JOURNAL OF VIROLOGY, Feb. 1995, p. 994–1000 Vol. 69, No. 2
0022-538X/95/$04.00+0

Characterization of Nuclear Protein Binding to a Site in the Long Terminal Repeat of a Murine Leukemia Virus: Comparison with the NFAT Complex

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Received 11 July 1994/Accepted 4 November 1994

We previously identified a protein-binding site (MLPal) that is located downstream of the enhancer element in the long terminal repeat (LTR) of a mink cell focusing-forming (MCF) murine leukemia virus (F. K. Yoshimura, K. Diem, H. Chen, and J. Tupper, J. Virol. 67:2298–2304, 1993). We determined that the MLPal site regulates transcription specifically in T cells and affects the lymphomagenicity of the MCF isolate 13 murine leukemia virus with a single enhancer repeat in its LTR. In this report, we present evidence that two different proteins, a T-cell-specific protein and a ubiquitous protein, bind the MLPal site in a sequence-specific manner. By mutational analysis, we determined that the T-cell-specific and the ubiquitous proteins require different nucleotides in the MLPal sequence for DNA binding. By competitive electrophoretic mobility shift assays, we demonstrated that the T-cell-specific protein that binds MLPal is identical or similar to a protein from nonactivable T cells that interacts with the binding site of the nuclear factor of activated T cells (NFAT). Unlike the NFAT-binding site, however, the MLPal site does not bind proteins that are inducible by T-cell activation. We observed that the MLPal sequence is conserved in the LTRs of other mammalian retroviruses that cause T-cell diseases. Furthermore, the MLPal sequence is present in the transcriptional regulatory regions of cellular genes that either are expressed specifically in T cells or are commonly rearranged by provirus integration in thymic lymphomas. Thus, the MLPal-binding proteins may play a role in the transcriptional regulation not only of the MCF virus LTR but also of cellular genes involved in T-cell development.

The long terminal repeat (LTR) of slowly transforming retroviruses is a major determinant of the type of neoplastic disease that these viruses produce (11, 14, 15, 17, 25, 26). Studies of murine leukemia viruses (MLVs) differing in their pathogenic characteristics have demonstrated a direct correlation between the cell-type-specific transcriptional activity of the LTR and the cell type involved in disease (15, 27, 42).

The best-studied region of the LTR for transcriptional regulation is the enhancer element. Protein-binding sites within the enhancer that contribute to T-cell-specific transcription have been identified for murine retroviruses that cause thymic lymphoma (4, 14, 15, 27, 41, 43, 45). It has been demonstrated that these protein-binding sites also contribute to the disease specificity of these MLVs (15, 42). Besides the enhancer element, the region downstream of it also has been shown to be important for specificity of retroviral pathogenesis and transcriptional regulation (16, 17, 24, 26, 46). We recently observed that deletion of the entire region between the promoter and enhancer of the LTR of the mink cell focus-forming (MCF) MLV significantly reduced the incidence of thymic lymphoma and increased the latency of disease (46). Furthermore, we observed that deletion of this region resulted in a marked reduction of transcription as assessed by transient expression assays (46). Within this LTR region, we identified a 25-bp protein-binding site that contributes to T-cell-specific transcription (52, 53). We refer to this protein-binding site as the MCF virus LTR palindrome (MLPal) because it contains an 18-bp palindromic sequence.

Our studies of the mechanism by which the MLPal site regulates transcription revealed that its activity depends on interactions with the enhancer, which is located 14 bp upstream of the MLPal site. Although multimerized copies of MLPal had no effect on the MCF virus promoter, the addition of a single copy of MLPal increased transcription of one copy of the viral enhancer repeat and promoter by 10-fold (52). We further observed that the MLPal site partly overlaps a GATA consensus sequence, which is a binding site for a multigene family of transcription factors that regulate gene expression in a cell-type-specific manner (34). Our preliminary analysis of interactions between the MLPal- and GATA-binding sites suggest that the GATA site inhibits transcriptional activation by MLPal, most likely by interfering with protein binding to the MLPal site (unpublished data).

In this report, we describe studies that examine protein binding to the MLPal site. We have detected an MLPal-binding protein that is present only in T cells. By mutational analysis, we identified the bases in the MLPal sequence that are involved in protein binding. We present evidence that the MLPal site binds a nuclear protein that may also be part of the multisubunit nuclear factor of activated T cells (NFAT) (8, 40). We also discuss our detection of sequences with homology to MLPal that are present in other mammalian retroviruses that cause T-cell diseases and in the transcriptional regulatory regions of several cellular genes.

MATERIALS AND METHODS

Cell lines. Murine T-lymphoma cell lines L691, Ti6, EL4, and BW5147.3 were grown in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) (GIBCO). The murine B-cell line S194 and human T-cell line Jurkat were grown in RPMI 1640, 10% FBS, and 5×10^{-5} M β-mercaptoethanol. NIH 3T3 mouse and AH927 feline fibroblast cell lines were propagated in Dulbecco's modified Eagle's medium (GIBCO) plus 10% FBS. Jurkat cells were activated by the addition to the medium of 25 ng of 12-*O*-

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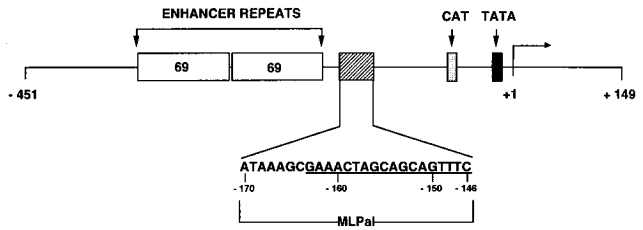

FIG. 1. MCF13 LTR. Open boxes correspond to 69-bp direct repeats; the hatched box indicates the MLPal protein-binding site; CAT and TATA boxes are indicated; the horizontal arrow marks the start site of transcription. Numbering of nucleotides is relative to the start site of transcription. The nucleotide sequence of the MLPal site is shown below; the palindromic sequence is underlined.

tetradecanoylphorbol-13-acetate (TPA; Sigma) per ml and 1.4 μg of ionomycin (Calbiochem) per ml for 3.5 h.

Nuclear extract preparation. Cells were collected by centrifugation at 500 × *g* for 10 min at 4°C and rinsed once with phosphate-buffered saline (2 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl); lysis buffer (LB; 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 8.0], 50 mM NaCl, 7 mM β-mercaptoethanol, 0.5 M sucrose, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine) was then added. Cells were lysed with LB containing 0.5% Nonidet P-40, with a 5- to 10-min incubation on ice. After this step, all buffers contained 0.5 mM phenylmethylsulfonyl fluoride, aprotinin (3 μg/ml), leupeptin (1 μg/ml), and pepstatin A (1 μg/ml) as protease inhibitors. After centrifugation, nuclei were washed with extraction buffer (EB; identical to LB except that 10% glycerol is substituted for sucrose). Nuclei were resuspended in 5 to 10 ml of EB to which was added an equal volume of EB that contained 0.55 N NaCl dropwise with constant swirling. Nuclei were incubated on ice for 30 min with gentle shaking every 5 min. Treated nuclei were centrifuged at 10,800 × *g* for 20 min at 4°C. The supernatant was decanted, and ammonium sulfate was slowly added to a final concentration of 70% saturation for protein precipitation. The ammonium sulfate precipitate was collected by centrifugation at 17,200 × *g* for 20 min at 4°C. Protein was resuspended in 500 μl of EB and dialyzed against 100 ml of buffer B (EB without the spermine and spermidine) in the cold. Nuclear extracts were clarified by centrifugation at 15,000 × *g* for 10 min before storage at −70°C. Protein was measured by the Bradford method (5).

EMSA. The electrophoretic mobility shift assay (EMSA) is based on the method devised by Garner and Revzin (13). One nanogram of ^{32}P-labeled synthetic double-stranded oligonucleotides (approximately 50,000 Cerenkov cpm) was incubated with 2 to 4 μg of nuclear extract and 1 μg of poly(dI-dC) (Boehringer Mannheim) in 1× EB buffer for 30 min on ice. Unlabeled double-stranded oligonucleotide competitors were preincubated with the nuclear extract for 10 min prior to addition of the labeled probe. Samples were electrophoresed through a 5% polyacrylamide gel in 0.3× TBE buffer (1× TBE buffer is 81 mM Tris base, 81 mM boric acid, and 1.8 mM EDTA) at 17 V/cm for 2 h at 4°C. Gels were dried and exposed for various times to Kodak X-Omat film with a DuPont Lightning Plus intensifying screen at −70°C.

Complementary oligonucleotide strands were synthesized with an Applied Biosystems 380B synthesizer by a Howard Hughes Medical Institute core facility at the University of Washington. Coding strand sequences of the oligonucleotides used in EMSAs were as follows: MLPal, ATAAAGCGAAACTAGCAG CAGTTTC; Pal-1, TACAGTCGCTGCACTGTA; Pal-2, GAAACAGCACCG ACAGAA; P sequence, CGAAAATTTCC; and NFAT, TGCCCAAAGAGGA AAATTTGTTTCATACAG. Nucleotides that are underlined in Pal-1 and Pal-2 sequences are identical to the nucleotides in the same positions in the MLPal sequence.

Preparation of DNA probes for EMSAs. Double-stranded oligonucleotides were ^{32}P labeled by annealing both strands and labeling with [α-^{32}P]dATP and [α-^{32}P]dCTP with the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) at room temperature for 30 min. Specific activities ranged from 1×10^7 to 5×10^7 cpm/μg. Labeled probes were purified by electrophoresis through a 4% polyacrylamide gel. After autoradiography, the DNA was excised from the gel and eluted by overnight incubation in 10 mM Tris-Cl (pH 7.5)–1 mM EDTA at 37°C. The DNA was ethanol precipitated with yeast tRNA as the carrier before further use.

RESULTS

A T-cell-specific protein and a ubiquitous protein bind the MLPal site. Our previous studies of the MLPal protein-binding site (Fig. 1) involved the use of nuclear extracts isolated only from T cells (52). To determine whether the MLPal site binds a T-cell-specific or a ubiquitous protein, we compared

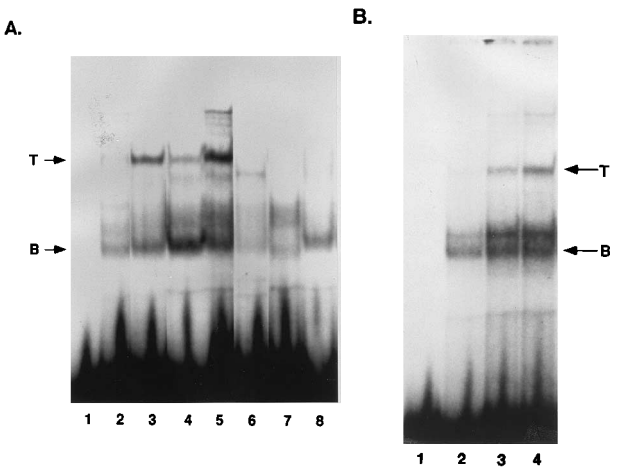

FIG. 2. (A) Cell line comparison of nuclear protein binding to the MLPal sequence. Two nanograms of ^{32}P-labeled MLPal oligonucleotide probe (lane 1) was reacted with 4 μg each of nuclear extracts isolated from various cell lines. T-cell lanes: 2, BW5147.3; 3, L691; 4, Ti6; 5, EL4. B-cell lane: 6, S194. Feline and murine fibroblast lanes: 7, AH927; 8, NIH 3T3. Arrows indicate the top (T) and bottom (B) bands that correspond to the two major protein-DNA complexes detectable with T-cell nuclear extracts. Binding reactions were performed in EB for 30 min at 4°C. Polyacrylamide gel electrophoresis and processing of the gel were performed as described in Materials and Methods. (B) Titration of protein-DNA complexes generated by binding of T-cell nuclear protein to the MLPal sequence. Two nanograms of ^{32}P-labeled MLPal oligonucleotide probe (lane 1) was reacted with increasing amounts of L691 T-cell nuclear extract (2 [lane 2], 4 [lane 3], and 6 [lane 4] μg) as described for panel A. T, top band; B, bottom band. Polyacrylamide gel electrophoresis was performed as described in Materials and Methods.

EMSA reactions with nuclear extracts isolated from different cell types. For these EMSAs, we used a ^{32}P-labeled oligonucleotide probe that contained the MLPal sequence (see Materials and Methods). Nuclear extracts from four different murine T-cell lines, BW5147.3, L691, Ti6, and EL4, produced protein-DNA complexes with similar mobilities (Fig. 2A, lanes 2 to 5). We detected two major bands, top and bottom, that were generated by nuclear extracts from all of the T-cell lines that we examined. Two additional bands with intermediate mobilities were most noticeable with nuclear extracts from BW5147.3 (lane 2) and EL4 (lane 5) cells. Bands lower in mobility than the top band were also detectable with nuclear extract from two T-cell lines (lanes 2 and 5). Nuclear extracts from either a murine B-cell line, S194 (lane 6), or a feline (AH927; lane 7) or murine (NIH 3T3; lane 8) fibroblast cell line produced protein-DNA complexes with mobilities corresponding to the bottom and/or intermediate bands that were detectable with the T-cell extracts. The bottom band was detectable with nuclear extracts from all cell types that we analyzed. Non-T cells lacked the top band that was generated by the T-cell nuclear extracts. For S194 B cells (lane 6), we detected a band that migrated slightly faster than the T-cell-specific complex.

We observed that the signals corresponding to the top and bottom bands increased in intensity when we added increasing amounts of L691 T-cell nuclear extract to the reaction mixture (Fig. 2B). This result indicated that binding of the nuclear factors that produce the top and bottom bands to the MLPal probe was not due to nonspecific protein binding to DNA. We further address this point below.

Specificity of protein binding to the MLPal sequence. To determine whether the protein-DNA complexes corresponding to the top and bottom bands that were detectable with T-cell

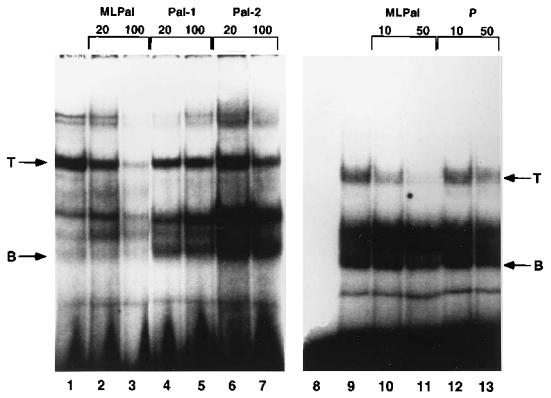

FIG. 3. Competition for T-cell nuclear protein binding to MLPal by unlabeled oligonucleotides containing various sequences. Excess amounts of unlabeled double-stranded oligonucleotides were reacted with 4 μg of L691 nuclear protein for 10 min prior to addition of 4 ng of ^{32}P-labeled MLPal oligonucleotide in an EMSA binding reaction. Lanes 1 and 9, MLPal probe and nuclear protein with no competitor oligonucleotide. Unlabeled competitor oligonucleotides at indicated molar ratio excesses were in lanes 2 (20× MLPal), 3 (100× MLPal), 4 (20× Pal-1), 5 (100× Pal-1), 6 (20× Pal-2), 7 (100× Pal-2), 10 (10× MLPal), 11 (50× MLPal), 12 (10× P sequence), and 13 (50× P sequence). Lane 8 is the MLPal probe with no protein. Sequences of oligonucleotides used for competition are listed in Materials and Methods. T, top band; B, bottom band. Gel electrophoresis and processing were as described in the legend to Fig. 2A.

nuclear extracts were generated by specific DNA binding, we performed EMSA competition assays (Fig. 3). For this determination, we included in the EMSA binding reaction mixture competing unlabeled double-stranded oligonucleotides that contained either the wild-type MLPal sequence (lanes 2 and 3) or two different sequences that correspond to mutations of only the palindromic region of MLPal (Pal-1 [lanes 4 and 5] and Pal-2 [lanes 6 and 7]) at 20- or 100-fold molar excess compared with the labeled MLPal probe. The Pal-1 oligonucleotide contains base changes that maintain a palindromic structure with the same GC content as the wild-type MLPal sequence. Pal-2 has mutations of the palindromic sequence that eliminate the twofold symmetry. In a separate experiment, we also used an oligonucleotide that contained the sequence of a *cis*-acting element that is located upstream of the promoter of the interleukin-4 gene (P sequence [1]) at 10- or 50-fold molar excess as a competitor (lanes 12 and 13). We observed that the oligonucleotide with the MLPal sequence was an effective competitor in both experiments (lanes 2, 3, 10, and 11). Competition for the top band was more efficient that competition for the bottom band at all molar ratios used. Competition for the bottom band is seen more easily in lane 3 than in lane 11. We detected no comparable competition by oligonucleotides that contained either the Pal-1 (lanes 4 and 5), Pal-2 (lanes 6 and 7), or P (lanes 12 and 13) sequence. For unknown reasons, inclusion of the Pal-1 and Pal-2 oligonucleotides increased the intensity of the bottom band compared with the reaction with no competitor (lanes 4 to 7). Competition of the bands migrating more slowly than the top band by Pal-1 and Pal-2 (lanes 4 and 7, respectively) indicated that these bands were generated by nonspecific DNA binding. From the results of these competition assays, we concluded that the two major protein-DNA complexes detectable with the wild-type MLPal probe represented specific binding of T-cell nuclear proteins to the MLPal sequence.

Mutational analysis of protein binding to the MLPal site. To identify the bases within the MLPal site that participate in protein binding, we mutated the wild-type sequence as shown

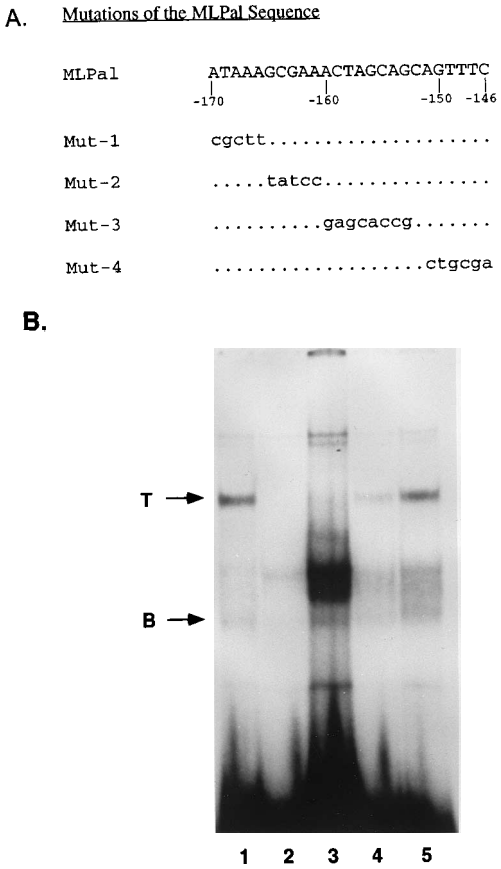

FIG. 4. (A) Sequences of oligonucleotides containing mutations of the MLPal sequence used for binding to T-cell nuclear extract in EMSA reactions. Mutated bases are identified by lowercase letters; dots correspond to identical bases. (B) Identification of bases in the MLPal sequence that are involved in the generation of the top and bottom protein-DNA complexes. Wild-type and mutant MLPal oligonucleotides in lanes 1 (wild type), 2 (Mut-1), 3 (Mut-2), 4 (Mut-3), and 5 (Mut-4) were ^{32}P labeled, reacted with 4 μg of T-cell nuclear extract, and electrophoresed as described in the legend to Fig. 2A. Arrows indicate top (T) and bottom (B) bands.

in Fig. 4A. Synthetic double-stranded oligonucleotides containing each of the indicated mutations were ^{32}P labeled and used as probes in binding reactions with L691 T-cell nuclear extract (Fig. 4B). We observed that mutations of nucleotides (nt) −166 to −170 (Mut-1) resulted in the loss of the top and bottom bands corresponding to the T-cell-specific and ubiquitous protein-DNA complexes (lane 2). Mutations of nt −161 to −165 (Mut-2) eliminated the top but not the bottom band and increased the intensity of the intermediate bands (lane 3). Mutations of nt −153 to −160 in the middle of the palindrome (Mut-3) did not completely eliminate but significantly reduced the intensity of the top band (lane 4). These mutations did not affect the bottom band. Mutations of nt −146 to −149 (Mut-4) did not significantly affect the top band and produced a moderate increase in the intensity of the bottom and intermediate bands (lane 5). These data indicated that the sequence of the first 10 bases of MLPal is essential for T-cell-specific protein-binding and that the precise sequence of the last 6 bases of MLPal that lie within the palindrome is not important. We also concluded that the nucleotides involved in binding of the ubiquitous protein differed from those essential for binding of the T-cell-specific protein.

The MLPal site binds a nuclear protein related to NFAT. A

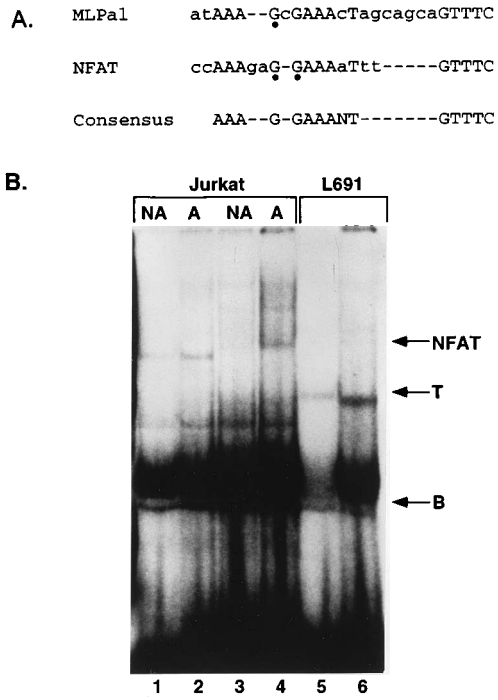

FIG. 5. (A) Consensus sequence shared by the MLPal and NFAT protein-binding sites. The NFAT nucleotide sequence corresponds to nt −296 to −267 of the distal NFAT site of the murine interleukin-2 gene promoter (18). Identical bases are indicated by uppercase letters, and lowercase letters indicate differences. Dashed lines correspond to gaps in the sequence. Filled circles identify the methylated guanines in the coding strand that interfere with protein binding (reference 18 and unpublished data). The consensus sequence derived for the MLPal and NFAT sites is shown. (B) Comparison of protein binding to MLPal and NFAT sequences for nuclear extracts isolated from activable and nonactivable T cells. A ^{32}P-labeled MLPal or NFAT double-stranded oligonucleotide was reacted with 4 μg of nuclear protein isolated from L691 cells (lanes 5 and 6) or Jurkat cells that were either treated with 25 ng of TPA per ml and 1.4 μg of ionomycin per ml (lanes 2 and 4) or untreated (lanes 1 and 3). Binding conditions were as described in the legend to Fig. 2A. Lanes: 1, 2, and 5, MLPal probe; 3, 4, and 6, NFAT probe. The arrow labeled NFAT indicates the band in lane 4 that corresponds to the NFAT complex that is inducible in activated T cells. Arrows labeled T and B correspond to the top and bottom bands, respectively, in lane 5 that are detectable with the MLPal probe and L691 nuclear extract. NA and A designate nonactivated and activated Jurkat cells, respectively.

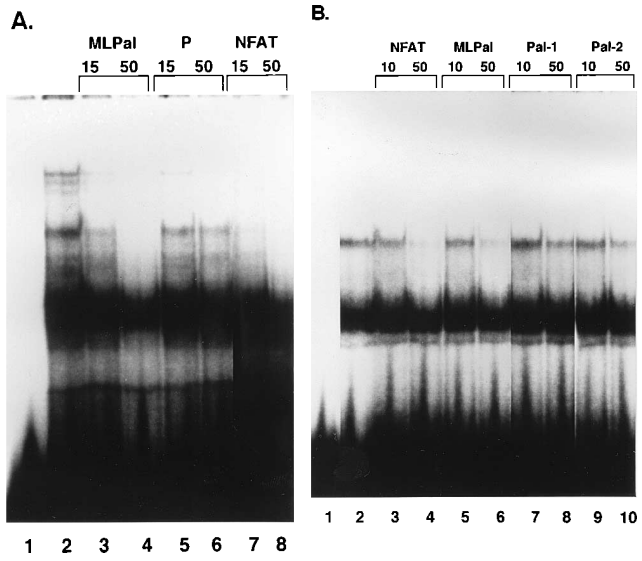

FIG. 6. (A) Competition by the NFAT oligonucleotide for T-cell nuclear protein binding to the MLPal sequence. Conditions for the binding reaction were as described in the legend to Fig. 2A. Unlabeled competitor oligonucleotides at either 15- or 50-fold excess molar ratio were preincubated with nuclear protein for 10 min before the addition of the ^{32}P-labeled MLPal probe. Lanes: 1, MLPal labeled probe without protein; 2, MLPal labeled probe with 6 μg of protein; 3, 15× unlabeled MLPal; 4, 50× unlabeled MLPal; 5, 15× unlabeled P sequence; 6, 50× unlabeled P sequence; 7, 15× unlabeled NFAT; 8, 50× unlabeled NFAT. (B) Competition by the MLPal oligonucleotide for L691 T-cell nuclear protein binding to the NFAT sequence. Unlabeled competitor oligonucleotides at either 10- or 50-fold excess molar ratio were preincubated with nuclear protein before the addition of the ^{32}P-labeled NFAT probe. Lanes: 1, NFAT probe without protein; 2, NFAT probe with 6 μg of protein; 3, 10× NFAT competitor; 4, 50× NFAT competitor, 5, 10× MLPal competitor; 6, 50× MLPal competitor; 7, 10× Pal-1 competitor; 8, 50× Pal-1 competitor; 9, 10× Pal-2 competitor; 10, 50× Pal-2 competitor.

nucleotide sequence comparison of the MLPal site with known *cis*-acting transcriptional regulatory elements revealed homology with the binding site for NFAT (12, 40) (Fig. 5A). NFAT is a multisubunit complex consisting of at least three identified proteins (3, 18, 19, 28a, 29, 32, 33) and is required for the transcriptional activation of the interleukin-2 gene (8, 12, 40). We observed that the sequence motif AAA--G-GAAANT-------GTTTC was shared by the MLPal and NFAT sites. An additional similarity that we detected between these two binding sites is the location of methylated guanines that interfere with protein binding (indicated by the filled circles in Fig. 5A) (reference 18 and unpublished data).

To determine whether these two sites bind similar proteins, we compared protein binding to the MLPal and NFAT sites by EMSA analysis. When L691 T-cell nuclear extract was used in the binding reaction, we detected a band for the NFAT probe (Fig. 5B, lane 6) with mobility similar but not identical to that of the MLPal top band (Fig. 5B, lane 5). The slight difference in mobilities between the MLPal and NFAT bands was reproducibly detectable. The L691 cells that were used for these binding assays are T cells that are not activable and do not produce the NFAT nuclear complex in response to agents that mimic antigen activation of T cells (unpublished data). The protein-DNA complex that was detectable with the NFAT probe and L691 nuclear extract had a mobility different from that of the NFAT complex detectable with nuclear extract from activated Jurkat cells (Fig. 5B; compare lanes 4 and 6). With the L691 nuclear extract, we also detected NFAT complexes with mobilities similar to those of the intermediate and bottom bands that were detectable with the MLPal probe.

We performed EMSA competition experiments to test the possibility that the MLPal and the NFAT sites bind the same protein in L691 nuclear extracts. When we used the MLPal oligonucleotide as a probe, we detected effective competition of the top band by the unlabeled double-stranded NFAT oligonucleotide (Fig. 6A). Competition by the NFAT oligonucleotide (lanes 7 and 8) was comparable to that by the MLPal oligonucleotide (lanes 3 and 4). The oligonucleotide containing the P sequence did not compete (lanes 5 and 6). When we performed the converse experiment, we observed that the unlabeled MLPal oligonucleotide effectively competed with the NFAT band with mobility similar to that of the MLPal top band (Fig. 6B, lanes 5 and 6). Competition by MLPal was about as effective as that by the cognate NFAT competitor oligonucleotide (lanes 3 and 4). We did not detect comparable competition for the top NFAT complex by the Pal-1 (lanes 7 and 8) or Pal-2 (lanes 9 and 10) oligonucleotide. Effective competition for the additional bands that were generated by the NFAT probe was not observed for any of the oligonucleotides at the excess molar ratios used in this experiment.

Because NFAT corresponds to a multiprotein-DNA complex that is specifically induced when T cells are activated, we examined the possibility that MLPal can bind similarly inducible proteins. To do this, we prepared nuclear extracts from a Jurkat T-cell line that is activable by exposure to phorbol ester (TPA) and calcium ionophore (ionomycin). Others have shown that these two reagents together mimic activation of T cells by antigen presentation (50). Therefore, they have been used to stimulate T cells to detect the NFAT nuclear complex (3). When we compared EMSAs of nuclear extracts from activated and nonactivated Jurkat cells, we observed identical band patterns with the MLPal probe (Fig. 5B, lanes 1 and 2). In contrast, a comparison of the binding of these nuclear extracts to the NFAT probe resulted in our detection of the NFAT nuclear complex only for activated Jurkat cells (Fig. 5B, lanes 3 and 4). Our results suggested that a protein or proteins that are inducible by the activation of T cells do not bind MLPal. In contrast, MLPal binds nuclear proteins that are constitutively expressed in mouse and human T cells.

Conservation of the MLPal sequences in the LTRs of other MLVs and transcriptional regulatory regions of cellular genes. We compared the nucleotide sequences located at a site analogous to MLPal in the LTRs of other retroviruses and detected a strong conservation of the MLPal sequence in mammalian viruses that induce T-cell diseases (Fig. 7A). For MLVs that induce thymic lymphoma (radiation leukemia virus [20], MCF virus isolate 247 [MCF247] [21], and Tikaut [6]), there were one to three base pair differences. The analogous site in the LTR of feline leukemia virus (44), which induces T-cell lymphoma, also has sequence similarity with MLPal. MLVs that are either nononcogenic (Akv [47]) or induce diseases involving non-T cells (Friend [22]) had 7-of-25 base pair differences for sites that were analogous to MLPal.

We searched the GenBank nucleotide database to determine whether we could detect the MLPal sequence in the transcriptional regulatory regions of cellular genes. For this search, we compared only nt −161 to −170 of the MLPal site, which our mutational studies demonstrated were most critical for T-cell-specific protein binding as described above (Fig. 4B). We detected significant homology with these MLPal bases in the transcriptional regulatory regions of eight cellular genes that either are of significance to T cells or are proto-oncogenes that are frequently rearranged in T-cell lymphomas (7, 9, 48) (Fig. 7B). Five of these cellular genes, the T-cell receptor (TCR) Cβ2, Vβ2, and δ genes, CD8 β-chain gene, and CD3 ε-chain gene (6, 28, 31, 35–37), are normally expressed only in T cells. The N-*myc* gene (10) is expressed preferentially in kidney, intestine, and newborn brain (54), and the c-*myc* gene (2) is expressed in all cell types (54). *pim-1* (39) expression occurs in hematolymphoid tissues and testis (30). The upstream region of N-*myc* (10) and the intron between the TCR Jβ2 and Cβ2 (28) genes each possessed two sites with homology to MLPal. A total of 11 sites with significant sequence homology with MLPal were identified for the cellular genes cited above. Six of the homologous sequences were in the forward orientation, and five were reversed.

A.

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MCF13     ATAAAGC-GAAACTAGCAGCAGTTTC
MCF247    .......-.........a.......
Tikaut    .......-.......a..a.......
RadLV     ......t-.......a..a.......
FeLV      ...t...t.....-.....a......
Akv       .a.t...-t...ac.a..a.......
Friend    ...tg..-cc...cct..........
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B.

MLPal	ATAAAGCGAA
TCR Jβ2-Cβ2	......aa..
	..t......t
TCR Vβ2	c.....t...
c-*myc*	.g....g...
N-*myc*	.g......g.
	.g......gt
pim-1	....g...tt
TCR/CD3-ε	.c......ct
TCR δ	.g....t..t
CD8 β-chain	g......ctc

FIG. 7. (A) Nucleotide sequence comparison of the MLPal sites in the LTRs of various mammalian retroviruses. Identical bases are indicated by dots, dashes represent missing bases, and lowercase letters correspond to differing bases. Sequences shown: MCF13 (51); MCF247 (21); Tikaut MLV (7); radiation-induced leukemia virus (RadLV) (20); feline leukemia virus (FeLV) (44); Akv 614 murine nonleukemogenic virus (47); Friend MLV clone 57 (22). (B) Nucleotide sequences in transcriptional regulatory regions of cellular genes with homology to the first 10 bases of the MLPal sequence. Identical bases are represented by dots; differing bases are indicated by lowercase letters. TCR Jβ2-Cβ2, the Jβ2-Cβ2 intron of the murine TCR β-chain gene (28); TCR Vβ2, 91 to 100 nt upstream of the transcriptional start site of the murine Vβ2 gene (36); c-*myc*, 252 to 261 nt upstream of the P1 promoter of the murine c-*myc* proto-oncogene (2); N-*myc*, 723 to 732 and 681 to 690 nt upstream of the transcription initiation site of the murine N-*myc* proto-oncogene (10); *pim-1*, GC-rich promoter region of the murine *pim-1* proto-oncogene (39); TCR Cβ2, 3′ enhancer of the human TCR Cβ2 gene (35); TCR/CD3-ε, 3′ enhancer of the human TCR/CD3-ε gene (6); TCR δ, enhancer of the human TCR δ gene (37); CD8 β-chain, 5′-flanking region of the murine CD8 β-chain gene (31).

DISCUSSION

We previously identified a protein-binding site (MLPal) that is located near the 3′ end of the enhancer element in the LTR of the MCF13 MLV. We demonstrated that the MLPal sequence is a *cis*-acting element that potentiates transcription specifically in T cells (46, 52). In this report, we describe our detection of a T-cell-specific protein that binds the MLPal site. A sequence search uncovered homology between the MLPal and NFAT sites, and EMSAs demonstrated that both sequences competed for binding by a T-cell nuclear protein. It is not clear, however, which, if any, of the multiple proteins that have been identified in the NFAT complex binds MLPal. These NFAT-binding proteins include Fos, Jun, and NFATp (3, 18, 19, 32, 33). Unlike NFAT, however, the MLPal sequence did not bind proteins that are inducible by T-cell activation. It has been shown that Fos and Jun are the inducible proteins in the NFAT complex (3, 18). Additional reasons why it is unlikely that Fos and Jun bind MLPal include the absence of an AP-1 motif in MLPal and our observation that mutations of the analogous bases that affect Fos and Jun binding to NFAT (3) had no effect on protein binding to MLPal. Furthermore, we have not been able to detect an effect on the mobility or generation of the MLPal complex by the addition of antibodies to Fos and Jun (unpublished data). Our current data indicate that the same or a closely related nuclear protein that is present in nonactivable T cells binds both the MLPal- and NFAT-binding sites. However, in activated T cells, differ-

ent proteins bind these two sites. Thus, we conclude that the MLPal- and NFAT-binding sites bind similar but not identical proteins. The MLPal-binding protein may be a novel member of the family of NFAT-binding proteins that has recently been identified, but one which is constitutively expressed in the nucleus (28a, 32). Although there is a constitutive component (NFATp) of the NFAT multisubunit complex, it is localized in the cytoplasm (19, 29, 32, 33) and hence should be absent from our T-cell nuclear extracts.

Our data from both EMSA and mutational analysis of the MLPal sequence indicated that more than one protein binds this site. We detected two major protein-DNA complexes that corresponded to specific protein binding as determined by competitive EMSAs. One band (bottom) appeared with nuclear extracts from various cell types. This observation suggested that the protein that generates this complex is ubiquitous. The second specific complex (top band) was detectable only with T-cell nuclear extracts. Mutational analysis of the MLPal sequence revealed that the ubiquitous protein requires nt −166 to −170 for binding (Fig. 4A). In contrast, nt −153 to −170 of MLPal were found to be important for binding of the T-cell protein, although nt −161 to −170 were more critical for binding than nt −153 to −160. Single base mutations of these nucleotides will be required to determine whether all or only a few of them are essential for protein binding. We observed that disruption of the palindrome by mutations of nt −146 to −151 had no effect on the generation of the top band, suggesting that the specific sequence and not solely secondary structure is required for binding of the protein from T cells.

The presence of sequences similar to MLPal in an analogous site of the LTRs of several mammalian retroviruses that induce T-cell diseases suggests that this protein-binding site is involved in viral pathogenesis. However, it is not an absolute requirement since other lymphomagenic viruses do not have this protein-binding site (25). In a previous study, we observed that deletion of MLPal had a moderate effect on MCF13 MLV pathogenicity only when the virus contained a single enhancer element. Our detection of sequences with close homology to the first 10 bases of MLPal, which are most stringently required for generation of the T-cell-specific complex, in the transcriptional regulatory regions of cellular genes is intriguing. Several of these genes are those that encode proteins expressed specifically in T cells, such as the TCR Cβ2, Vβ2, and δ, CD3 ε-chain, and CD8 β-chain proteins (6, 28, 31, 35–37). Moreover, it has been shown that the sites with homology to MLPal in the TCR δ gene enhancer and the intron between Jβ2 and Cβ2 are bound by protein (28, 37). It is particularly intriguing that the cellular genes c-*myc*, *pim-1*, and N-*myc*, which are frequently rearranged in T-cell lymphomas (7, 9, 48), have sequences with homology to MLPal in upstream regions of their promoters. For c-*myc*, these homologous sequences are found immediately upstream of the P1 promoter (2). It has been observed that deregulation of the c-*myc* proto-oncogene in T-cell tumors correlated with a shift in usage of the *myc* promoter from P2 to P1 (38). We speculate that protein binding to the MLPal site may be required for the activation of the P1 promoter. For *pim-1*, the MLPal sequence is also present in the promoter region, where it partly overlaps an Oct-1-binding site (39). Studies with transgenic mice have suggested a strong cooperation between *pim-1* and c-*myc* or N-*myc* in lymphomagenesis (49). The MLPal protein-binding site may contribute to the coordinate expression of these proto-oncogenes. Thus, the proteins that bind the MLPal site may have an important role in both the normal regulation of cellular genes and their deregulation in tumor cells. The identification and isolation of these MLPal-binding proteins will allow us to make this determination.

ACKNOWLEDGMENTS

We are grateful to Ted Young for critically reading the manuscript.

This work was supported by Public Health Service grant CA44166 from the National Institutes of Health and a grant from the Royalty Research Fund of the University of Washington to F.K.Y.

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