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Identification of ETS Domain Proteins in Murine T Lymphocytes That Interact with the Moloney Murine Leukemia Virus Enhancer

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The enhancer of Moloney murine leukemia virus (Mo-MuLV) contains an array of transcriptional control elements that direct viral gene expression in diverse cell types. The murine transcription factor Ets-1 was shown to bind to the LVb and LVc elements of the enhancer by DNase I protection and methylation interference assays. Enhancers containing disrupted Ets-1 binding sites were tested in transient expression assays in the murine T-cell line EL4.E1; alterations in the LVb element affected constitutive enhancer activity, while mutation of either the LVb or LVc element disrupted phorbol ester-induced enhancer activity. Members of the *ets* gene family of proteins display similar DNA-binding properties; therefore, we speculated that *ets* proteins other than Ets-1 also might bind these elements. Crude nuclear extracts of EL4.E1 cells were assayed to identify the protein(s) that potentially functions at the LVb element. The predominant binding activity was not Ets-1 but rather two independent DNA-protein complexes that comigrated in mobility shift assays. UV cross-linking and denaturing gel electrophoresis sized the two DNA-binding species, which we denoted p55 and p100. Immunoprecipitation combined with UV cross-linking identified p55 as the alpha subunit of GA-binding protein. The DNA-binding properties of p100 and several *ets* proteins were compared. Similarities suggested that p100 is also an ETS domain protein, possibly Elf-1. This strategy could be used to identify other ETS domain proteins in crude nuclear extracts. These findings suggest multiple ETS domain proteins could regulate gene expression of Mo-MuLV.

Moloney murine leukemia virus (Mo-MuLV) provides a useful model for the study of eukaryotic transcriptional machinery. The transcriptional control elements in the long terminal repeat (LTR) include a TATA box and an extensive array of positive-acting elements arranged as a proximal promoter and a more distal enhancer (13, 25). Many cellular proteins that bind these activator elements have been identified (7, 14, 17, 38, 50). Transcription factors that regulate the Mo-MuLV enhancer in T cells are of particular interest since Mo-MuLV causes T-cell lymphomas and leukemias in mice. Regulatory sequences within the enhancer have been identified as determinants for both thymotropism and T-cell transformation (5, 8, 11, 28, 39, 40). However, the T-cell specificity of virus-induced disease does not preclude the function of the Mo-MuLV LTR in many other cell types; LTR-driven transcription as well as viral replication have been observed in numerous tissue types and mammalian cell lines in culture (56). These data suggest that Mo-MuLV can use different combinations of transcription factors, depending on the cell type. Insights bearing on this issue come from the discovery that members of the *ets* family of eukaryotic transcription factors bind key regulatory elements in the Moloney LTR.

The *ets* gene family of proteins includes transcription factors that display conservation within an 85-residue DNA-binding domain, termed the ETS domain (22, 29, 51). More than a dozen *ets* family members, including tissue-restricted members of the family as well as more ubiquitous representatives, have been identified in vertebrate species. The majority of ETS domain proteins bind a GGA motif that is centrally located within a 9-bp region of close DNA contact. Sequence preferences that extend beyond this central GGA core have been determined for five *ets* proteins by biochemical selection experiments (4, 10, 34, 46, 57). The preferences in the central 9 bp are surprisingly similar, suggesting that *ets* proteins could display significant overlap in recognition of binding sites. Nevertheless, GGA motifs that distinguish between the binding of Ets-1 and Elf-1 (48) as well as between Ets-1 and PU.1 (31) have been found. The binding specificity of *ets* proteins also can be influenced by association with additional proteins. For example, the *ets* protein Elk-1 binds DNA cooperatively with SRF on the c-*fos* promoter (19, 21) while the *ets* protein PU.1 interacts with NF-EM5 on the immunoglobulin κ-chain 3′ enhancer (35). How functional specificity is achieved within the *ets* family is an issue for continuing investigations.

In this report, we describe two binding sites for ETS domain proteins within the 75-bp repeat of the Mo-MuLV enhancer. These sites correspond to the LVb and LVc elements that were originally reported by Speck and Baltimore as binding sites for uncharacterized leukemia virus factors b and c (38). A third activator element, termed core, lies between LVb and LVc. The functional importance of these three elements in the replication of Mo-MuLV is supported by several lines of evidence. First, the sequences of LVb and core are highly conserved among the type C retroviruses (12). Second, LVb and core function as transcriptional regulatory elements in both infectious virus assays and transient expression assays (39, 40). Furthermore, LVc, LVb, and core are required for the inducibility of the Mo-MuLV enhancer by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (40). Finally, viruses bearing mutations in the LVb and core elements no longer transform predominantly thymocytes in infected mice; thus, these elements are described as disease determinants for Mo-MuLV (39).

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Our study is based on the proposal that the dominant enhancer-binding proteins in crude nuclear extracts would be candidate regulators of the Mo-MuLV enhancer. Biochemical and immunological analyses demonstrate that *ets* proteins are responsible for the predominant LVb-binding activity in a murine T-cell line EL4.E1; GA-binding protein (GABP) and a possibly new *ets* protein were identified by our investigation. In contrast to cellular genes, the enhancer in Mo-MuLV may have evolved to be promiscuous, exploiting the similarities among ETS domain proteins to extend the diversity of potential host cells. This possibility highlights the Mo-MuLV enhancer as a unique system with which to investigate the use of the *ets* family.

MATERIALS AND METHODS

Cell culture. EL4.E1 (kindly provided by Ellen Rothenberg, California Institute of Technology) is a subclone of the EL4 murine T-cell line that was selected for high-level interleukin 2 production in response to combined treatment with TPA and A23187, a calcium ionophore (32). Cells were maintained in suspension culture in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 250 μg of streptomycin per ml, and 100 U of penicillin per ml at 37°C under 8% CO_2. TPA was prepared as a 10-μg/ml stock solution in dimethyl sulfoxide and stored at −20°C.

Transfections and chloramphenicol acetyltransferase (CAT) assays. Transfection of EL4.E1 cells was performed by the DEAE-dextran method. Cells were collected by centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered DMEM at a density of 10^7 cells per ml. Cells were incubated with 250 μg of DEAE-dextran (Pharmacia), 8 μg of supercoiled plasmid DNA, and 1 mM chloroquine in a final volume of 1 ml in uncapped tubes for 30 min at 37°C under 8% CO_2. Washed cells were resuspended in 20 ml DMEM plus 10% heat-inactivated fetal calf serum at a final concentration of 5×10^5 cells per ml, incubated for 24 h, and then split into duplicate dishes. One dish was treated with 10 ng of TPA per ml for 20 h. The duplicate dish was left untreated. At 44 h posttransfection, cells were pelleted, washed in PBS, resuspended in 100 μl of 250 mM Tris-Cl (pH 7.5), and stored at −80°C. Duplicate transfections were performed for each test plasmid in each of four experiments.

CAT activity was assayed as described by Seed and Sheen (37). Briefly, each 100-μl reaction mixture included 50 μg of cellular protein (Bradford assay; Bio-Rad), 0.2 μCi of D-*threo*-[dichloroacetyl-1,2-^{14}C]chloramphenicol (60 mCi/mmol; New England Nuclear), 250 μM *n*-butyryl coenzyme A (Sigma), 250 mM Tris-Cl (pH 7.9), and 1% glycerol. Reaction mixtures were incubated at 37°C for 1 h and subsequently extracted three times with a 2:1 mixture of 2,4,10,14-tetramethylpentadecane and xylenes to extract acylated chloramphenicol away from unreacted substrate. The amount of [^{14}C]chloramphenicol in the organic and aqueous phases was determined by measuring the radioactivity of an aliquot of each phase in scintillant.

DNA probes. DNA-binding-site probes were radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as previously described (33). Enhancer probes for DNase I protection assays were derived from plasmids that contain a wild-type or mutant *Pvu*II (7938)-*Pvu*II (8013) fragment of the Mo-MuLV enhancer subcloned into the *Sma*I site of pUC13 (numeration from the 5′ end of the viral RNA genome) (39, 56). The 130-bp *Eco*RI-*Hin*dIII fragments were radiolabeled at the *Eco*RI site.

The rfSC1 and rfLVb restriction fragment probes were derived from two high-affinity Ets-1-binding sites (SC1 and SC13) that were selected from a random pool of oligomeric DNA and then cloned into pKS vector (Stratagene) (34). Probes for mobility shift assays were 29-bp *Xho*I-*Sal*I fragments labeled at the *Xho*I site. Probes for ethylation interference and DNase I protection were 47-bp *Xba*I-*Xho*I fragments labeled at the *Xba*I (GGA strand) or *Xho*I (TCC strand) site. The sequences of synthetic oligomeric probes used in mobility shift assays are as follows: SC1, 5′-CGGCCAAGCC GGAAGTGAGTGCC-3′; LVb, 5′-CCAAACAG GATATCTGTGGTAAGCA-3′; and nonspecific, 5′-GCCT GATTGCCCAATTGT-3′. In each case, only one strand of the doubled-stranded probe is shown. Single-stranded extensions that contained restriction enzyme cohesive ends were present at the 5′ end of each oligonucleotide (SC1, TCGA; LVb, GATC; nonspecific, AATT). The SC1 oligomeric probe sequence represents the SC1 clone as described above (34). The LVb oligomeric probe sequence represents positions 8024 to 8048 in the Mo-MuLV genome which includes the LVb and core elements. In the LVb$^-$ probe, the two G residues of the GGA motif were replaced by T residues.

UV cross-linking probes. The DNA substrates used in UV cross-linking reactions were modified versions of the SC1 and LVb oligomeric probes. Short oligonucleotides (for SC1, 5′-GGCACT-3′; for LVb, 5′-TGCTTACC-3′) primed the synthesis of the bottom strand (TTC containing) from the SC1 or LVb template. The two probes were made in an identical manner except for the labeling method. For the LVb probe, primer and template oligomers were radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP prior to annealing. In the case of SC1, the only dCTP in the synthesis reaction was provided by 0.5 mCi of [α-^{32}P]dCTP (3,000 Ci/mmol). SC1 and LVb primers (100 ng) were mixed with their respective GGA strand templates (100 ng), boiled for 3 min, cooled, and incubated on ice in a total volume of 20 μl of synthesis buffer (70 mM Tris [pH 7.6], 10 mM $MgCl_2$, 50 mM NaCl, 5 mM dithiothreitol, 1 mM spermine). DNA was then incubated for 3 h at 25°C with 5 U of *Escherichia coli* DNA polymerase I Klenow fragment and 125 μM dGTP, dATP, bromo-dUTP (BrdUTP), and dCTP (LVb only) in a final volume of 30 μl. Full-length, double-stranded products were resolved on a 10% nondenaturing polyacrylamide gel and then gel purified by electroblotting onto NA45 paper (Schleicher & Schuell), elution with 1 M NaCl, and ethanol precipitation.

DNA binding assays. DNase I protection was performed as previously described (14). Binding reaction mixtures included 5 to 10 fmol of radiolabeled restriction fragment probe and 100 ng of poly(dI-dC) in a final volume of 50 μl of TM buffer (25 mM Tris-Cl [pH 7.9], 6 mM $MgCl_2$, 0.25 mM EDTA, 10% glycerol) adjusted to 65 mM KCl. DNA binding reaction mixtures for mobility shift assays included 6 to 12 fmol of radiolabeled oligomer probe or 25 fmol of restriction fragment probe in a final volume of 25 μl of TM buffer. Poly(dI-dC) was included as a nonspecific competitor at a ratio of 0.5 μg/μg of protein in crude bacterial or EL4.E1 extracts. Binding reaction mixtures were incubated on ice for 20 min, and then complexes were resolved by electrophoresis in 5% native polyacrylamide gels with 45 mM Tris-borate buffer (pH 8.3) as previously described (34). Ethylation interference analysis on the rfSC1 probe was performed as previously described (33).

Binding reaction mixtures for UV cross-linking contained 5 μg of poly(dA-dT), poly(dI-dC) (1 μg/μg of protein), and [α-^{32}P]CTP-labeled SC1 probe or [γ-^{32}P]ATP-labeled LVb probe in a total volume of 25 μl of TM buffer. Protein-DNA complexes were resolved by mobility shift assay. A transillumi-

nator emitting 300-nm UV light (Fotodyne 3-3000) was used to irradiate the bandshift gel for 15 min from a distance of 1 cm. Following cross-linking, the binding complexes were viewed by autoradiography. In experiments that did not include nuclease treatment, the cross-linked products were recovered from the gel slices by electroelution at 200 V into a 1-ml volume of buffer (50 mM Tris-borate [pH 8.3], 1 mM EDTA) in a dialysis bag. Eluted complexes were precipitated with 5 volumes of ice-cold acetone, resuspended in sample buffer, and resolved by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels (24). For nuclease treatment, gel slices were incubated for 1 h at 37°C in 40 μl of digestion buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA, 12.5 mM $CaCl_2$) containing 5 μg of DNase I and 1 U of micrococcal nuclease. Gel slices were then placed into the well of the resolving gel for electroelution.

For immunoprecipitation of cross-linked products, the acetone precipitate was resuspended in 250 μl of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), 3 μl of protein A–Sepharose CL-4B (Pharmacia) suspension (50% [vol/vol] with RIPA buffer), and specific antibodies and incubated at 4°C overnight. Protein A-Sepharose was pelleted, and the immunosupernatant was removed. Unreactive proteins were precipitated with 5 volumes of ice-cold acetone, pelleted at 12,000 × *g* for 20 min, and then dissolved in sample buffer. The immunoprecipitate was washed once with RIPA buffer for 30 min and then released from the Protein A-Sepharose by boiling in sample buffer. Supernatant and precipitate fractions were resolved on SDS–10% polyacrylamide gels and visualized by autoradiography at −80°C with the aid of an intensifying screen.

Protein expression and purification. The 50-kDa Ets-1 protein, encoded by the murine cDNA, was synthesized in *E. coli* with the pET3 vector system (41) and purified as previously described (34). Purity of the protein, as estimated by Coomassie blue staining, was greater than 90%. The concentration of Ets-1 was estimated by absorbance at an optical density of 278 nm, using an extinction coefficient of 71,000 M^{-1} cm^{-1}. The cDNA for the murine *fli-1* gene (provided by Richard Maki, La Jolla Cancer Research Foundation) (1, 23) was subcloned into the pET3 vector system for expression in *E. coli*. A PCR-generated adapter facilitated the placement of an *Nde*I site at the start codon of the *fli-1* open reading frame for cloning into the pET vector. Bacterial cells containing Fli-1 were lysed by sonication as described for Ets-1 in a nondenaturing lysis buffer (1 M NaCl, 50 mM Tris-Cl [pH 7.9], 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The extract, cleared by centrifugation at 10,000 × *g*, was dialyzed against TM buffer adjusted to 100 mM KCl. The protein concentration of bacterial extracts was determined by the Bradford assay (Bio-Rad), with bovine serum albumin as a standard. GABPα and GABPβ1 proteins that were synthesized in *E. coli* and purified (44) were generously provided by Thomas Brown (Pfizer Incorporated).

EL4.E1 nuclear extracts. Nuclear extracts were made from logarithmically growing cultures of EL4.E1 cells as described previously (9), but with the following modifications. Buffers A and B included the following supplements: 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2.5 mM benzamidine, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 10 mM β-glycerophosphate, 1 mM NaF, and 1 mM Na_3VO_4. Following extraction of nuclei in buffer B, the extract was incubated on ice with moderate stirring by a micro-stir bar for 30 min prior to clearing by centrifugation. The nuclear protein supernatant was dialyzed against 50 volumes of buffer D (20 mM HEPES [pH 7.9]–20% glycerol–50 mM KCl–0.2 mM EDTA with 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol).

For fractionation, approximately 50 mg of crude EL4.E1 nuclear extract was loaded onto a 10-ml DEAE-cellulose column (Pierce) in buffer D. Bound protein was eluted with a 50-ml linear gradient (50 to 750 mM KCl) and collected in 2.5-ml fractions. Fractions were assayed for binding activity by mobility shift assay. Active fractions (100 to 500 mM KCl) were pooled and dialyzed in buffer D. The dialyzed pool was then loaded onto a 7-ml heparin–Sepharose CL-6B column (Pharmacia) in buffer D. Bound protein was eluted with a 70-ml linear gradient (50 to 700 mM KCl) and collected in 1.4-ml fractions. Fraction 11 (225 mM KCl) and fraction 19 (425 mM KCl) were concentrated 5- to 10-fold with a Centricon 10 microconcentrator (Amicon) for use in UV cross-linking, ethylation interference, and DNase I protection assays. All operations were performed at 4°C. Extracts and fractions were stored at −80°C. Protein concentration of nuclear extracts was determined by the Bradford assay (Bio-Rad).

Antibody preparation. Polyclonal antibodies specific to Ets-1 were generated in New Zealand White male rabbits (Ellis & Pritchard) against a synthetic peptide antigen that represented the 13 carboxyl-terminal amino acids of Ets-1 and an amino-terminal cysteine for thiol linkage to carrier protein or peptide affinity resin, CELHAMLDVKPDAD. The peptide was coupled to maleimide-activated keyhole limpet hemocyanin protein as specified by the manufacturer (Pierce). For immunizations, 250 μg of the keyhole limpet hemocyanin-peptide conjugate was emulsified in complete Freund's adjuvant and injected intradermally. Boosts of conjugate in incomplete Freund's adjuvant were administered every 4 to 6 weeks. The Ets-1-specific antibody was affinity purified on SulfoLink gel (Pierce) coupled with Ets-1 peptide according to the manufacturer's guidelines. The antiserum shows specificity for Ets-1, failing to react with either the highly related *ets* protein Ets-2 (data not shown) or GABP (see Fig. 5). GABPα and -β polyclonal antisera (a kind gift of T. Brown) were produced in rabbits by immunization with mouse GABP subunits purified after expression in *E. coli*.

Data reproduction. Primary detection of radioactivity was by autoradiography. The presentation of film densities in Fig. 1 and 3 to 8 was computer assisted, using Adobe Photoshop (version 2.5). Autoradiographs were scanned (Bio-Rad model GS-670) to convert autoradiographic images to TIFF files. In most cases, the images were scaled for final reproduction.

RESULTS

LVb and LVc elements of the Mo-MuLV enhancer bind Ets-1. The binding of Ets-1 to the Mo-MuLV enhancer was tested in DNase I protection assays. The protected regions spanned two elements reiterated within each 75-bp repeat of the Moloney LTR, previously termed LVb and LVc. Figure 1 shows the protection within one 75-bp repeat. Methylation interference analyses demonstrated that Ets-1 contacted the two G positions within both the LVb and LVc elements (Fig. 1B). Furthermore, the pairs of guanines were absolutely required for Ets-1 binding, as demonstrated by the loss of protection on mutant enhancers with transversions at these positions (Fig. 1A). (In each case, the degree of protection from DNase I at the second site, which had not been altered, was also compromised, suggesting that Ets-1 interactions at LVb and LVc elements were not independent events in this assay.) Experiments in this study address the relevance of Ets-1 binding at the LVb and LVc elements to the function of the Mo-MuLV enhancer.

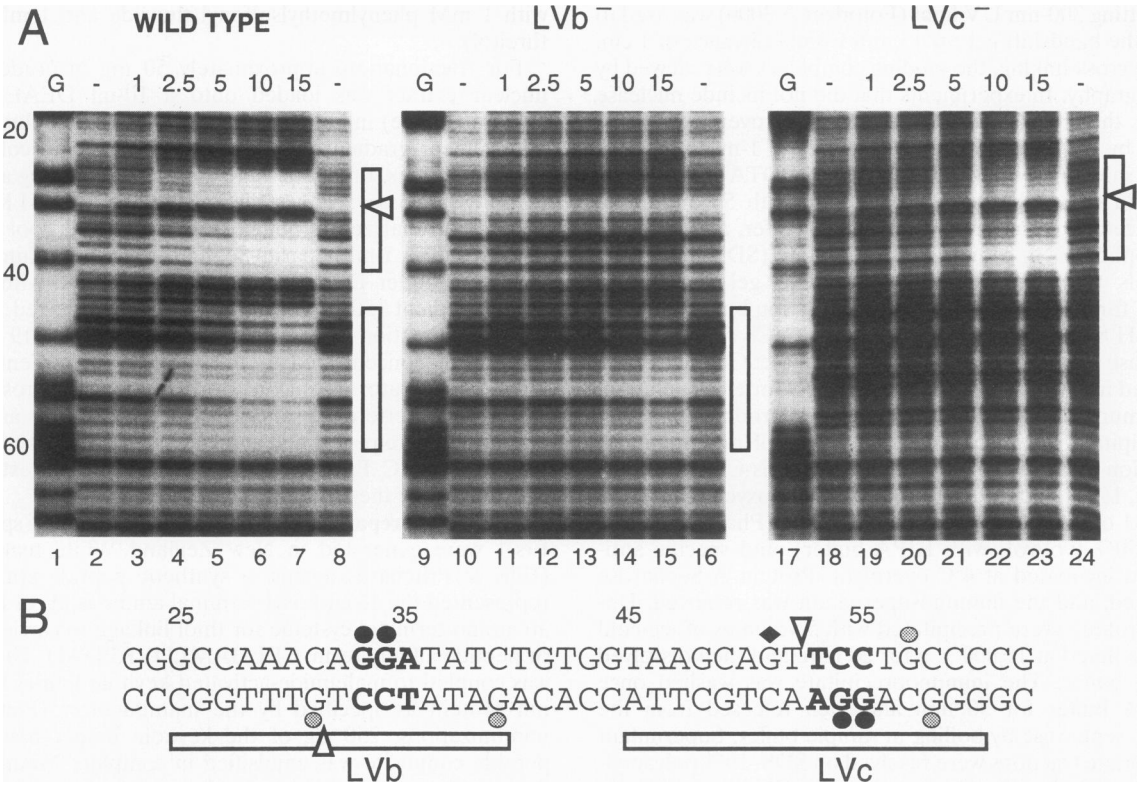

FIG. 1. Ets-1 binding to the LVb and LVc elements of the Mo-MuLV enhancer. (A) DNase I cleavage pattern on the minus strand of the LTR in the absence (−) and presence of murine Ets-1. Numbers above lanes indicate units of purified Ets-1 (1 U = 500 ng). Products of chemical sequencing of guanines are displayed as markers (G lanes). Coordinates refer to positions 1 through 75 of the promoter-distal 75-bp repeat of the Mo-MuLV LTR. Analysis was performed on the wild-type enhancer (lanes 1 to 8) and mutant repeats in which the GGA motif at position 33 (lanes 9 to 16) or at position 55 (lanes 17 to 24) was altered to TTA. (B) Summary of methylation interference analyses and DNase I protection assays. Methylation interference analysis of LVb was performed on an enhancer with mutations in LVc (see also reference 34). LVc analysis was performed on the enhancer with LVb mutations. Circles, guanines at which methylation interferes with binding (gray shading indicates less severe interference than black); diamond, a guanine at which methylation enhanced the binding; open bars, zones of protection from DNase I cleavage; arrowhead, DNase I-hypersensitive sites.

Ets-1-binding sites correspond with activator elements in the Mo-MuLV enhancer. The correlation between Ets-1 binding and enhancer function was investigated by transient expression assays in EL4.E1 cells, a murine T-cell line which is responsive to TPA (32). The reporter plasmids (kindly provided by N. Speck) contained the entire Mo-MuLV LTR with either wild-type or mutant enhancer repeats fused upstream of the bacterial CAT gene (40). Enhancer function was assayed in both untreated and TPA-treated cells. TPA treatment of EL4.E1 cells induced the activity of the wild-type enhancer fivefold above constitutive levels (Fig. 2B). The LVb element regulated constitutive as well as TPA-inducible transcription activation (Fig. 2A), while LVc did not function in activation until intracellular signaling pathways were stimulated (Fig. 2B). These data confirmed previous studies of the Mo-MuLV enhancer in the human Jurkat T-cell line (40). The results also were consistent with the function of these elements during virus infection (39). Most important for the current report, the identical mutations that disrupted Ets-1 DNA binding (Fig. 1) reduced the enhancer activity in EL4.E1 cells, providing a correlation between in vitro binding studies and in vivo function.

An LVb-binding activity in EL4.E1 extracts has properties of an ETS domain protein. The LVb and LVc elements include the purine-rich GGA motif conserved in almost all known *ets* protein-binding sites (51). The flanking sequences of the central GGA trinucleotide also fit within the consensus binding sites for several ETS domain proteins (Table 1). These data suggested that *ets* proteins other than Ets-1 also might bind the Mo-MuLV enhancer and function at these elements in vivo. We speculated that the most likely regulatory protein(s) would be responsible for the dominant binding activity in crude nuclear extracts. The relative binding activity will depend on both the concentration of active proteins and the affinity of the particular DNA-protein interaction. Because of the importance of LVb in all assays of Mo-MuLV enhancer function, this element became the focus of further study.

Nuclear extracts were prepared from EL4.E1 cells and tested for DNA-binding activity in mobility shift assays with an oligomeric probe spanning the LVb and core elements of the Mo-MuLV enhancer (Fig. 3, lanes 1 and 2). Three shifted bands were detected; however, only the upper band was disrupted by mutation of the LVb GGA motif (lane 2). In further analysis, the two faster bands could not be shown to be specific for Mo-MuLV sequences. Because the identical mutations disrupted the extract binding activity and the function of the LVb element (Fig. 2), we tentatively concluded that the nuclear protein(s) in the slowest band was a candidate regulator of the Mo-MuLV enhancer. Furthermore, the GG requirement of the binding activity was consistent with the nuclear protein(s) being an *ets* family member(s).

The binding activity was analyzed further for *ets*-type prop-

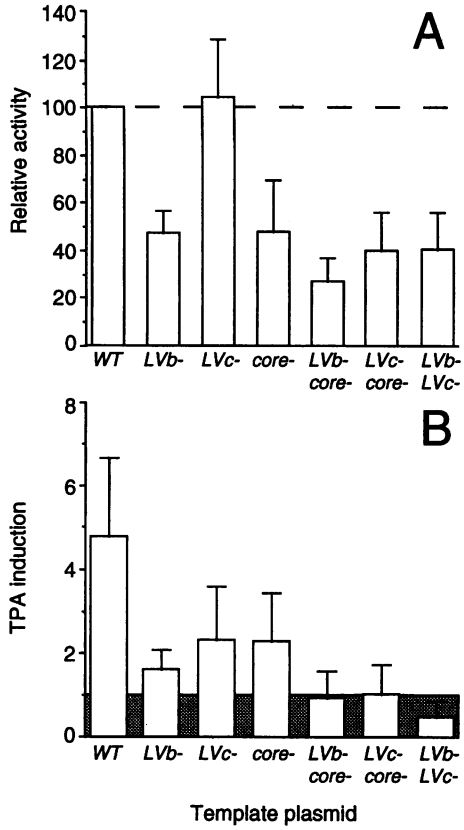

FIG. 2. Genetic analysis of Mo-MuLV enhancer function in EL4.E1 cells. Template plasmids, provided by Speck and colleagues (40), are named according to the mutated element(s) within each copy of the 75-bp repeat. Transcriptional activity of each template was determined by CAT assay (37). The percent conversion of [^{14}C]chloramphenicol to acylated forms was calculated as [$cpm_{(organic\ phase)}$/$cpm_{(organic + aqueous\ phases)}$] × 100. Percent conversion for the wild-type (WT) template ranged from 14 to 30% in four experiments. The means ± standard deviations of eight values from four experiments are indicated. (A) The relative activity for each mutant template was calculated as (% conversion for mutant/% conversion for wild type) × 100. The dashed line marks the activity of the wild-type template, defined as 100% for each experiment. (B) TPA induction was calculated as the ratio of the percent [^{14}C]chloramphenicol conversion for each template following TPA treatment relative to untreated cells. Shading marks values less than one, indicating no TPA induction.

TABLE 1. Comparison of the LVb element and SC1 site with the consensus binding sites for five ETS domain proteins

Protein or element	Consensus sequence[a]
ETS domain proteins	
Ets-1	GCCGGAAGT
	A a Tac
GABPα	GCCGGAAGT
	Aga tac
ER81	GGCGGAAGT
	aca Tac
ER71	GGCGGAAGT
	CCa Tac
SAP-1	aCCGGAAGT
	gaa tAc
Elements[b]	
rfLVb	ACAGGATAT
rfSC1	GCCGGAAGT

[a] Each consensus sequence was derived by in vitro selection (3). Capital letters designate strong selection for a particular base; lowercase letters indicate a weaker preference. Data for Ets-1 (34), GABPα, ER81, and ER71 (4), and SAP-1 (46) are from the indicated references.

[b] Sequences of the central 9 bp of LVb (SC13)- and SC1-binding sites, which are two selected and cloned sites in the Ets-1 analysis (34). Outside of the central 9 bp, the sequences were identical to the Mo-MuLV LVb element flanking sequences for 6 bp on the left and 5 bp on the right.

erties on two restriction fragment probes bearing GGA motifs with divergent flanking sequences (Table 1). The rfLVb probe contained 20 bp corresponding to the LVb element of Mo-MuLV, while rfSC1 contained a high-affinity binding site for Ets-1, termed SC1 (34). A single predominant band of identical mobility was detected on each probe (Fig. 3, lanes 3 to 16), suggesting that both probes detected the same binding activity. The GGA specificity of the binding activities was confirmed by competition with DNA probes containing either the SC1 GGA motif or a nonspecific sequence. In addition, methylation interference analysis demonstrated that the central guanines of the SC1 and LVb probes were in close contact with DNA (15).

The GGA-binding species displayed a higher affinity for the rfSC1 probe than the rfLVb probe, since less rfLVb than rfSC1 was shifted into complex despite four times more extract in the rfLVb binding reaction (Fig. 3, lanes 3 and 10). Ets-1 displays a similar preference for the SC1 site relative to LVb (34). This sequence preference also is expected for four other ETS domain proteins, based on the consensus sites derived from in vitro selection experiments (Table 1). In conclusion, the GGA specificity and flanking sequence preferences of the EL4.E1 DNA-binding complexes suggested that the LVb-binding activities were encoded by an *ets* gene(s).

Two polypeptides with LVb-binding activities are detected. As a first step toward identifying the putative *ets* protein(s) in the crude nuclear extracts, proteins in the shifted bands were sized. The polypeptides were cross-linked to radiolabeled probe DNA by UV irradiation and then visualized by SDS-polyacrylamide gel electrophoresis (PAGE). As a control, Ets-1 was analyzed (Fig. 4A, lanes 1 and 2). Nuclease treatment reduced the mass of the Ets-1–DNA complex to approximately 54 kDa, matching the apparent molecular mass of Ets-1. With the EL4.E1 extract, two cross-linked species were detected on both the SC1 and LVb probes (lanes 3 and 5). The similarity in the mobilities suggested that the same polypeptides were binding the two GGA-bearing probes. (The difference in relative specific activity of the larger and smaller species on the two probes was likely caused by differences in cross-linking efficiencies, since the positions of BrdU in the two probes differ.) Nuclease treatment of the SC1 complexes generated species with estimated molecular weights of 100,000 and 55,000 (lane 4); therefore, the polypeptides were denoted p100 and p55. The SC1 as well as LVb probe detected both p100 and p55, suggesting that both species were dependent on the GGA sequence. In addition, neither cross-linked product was detected when the mutant LVb oligomeric probe was used (15).

Immunological assays identify GABP as an LVb-binding protein. The p55 and p100 species cross-linked to the LVb probe were challenged with specific antisera to test their identity. The UV-cross-linked species were recovered from the native gel, treated with detergent-containing buffers, and challenged with antisera in immunoprecipitation assays. These stringent conditions dissociated any noncovalently bound partners from the cross-linked protein. Both the reactive (precip-

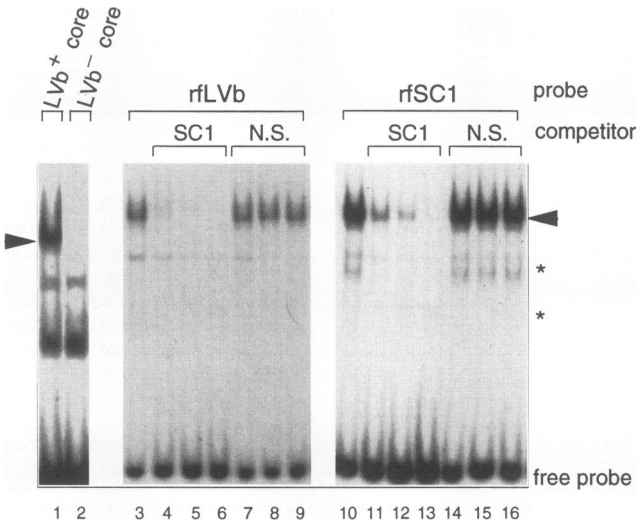

FIG. 3. Mobility shift analysis of GGA motif-binding activities in EL4.E1 nuclear extract. Standard DNA binding conditions are described in Materials and Methods. Reaction mixtures in lanes 1 and 2 included 8 μg of EL4.E1 extract and 25 fmol of oligomeric probes which span the LVb and core elements. In the mutant version (LVb$^-$), the GGA motif was altered to TTA. Reaction mixtures in lanes 3 to 16 included 25 fmol of restriction fragment probe DNA (either rfLVb or rfSC1; see Table 1), EL4.E1 nuclear extract (8 μg for rfLVb and 2 μg for rfSC1), and either no competitor or competitor (as indicated) in one-, three-, and ninefold molar excess. Nucleoprotein complexes were resolved on native polyacrylamide gels and detected by autoradiography (shown). Arrowhead, dominant GGA-dependent nucleoprotein complexes; asterisks, minor GGA-dependent activities that were not analyzed further; N.S., nonspecific.

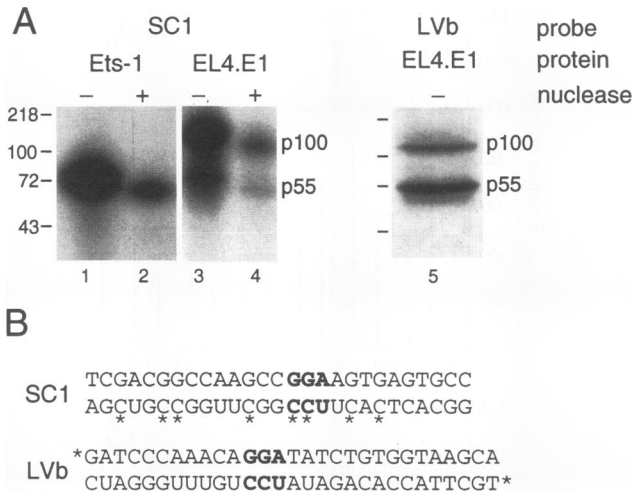

FIG. 4. Sizing of GGA-binding polypeptides. (A) Protein preparations were incubated in standard DNA binding conditions with radiolabeled probes containing BrdU substitutions at thymine residues. Nucleoprotein complexes were resolved on native gels, exposed to UV irradiation, and then visualized by autoradiography. The predominant GGA-binding complex was isolated by electroelution, resolved by SDS-PAGE, and viewed by autoradiography (shown). Molecular weights of prestained size markers are indicated in thousands. Reaction mixtures in lanes 1 to 4 included 10 fmol of SC1 probe, radiolabeled by incorporation of [α-^{32}P]CTP by using DNA polymerase, and either 60 ng of purified Ets-1 or 12 μg of EL4.E1 extract. Reaction mixtures in lane 5 included 300 fmol of LVb oligomeric probe, radiolabeled by phosphorylation with T4 kinase and [γ-^{32}P]ATP, and 20 μg of EL4.E1 nuclear extract. Nuclease treatment was performed prior to SDS-PAGE as indicated. (B) Sequences of SC1 and LVb oligomeric probes. Sites of BrdU substitutions (U) and ^{32}P incorporation (*) are indicated. The LVb probe spans the LVb and core elements as in Fig. 3.

itate) and the unreactive (supernatant) cross-linked species were resolved by SDS-PAGE. In a control experiment, Ets-1 reacted with the Ets-1 but not GABPα or GABPβ1 antiserum (Fig. 5, lanes 1 to 6). The p55 cross-linked product reacted specifically with the GABPα (lanes 9 and 10) but not Ets-1 or GABPβ1 (lanes 11 to 14) antiserum. These findings were consistent with the reported functions of GABP subunits. DNA binding of this heteromeric protein is mediated by the ETS domain-containing alpha subunit rather than the ankryin repeat-containing beta subunit (44). The p55 species did not partition completely into the nonreactive and reactive fractions as in the Ets-1 control, possibly because of limiting amounts of antisera. The failure of p100 to react with either the GABPα or GABPβ1 antibody eliminated the unlikely possibility that p100 represented a ternary complex of GABPα and -β simultaneously cross-linked to DNA. This experiment was repeated on the SC1 probe with identical results, confirming our proposal that the SC1 and LVb probes detect the same species (15) (see Fig. 6).

To detect the GABP beta subunit in the GGA-dependent complexes, the immunological challenge was performed under nondenaturing conditions in supershift assays on the SC1 probe. The GGA-binding activity from EL4.E1 extracts was reduced in intensity, and nucleoprotein complexes were excluded from gel entry (Fig. 6, lanes 8 and 9) in the presence of either the GABPα or GABPβ1 but not Ets-1 antiserum. Purified GABP in complex with DNA showed the same reactivity (lanes 13 and 14). In controls, no cross-reactivity between the Ets-1 antibody and GABP or between the GABP antibody and Ets-1 was observed (lanes 12, 18, and 19). The reactivity with antibodies specific to both α and β1 subunits of GABP strongly indicated that GABP was binding the SC1 probe. Similar results were obtained for the LVb probe (15). Thus, these immunological studies confirmed the hypothesis that an *ets* protein was responsible for the LVb-binding activity in EL4.E1 extracts and implicated GABP as a Mo-MuLV transcriptional regulator.

GABP and p100 display independent binding activities. The failure of the GABP antiserum to supershift all of the GGA-binding activity in EL4.E1 extracts (Fig. 6, lanes 8 and 9) suggested that the remaining signal was due to binding of the p100 species as a second, independent DNA-binding activity. To test this hypothesis, EL4.E1 nuclear extracts were fractionated and analyzed on the rfSC1 (Fig. 7A, upper panel) and rfLVb (15) probes. Individual chromatographic fractions from the heparin-Sepharose elution were challenged with the GABPα antiserum in supershift assays. Only the GGA-binding activity that eluted in the first half of the gradient reacted with the GABPα antibody (Fig. 7A, lower panel), suggesting that the p100 species eluted later in the gradient. To test this hypothesis, a GABP-containing band (lane 11) and the shifted band in lane 19 were analyzed by UV cross-linking and SDS-PAGE (Fig. 7B). The GABP-containing fraction corresponded with p55, while the shifted species from lane 19 had the same mobility as p100. These data demonstrated that the two GGA-binding activities present in EL4.E1 extracts bound DNA independently.

p100 resembles an ETS domain protein. The GGA-binding properties of p100 suggested that this species was also an ETS

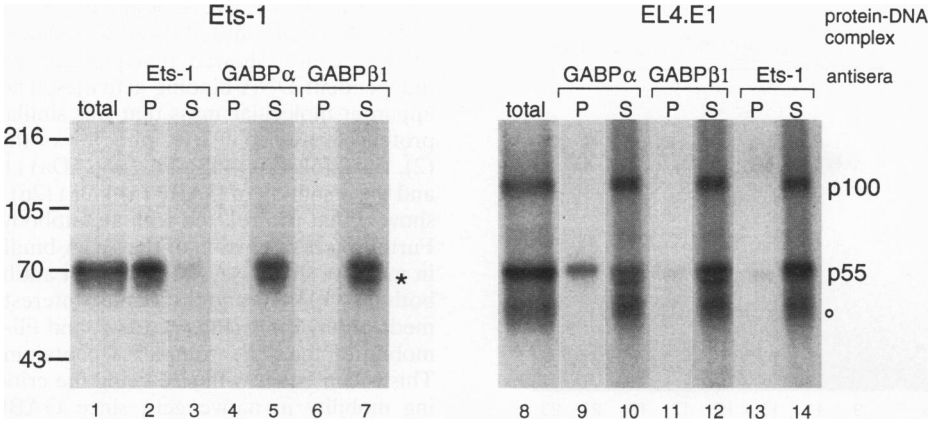

FIG. 5. Reactivity of UV-cross-linked protein-DNA complexes with ETS domain protein antisera. Cross-linked species containing either Ets-1 (lanes 1 to 7)- or EL4.E1 (lanes 8 to 14)-binding activities were prepared as for Fig. 4 on radiolabeled, BrdU-substituted LVb probe. Following electroelution of cross-linked complexes from native gels, the protein components were either resolved as total cross-linked species (lanes 1 and 8) or immunoprecipitated with an Ets-1, GABPα, or GABPβ1 antiserum. Cross-linked species in the precipitate (P lanes) and supernatant (S lanes) were resolved by SDS-PAGE and detected by autoradiography (shown). Open circle, a cross-linked product that was not reproducibly detected; asterisk, a subband that is generated in the supernatant of each GABP antiserum immunoprecipitation. Molecular weights are indicated in thousands.

domain protein. To provide further support for this hypothesis, the DNA-binding properties of partially purified p100 and three other *ets* proteins were compared. Ets-1 contains a sufficiently novel DNA-binding domain that the pattern of DNA contacts is distinct from the pattern observed with many other families of DNA-binding proteins (34). We predicted that DNA-binding contacts, as detected in ethylation interference and DNase I protection assays, would provide a distinctive pattern by which to identify *ets*-type binding activities.

Sites of ethylation interference for p100 binding and three ETS domain proteins, Fli-1, Ets-1, and GABP, were compared (Fig. 8). This technique tested the effect of ethylation of specific phosphates on DNA-binding activity. The pattern of interference observed in the p100 analysis closely resembled the ETS domain binding pattern. Next, the DNase I cleavage pattern caused by Fli-1, Ets-1, and GABP binding to the SC1 probe was examined (Fig. 9A). All of the ETS domain proteins and p100 caused the appearance of the characteristic DNase I-hypersensitive site in the center of the protected region. A helical model of the SC1-binding site with the position of the enhanced cleavage site and the predominant sites of ethylation interference is illustrated (Fig. 9B). As previously noted, the accessibility of DNase I, as a minor groove-binding protein, probably occurs on the opposite face of the helix from the proposed major groove binding of the ETS domain proteins (34). These comparative studies illustrated the similarities between the DNA-binding properties of all ETS domain proteins. Furthermore, several features of p100 DNA binding strongly suggested that this species was an *ets* family member.

DISCUSSION

The transcriptional control elements in the Mo-MuLV enhancer that regulate constitutive and TPA-responsive tran-

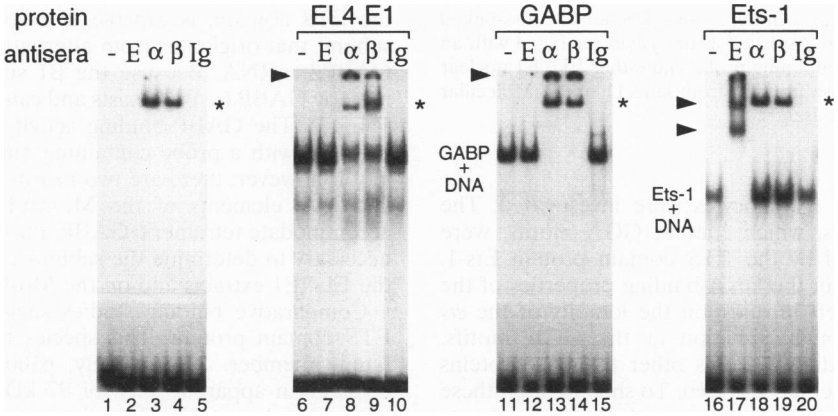

FIG. 6. Protein-DNA complexes challenged with specific antisera in mobility shift assays. DNA binding reaction mixtures included 6 fmol of radiolabeled SC1 oligomeric probe with no protein (lanes 1 to 5), 2 μg of EL4.E1 nuclear extract (lanes 6 to 10), 12 ng of purified GABPα/β1 (lanes 11 to 15), or 10 ng of purified Ets-1 (lanes 16 to 20). Reaction mixtures were prepared in the absence (−) or presence of immunoglobulins (E, affinity-purified Ets-1 antibody; α, GABPα antiserum; β, GABPβ1 antiserum; Ig, purified rabbit immunoglobulin G) and analyzed on native gels. Autoradiographs of gels are shown. Asterisk, nonspecific DNA-binding complexes formed between proteins present in the GABP antisera and the SC1 probe; arrowheads, DNA-protein-antibody complexes.

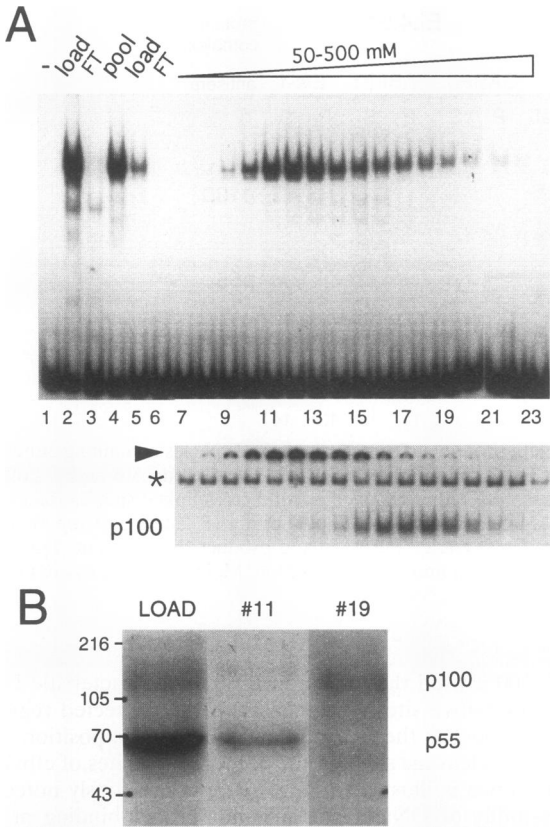

FIG. 7. Independent DNA-binding activity of p55 and p100. (A) Chromatographic fractionation of EL4.E1 extracts and identification of GABP-containing fractions. Crude EL4.E1 nuclear extract was fractionated by DEAE-cellulose and heparin-agarose chromatography. The upper panel shows a mobility shift assay of GGA-binding activities on the SC1 probe. Lanes: 1, free DNA; 2 to 4, DEAE chromatography (load, flowthrough [FT], and active pool from a 50 to 750 mM KCl gradient elution); 5 to 23, heparin-Sepharose chromatography (load, flowthrough, and 50 to 500 mM KCl gradient fractions). (An equal volume of each heparin-Sepharose fraction was tested.) The lower panel shows mobility shift assays of heparin-Sepharose fractions challenged with a GABPα-specific antiserum in DNA binding reactions. Asterisk, a binding complex containing SC1 and nonspecific DNA-binding serum proteins; arrowhead, supershifted DNA-protein-antibody complexes. (B) UV cross-linking of GGA-binding species in heparin-Sepharose fractions. Cross-linked products were prepared and displayed as described for Fig. 4 with an LVb-radiolabeled, BrdU-containing probe and either EL4.E1 nuclear extract or heparin-Sepharose fraction from lane 11 or 19. Molecular weights are indicated in thousands.

scriptional activity in T lymphocytes were investigated. The LVb and LVc elements, which contain GGA motifs, were shown to be recognized by the ETS domain protein Ets-1. However, the similarity in the DNA-binding properties of the ETS domain proteins left in question the identity of the *ets* family members that might function at the GGA motifs. Furthermore, DNA-binding proteins other than *ets* proteins might recognize these elements in vivo. To shed light on these issues, we investigated proteins from crude nuclear extracts with the highest enhancer-binding activity as candidate activators of Mo-MuLV.

The investigation focused on the GGA motif in the LVb element. Initially, there appeared to be only one predominant LVb-binding activity detected by mobility shift assays with EL4.E1 nuclear extracts. However, two species were detected within the single shifted band. Chromatographic fractionation of the extracts showed that p55 and p100 represented two independent DNA-binding activities. The p55 species had an apparent molecular mass that was similar to those of five *ets* proteins expressed in T lymphocytes or thymus: Ets-1 (50 kDa) (2), Ets-2 (52 kDa) (55), Fli-1 (56 kDa) (1), Tel (54 kDa) (45), and the α subunit of GABP (51 kDa) (26). Immunological tests showed that p55 reacted with an antibody specific to GABPα. Furthermore, a portion of the GGA-binding activity visualized in mobility shift assays reacted with antibodies that recognize both the GABP α and β subunits. Interestingly, the band shifts mediated by Ets-1 (Fig. 6), Ets-2, and Fli-1 (15) all have faster mobilities than the complexes containing GABP and p100. This is consistent with size being the critical parameter affecting mobility in native gels, since GABP with a 51- and a ~40-kDa subunit might comigrate with a 100-kDa species.

Criteria for identifying ETS domain proteins. Four approaches detected similarities in the DNA-binding properties of p100 and three ETS domain proteins. The specificity of p100 for the GGA motif in the LVb probe was demonstrated in competition experiments, methylation interference analysis, and genetic studies in which binding to the mutated LVb element was tested. Next, partially purified p100 was shown to have a preference for the SC1 probe over the LVb element probe (15) as was observed with the LVb-binding complex in crude extracts. The p100 DNase I footprint showed the same cleavage pattern as three other ETS domain proteins, Ets-1, Fli-1, and GABPα. Most importantly, p100 binding caused a DNase I-hypersensitive site on the TCC strand of the binding site characteristic of other ETS domain proteins. Finally, the ethylation interference analysis of the p100-DNA complex identified putative backbone contacts that were similar to those of three other ETS domain proteins. This combination of approaches should be appropriate for identifying ETS domain proteins in crude or fractionated extracts.

Specific *ets* family members as potential regulators of Mo-MuLV transcription. GABP was responsible for a significant portion of the LVb-binding activity in EL4.E1 extracts. These findings led us to propose that GABP functions as a viral activator. Consistent with this proposal, GABP has been shown to stimulate transcription of a cellular promoter (47). This result also is consistent with the high levels of GABP found in the thymus (26). GABP is a heteromeric protein encoded by two separate genetic loci (26). The α subunit, which contains the ETS domain, is capable of associating with a β1 or β2 subunit that originates from alternatively spliced forms of the GABPβ mRNA. Because the β1 subunit can dimerize, tetrameric GABP ($\alpha_2\beta1_2$) exists and can bind to two GGA motifs (44, 47). The GABP binding activity in EL4.E1 extracts was detected with a probe containing only one GGA recognition motif; however, there are two nearby GGA motifs in the LVb and LVc elements of the Mo-MuLV enhancer that could accommodate tetrameric GABP. Further investigations will be necessary to determine the subunit configuration of GABP in the EL4.E1 extracts and on the Mo-MuLV enhancer.

Comparative binding studies suggested that p100 was an ETS domain protein. This species may represent a new *ets* family member. Alternatively, p100 might be Elf-1, which displays an apparent mass of 97 kDa, even though the *elf-1* gene encodes a 67-kDa protein (49). In support of this possibility, the LVb element fits with the reported sequence preferences of Elf-1 (48). The putative role of Elf-1 in T-cell signal transduction corresponds with the function of LVb during TPA induction of Mo-MuLV transcription (27, 43).

Because *ets-1* is reported to be highly expressed in thymus

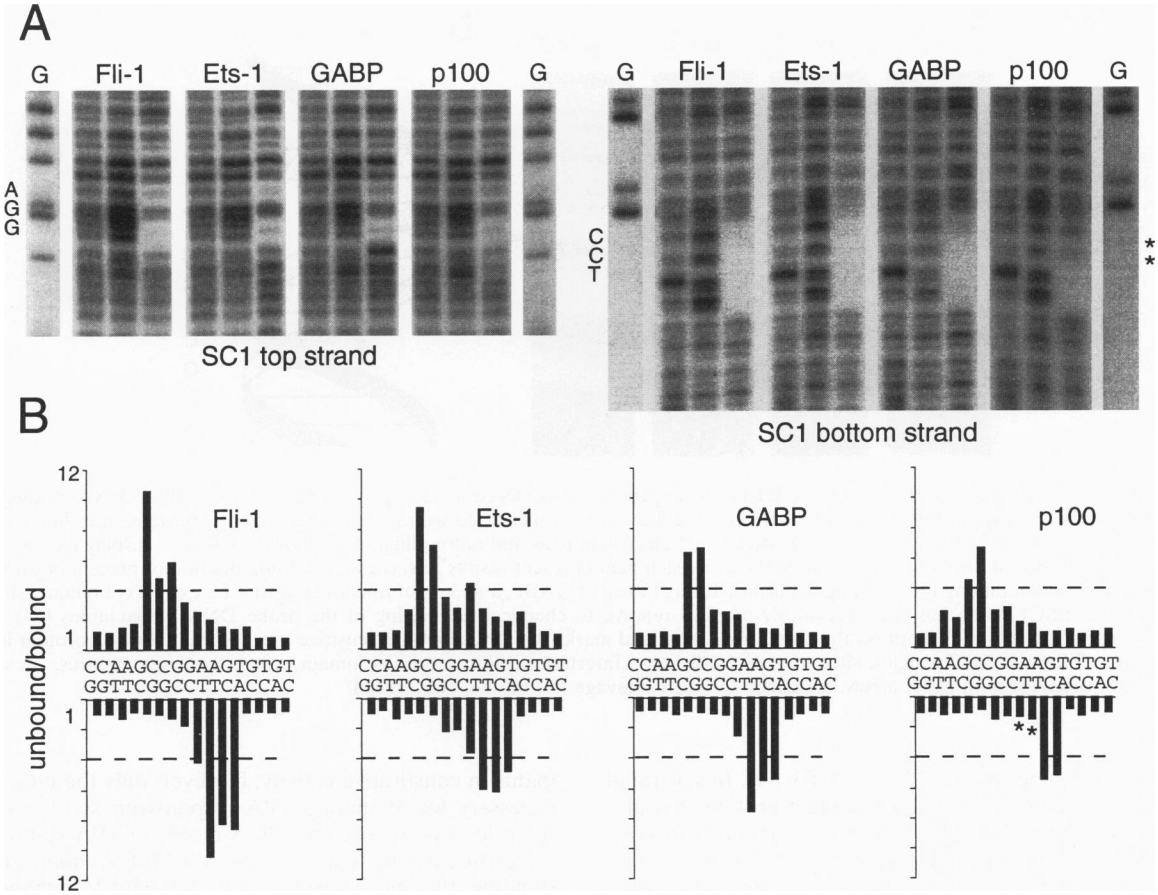

FIG. 8. Ethylation interference analysis of the DNA binding of p100 and three ETS domain proteins. (A) Restriction fragment probe rfSC1 was end labeled on either the GGA strand (left) or the TCC strand (right) and then ethylated with *N*-ethyl-*N*-nitrosourea. Mobility shift reaction mixtures contained 100 fmol of probe and 15 μg of bacterial extract containing Fli-1, 30 ng of purified Ets-1, 13 ng of purified GABP, or 8 μg of an EL4.E1 heparin-Sepharose fraction containing p100. Unbound probe DNA was separated from DNA-protein complexes by native gel electrophoresis. DNA recovered from both fractions was cleaved at ethylated phosphates and resolved by denaturing PAGE and autoradiography (shown). Each set of three lanes contains ethylated and cleaved DNA that was never exposed to protein (left lanes), DNA that failed to bind protein (middle lanes), and DNA from the nucleoprotein complexes (right lanes). Chemical sequencing of the probe DNA for guanines provided markers (G lanes). (B) Quantitation of ethylation interference data. Individual lanes were scanned with a PhosphorImager (Molecular Dynamics). Bar graphs display the relative intensities of bands in the unbound and bound DNA fractions from panel A. Bars are positioned over the 5′ phosphate of each base pair in the SC1 sequence. Asterisks indicate distinguishing sites in p100 analysis. Dashed lines intersect sites of severe interference, as defined by a ratio of unbound to bound that is greater than 4.

and *ets-1* RNA and protein can be detected in EL4.E1 cells (16), we were surprised that the predominant GGA-binding complexes did not contain Ets-1. However, Ets-1 was detected in the fractionated extracts by supershift assays, following removal of the p55 and p100 species (15). It is possible that other *ets* family members, like *ets-1*, contribute to the transcriptional control of Mo-MuLV in other cell types or T-cell lines. Additional functional tests are in progress to resolve these issues.

Earlier studies characterized two LVb-binding activities by partial purification and alkylation interference analysis. Leukemia virus factor from T cells is likely to be an *ets* family member that is highly expressed in T and B lymphocytes (30), while the mammalian C-type enhancer factor 1 (MCREF-1) recognizes DNA differently than an ETS domain protein (42). MCREF-1 binding activity was not detected in the unfractionated nuclear extracts of EL4.E1 cells. Additional studies are required to determine the tissue distribution of MCREF-1 and its relevance to Mo-MuLV enhancer function.

Constitutive and induced activity of the Mo-MuLV enhancer. Transient expression assays of the Mo-MuLV enhancer function indicated that the LVb and core elements, but not the LVc element, were necessary for constitutive transcription activity. Biochemical studies suggest a specific model for this active state. GABP and p100 from fractionated EL4.E1 extracts, as well as purified Ets-1, bound the LVb element. The core element is recognized by core-binding factor (CBF), a heterodimeric protein (50). Thus, we propose that an ETS domain protein binds the GGA motif, while CBF binds the core element during constitutive function of the enhancer (Fig. 10A). TPA treatment of several T-cell lines stimulates the transcriptional activity of the Mo-MuLV LTR (reference 40 and this report) via not only the two constitutive elements, LVb and core, but also the LVc element. Figure 10B presents a model of the activated state in which all three sites are occupied by CBF and two ETS domain proteins. A single GABP tetramer also would fit this configuration. Biochemical analyses are in progress to test these models.

Transcriptional control elements that confer TPA responsiveness have been mapped to GGA motifs in a variety of

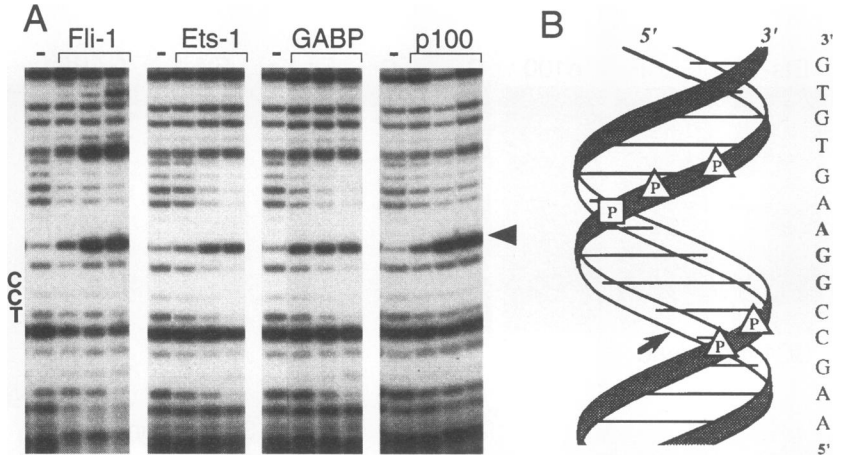

FIG. 9. DNase I protection by p100 and three ETS domain proteins. (A) DNase I cleavage pattern with and without DNA-binding proteins. The restriction fragment probe rfSC1 was end labeled on the TCC strand, incubated with increasing amount of protein, and then cleaved with DNase I. Cleavage products were resolved by denaturing gel electrophoresis and autoradiography (shown). Lanes − display cleavage products generated in the absence of protein. The next three lanes in each panel present results of successive half-log dilution of protein beginning with 6 μg of bacterial extract containing Fli-1, 150 ng of purified Ets-1, 130 ng of GABP, and 3 μg of fractionated EL4.E1 extract containing p100. DNase I cleavage on the rfSC1 probe has been previously mapped relative to chemical sequencing of the probe DNA for guanines (34), providing information to position the TCC motif on the panel. An arrowhead marks the DNase I-hypersensitive site. (B) Model of *ets* protein interaction with DNA on a B-form helix. Triangles, sites of severe ethylation interference in three ETS domain protein analyses; squares, sites of severe interference not observed with p100; arrow, enhanced DNase I cleavage site on the TCC strand.

cellular and viral genes (6, 18, 27, 36, 43, 53, 54). In several of these cases, in vitro binding of ETS domain proteins has also been demonstrated. In the TPA-responsive enhancers of polyomavirus (20, 52, 59) and the T-cell receptor β-chain gene (36, 58), both GGA and core motifs have been mapped as participants in constitutive activity; however, only the GGA motif is necessary for mediating TPA responsiveness (20, 36). Thus, the role of both core and GGA motifs in TPA responsiveness is a distinguishing feature of the Mo-MuLV enhancer. Understanding the mechanism of the Mo-MuLV enhancer TPA responsiveness should provide unique insights into the role of ETS domain proteins in signal transduction.

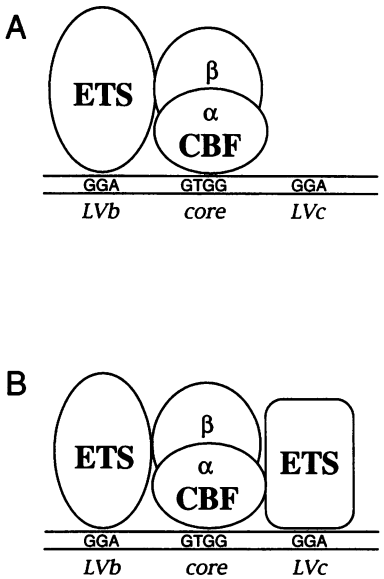

FIG. 10. Models of the occupancy of the LVb, core, and LVc elements in Mo-MuLV enhancer by host transcription factors. (A) Uninduced, constitutive state of the enhancer with the LVb element occupied by an ETS domain protein and the core element occupied by a two-subunit protein, CBF. (B) The TPA-induced state of the enhancer with both the LVb and LVc elements occupied by ETS domain proteins. The core element retains CBF binding as in the constitutive state.

Deciphering the specificity of action of a family of transcription factors that have a common set of DNA recognition properties is a challenging problem. It is presumed that in the case of cellular genes, a particular GGA motif would interact with a single or small number of the family members. The possibilities in the case of a viral transcriptional control element are more diverse. Although the Mo-MuLV LTR might be designed to function optimally with a particular *ets* family member, a more intriguing possibility is that the activation potential of many members of the *ets* family are exploited by a viral enhancer.

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