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Nuclear Factors That Bind to the Enhancer Region of Nondefective Friend Murine Leukemia Virus

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Nondefective Friend murine leukemia virus (MuLV) causes erythroleukemia when injected into newborn NFS mice, while Moloney MuLV causes T-cell lymphoma. Exchange of the Friend virus enhancer region, a sequence of about 180 nucleotides including the direct repeat and a short 3′-adjacent segment, for the corresponding region in Moloney MuLV confers the ability to cause erythroid disease on Moloney MuLV. We have used the electrophoretic mobility shift assay and methylation interference analysis to identify cellular factors which bind to the Friend virus enhancer region and compared these with factors, previously identified, that bind to the Moloney virus direct repeat (N. A. Speck and D. Baltimore, Mol. Cell. Biol. 7:1101–1110, 1987). We identified five binding sites for sequence-specific DNA-binding proteins in the Friend virus enhancer region. While some binding sites are present in both the Moloney and Friend virus enhancers, both viruses contain unique sites not present in the other. Although none of the factors identified in this report which bind to these unique sites are present exclusively in T cells or erythroid cells, they bind to three regions of the enhancer shown by genetic analysis to encode disease specificity and thus are candidates to mediate the tissue-specific expression and distinct disease specificities encoded by these virus enhancer elements.

Nondefective murine leukemia viruses (MuLVs) display a variety of phenotypes when injected into mice; some cause no disease, while others induce one or more types of hematopoietic tumors. Genetic studies have shown that the nature of the disease induced by a particular virus, including the incidence, latency, and type of leukemia or lymphoma induced, can be influenced by many regions of the viral genome; however, in a number of studies a primary determinant of these disease-related phenotypes is the virus enhancer, located in the U3 region of the long terminal repeats (57, 58).

MuLV enhancer elements have been shown to be determinants of leukemogenicity (12, 25, 34, 38), tissue tropism (3, 4, 13, 16, 18, 24, 26, 37, 47, 51, 60), and disease specificity (5, 6, 11, 27, 29, 36, 44, 45). Presumably, many of these functions are mediated by cellular proteins binding to the virus enhancer, as MuLVs are not known to encode a *trans*-activator function. Analysis of the retrovirus enhancer thus serves to identify interesting cellular factors and elucidate viral mechanisms of disease induction.

Moloney and Friend MuLVs cause T-cell lymphoma and erythroleukemia, respectively, when injected into newborn NFS mice. The distinct disease specificities of Moloney and Friend MuLVs have been shown by genetic studies to be determined primarily by the enhancer region, which includes the direct repeats and a short GC-rich region immediately 3′ of the repeats in the U3 region of the long terminal repeat (5, 6, 20, 27, 32, 35, 36, 45, 52). As Moloney and Friend viruses have approximately 85% DNA sequence homology in this region, the distinct disease-inducing phenotypes are determined by a small number of sequence differences. It is thought that differences in the transcriptional activities of the virus enhancers in specific target cell populations in vivo contribute to the viral disease phenotype. Indeed, there is evidence from in vitro studies that the Moloney virus enhancer is more active than the Friend virus enhancer in T cells and that the Friend virus enhancer has higher activity in erythroid cells than does that of the Moloney virus (3, 8, 24, 51, 54) although their relative activities are similar in fibroblasts. Also, while both viruses replicate equally well in the spleen after injection into newborn mice, Moloney MuLV replicates to a higher titer in the thymus than does Friend MuLV (16; J. Hartley, unpublished results).

Golemis et al. (22) constructed recombinant viruses in which segments of the Moloney and Friend virus enhancer regions were switched in order to further pinpoint the location of disease specificity determinants, particularly determinants of erythroleukemogenicity in the Friend virus enhancer. The results indicate that three regions, the 5′ and 3′ halves of the repeated sequence and the GC-rich region, which together include the entire enhancer, contribute to this phenotype, possibly acting in a cooperative manner. We have used the electrophoretic mobility shift assay (19) coupled with methylation interference analysis to identify the binding sites for cellular factors which may be involved in the regulation of this disease specificity, in particular those which bind to the Friend virus enhancer. Our results show that, although the Moloney and Friend virus direct repeats share some binding sites in common, base changes between the two viruses in both the direct repeat and GC-rich regions define binding sites specific to either the Friend or Moloney virus enhancer (53).

MATERIALS AND METHODS

Extracts and cell lines. Nuclear extracts were made from cell lines and primary cells by the method of Dignam et al. (15). Protein concentrations were determined using the Bio-Rad protein assay. EL4 and BW are mouse T-cell lines. TSA9, a CFU-E stage erythroleukemia cell line, and CB5, a BFU-E stage erythroleukemia cell line, were provided by T. Mak (49). Spleen nuclear extract was made from spleen cells isolated from adult BALB/c mice which were washed on Ficoll-Isopaque and cultured in a solution containing 5% fetal calf serum, 50 mM β-mercaptoethanol, 2 μg of conca-

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navalin A per ml, and 10 U of recombinant interleukin-2 per ml for 3 days before extracts were made (materials for the spleen culture were kindly provided by D. Raulet). Rat brain extracts were prepared from homogenized brain tissue from Fischer rats. The resulting crude extract was fractionated over a Sephacryl S-300 column (Pharmacia) and assayed for binding activity, using the gel shift assay described below. The binding activities assayed were all present in the major protein peak in the flowthrough fraction, designated in the text as "rat brain S-300." Other extracts were kindly provided as follows: WEHI 231 mouse B-cell line and F9 undifferentiated mouse embryonal carcinoma cell line nuclear extracts from N. Speck; HeLa human cervical carcinoma cell line whole-cell extract and calf brain primary cell nuclear extract from R. Carthew and L. Chodosh; U937 human macrophage precursor cell line nuclear extract from Y. Li; BALB/3T3 A31 mouse fibroblast cell line nuclear extract from T. Hayes.

Probes and competitors. Probes and competitors were either restriction enzyme-generated fragments or double-stranded synthetic oligonucleotides. Radioactive probes were prepared from plasmids by digestion with an appropriate restriction endonuclease, treatment with alkaline phosphatase (Boehringer Manneheim Biochemicals), labeling with [γ-^{32}P]ATP, using polynucleotide kinase, and redigesting with a second restriction endonuclease. The resulting fragments were gel purified in 10 to 20% native polyacrylamide gels and eluted overnight at room temperature in 1 M NaCl–20 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (pH 8.0). Plasmids used were described previously (36). The following probes were generated (see Fig. 1): Moloney *Sau*3AI-*Eco*RV and *Eco*RV-*Ava* 2 from pModrA(ΔRV), Friend *Sma*I-*Eco*RV from pFrdrD, and Friend *Sau*3AI-*Eco*RV and *Eco*RV-*Ava* 2 from pFrdrD(ΔRV).

Complementary synthetic oligonucleotides were made using an Autogen 6500 model genetic design DNA synthesizer. Oligonucleotide probes were generated by end labeling one strand with [γ-^{32}P]ATP and polynucleotide kinase and annealing with the complementary strand as described above. Double-stranded oligonucleotides for competitions were prepared by annealing complementary strands in 1× gel binding buffer (10 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol) at a final double-stranded DNA concentration of 100 ng/μl. Annealing and concentration were verified by comparison with *Hae*III-digested ϕX174 marker DNA (New England BioLabs, Inc.) and single-stranded oligonucleotides on 4% low-melting-agarose gels.

Gel binding. Protein-DNA binding reactions (19) were carried out using the specifications described by Speck and Baltimore (53) with the following modifications. Reactions were carried out in 1× gel binding buffer at 30°C for 30 min. Typical reactions contained 50,000 cpm of end-labeled probe and 10 to 15 μg of extract, with 1 to 2 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia) added as a nonspecific carrier in a total volume of 20 μl. Amounts of extract and carrier were determined empirically for each probe. Binding reactions were loaded directly onto 4% polyacrylamide gels (acrylamide/bis ratio, 29:1) containing 22 mM Tris borate, 22 mM boric acid, 0.5 mM EDTA, and 3% glycerol and run at 10 V/cm. Competition experiments were carried out as described above, with the addition of 10 to 100 ng of unlabeled competitor fragments or oligonucleotides before the addition of extract.

Methylation interference. End-labeled DNA fragments were partially methylated at guanine residues by the method of Maxam and Gilbert (39). Methylated DNA was ethanol precipitated twice, rinsed with 70% ethanol, and used in binding reactions. Preparative binding reactions were performed by scaling up regular binding conditions by 10 to 20-fold and electrophoresing samples as usual. After electrophoresis, gels were wrapped in Saran Wrap and exposed wet for 2 to 15 h at 4°C. The protein-DNA complex and free DNA bands were cut from the gel, and DNA was eluted by incubation at 50°C for 2 h or at room temperature overnight in a solution containing 0.5 M sodium acetate, 10 mM magnesium acetate, 10 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, and 1% sodium dodecyl sulfate. Eluted DNA was phenol extracted twice, chloroform extracted twice, ethanol precipitated twice, and rinsed with 70% ethanol. The dried pellet was cleaved in 1 M piperidine at 90°C, lyophilized overnight, rinsed with distilled water, and relyophilized twice. Resulting fragments were analyzed on 10 to 15% denaturing polyacrylamide gels and autoradiographed at −70°C with an intensifying screen.

RESULTS

A diagram of the long terminal repeat and the nucleotide sequences of the enhancer regions of the Friend and Moloney viruses employed in these studies are shown in Fig. 1A (31, 50, 58). The direct repeat of Moloney virus is a 9-65-9-65-9 nucleotide structure in which two identical copies of a 65-base-pair (bp) repeat are flanked by three copies of a 9-bp consensus sequence for the glucocorticoid response element (10, 53). The Friend virus direct repeat has a slightly shorter sequence, and the two copies are not identical; there are several base changes between them and a 9-bp insertion in the second copy (31). The repeated sequences of these clones of the Friend and Moloney viruses are identical in a central region 32 bp long surrounding an *Eco*RV site. The GC-rich region, defined as being involved in transcriptional activation (32) and shown in genetic studies to encode a determinant of disease specificity (22), lies between the *Ava*II and *Dde*I sites immediately 3′ of the direct repeats and includes four base differences between the two viruses.

To identify nuclear factors that bind to the Friend virus enhancer region and compare these with factors that bind to the Moloney virus enhancer, particularly in the regions that differ in sequence between the two viruses, we began by dividing the direct repeat at the central *Eco*RV site (Fig. 1). We used either restriction endonuclease-generated fragments cut from appropriate plasmids or double-stranded synthetic oligonucleotides as probes in gel shift assays (see Materials and Methods). The fragments and oligonucleotides used are represented schematically in Fig. 1B, and the sequences of the oligonucleotides are given in Table 1. Fragments and oligonucleotides are designated F if their sequence is unique to the Friend Virus, M if unique to Moloney virus, and MF if derived from a region of sequence identity between the Friend and Moloney viruses. We designated the 5′ copy of the direct repeat I and the 3′ copy II. The 5′ and 3′ halves of each repeat are designated (A) and (B), respectively. The GC-rich region 3′ of the direct repeat is designated (C). Because of the complexity of the data, some readers may find it helpful to consult the summary figure (Fig. 10) while reading some of the following sections of the results.

Binding to the NF1 consensus sequences. The Friend virus enhancer contains two copies and the Moloney enhancer contains four copies of sequences highly related to the consensus sequence for the binding site for nuclear factor 1 (NF1) [5′-T/CGG(N6-7)GCCAA-3′] (33, 41, 53).

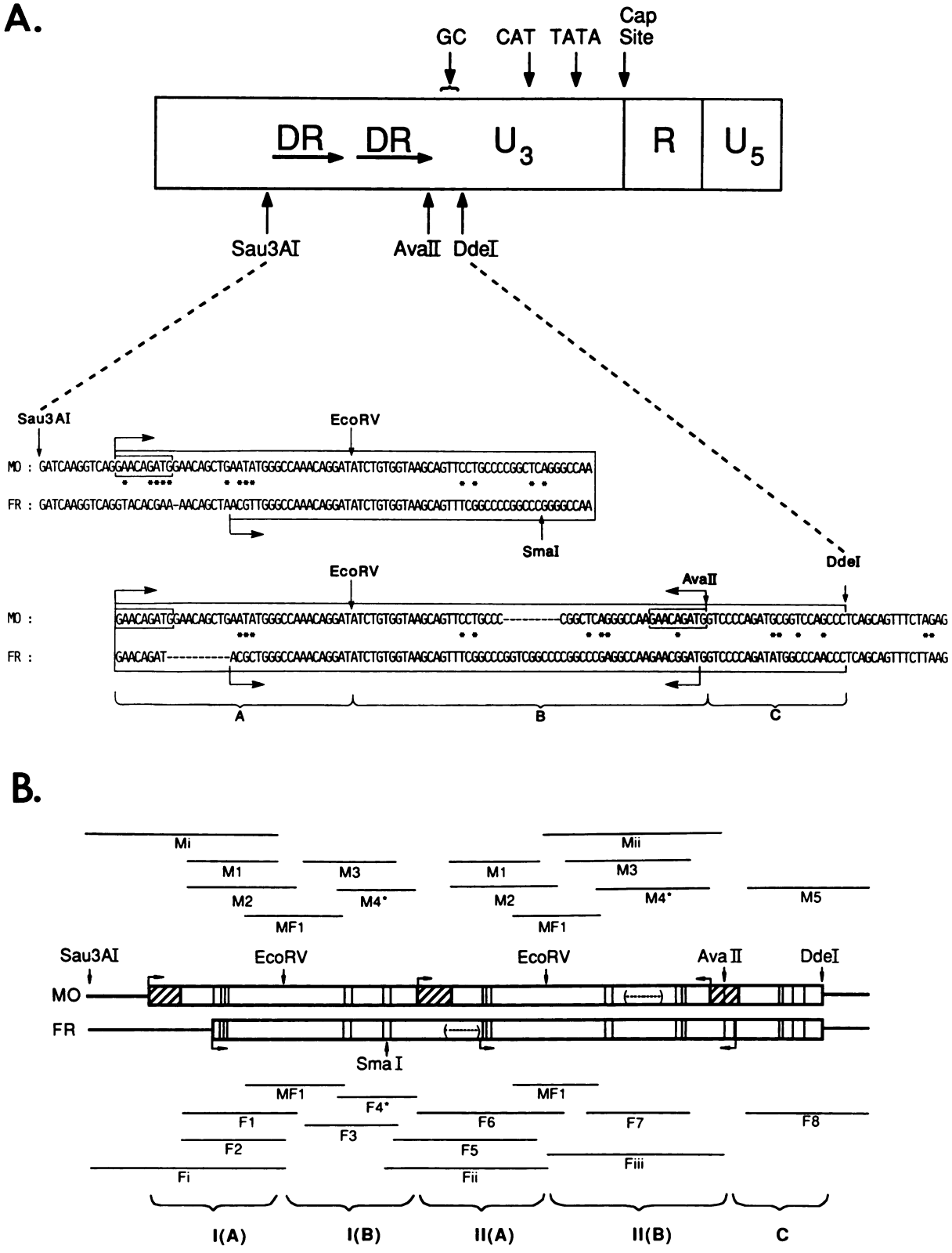
A.
GC
CAT
TATA
Cap Site
DR
DR
U3
R
U5
Sau3AI
AvaII
DdeI
Sau3AI
EcoRV
MO : GATCAAGGTCAGGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAA
FR : GATCAAGGTCAGGTACACGAA-AACAGCTAACGTTGGGCCAAACAGGATATCTGTGGTAAGCAGTTTCGGCCCCGGCCCGGGGCCAA
SmaI
DdeI
EcoRV
AvaII
MO :
FR :
GAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGCCC---------CGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGAG
GAACAGAT----------ACGCTGGGCCAAACAGGATATCTGTGGTAAGCAGTTTCGGCCCGGTCGGCCCCGGCCCGAGGCCAAGAACGGATGGTCCCCAGATATGGCCCAACCCTCAGCAGTTTCTTAAG
A
B
C
B.
Mi
Mii
M1
M3
M2
M4*
M5
MF1
Sau3AI
EcoRV
EcoRV
Ava II
DdeI
MO
FR
Sma I
F4*
F1
F6
F7
F8
F3
F2
F5
Fiii
Fi
Fii
I(A)
I(B)
II(A)
II(B)
C

TABLE 1. Sequences and locations of synthetic oligonucleotides

Oligonucleotide	Sense strand sequence (5′–3′)[a]	Location[b]
M1	AACAGCTGAATATGGGCCAAACAGGAT	7933–7959, 8008–8034
M2	AACAGCTGAATATGGGCCAAACAGGATATCTG	7933–7964, 8008–8039
M3	GGTAAGCAGTTCCTGCCCCGGCTCAGG	7966–7982, 8041–8067
M4*	gatccTCCTGCCCCGGCTCAGGGCCttG	7976–7998, 8051–8073
M5	CCCCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGAG	8084–8119
MF1	CCAAACAGGATATCTGTGGTAAGCAGTT	7949–7976, 8024–8051 (Moloney); 2914–2941, 2979–3006 (Friend)
F1	AACAGCTAACGTTGGGCCAAACAGGATATCTG	2898–2929
F2	AACAGCTAACGTTGGGCCAAACAGGAT	2898–2924
F3	GGTAAGCAGTTTCGGCCCCGGCCCGGG	2931–2957
F4*	gatccTTCGGCCCCGGCCCGGGGCCttG	2941–2963
F5	GGCCAAGAACAGATACGCTGGGCCAAACAGG	2957–2987
F6	AACAGATACGCTGGGCCAAACAGGATATCTG	2964–2994
F7	CAGTTTCGGCCCGGTCGGCCCCGGCCCGAGG	3002–3032
F8	CCCCAGATATGGCCCAACCCTCAGCAGTTTCTTAAG	3048–3083

[a] Wild-type viral sequences are shown in uppercase letters. Lowercase letters indicate single-stranded restriction endonuclease linker sequences on the 5′ side and the introduction of two A-to-T mutations near the 3′ ends of oligonucleotides M4* and F4*.

[b] Locations for M1, M2, M3, and M4* are given for both copies of the Moloney virus direct repeat and for MF1 for both the Moloney and Friend virus direct repeats. As the two copies of the Friend virus direct repeat have some sequence differences, each of oligonucleotides F1 through F7 is unique within the Friend virus enhancer. Locations given are taken from reference 58.

The 5′ halves of the Moloney virus direct repeat contain the sequence 5′-TGA(N6)GCCAA-3′, which was identified by Speck and Baltimore (53) as an NF1 binding site by sequence homology. Using radioactively labeled fragment Mi from Moloney virus region I(A) as a probe in gel shift assays, we found specific binding which was competed for by oligonucleotide M1 but not by F2, the corresponding oligonucleotide from Friend virus segment I(A) (data not shown). Methylation interference analysis showed interference at G residues in the consensus sequence consistent with NF1 binding. That this binding activity represents NF1 binding is further supported by competitions using a high-affinity NF1 site derived from the adenovirus origin of replication (7; data not shown; NF1 DNA kindly provided by L. Chodosh). Binding using radioactively labeled fragments Fi and Fii, which include the corresponding Friend virus sequences from segments I(A) and II(A), did not show any evidence of NF1 binding. Binding to these fragments is discussed in detail in a later section. As these fragments do not contain a good match for the consensus site and do not compete binding to fragment Mi or demonstrate NF1 binding, we conclude that the Friend virus enhancer does not bind NF1 at this site.

Both Moloney and Friend viruses contain the NF1 consensus sequence in the 3′ halves of their direct repeat sequences. Binding to these sequences was analyzed using fragments Mii and Fiii in gel shift assays with the WEHI 231 nuclear extract. A specific complex is seen which is shared by both fragments (Fig. 2A, lane 1, and Fig. 2B, lane 1, arrow). Oligonucleotide M1, which contains the NF1 site from the 5′ half of the Moloney virus direct repeat, can compete for complex formation on Mii and Fiii (Fig. 2A, lane 2, and Fig. 2B, lane 2); two other oligonucleotides, F4* and M4*, whose sequences are variants of sequences present in the 3′ halves of the Friend and Moloney virus direct repeats and which contain mutations in the NF1 consensus sequence (5′-GCCAA-3′ to 5′-GCCTT-3′), cannot (Table 1; Fig. 2A, lane 3, and Fig. 2B, lanes 3 and 5). The results of these competitions are consistent with the complex marked by arrows in Fig. 2 being due to NF1 binding. Methylation

FIG. 1. (A) Schematic diagram of the MuLV long terminal repeat and sequence alignment of the Moloney (MO) and Friend (FR) virus enhancer regions. U3 is approximately 450 bases long and contains the enhancer region, including the direct repeat (DR) and adjacent GC-rich region (GC), and the CAT and TATA sequences of the promoter region. Useful restriction sites near the boundaries of the enhancer region are shown. The sequences of the Friend and Moloney virus enhancer regions from *Sau*3AI to 16 bp beyond the *Dde*I restriction sites are shown, with sequence differences indicated by asterisks and gaps needed to maximize the sequence alignment indicated by dashes. Horizontal arrows indicate the boundaries of the direct repeats. The (A), (B), and (C) segments of the enhancer regions are indicated with braces below the figure. Consensus sequences for the glucocorticoid response elements in the Moloney virus enhancer are boxed, with the larger boxes outlining the region of interest in these studies. (B) Diagram showing locations within the Moloney and Friend virus enhancer regions of fragments and synthetic oligonucleotides used for binding and methylation interference experiments. The two copies of the direct repeat and the GC-rich region are shown as open boxes, with the direct repeat boundaries indicated by horizontal arrows. The (A) and (B) segments in each copy of the direct repeat and the (C) segment are indicated with braces below the diagram. The restriction sites shown are those used to generate probes for gel binding and methylation interference experiments. The *Sma*I site is present only in the Friend virus direct repeat; all other sites are present in both viruses. The Moloney virus direct repeat includes a nine-base glucocorticoid response element which is present in three copies and is indicated by hatched boxes. Sequence differences between the two enhancers are indicated by vertical bars. Dotted lines within parentheses indicate gaps in the sequence alignment. Fragments and synthetic oligonucleotides whose sequences are derived from the Moloney virus enhancer sequence are shown above the diagram; those whose sequences are derived from the Friend virus enhancer are shown below the diagram. The sequence of oligonucleotide MF1 is shared between Moloney and Friend virus. Fragments Mi, Mii, Fi, Fii, and Fiii were generated by cutting at the restriction sites indicated. Oligonucleotides M4* and F4* were made with *Bam*HI linkers on each end and identical A-to-T mutations in the NF1 consensus sequence, from 5′-GGCCAAG-3′ to 5′-GGCCTTG-3′ (Table 1); all other oligonucleotides were made with blunt ends. The locations of restriction fragments in the Moloney virus direct repeat are Mi (7911 to 7959) and Mii (8035 to 8081) and in the Friend direct repeat are Fi (2877 to 2924), Fii (2955 to 2989), and Fiii (2990 to 3045) (reference 58, p. 782 and 818). Locations and sequences of synthetic oligonucleotides are given in Table 1.

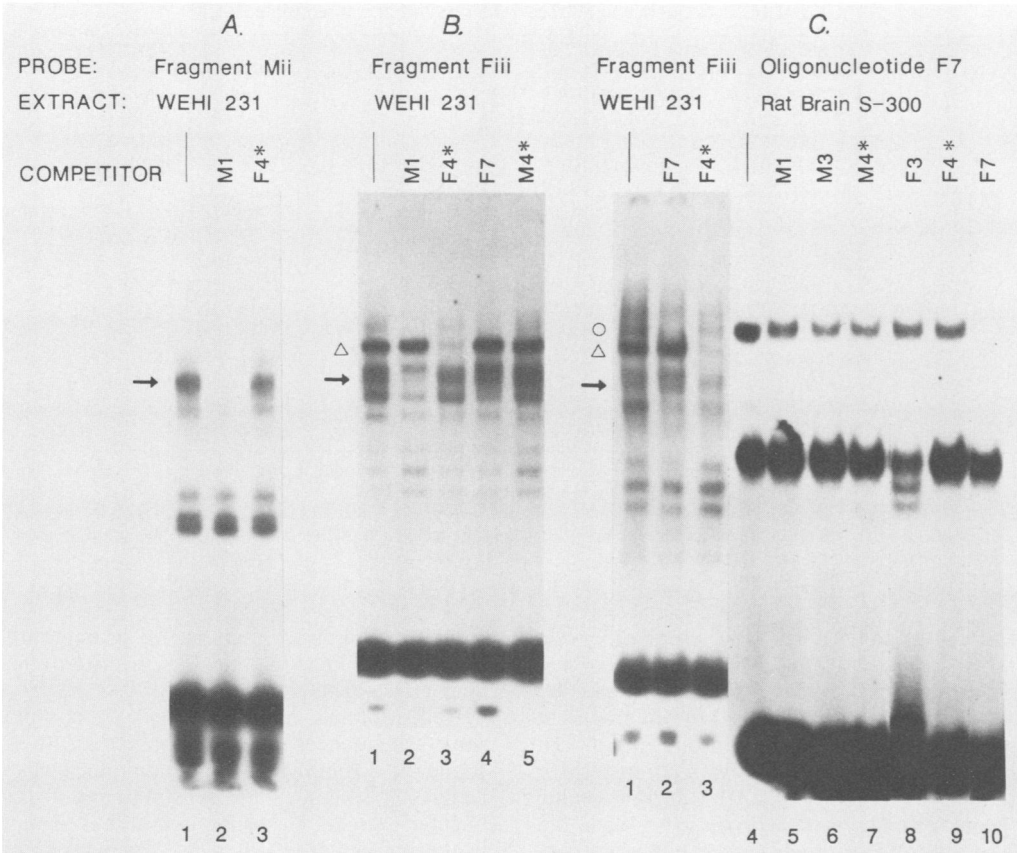

FIG. 2. Binding to region II(B) of the Moloney and Friend virus direct repeats: NF1, FVb2, and FVb1. Binding reactions contained 50,000 cpm of end-labeled fragment or oligonucleotide DNA as a probe, incubated with nuclear extract, poly(dI-dC)-poly(dI-dC) carrier DNA, and unlabeled oligonucleotide competitor DNAs as specified at the top of the figure. Reaction mixtures were electrophoresed through a low-ionic-strength 4% polyacrylamide gel as described in Materials and Methods. Free probe runs at the bottom of the gel. (A) Binding to Moloney virus fragment Mii: NF1. The end-labeled *Eco*RV-*Ava*II 47-bp fragment Mii was incubated with 15 μg of WEHI 231 nuclear extract, 1 μg of poly(dI-dC)-poly(dI-dC), and 100 ng of competitor oligonucleotides. Competitors: lane 1, no competitor; lane 2, oligonucleotide M1; lane 3, oligonucleotide F4*. Formation of the upper band indicated by the arrow is specifically inhibited by oligonucleotide M1, which includes an NF1 binding site. The lower bands in the gel were not competed for by the addition of any competitor DNAs. (B) Binding to Friend virus fragment Fiii: NF1 and FVb2. The end-labeled 56-bp fragment Fiii was incubated with 15 μg of WEHI 231 nuclear extract, 1 μg of poly(dI-dC)-poly(dI-dC), and 100 ng of competitor oligonucleotides, identical to binding reactions shown in panel A with Moloney virus fragment Mii as a probe. Competitors: lane 1, no competitor; lane 2, oligonucleotide M1; lane 3, oligonucleotide F4*; lane 4, oligonucleotide F7; lane 5, oligonucleotide M4*. The band indicated by the arrow is competed for specifically by oligonucleotide M1, but not by other competitor DNAs. The band indicated by an open triangle is competed for specifically by oligonucleotide F4*. (C) Binding to fragment Fiii and oligonucleotide F7: FVb1. Lanes 1, 2, and 3, binding to fragment Fiii as described in panel B. Competitors: lane 1, no competitor; lane 2, oligonucleotide F7; lane 3, oligonucleotide F4*. Bands indicated by the open triangle and the arrow are those described in panel B. Formation of the band indicated by the open circle is inhibited specifically by oligonucleotide F7, but not by oligonucleotide F4*; the autoradiograph of this gel was exposed longer than that shown in panel B to allow this band to be more visible. Lanes 4 to 10, End-labeled oligonucleotide F7 was incubated with 7 μg of rat brain S-300 (described in Materials and Methods), 1 μg of poly(dI-dC)-poly(dI-dC), and competitor oligonucleotides. Formation of the upper band was inhibited specifically by oligonucleotide F7 and has a mobility similar to that of the band indicated by the open circle which is formed with the fragment Fiii probe. The strong band in the middle of the gel is the result of binding to single-stranded DNA present in the probe preparation. Competitors: lane 4, no competitor; lane 5, oligonucleotide M1; lane 6, oligonucleotide M3; lane 7, oligonucleotide M4*; lane 8, oligonucleotide F3; lane 9, oligonucleotide F4*; lane 10, oligonucleotide F7.

interference analysis of this complex, using fragments Mii and Fiii as probes, also shows interference patterns consistent with NF1 binding (D. Mbangkollo and N. Manley, unpublished results). These data confirm the report of NF1 binding to the Moloney virus enhancer at this site and extend it to include the corresponding region of Friend virus.

Identification of the FVb2 binding site. We identified a complex which forms over a sequence in both 3′ halves of the Friend virus direct repeat but cannot bind to the corresponding region of Moloney virus. Using the WEHI 231 extract and fragment Fiii as a probe, we detected a major band (Fig. 2B, lane 1, △) that is not detected using fragment Mii (Fig. 2A, lane 1). This complex, which we refer to as FVb2, is competed for by oligonucleotide F4* (Fig. 2B, lane 3), which is derived from the 3′ half of the first copy of the Friend virus direct repeat. M1, which contains the 5′ Moloney NF1 site, or M4*, which covers the Moloney virus sequence corresponding to F4*, cannot compete with Fiii for formation of the complex (Fig. 2B, lanes 2 and 5). The F7 oligonucleotide, which includes most of the sequence in Friend virus segment II(B) which corresponds to the F4* sequence, also does not compete for binding of this complex (Fig. 2B, lane 4). Methylation interference analysis of this complex, using fragment Fiii as a probe, shows that meth-

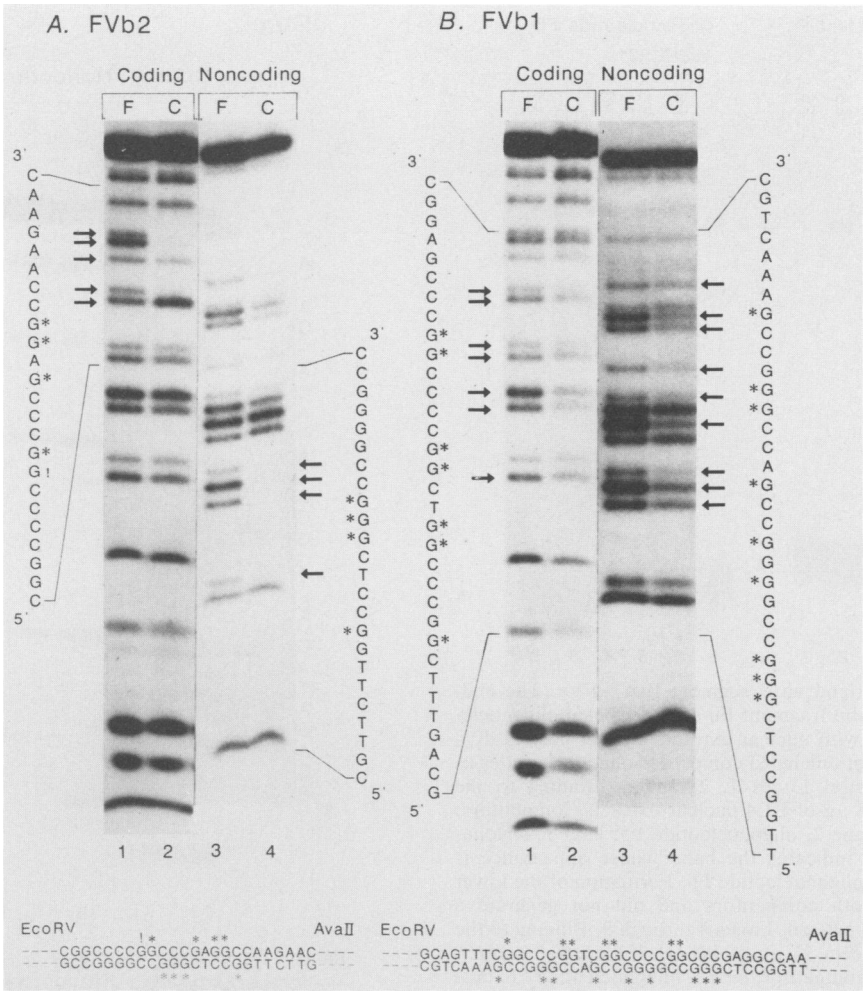

FIG. 3. Methylation interference analysis of novel factors binding to the Friend virus enhancer in segment II(B): methylation interference patterns for the FVb2 and FVb1 complexes (△ and ○, Fig. 2C, lane 1), using the WEHI 231 nuclear extract. Typical preparative binding reactions contained 750,000 to 2,000,000 cpm and 80 to 150 μg of nuclear extract in a final volume of 100 to 200 μl. Fragment Fiii was end labeled at the *Eco*RV site to visualize the coding strand and at the *Ava*II site for the noncoding strand. Free DNA and protein-DNA complex bands are indicated by F and C above the lanes; lanes 2 and 4 in each panel correspond to the protein-DNA complex; lanes 1 and 3 correspond to the free DNA bands from the same binding reactions. Arrows indicate bands in the sequence ladder involved in the methylation interference pattern. Methylation of G residues indicated by asterisks results in the inhibition of specific complex formation. The exclamation point indicates the location of a G residue whose methylation enhances binding of the specific complex. Bands in the complex lane which appear to be reduced but are outside the indicated limits of the interference pattern are due to underloading of the lane and are not reproducible. (A) Analysis of the FVb2 complex formed with fragment Fiii (△, Fig. 2C, lane 1). Preparative binding reactions contained 200,000 cpm, 30 μg of WEHI 231 nuclear extract, and 7 μg of poly(dI-dC)-poly(dI-dC). (B) Methylation interference pattern from the FVb1 complex formed with fragment Fiii (○, Fig. 2C, lane 1). Reactions contained 200,000 cpm, 24 μg of WEHI 231 nuclear extract, and 4 μg of poly(dI-dC)-poly(dI-dC). A 200-ng amount of unlabeled oligonucleotide M1 was also included to minimize NF1 complex formation. Partial interference at G residues involved in specific binding was routinely obtained; this result may be due to the low intensity of the specific band relative to the background signal.

ylation of G residues over a 9-bp sequence located entirely within the 3′ NF1 site interferes with specific binding, while methylation of a base on the 5′ border of this sequence results in enhanced binding (Fig. 3A). Binding experiments performed using the F4* oligonucleotide as a probe, which has a one-nucleotide difference in its putative FVb2 binding site relative to the corresponding sequence in the second copy, show a single major band which is indistinguishable by competition and methylation interference analysis from that formed with the larger fragment Fiii (data not shown). These experiments demonstrate that the single base change in the FVb2 binding site between the first and second copies of the Friend virus direct repeat does not appreciably affect the specific binding of the FVb2 complex.

Identification of the FVb1 binding site. Segment II(B), in addition to several base differences between Moloney and Friend virus, contains a 9-bp insertion in Friend virus relative to Moloney virus, which is not present in Friend virus segment I(B). The insertion results in a near-perfect duplication of a 10-bp sequence that is present just once in Friend virus segment I(B), 5′-TCGGCCCCGG-3′. Gel binding using the WEHI 231 extract with Friend virus fragment Fiii shows a specific band unique to this Friend virus segment II(B) probe (Fig. 2C, lane 1, ○). As this band is much fainter than the FVb2 band, a longer autoradiograph exposure is needed to clearly visualize it. Formation of this low-mobility Friend virus-specific band can be inhibited by unlabeled oligonucleotide F7, whose sequence includes the

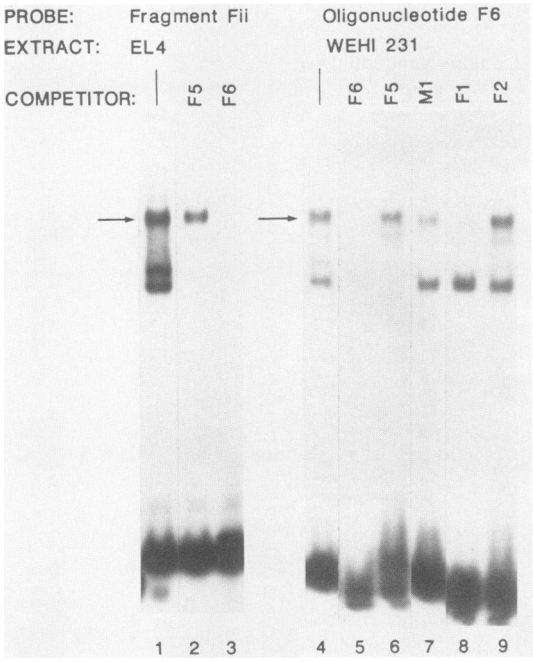

FIG. 4. Binding to Friend virus segment II(A): FVa. The end-labeled *Sma*I-*Eco*RV 35-bp fragment Fii or end-labeled oligonucleotide F6 was incubated with nuclear extract, 1 μg of poly(dI-dC)-poly(dI-dC), and 100 ng of unlabeled competitor oligonucleotides as indicated above the figure. Lanes 1, 2, and 3, Binding to the fragment Fii probe in 15 μg of EL4 nuclear extract. Competitors: lane 1, no competitor; lane 2, oligonucleotide F5; lane 3, oligonucleotide F6. The arrow indicates the band whose appearance is specifically inhibited by oligonucleotide F6. Formation of the lower band is inhibited by both competitors and did not generate a methylation interference pattern. Lanes 4 through 9, Binding to the oligonucleotide F6 probe in 16 μg of WEHI 231 nuclear extract. Competitors: lane 4, no competitor; lane 5, oligonucleotide F6; lane 6, oligonucleotide F5; lane 7, oligonucleotide M1; lane 8, oligonucleotide F1; lane 9, oligonucleotide F2.

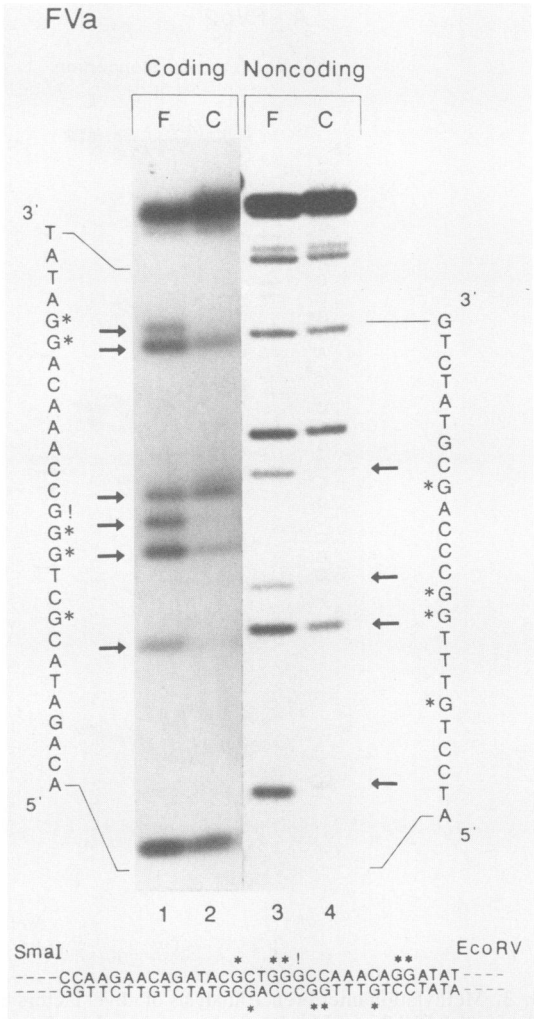

FIG. 5. Methylation interference analysis of FVa binding to Friend virus enhancer segment II(A). The methylation interference pattern on the coding strand, using the F6 oligonucleotide as a probe (Fig. 4, lane 4) and the EL4 nuclear extract, is shown; the pattern on the noncoding strand is shown using fragment Fii end labeled at the *Eco*RV site as a probe (Fig. 4, lane 1) and the TSA9 nuclear extract (results for both strands of both oligonucleotide and plasmid-derived probes are indistinguishable in EL4 and TSA9 extracts). Coding strand reactions contained 500,000 cpm, 30 μg of EL4 nuclear extract, and 4 μg of poly(dI-dC)-poly(dI-dC). Noncoding strand reactions contained 250,000 cpm, 28 μg of TSA9 nuclear extract, and 5 μg of poly(dI-dC)-poly(dI-dC). F and C, arrows, asterisks, and the exclamation point are as in Fig. 3.

insertion and all but the most 3′ of the base differences between Moloney and Friend virus in this II(B) segment (Fig. 2C, lane 2). This complex is not competed for by the F4* oligonucleotide, which contains the FVb2 site from segment I(B) (Fig. 2C, lane 3). Methylation interference analysis, using fragment Fiii as a probe, identifies G residues involved in specific binding extending over the entirety of the duplicated 10-bp sequence (Fig. 3B). We will refer to this as the FVb1 binding site. To further characterize the specificity of binding to the FVb1 site, binding experiments were performed using the F7 oligonucleotide as a probe, thereby isolating the FVb1 complex from the FVb2 and NF1 complexes which also form on fragment Fiii. The F7 oligonucleotide probe generated a specific band of the same mobility and specificity as that formed with the fragment Fiii probe (Fig. 2C, lane 4). Binding of this complex on F7 is inhibited efficiently by unlabeled F7 (Fig. 2C, lane 10), but not by M1, which binds NF1, M3, which covers the Moloney virus sequence corresponding to F7, or M4*, which overlaps the sequence considerably (Fig. 2C, lanes 5, 6, and 7).

To date, we have been unable to detect any binding over the 10-bp sequence, 5′-TCGGCCCCGG-3′, in Friend virus segment I(B) which is included in the FVb1 site in segment II(B). The F3 and F4* oligonucleotides, both of which contain this 10-bp sequence, do not block binding to the FVb1 site (Fig. 2C, lanes 8 and 9). Direct binding to the F3 oligonucleotide or to a plasmid-derived Friend virus *Eco*RV-*Eco*RV probe generates no observable specific complexes binding over this sequence (data not shown). These results show that there is a difference in the proteins which can bind to the two copies of the Friend virus direct repeat, determined by the presence of the 9-bp insertion in the second copy. Although we did not detect it, it is possible that there is a binding activity which recognizes the sequence present in the first copy, perhaps requiring more extensive flanking sequences or different binding conditions to be detected in the mobility shift assay.

Characterization of FVa binding. To analyze the binding of factors to the 5′ halves of the Friend virus direct repeat sequence, gel shift assays were performed using fragments

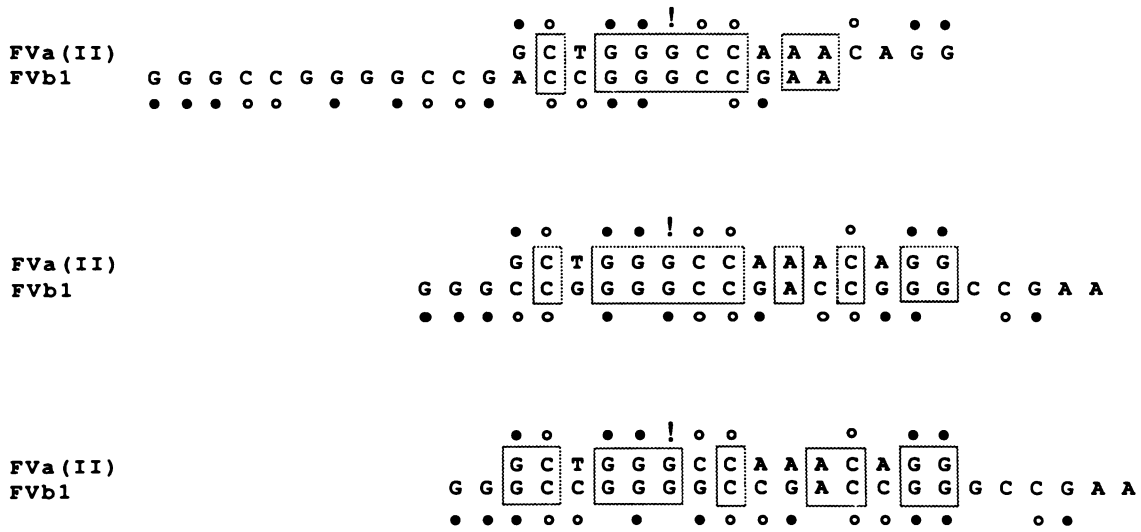

FIG. 6. Sequence alignments of the FVaII and FVb1 binding sites. The coding strand sequence of the FVa site in segment II(A) is shown in three different alignments with the noncoding strand sequence of the FVb1 binding site. Both sequences are written in a 5′-3′ orientation. Sequence identities between the two binding sites are shown by boxes. Symbols: ●, G residues on the strand shown important for binding as identified by methylation interference experiments; ○, important G residues on the opposite strands.

Fi and Fii. These fragments include segments I(A) and II(A), respectively, of the Friend virus enhancer. A strong specific complex is seen on fragment Fii (Fig. 4, lane 1, arrow) where only a faint smear is seen on fragment Fi (data not shown). The fragment Fii complex is competed for by oligonucleotide F6, which extends just beyond the *Eco*RV site in the second copy of the direct repeat, but not by F5, which ends just before the *Eco*RV site (Fig. 4, lanes 2 and 3; Table 1). Binding experiments using oligonucleotide F6 as a probe yield a complex of mobility and specificity similar to that seen with the fragment Fii probe (Fig. 4, lane 4); F6 inhibits formation of this complex, but F5 does not (Fig. 4, lanes 5 and 6). Oligonucleotide M1, which includes the Moloney (A) sequence terminating at the *Eco*RV site and which corresponds to the Fii probe, also does not efficiently compete with F6 in complex formation (Fig. 4, lane 7). To more precisely identify the sequences involved in binding, methylation interference analysis was performed using both fragment Fii and oligonucleotide F6 as probes. Both probes gave the same result; data are shown for the oligonucleotide F6 probe for the sense strand and for the fragment Fii probe for the antisense strand (Fig. 5). We will refer to this sequence as the FVa binding site.

Comparison of the FVa site identified in segment II(A) with the corresponding sequence in the first copy of the Friend virus direct repeat shows a single base difference between the two, a C-to-T change in the second base of the binding site. The base change occurs at a residue which is identified by methylation interference analysis as being involved in binding in the segment II(A) FVa binding site. This sequence difference may lower the affinity of the binding site in segment I(A) such that cutting at the *Eco*RV site in the first copy of the direct repeat drastically reduces FVa binding. Oligonucleotide F2, which ends at the first-copy *Eco*RV site, does not inhibit binding to labeled F6 (Fig. 4, lane 9), although weak binding is seen using either oligonucleotide F2 or fragment Fi as a probe, and methylation interference analysis of this binding shows weak interference consistent with FVa binding (data not shown). However, oligonucleotide F1, which extends beyond the *Eco*RV site in the first copy of the direct repeat, comparable to the F6 oligonucleotide from the second copy, is able to compete with F6 for FVa binding (Fig. 4, lane 8) and generates a band of the same mobility and specificity when used as a probe in the gel shift assay (data not shown). As F1 and F6 behave indistinguishably in binding and competition experiments, the sequence difference in the segment I(A) FVa binding site only significantly affects binding in the gel shift assay when the binding site is terminated at the *Eco*RV site. In the context of the second-copy sequence, binding is not affected by *Eco*RV digestion. In the intact enhancer, segments I(A) and II(A) might be equivalent in their ability to bind FVa.

The above data raise the question of whether the corresponding Moloney virus sequence, which has two base differences with both of the Friend FVa sequences, would be able to bind this activity if the Moloney virus sequence were extended past the *Eco*RV site. Binding to this region of the Moloney virus direct repeat with respect to FVa binding is discussed in the next section.

Cross competitions between binding sites. A comparison of the FVa and FVb1 binding sites reveals an interesting sequence similarity between the two. Two different alignments of the sense strand of the FVa site and the antisense strand of the FVb1 site produce matches of 10 of 15 bases; another alignment produces a partial match of 8 of 11 bases (Fig. 6). To investigate the possible relationship between these binding sites, a competition analysis was performed in which a panel of oligonucleotides were tested for their ability to inhibit complex formation on the F7 oligonucleotide containing the FVb1 binding site (Fig. 7A). Binding of the FVb1 complex was competed for efficiently by F1 and F6, which contain the FVa binding sites from the two copies of the direct repeat (Fig. 7A, lanes 3 and 5). F2 and F5, which do not bind FVa (Fig. 4), also do not interfere with formation of the FVb1 complex (Fig. 7A, lanes 4 and 6). Surprisingly, the MF1 oligonucleotide, which contains sequences around the *Eco*RV site common to both Moloney and Friend virus and has no obvious sequence homology to the F7 oligonucleotide, inhibits FVb1 complex binding (Fig. 7A, lane 7).

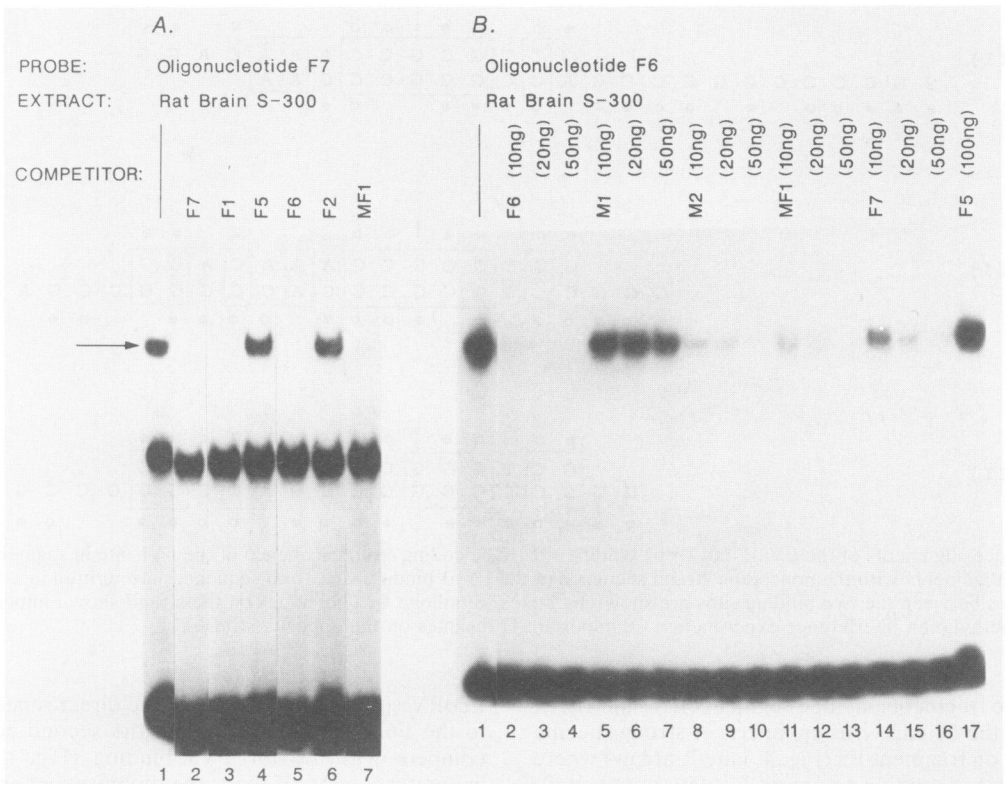

FIG. 7. Cross-competition analysis of binding to oligonucleotides containing FVa, FVb1, and LVb binding sites. (A) Binding to end-labeled oligonucleotide F7, containing the FVb1 binding site, was competed for by unlabeled oligonucleotides identified above the figure. The specific band is indicated by the arrow. Binding reactions contained 7 μg of rat brain S-300, 1 μg of poly(dI-dC)-poly(dI-dC), and 100 ng of unlabeled competitor oligonucleotides. Competitors: lane 1, no competitor; lane 2, oligonucleotide F7; lane 3, oligonucleotide F1; lane 4, oligonucleotide F5; lane 5, oligonucleotide F6; lane 6, oligonucleotide F2; lane 7, oligonucleotide MF1. (B) Titration of specific competitions of binding to oligonucleotide F6. Binding reactions contained end-labeled oligonucleotide F6, which contains the FVa binding site from Friend virus segment II(A), 7 μg of rat brain S-300, 1 μg of poly(dI-dC)-poly(dI-dC), and unlabeled competitor oligonucleotides as indicated above the figure. The reaction in lane 1 contained no competitor. Reactions in lanes 2 through 16 included 10, 20, and 50 ng of unlabeled oligonucleotides F6, M1, M2, MF1, and F7 as indicated above the figure. The reaction in lane 17 contains 100 ng of unlabeled oligonucleotide F5, which contains an FVa site which is truncated on its 3′ side and does not inhibit binding of the FVa complex.

The sequences in this oligonucleotide include a binding site for a ubiquitous factor, LVb, identified by Speck and Baltimore (53) as binding to the Moloney virus enhancer at the *Eco*RV site, as well as a simian virus 40-like "core" sequence (8, 28, 53). Binding to the LVb binding site was not previously seen in this study as our plasmid-derived probes all terminate at the *Eco*RV site, which eliminates LVb binding (53). It was also observed that the specific complexes formed with the MF1, F6, and F7 oligonucleotide probes have similar mobilities in the gel shift assay.

To determine the relative affinities of the F6, F7, and MF1 oligonucleotides for binding of the FVa-FVb1 complex, competitions were titrated from 10 to 100 ng of unlabeled competitor, testing the ability of each oligonucleotide to compete for binding to the other two. Oligonucleotide M2, which contains the Moloney virus sequence extending beyond the *Eco*RV site corresponding to Friend virus oligonucleotides F1 and F6 (see previous section), was also included. Results shown in Fig. 7B for the F6 probe are representative. All four oligonucleotides, F6, F7, MF1, and M2, show significant ability to inhibit complex formation at the lowest level of competition, 10 ng (Fig. 7B, lanes 2, 8, 11, and 14). Competitors which contain only the Moloney virus 5′ NF1 binding site (terminating at the *Eco*RV site) or a truncated FVa site do not compete for binding appreciably, even at 100 ng of competitor (Fig. 7B, lanes 5, 6, 7, and 17). These results suggest that the FVa, FVb1, and LVb binding sites are being recognized by the same factor or that the binding activities share a common limiting factor.

The question then remains whether the competition by Moloney virus oligonucleotide M2 is due to the presence of the LVb site or to binding to the Moloney virus sequence corresponding to the FVa site, which contains two-base differences from the Friend virus FVaI and FVaII sequences, both at residues identified by methylation interference as being important for binding. Methylation interference analysis of binding to this region of the Moloney virus enhancer is inconclusive; binding to the NF1 site largely obscures any potential binding over the putative FVa sequence, as the complexes have similar mobilities when oligonucleotide M2 is used as a probe. Although the competition results rule out the possibility that the competition of binding to the F6 probe by oligonucleotide M2 is due to NF1 binding, conclusive evidence concerning the binding of FVa-FVb1-LVb to the Moloney virus enhancer I(A) region will have to await the availability of a more purified protein fraction.

Identification of the FVc binding site. There are four-base differences between the Moloney and Friend virus sequences in segment C, which is defined as a 22-bp segment immediately 3′ of the direct repeat, from the *Ava*II site to the *Dde*I site. Because of the lack of convenient restriction sites

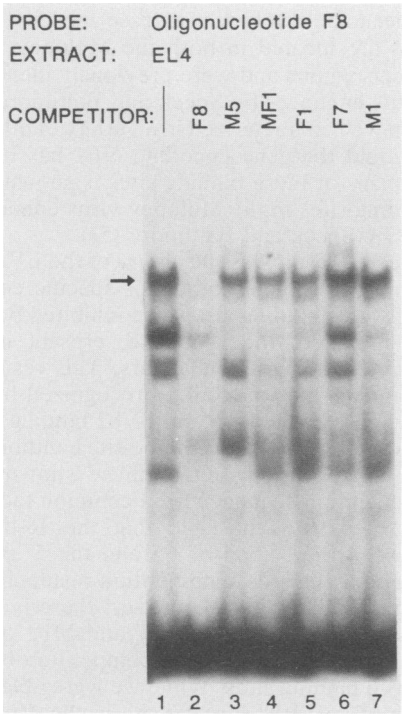

FIG. 8. Binding to Friend virus enhancer segment C: FVc. End-labeled oligonucleotide F8 (50,000 cpm) was incubated with 12 μg of EL4 extract, 1 μg of poly(dI-dC)-poly(dI-dC), and 100 ng of unlabeled competitor oligonucleotides as indicated above the figure. The specific band is indicated with an arrow. Competitors: lane 1, no competitor; lane 2, oligonucleotide F8; lane 3, oligonucleotide M5; lane 4, oligonucleotide MF1; lane 5, oligonucleotide F1; lane 6, oligonucleotide F7; lane 7, oligonucleotide M1.

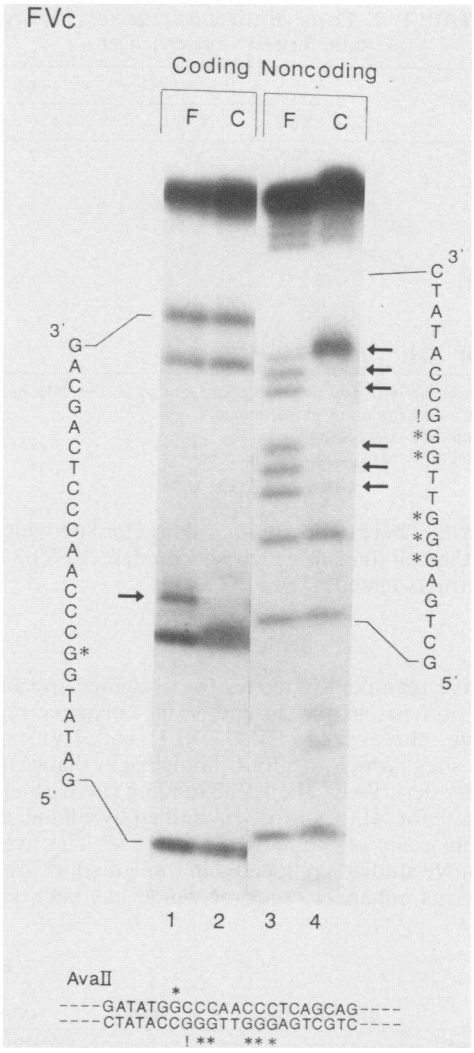

FIG. 9. Methylation interference analysis of specific binding to Friend virus enhancer segment C: methylation interference assay on the FVc complex formed with oligonucleotide F8 (arrow, Fig. 8, lane 1). Reactions contained 450,000 cpm, 30 μg of EL4 nuclear extract, and 3 μg of poly(dI-dC)-poly(dI-dC). F and C, arrows, asterisks, and the exclamation point are as in Fig. 3.

in the two viruses in this region, double-stranded synthetic oligonucleotides M5 and F8 were made containing a 36-bp sequence from downstream of the direct repeats of the Moloney and Friend virus enhancers, respectively (Fig. 1B; Table 1). Gel shift assays were performed using radioactively labeled F8 as a probe. A single specific complex is formed and this complex formation is inhibited by the addition of unlabeled oligonucleotide F8 but not by oligonucleotide M5, containing the corresponding Moloney virus sequence (arrow, Fig. 8, lanes 1, 2, and 3). This complex is also not competed for by MF1, which contains the binding sites for LVb and core (see previous section), by F1, which contains the FVa site from Friend segment I(A), by F7, which contains the FVb1 site from Friend segment II(B), or by M1, which contains the NF1 binding site from the first half of the Moloney virus direct repeat (Fig. 8, lanes 4, 5, 6, and 7). Methylation interference analysis identifies G residues important for binding in a 9-bp sequence, 5′-GCCC AACCC-3′ (Fig. 9), designated the FVc binding site.

Tissue distribution of factors binding to the Friend virus enhancer. Extracts from cell lines and primary tissues representing a variety of hematopoietic lineages and other tissue types were tested for the presence of the FVa, FVb1, FVb2, and FVc complexes. Binding was tested using approximately 16 μg of nuclear extract from the following hematopoietic extracts: CB5 (BFU-E), TSA9 (CFU-E) BALB/c spleen, EL4 (T cell), BW (T cell), WEHI 231 (B cell), and U937 (human macrophage precursor). Binding to rat brain and calf brain primary cell extracts and HeLa, F9, and BALB/c 3T3 cell line extracts was also tested. Probes made from oligonucleotides F4* and F7 were used to detect FVb2 and FVb1 complex binding to avoid complications which could arise from multiple factors competing for binding to the longer fragment Fiii probe. Oligonucleotides F5 and F8 were used to detect FVa and FVc complex binding, respectively. Bands with mobilities corresponding to FVa, FVb1, and FVc complex binding were detected in all extracts tested, with no significant differences in abundance (Table 2).

The results of the tissue distribution analysis of FVb2 yielded quite a different result. The FVb2 complex was detected strongly only in the WEHI 231 (mouse B-cell), HeLA (human epithelial), and BALB/c 3T3 (mouse fibroblast) extracts (Table 2). No specific binding to this site was seen in a variety of other cell lines, including, in addition to those listed in the previous paragraph, PD, 38B9, and lipopolysaccharide-induced and uninduced 70Z (pre-B-cell lines), ED2 (B cell), SP2/D (myeloma), ψ2 (fibroblast), and L cells (additional extracts kindly provided by N. Speck) (data

TABLE 2. Tissue distribution of factors binding to the Friend virus enhancer

Extract	Binding activity[a] of factor:			
	FVa	FVb1	FVb2	FVc
CB5	+[b,c]	+[b]	−	+[b]
TSA9	+[b,c]	+[b,c]	−	+[c]
Spleen	+[b]	+	−	+
EL4	+[b,c]	+[b,c]	−	+[b,c]
BW	+	+[b]	−	+
WEHI 231	+[b,c]	+[b,c]	+[b,c]	+
U937	+	+	−	+
HeLa	+	+	+	ND
BALB/3T3 A31	+[b]	+	+[b]	+

[a] +, Binding activity present; −, binding activity absent; ND, not done. See Results for a description of extracts used.
[b] Confirmed by competitions.
[c] Confirmed by methylation interference.

not shown). There is no obvious characteristic which distinguishes the cell lines in which we can detect FVb2 from the other extracts tested.

DISCUSSION

We have identified five sites for sequence-specific DNA-binding proteins in the Friend virus enhancer region. Of these five, three sites, FVa, FVb1, and FVc, represent binding sites which, to our knowledge, have not been previously described. The FVb2 binding site is similar to the binding site for NF(m)-c-fos2, identified by Gilman et al. (21) 138 bp upstream of the mouse c-*fos* gene. The five binding sites that we studied are located in three distinct areas of the Friend virus enhancer, each of which has been shown by genetic studies to be involved in disease specificity (22). NF1 binding sites are located in both the Moloney and Friend virus enhancer regions and were previously identified in the Moloney virus enhancer by Speck and Baltimore (53). NF1 has also been well characterized in a variety of other systems (30, 33, 42), and the gene encoding NF1 has been cloned (48). A summary of these binding sites is shown in Fig. 10, with the binding sites in the Moloney virus enhancer shown as identified by Speck and Baltimore (53).

An unexpected result was the ability of the FVa and FVb1 sites to compete with each other for specific complex formation and for this binding also to be inhibited by a competitor corresponding to the LVb site, present in both the Moloney and Friend virus enhancers. This result suggests that these binding sites may all be recognized by the same factor. In the case of the FVa and FVb1 binding sites, there are sequence similarities between the sites, although it is not clear which alignment, if any of those shown in Fig. 6, reflects a recognition sequence for a common factor binding to the two sites. It is possible that the 16-bp-sequence overlap between the 3′ end of F6 and the 5′ end of MF1 could account for the cross-competition results between the LVb and FVa binding sites. However, there is no obvious sequence similarity between the F7 and MF1 oligonucleotides which could account for the competition between the FVb1 and LVb binding sites. While we were able to rule out the presence of an FVb1 binding site in the Moloney virus enhancer, the presence of an FVa site in the Moloney virus enhancer was more difficult to determine. Although we were unable to conclusively demonstrate binding to the sequence in the 5′ half of the Moloney virus enhancer corresponding to the FVa site, the presence of the NF1 and LVb sites in this region of the Moloney virus enhancer overlapping the putative FVa sequence made competition and methylation inter-

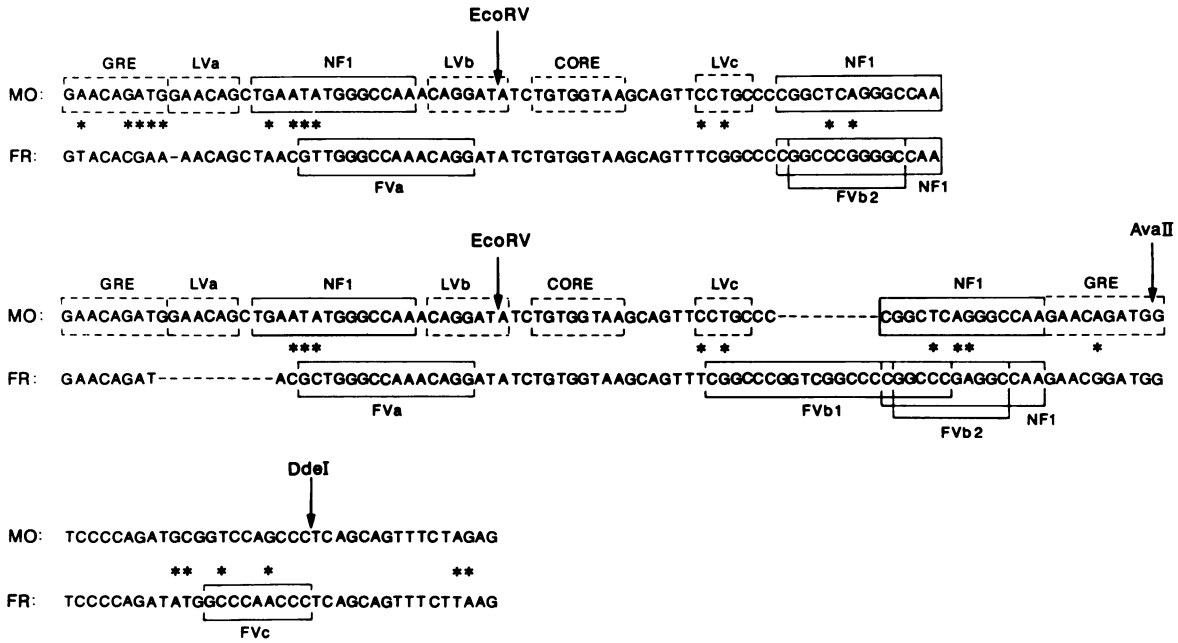

FIG. 10. Comparison of binding sites in the Moloney (MO) and Friend (FR) virus direct repeats. The aligned sequences of the Moloney and Friend virus direct repeats are shown; sequence differences are indicated by asterisks; gaps needed to optimize the alignment are indicated by dashes. Broken lines delineate binding sites in Moloney virus for the glucocorticoid receptor (GRE), core-binding protein (core), LVa, LVb, and LVc as reported by others (10, 28, 53). Solid lines represent binding sites confirmed or identified in this report. Note that although the LVb and core-binding sites are present in the Friend virus enhancer, binding to these sequences was not directly tested in this study.

ference results difficult to interpret. We were thus unable to rule out the possibility of specific binding of the FVa factor to this Moloney virus sequence. Further purification and characterization of the FVa and LVb binding activities will be necessary to conclusively address this issue.

Attempts to align the sequences of the FVa, FVb1, and LVb binding sites to account for the competition results obtained could be misleading. Even for FVa and FVb1, which can be aligned to some degree of homology, the binding sites as defined by methylation interference are different both in size and in the pattern of G residues important for complex formation. Specific binding of a single protein to two different recognition sequences has been described in several cases (1, 9, 40, 46), and multiple proteins which recognize the same or similar binding sites have also been described (14, 17, 56, 59). It is also possible that the competitions observed between the FVa, FVb1, and LVb sites could be the result of strong cooperative interactions between a common factor in each complex and a distinct factor or factors which change the sequence specificity of the binding complex. This type of cooperative interaction which alters binding specificity has been described in yeast cells (2, 23).

A comparison of the binding sites identified in the Friend MuLV enhancer with those reported by Speck and Baltimore (53) in the Moloney virus enhancer shows that there are both common and distinct binding sites between the two enhancers (Fig. 10). Both enhancers share a region of sequence identity in the center of each direct repeat, including the binding sites for the LVb and core factors, previously identified in the Moloney virus enhancer (8, 28, 53). Both sites are present in the Friend virus enhancer by sequence homology, although binding to these two sites in the context of flanking Friend virus sequences was not tested directly in this study, except for the experiments involving FVa binding. Besides, potentially, the LVb and core sites, both enhancers also share an NF1 site in the 3′ half of each direct repeat. A sequence comparison of the enhancers of a wide variety of mammalian type C retroviruses shows that these three sites, including their relative positions within the enhancer, are widely conserved (E. Golemis, N. A. Speck, and N. Hopkins, manuscript in preparation).

It is possible that differences in the binding of factors to adjacent sequences in the enhancer could affect the pattern of binding over the central region of sequence identity between the two viruses. The FVa site, for example, extends for 13 bp into this region, overlapping the LVb site and ending only 5 bp from the core homology. Thus, although the Moloney and Friend virus enhancers are highly homologous at the nucleotide sequence level and share some common binding sites, the patterns of proteins binding to them, and possibly the mechanisms by which they influence gene expression, may be quite different.

In the 5′ (A) and 3′ (B) segments flanking the LVb-core region in each repeat, there are several sequence differences between the Friend and Moloney virus enhancers. In the (A) and (B) segments, Moloney virus contains binding sites for factors LVa and LVc (53). We failed to detect LVa or LVc complex binding to the Friend virus direct repeat under the binding conditions used in this study. As binding to these two sites in the Moloney virus enhancer was only detected clearly in column fractions by Speck and Baltimore (53), and not in crude extracts, we cannot exclude the possibility that these sites are present in the Friend virus enhancer. However, the sequences in the Friend virus enhancer corresponding to the LVa and LVc binding sites both contain base differences at G residues shown by methylation interference to be important for specific binding and thus are unlikely to bind these factors. In the case of the LVc site in the second copy, these base differences include the 5′ end of the FVb1 binding site.

A striking feature of the Moloney virus enhancer is the presence of three glucocorticoid response elements (10, 53). In the Friend virus enhancer the most 5′ of these is entirely absent, while the central and 3′ sequences both contain base differences from the consensus sequence 5′-AGAACA GATG-3′ (10). Whether these base differences affect glucocorticoid receptor binding to the Friend virus enhancer was not determined in our studies.

A computer-generated sequence comparison of the enhancer regions of 35 mammalian type C retroviruses shows an interesting distribution for the binding site sequences present in the Friend virus enhancer. The enhancer of Rauscher MCF virus, which causes almost exclusively erythroid disease, contains perfect matches for the FVa, FVb2, and FVc binding sites. While the FVb1 site is unique to the Friend virus enhancer because of the 9-bp insertion, the 10-bp sequence that is imperfectly duplicated in the FVb1 site is also present in the Rauscher MCF virus enhancer. Perfect matches or single-base variations of the FVa, FVb2, and FVc sites and the 10-bp sequence are also present in the enhancers of Rauscher leukemia virus, CasBRE, and amphotrophic 4070A virus, all of which have been shown to induce erythroid disease under some conditions with various frequencies (43, 55; Hartley, unpublished observations). This distribution supports the hypothesis that these binding sites are involved in influencing the ability of Friend MuLV to induce erythroid disease.

As tissue distribution analysis of the FVa, FVb1, and FVc complexes shows them to be present in all of the hematopoetic lineage tumor cell lines tested, the way in which these sites could influence erythroid disease specificity is not straightforward. It seems unlikely that the unusual cell line distribution of the FVb2/NF(m)-c-fos2 factor is the sole determinant for the Friend virus enhancer specificity, as the cell lines in which its binding activity is present have no obvious common characteristic linking them to the disease specificity phenotype of the Friend virus enhancer, and the binding site is present in only one of the segments shown to contribute to disease specificity. Relatively small differences in the abundance of other binding factors may have a large tissue-specific effect on enhancer activity in vivo. It is also possible that the use of tumor cell lines or limitations of the binding conditions used in this study prevented us from detecting a restricted distribution which may be present in primary cells or possibly in a small population of erythroid precursor cells critical to the development of erythroid disease. A determination of the role, it any, that the binding sites identified in this report have in determining the disease specificity of the Friend virus enhancer will be more directly addressed by the construction of mutant viruses containing point mutations in these binding sites, coupled with biochemical characterization of the binding activities.

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LITERATURE CITED

1. **Baumruker, T., R. Sturm, and W. Herr.** 1988. OBP100 binds remarkably degenerate octamer motifs through specific interactions with flanking sequences. Genes Dev. **2**:1400–1413.
2. **Bender, A., and G. F. Sprague, Jr.** 1987. MAT α1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. Cell **50**:681–691.
3. **Bosze, Z., H. J. Thiesen, and P. Charnay.** 1986. A transcriptional enhancer with specificity for erythroid cells is located in the long terminal repeat of the Friend murine leukemia virus. EMBO J. **5**:1615–1623.
4. **Celander, D., and W. A. Haseltine.** 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukemogenic potential of murine retroviruses. Nature (London) **312**:159–162.
5. **Chatis, P. A., C. A. Holland, J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1983. Role for the 3′ end of the genome in determining disease specificity of Friend and Moloney murine leukemia viruses. Proc. Natl. Acad. Sci. USA **80**:4408–4411.
6. **Chatis, P. A., C. A. Holland, J. E. Silver, T. N. Frederickson, N. Hopkins, and J. W. Hartley.** 1984. A 3′ end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukemia virus. J. Virol. **52**:248–254.
7. **Chodosh, L. A., A. S. Baldwin, R. W. Carthew, and P. A. Sharp.** 1988. Human CCAAT-binding proteins have heterologous subunits. Cell **53**:11–24.
8. **Clark, S. P., R. Kaufhold, A. Chan, and T. W. Mak.** 1985. Comparison of the transcriptional properties of the Friend and Moloney retrovirus long terminal repeats: importance of tandem duplications and of the core enhancer sequence. Virology **144**:481–494.
9. **Davidson, I., J. H. Xiao, R. Rosales, A. Staub, and P. Chambon.** 1988. The HeLa cell protein TEF-1 binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. Cell **54**:931–942.
10. **DeFranco, D., and K. R. Yamamoto.** 1986. Two different factors act separately or together to specify functionally distinct activities at a single transcriptional enhancer. Mol. Cell. Biol. **6**:993–1001.
11. **DesGroseillers, L., and P. Jolicoeur.** 1984. Mapping the viral sequences conferring leukemogenicity and disease specificity in Moloney and amphotropic murine leukemia viruses. J. Virol. **52**:448–456.
12. **DesGroseillers, L., and P. Jolicoeur.** 1984. The tandem repeats within the long terminal repeat of murine leukemia viruses are the primary determinant of their leukemogenic potential. J. Virol. **52**:945–952.
13. **DesGroseillers, L., E. Rassart, and P. Jolicoeur.** 1983. Thymotropism of murine leukemia virus is conferred by its long terminal repeat. Proc. Natl. Acad. Sci. USA **80**:4203–4207.
14. **Desplan, C., J. Theis, and P. H. O'Farrell.** 1988. The sequence specificity of homeodomain-DNA interaction. Cell **54**:1081–1090.
15. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
16. **Evans, L. H., and J. D. Morrey.** 1987. Tissue-specific replication of Friend and Moloney murine leukemia viruses in infected mice. J. Virol. **61**:1350–1357.
17. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240**:889–895.
18. **Fan, H., S. Mittal, H. Chute, E. Chao, and P. K. Pattengale.** 1986. Rearrangements and insertions in the Moloney murine leukemia virus long terminal repeats alter biological properties in vivo and in vitro. J. Virol. **60**:204–214.
19. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
20. **Fromm, M., and P. Berg.** 1983. Simian virus 40 early- and late-region promoter functions are enhanced by the 75-base-pair repeat inserted at distant locations and inverted orientations. Mol. Cell. Biol. **3**:991–999.
21. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6**:4305–4316.
22. **Golemis, E., Y. Li, J. W. Hartley, and N. Hopkins.** 1989. Distinct segments within the enhancer region collaborate to specify the type of leukemia induced by nondefective Friend and Moloney viruses. J. Virol. **63**:328–337.
23. **Goutte, C., and A. D. Johnson.** 1988. a1 protein alters the DNA binding specificity of α2 repressor. Cell **52**:875–882.
24. **Holland, C. A., P. Anklesaria, M. A. Sakakeeny, and J. S. Greenberger.** 1987. Enhancer sequences of a retroviral vector determine expression of a gene in multipotent hematopoetic progenitors and committed erythroid cells. Proc. Natl. Acad. Sci. USA **84**:8662–8666.
25. **Holland, C. A., J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1985. At least four viral genes contribute to the leukemogenicity of murine retrovirus MCF 247 in AKR mice. J. Virol. **53**: 158–165.
26. **Holland, C. A., J. Wozney, P. A. Chatis, N. Hopkins, and J. W. Hartley.** 1985. Construction of recombinants between molecular clones of murine retrovirus MCF 247 and Akv: determinant of an in vitro host range property that maps in the long terminal repeat. J. Virol. **53**:152–157.
27. **Ishimoto, A., M. Takimoto, A. Adachi, M. Kakuyama, S. Kato, K. Kakimi, K. Fukuoka, T. Ogiu, and M. Matsuyama.** 1987. Sequences responsible for erythroid and lymphoid leukemia in the long terminal repeats of Friend-mink cell focus-forming and Moloney murine leukemia viruses. J. Virol. **61**:1861–1866.
28. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
29. **Jolicoeur, P., N. Nicolaiew, L. DesGroseillers, and E. Rassart.** 1983. Molecular cloning of infectious viral DNA from ecotropic neurotropic wild mice retrovirus. J. Virol. **45**:1159–1163.
30. **Jones, K. A., J. T. Kadonga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
31. **Koch, W., W. Zimmerman, A. Oliff, and R. Friedrich.** 1984. Molecular analysis of the envelope gene and long terminal repeat of Friend mink cell focus-forming virus: implications for the functions of these sequences. J. Virol. **49**:828–840.
32. **Laimins, L. A., P. Gruss, R. Pozzatti, and G. Khoury.** 1984. Characterization of enhancer elements in the long terminal repeat of Moloney murine sarcoma virus. J. Virol. **49**:183–189.
33. **Leegwater, P. A. J., P. C. van der Vliet, R. A. W. Rupp, J. Nowock, and A. Suppel.** 1986. Functional homology between the sequence-specific DNA-binding proteins nuclear factor I from HeLa cells and the TGGCA protein from chicken liver. EMBO J. **5**:381–386.
34. **Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine.** 1984. Determination of the leukemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature (London) **308**:467–470.
35. **Levinson, B., G. Khoury, G. Vande Woude, and P. Gruss.** 1982. Activation of SV40 genome by 72 base pair tandem repeats of Moloney sarcoma virus. Nature (London) **295**:568–572.
36. **Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins.** 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61**:693–700.
37. **LoSardo, J. E., L. A. Cupelli, M. K. Short, J. W. Berman, and J. Lenz.** 1989. Differences in activities of murine retroviral long terminal repeats in cytotoxic T lymphocytes and T-lymphoma cells. J. Virol. **63**:1087–1094.
38. **Lung, M. L., J. W. Hartley, W. P. Rowe, and N. H. Hopkins.** 1983. Large RNase T_1-resistant oligonucleotides encoding p15E and the U3 region of the long terminal repeat distinguish two biological classes of mink cell focus-forming type C viruses of inbred mice. J. Virol. **45**:275–290.
39. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.

40. **Moitoso de Vargas, L., C. A. Pargellis, N. M. Hasan, E. W. Bushman, and A. Landy.** 1988. Autonomous DNA binding domains of λ integrase recognize two different sequence families. Cell **54**:923–929.
41. **Nagata, K., R. A. Guggenheimer, T. Inomoto, J. H. Lichy, and J. Hurwitz.** 1982. Adenovirus replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. USA **79**:6438–6442.
42. **Nowock, J., U. Borgmeyer, A. W. Puschel, R. A. W. Rupp, and A. E. Suppel.** 1985. The TGGCA-binding protein binds to the MMTV-LTR, the adenovirus origin of replication, and the BK virus enhancer. Nucleic Acids Res. **13**:2045–2061.
43. **Oliff, A., M. D. McKinney, and O. Agranovsky.** 1985. Contribution of the *gag* and *pol* sequences to the leukemogenicity of Friend murine leukemia virus. J. Virol. **54**:864–868.
44. **Oliff, A., and S. Ruscetti.** 1983. A 2.4-kilobase-pair fragment of the Friend murine leukemia virus genome contains the sequences responsible for Friend murine leukemia virus-induced erythroleukemia. J. Virol. **46**:718–725.
45. **Oliff, A., K. Signorelli, and L. Collins.** 1984. The envelope gene and long terminal repeat sequences contribute to the pathogenic phenotype of helper-independent Friend viruses. J. Virol. **51**: 788–794.
46. **Pfeifer, K., T. Prezant, and L. Guarente.** 1987. Yeast HAP1 activator binds to two upstream activation sites of different sequence. Cell **49**:19–27.
47. **Rosen, C. A., W. A. Haseltine, J. Lenz, R. Ruprecht, and M. W. Cloyd.** 1985. Tissue selectivity of murine leukemia virus infection is determined by long terminal repeat sequences. J. Virol. **55**:862–866.
48. **Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian.** 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature (London) **334**:218–224.
49. **Shibuya, T., and T. W. Mak.** 1983. Isolation and induction of erythroleukemic cell lines with properties of erythroid progenitor burst-forming cell (BFU-E) and erythroid precursor (CFU-E). Proc. Natl. Acad. Sci. USA **80**:3721–3725.
50. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine virus. Nature (London) **293**:543–548.
51. **Short, M. K., S. A. Okenquist, and J. Lenz.** 1987. Correlation of leukemogenic potential of murine retroviruses with transcriptional preference of the viral long terminal repeats. J. Virol. **61**:1067–1072.
52. **Sitbon, M., B. Sola, L. Evans, J. Nishio, S. F. Hayes, K. Nathanson, C. F. Garon, and B. Chesebro.** 1986. Hemolytic anemia and erythroleukemia, two distinct pathogenic effects of Friend MuLV: mapping of the effects to different regions of the viral genome. Cell **47**:851–859.
53. **Speck, N. A., and D. Baltimore.** 1987. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney murine leukemia virus enhancer. Mol. Cell. Biol. **7**:1101–1110.
54. **Thiesen, H.-J., Z. Bösze, L. Henry, and P. Charnay.** 1988. A DNA element responsible for the different tissue specificities of Friend and Moloney retroviral enhancers. J. Virol. **62**:614–618.
55. **Vogt, M.** 1982. Virus cloned from the Rauscher virus complex induces erythroblastosis and thymic lymphoma. Virol. **118**: 225–228.
56. **Von der Ahe, D., S. Janich, C. Scheidereit, R. Renkawitz, G. Schultz, and M. Beato.** 1985. Glucocorticoid and progesterone receptors bind to the same sites in two hormonally regulated promoters. Nature (London) **313**:706–709.
57. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. RNA tumor viruses, 2nd ed., vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. RNA tumor viruses, 2nd ed., vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
59. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**: 209–252.
60. **Yoshimura, F. K., B. Davison, and K. Chaffin.** 1985. Murine leukemia virus long terminal repeat sequences can enhance gene activity in a cell-type-specific manner. Mol. Cell. Biol. **5**: 2832–2835.