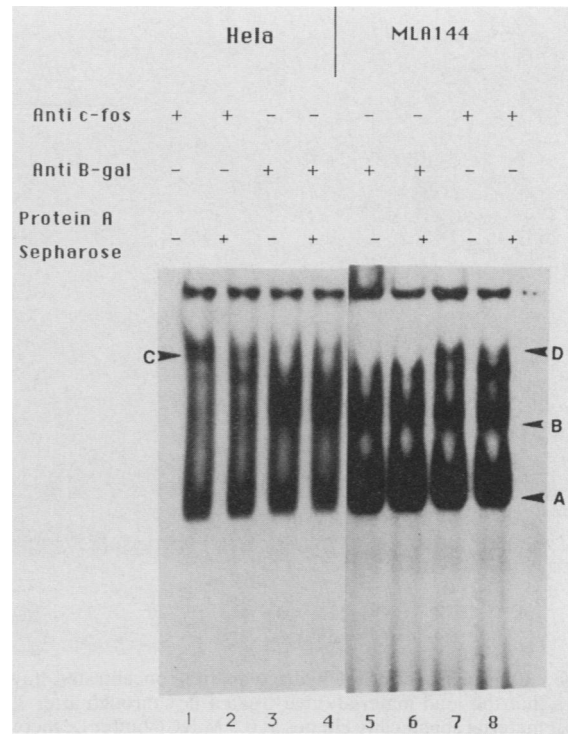

FIG. 9. The MLA144 reconstituted-specific complex is refractory to inhibition by the addition of the c-*fos* antibody. Lanes 1 to 4 contain 0.5 ng of GALV probe with 20 μg of HeLa extract. Lanes 1 and 2 have anti-c-*fos* immunoglobulin added; lanes 3 and 4 have anti-β galactosidase added. Lanes 5 to 8 have second-pass core complemented by flowthrough, with lanes 7 and 8 having anti-c-*fos* immunoglobulin added and lanes 5 and 6 having anti-β-galactosidase added. Antibodies were added to the nuclear extract before addition of probe (incubated with antibody for 1 h at 4°C). Reaction mixtures 2, 4, 7, and 8 were cleared by the addition of protein A-Sepharose and centrifugation before the addition of probe (lanes 2 and 7). The shifts are as follows: A, nonspecific complex; B, specific GALV complex; C, antibody-specific GALV complex with HeLa extract; D, antibody-specific GALV complex with MLA144 extract.

flowthrough fraction to the 0.5 M KCl–buffer D eluate, hereafter referred to as the core (Fig. 2); the flowthrough alone produced no shift, whereas the 0.5 M KCl fraction (core) yielded only the nonspecific shift. The core material itself could be repeatedly recycled on the GALV oligonucleotide column.

The intensity of the in vitro-reconstituted B shift was dependent on the quantity of flowthrough added to a constant amount of the 0.5 M KCl eluate (Fig. 3). Two experiments are shown; importantly, the flowthrough alone failed to produce any shift. Efficient reconstitution of the B shift was observed with the GALV wild-type oligonucleotide; heterologous sequences failed to support complementation in a standard assay. The complex making up the B shift was long lived in solution, with a half-life of approximately 10 min (Fig. 4).

To determine whether the reconstitution of the B shift occurred by a mechanism unrelated to specific protein-protein interaction, we performed several experiments. The addition of various ions (Mg^{2+}, Ca^{2+}, or Na^{+}), bovine serum albumin, or immunoglobulin G to the core failed to reconstitute the B shift. At no point in these studies was complementation achieved by altering the nature or abundance of the nonspecific competitor nucleic acid in the presence of

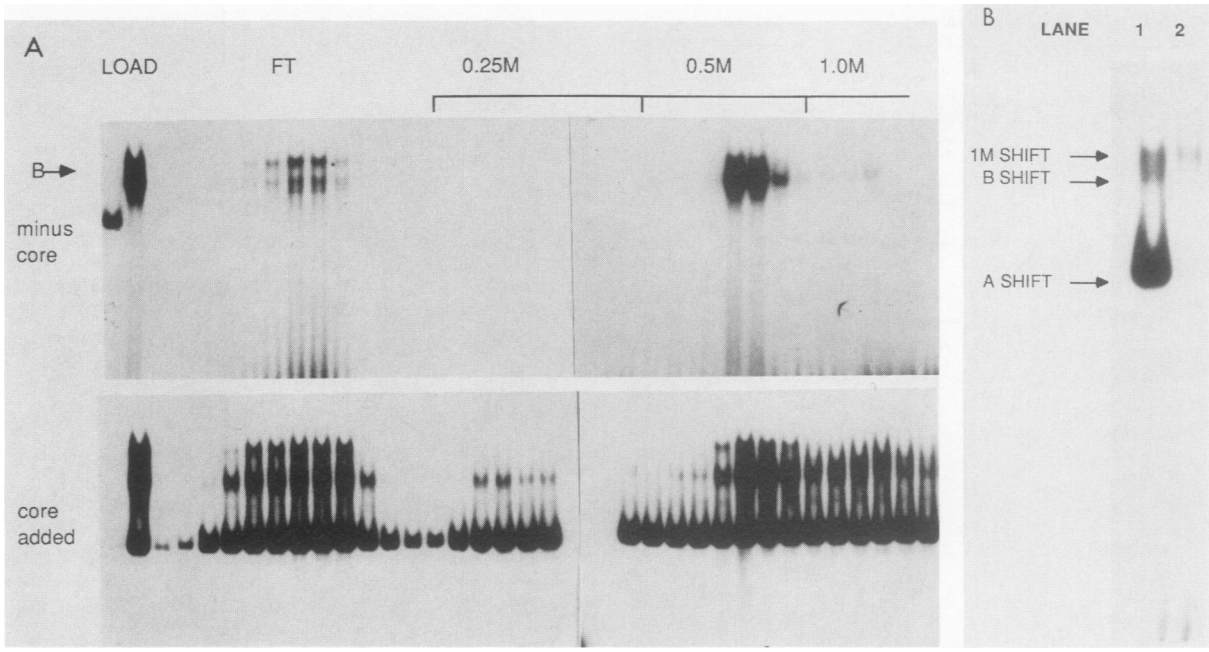

FIG. 10. Fractionation of hydroxyapatite-concentrated flowthrough over a GALV-specific oligonucleotide column. (A) The top panel shows that the load material (heat-treated flowthrough after 30-fold concentration on hydroxyapatite) gives rise to a specific B shift. This B-shift material specifically eluates at 0.5 M KCl–buffer D; there is also a complex that eluates at 1.0 M KCl–buffer D. The bottom panel shows the same elution profile as in the top panel, except that a constant amount of core material has been added. Note that addition of the core broadens and increases the peak of activity at 0.5 M KCl similar to the complementation seen with second-pass core and flowthrough prior to concentration on hydroxyapatite. Also note that an additional band appears above the specific complex (B→) with complementation of flowthrough by core; this band has the same contact point analysis as the specific complex B (data not shown), and we believe that it may represent an oligomeric form of this specific complex. (B) Lane 2 shows the complex eluted at 1.0 M KCl. Lane 1 shows the same 1.0 M KCl elution complemented by core material. Note that this complementation gives rise to the complex migrating faster than the 1.0 M KCl elution complex and comigrating with the 0.5 M KCl elution complex.

individual protein fractions (data not shown). Because the flowthrough activity was relatively heat stable, a 10-min treatment at 65°C followed by a brief centrifugation allowed the removal of most of the protein with little loss in core-complementing activity; the flowthrough activity did not survive heating to 90°C. Thus the generation of the specific B shift in vitro operationally required the interaction of two separable and distinct components.

Inclusion of a variety of competitor nucleic acids in binding assays revealed that addition of the non-DNA-binding flowthrough to the core generated the B complex, which possesses stricter sequence specificity than the nonspecific A shift. The GALV oligonucleotide competitor efficiently reduced the formation of the B shift (Fig. 5). Heterologous sequences were ineffective as competitors, except at very high concentrations.

Footprint analysis of the specific complex. Earlier experiments indicated that the binding activity producing the B shift was specific for the GALV-AP1 sequence. To confirm these observations with B-shift activity, reconstituted from core plus flowthrough, and to define the bases necessary for specific sequence recognition, we performed chemical footprinting and contact point analyses. The chemical footprinting technique used phenanthroline-copper to partially degrade DNA and DNA-protein complexes enmeshed in polyacrylamide gels following gel retardation (18). After the in situ cleavage, the degraded products were extracted from the gel, separated by electrophoresis under denaturing conditions, and visualized by autoradiography. In this manner, the footprints of proteins making up complexes A and B were each compared with the pattern of partial degradation displayed by the unbound probe. Complex B consistently yielded the footprint shown in Fig. 6. Protection of both strands was centered on the AP1 consensus site but included flanking sequences as well. No specific footprint was consistently detected for the A shift (data not shown).

To identify particular bases required for the formation of complex B, we chemically modified the GALV-AP1 site probe in a base-specific manner (Fig. 7), bound it to the reconstituted core plus flowthrough, and separated the specific complexes by mobility shift analysis. The bound and unbound DNAs were extracted from the gel, and the phosphodiester bonds at the sites of base modification were cleaved with piperidine. Although the unbound DNA yielded the expected base-specific ladders, some of the rungs of these ladders in complex B were absent or diminished. The critical contacts for complex B were well defined and tightly clustered; importantly, these contacts define virtually the same segment as the phenanthroline-copper footprint and include bases both within and flanking the AP1 consensus sequence. Contact point analysis of complex A did not yield evidence for base-specific contacts (data not shown). An additional series of experiments (data not shown) demonstrated that the A shift is composed of a complex of a previously purified nonspecific DNA binding Ku antigen and probe; ku causes a nonspecific complex frequently observed in a variety of human nuclear extracts (23, 24).

The presence or binding activity of the flowthrough factor may be regulated in different cells. Whereas MLA144 cells support GALV enhancer-driven transcription and possess a B shift reconstitutable from flowthrough and core, Jurkat human T cells show neither enhancer activity nor a B shift.

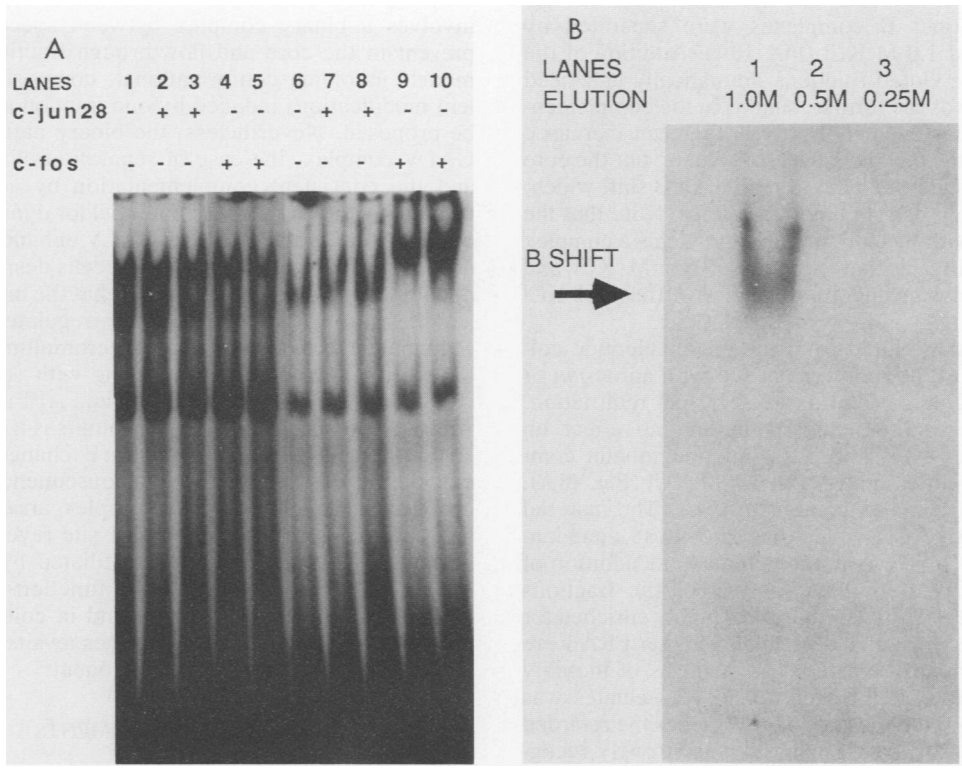

FIG. 11. (A) The complex eluted at 1.0 M KCl is antigenically related to c-*fos*. A standard gel retardation assay was performed with material from the 0.5 M KCl elution (lanes 1 to 5) and the 1.0 M KCl elution (lanes 6 to 10). Before addition of the probe and competitor poly(dI-dC), antibody was added to the corresponding reaction mixtures for 1 h at 4°C; lanes 2, 3, 7, and 8 contain c-*jun* 28, and lanes 4, 5, 9, and 10 contain c-*fos*. Addition of anti-c-*fos* immunoglobulin to the 1.0 M KCl elution material in lanes 9 and 10 abolished the specific shift and gave rise to a slower-migrating species owing to DNA-protein-antibody interaction. (B) Western blot analysis of the complexes formed on the GALV probe. The GALV probe was mixed under normal gel retardation conditions, with the 1.0 M KCl (lane 1), 0.5 M KCl (lane 2), and 0.25 M KCl (lane 3) eluates of the GALV oligonucleotide column loaded with hydroxyapatite-concentrated material. The resulting gel was transferred to nitrocellulose and probed with anti-c-*fos* immunoglobulin. Only the complex formed between the GALV probe and the 1.0 M KCl elution was positive with anti-c-*fos* immunoglobulin.

Speculating that the absence of the B shift was due to the absence of flowthrough activity, we supplemented nuclear extracts of Jurkat cells with the MLA144 flowthrough fraction and subjected them to mobility shift analysis. The results (Fig. 8) reveal strong biochemical complementation between the MLA144 flowthrough fraction and Jurkat nuclear extract to produce a B complex on the GALV probe. We conclude that Jurkat cells are deficient in the production or activation of the flowthrough factor necessary for formation of a specific shift with the GALV-AP1 site. The flowthrough fraction would not complement a nuclear extract derived from liver, suggesting that the core factor may also be modulated in a similar manner (data not shown). Experiments are in progress to determine whether the complex formed by complementation with Jurkat nuclear extract is composed of the same components as contained by the MLA144 B shift.

The major MLA144 complex binding the GALV-AP1 site is antigenically unrelated to c-*fos*/c-*jun* whereas a distinct minor complex is related to c-*fos*. The loss of GALV-AP1 site-binding activity following affinity chromatography of the Bio-Rex- fractionated MLA144 cell nuclear extract was unexpected. Stable complexes of *fos* and *jun* have been extracted from a variety of cells (10, 29) and assembled in vitro (12, 32, 33, 36). In contrast, the dissociation of the MLA144 GALV-AP1 site-binding complex into two components was promoted by mild, routine chromatographic procedures. It was not apparent, therefore, whether either constituent of the MLA144-GALV complex was related to *fos* or *jun*. The core and flowthrough fractions were subjected to immunoblot analysis to explore their relationship to *fos* and *jun*. In contrast to the crude nuclear extract, only traces of peptides with molecular sizes compatible with FRA were detected in the flowthrough, no *fos* immunoreactivity was noted with the core, and no p39 reactivity could be seen in either fraction (data not shown).

With amounts of anti-c-*fos* immunoglobulin sufficient to abolish the binding of HeLa factors to the GALV-AP1 site, no diminishing of the core plus flowthrough reconstituted complex binding was observed (Fig. 9). A small amount of a new complex (D) was produced by incubation of the flowthrough with anti-*fos* immunoglobulin, perhaps reflecting the presence of the same small quantities of FRA as seen by immunoblot analysis (Fig. 1).

After concentration of the flowthrough by hydroxyapatite chromatography, the B shift was again observed; since the principal GALV-AP1 binding complex from MLA144 cells was composed of easily dissociable components, we reasoned that this extensive concentration induced more GALV-specific B activity by mass action-driven association of subunits. The highly concentrated flowthrough was recycled onto the oligonucleotide affinity column, and only after this 30-fold concentration was the GALV-specific B activity retained more effectively on the affinity column. Two elec-

trophoretically distinct B complexes were separated by elution with 0.5 and 1.0 M KCl (Fig. 10A). Addition of the core material to the eluted fractions dramatically enhanced the binding activity of the former, similar to the complementation previously observed; not only is there an increased binding activity of the 0.5 and 1.0 M KCl eluate, but the core complemented the 0.25 M eluate to produce a B shift which, prior to this addition, had no binding activity. Note that the complementation with the core preparation yields a complex of the same mobility as that eluted as 500 mM KCl and distinct from the slower-migrating species in the 1 M KCl eluate (Fig. 10B).

The complexes that eluted from the oligonucleotide column at 0.5 M and 1.0 M KCl were mixed with anti-c-*jun* or anti-c-*fos* immunoglobulin and assayed by gel retardation. Addition of anti-c-*jun* immunoglobulin had no effect on either complex; however, anti-c-*fos* immunoglobulin completely shifted the eluted material at 1.0 M KCl (Fig. 11A), indicating that this complex contains a FRA. The material eluting at 500 mM KCl, which has the most abundant GALV-binding activity, was not affected by the addition of the *fos* antibody. Western blot analysis of the fractions determined that the 1.0 M KCl eluate was highly enriched for the FRA (data not shown). To determine whether FRA were in the retarded complex, Western blot analysis of mobility shift gels using the 0.25, 0.5, and 1.0 M KCl eluates was performed by using the c-*fos* antibody. Whereas the retarded complex from the 1.0 M KCl material was strongly recognized by this antibody (Fig. 11B), the complex from the 0.5 M KCl eluate was completely unreactive.

DISCUSSION

The activation of the GALV enhancer is mediated primarily through a 22-bp segment centered on a perfect AP1 consensus sequence. This segment has been shown to bind the c-*fos*/c-*jun* AP1 complex in human H9 and HeLa cells (10, 28). When antipeptide antibodies and immunoblot analysis were used, p55 c-*fos* and p39 c-*jun* were not detected in MLA144 nuclear extracts; in contrast, on the same blots, the presence of c-*fos* and c-*jun* in HeLa cells was readily confirmed. Because the abundance of *fos*/*jun* was reduced in MLA144 cells and GALV enhancer activity was very high, we inferred that a distinct or altered AP1 consensus recognition complex was present. Indeed, the GALV element has been shown previously to be unresponsive to PMA induction in MLA144 cells, in marked contrast to the simian virus 40 AP1 element (16). Chromatography of MLA144 nuclear extracts on a GALV enhancer oligonucleotide affinity column resolved the major GALV activity into two components; the flowthrough fraction, although alone possessing no significant binding activity, complemented a component present in the material retained on the column to reconstitute the B shift. Thus, it appears that the major MLA144 complex that specifically binds to the GALV element in MLA144 requires the use of at least two dissociable protein components. Another distinct complex was observed by concentrating the material before passing it over the oligonucleotide column; this minor complex is related antigenically to c-*fos*. Although c-*fos* and FRA have been shown to bind AP1 sequences (10), this study demonstrates the presence of separable complexes formed in vivo that are distinct from the c-*fos*/c-*jun* complex. This result is consistent with the formation of distinct heterodimeric complexes by using in vitro-translated members of the *fos* and *jun* families (25).

The mechanism of complementation between the core and flowthrough fractions is not known. The simplest model involves a binary complex between specific components present in the core and flowthrough fractions. Alternative models involving conformational, compositional, or covalent modifications induced by one fraction or the other may be proposed. Nevertheless, the binary nature of the major GALV complex, the ease of separation of its constituents, and the consistent complementation by addition of flowthrough to core suggest the potential for dynamic modulation of factors interacting with the GALV enhancer. The absence of the flowthrough factor in Jurkat cells despite the presence of complementable core indicates that the interaction of core and flowthrough is a biologically regulated phenomenon. Mammalian heterodimeric or heteromultimeric complexes have been identified as interacting with several elements, including CAAT boxes (5, 13, 26) and AP1 sites (14, 25, 30). Although combinatorial use of subunits is a compelling idea, so far the demonstration of subunit exchange in vivo and the elucidation of the physiologic consequences of such exchange remain elusive. The complex array of multimeric factors binding to the GALV-AP1 site reveals the potential for multiple regulatory modes mediated by this sequence. Determination of the biochemical functions of the core and flowthrough factors, separately and in combination, in the regulation of viral and cellular genes awaits further molecular characterization of each component.

ACKNOWLEDGMENTS

We thank M. Avigan and V. J. Bubb for critical reading of the manuscript, R. Franza for stimulating discussions, and M. Iadarola for preparation of the c-*fos* antibody.

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