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Regulation of the Long Terminal Repeat in Visna Virus by a Transcription

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The long terminal repeats of maedi visna virus strain 1514 contain a consensus AP-1 binding site which has been shown to be important in controlling virus transcription. However, this consensus site is absent in strain EV-1. Here, we have compared the ability of oligonucleotides corresponding to LTR sequences from EV-1 with those from 1514 to bind transcription factors in competitive gel retardation assays and activate reporter gene expression. The experiments demonstrated no observable binding of AP-1 to the EV-1-derived sequences and significant differences in the abilities of the 1514 and EV-1 sequences to activate transcription. However, both viral sequences interacted with a second, previously undetected, transcription factor. This factor gave specific gel shifts which were competed by an oligonucleotide containing the consensus sequence for the AML/PEBP2/CBF family of transcriptional factors, but not by control AP-1 or OCT-1 oligonucleotides. The factor was therefore denoted AML (vis). A second AML (vis) site, noted upstream of the TATA box proximal AP-1 site, gave single shifts which were competed by the downstream AML (vis) oligonucleotide. Both sites were functional in transfection assays. In gel shift retardation assays, polyclonal antisera directed against known runt domain proteins were able to supershift part of the AML (vis) binding activity in nuclear extracts from physiologically relevant cell types. The results thus suggest that the AML (vis) binding factor belongs to the AML/PEBP2/CBF family of transcription factors and may be important in controlling virus replication in these and other strains of ruminant lentiviruses.

Maedi-visna virus (MVV), an ovine lentivirus, causes a progressive inflammatory and degenerative disease after a prolonged incubation period (Sigurdsson *et al.,* 1957; Sigurdsson and Palsson, 1958). Inflammatory responses are observed in the lungs, joints, central nervous system (CNS), and other tissues (Hasse, 1986). The primary targets of infection *in vivo* are cells of the monocyte/macrophage lineage (Gendelman *et al.,* 1985, 1986). Expression of the viral genome within these cells appears to be controlled by both degree of differentiation and activation (Narayan *et al.,* 1983; Gendelman *et al.,* 1986; Small *et al.,* 1989). This restriction can be observed *in vivo,* where transcription of the MVV genome appears to be limited to tissue resident macrophages within the lungs, joints, and CNS (Gendelman *et al.,* 1986; Brodie *et al.,* 1995). The transcription of the genome occurs only in terminally differentiated macrophages, as monocytes do not accumulate significant quantities of viral transcripts or proteins. The cellular and viral factors that govern this restricted stage of the viral lifecycle are only poorly understood.

Using the long terminal repeat (LTR) from the MVV strain 1514 as a model system, the cellular transcription factor AP-1 has been proposed as a key regulator of MVV transcription (Gabuzda *et al.,* 1989; Hess *et al.,* 1989). The 1514 LTR carries a consensus AP-1 site (Faisst and Meyer, 1992), which has been demonstrated to bind the AP-1 complex (Shih *et al.,* 1992). A number of agents known to trigger AP-1 activity by posttranscriptional modification and to induce transcription of *jun, fos,* and *fra* genes are also potent inducers of monocyte differentiation and macrophage activation (Matsui *et al.,* 1990; Lord *et al.,* 1990, 1993; Liebermann and Hoffman, 1994). This consensus AP-1 site is believed to be important not only for basal transcription of the 1514 LTR, but also for transcriptional activation mediated by the transactivating protein tat (Gdovin and Clements, 1992; Hess *et al.,* 1989; Neuveut *et al.,* 1993).

In addition to 1514 (Sonigo *et al.,* 1985), an Icelandic isolate that is associated with the development of CNS disease, there are a number of other geographically distinct MVV isolates (Querat *et al.,* 1990; Sargan *et al.,* 1991). The EV-1 British strain was isolated from a sheep with the pneumonic form of the disease. The EV-1 LTR contains a number of base changes when compared to the 1514 LTR. Sequence divergence between the 1514 and EV-1 viruses and sequence variation within the EV-1 population leads to LTRs with distinct transcriptional activities (Sargan *et al.,* 1995). In both EV-1 and SA-OMVV (a South African MVV isolate) there is no consensus AP-1 site within the LTR. None of 54 EV-1 LTR sequences obtained directly from infected animals contained a consensus AP-1 site (Sargan *et al.,* 1995). In the present report, the effect of this sequence divergence on tran-

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scriptional factor binding to the 1514 and EV-1 viruses was examined using oligonucleotides corresponding to various regions of the LTR in gel retardation assays. Binding of transcription factors to these sequences was compared using gel retardation assays and by insertion of oligonucleotides upstream of a basal promoter linked to a bacterial chloramphenicol acetyl transferase (CAT) reporter gene.

MATERIAL AND METHODS

Cell lines and transfections

Ovine chondrocytes were isolated from cartilage discs by digestion with collagenase (1 mg/ml) (Sigma Chemical Co., Poole, Dorset) and cultured in DMEM supplemented with 10% FCS for 5 passes. All transfection experiments were performed using pass 5 frozen cell stocks. Ovine alveolar macrophages were obtained by bronchiolar lavage. Lungs were removed from animals at postmortem and washed extensively with light palpation with precooled (4°) Hank's balanced salt solution (HBSS). Recovered fluid was centrifuged for 15 min at 1500 *g* and the resulting cell pellets were resuspended and pelleted twice more through HBSS. When checked by cytospin these pellets contained approximately 90% macrophages. Cells (3×10^8) were resuspended in 50 ml 0.32 *M* sucrose containing 25 m*M* KCl, 3 m*M* $MgCl_2$, 0.1 m*M* DTT. Splenic extracts were obtained by first mincing 5 g of spleen tissue into the same buffer. To recover nuclei, alveolar macrophages or splenic tissue was homogenized using 20 passes of a Potter type Teflon-glass homogenizer with a mechanical pestel. After larger debris was removed by filtering through a double layer of cheesecloth, nuclei were pelleted by centrifugation (1500 *g,* 4°, 15 min), and the supernatant was discarded. The crude nuclear pellet was resuspended in extraction buffer and extracts were prepared as before.

Transfections were performed in 60-mm plastic dishes ($\sim 3 \times 10^6$ cells) using calcium phosphate precipitation (5 μg plasmid/plate) as described previously (Sargan *et al.,* 1995). Whole cell extracts were prepared from transfected cells by freeze–thawing. The cells were scraped from plates after washing in phosphate-buffered saline containing 1 m*M* ethylene diamine tetraacetic acid (EDTA), microcentrifuged, and then resuspended in 250 m*M* Tris–HCl, pH 8.0. Lysis was performed with three cycles of transferring from dry ice/methanol to a 37° water bath. Debris was removed by spinning at 13,500 rpm for 5 min.

Transfection efficiency was controlled using a vector that expressed β-galactosidase (pSVβgal; Promega, Enterprise Road, Southampton, UK). β-Galactosidase was measured by mixing 50 μl of cell extract with 50 μl of 2× β-galactosidase assay buffer (120 m*M* Na_2HPO_4, 80 m*M* NaH_2PO_4, 2 m*M* $MgCl_2$, 100 m*M* β-mercaptoethanol, 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside) in a flat-bottomed microtiter plate. The plate was incubated at 37° for 1 hr or longer, and then the reaction was stopped by adding 150 μl $NaCO_3$. The results were expressed as optical density at 405 nm.

CAT assays were performed as described by Seed and Sheen (1988). Briefly, 10 μl of cell extract was mixed with 5 μl *n*-butyryl CoA (5 mg/ml), 2 μl [^{14}C]-chloramphenicol (0.025 mCi/ml), and 113 μl 250 m*M* Tris–HCl, pH 8.0. After being incubated for 3 hr at 37° the reactions were placed on ice and extracted with 250 μl of mixed xylenes (Aldrich Chemical Co., Dorset, UK). After mixing, the tubes were spun at 13,500 rpm for 3 min. The upper organic phase was removed and extracted with 250 μl water. After this back-extraction, 125 μl of the organic phase was mixed with scintillation fluid and counted for radioactivity.

Preparation of nuclear extracts

Nuclear extracts were prepared as described by Schreiber *et al.* (1989). Briefly, 1×10^7 cells were resuspended in 400 μl ice-cold hypotonic buffer A (10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* dithiothreitol). After 15 min of incubation, the cells were lysed by the addition of 25 μl of 10% Triton X-100. The sample was vortexed for 10 sec and the nuclei were immediately spun out and resuspended in 50 μl of 20 m*M* HEPES, pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT (buffer B). The nuclei were rocked vigorously at 4° for 15 min, spun out, and then stored at −70°. Prior to freezing, the protein concentration was determined using a protein assay kit obtained from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts, UK), and the extract was diluted to 5 mg/ml in buffer B. All buffers contained the following protease inhibitors: 5 μg/ml aprotonin and leupeptin, 2 μg/ml pepstatin and 1 m*M* phenylmethylsulphonyl fluoride (Sigma).

Oligonucleotides and CAT vectors

Oligonucleotides, purified by high-pressure liquid chromatography, were supplied by OSWELL DNA Services, University of Edinburgh. The oligonucleotides corresponding to the EV-1 and 1514 LTRs are shown in Fig. 1 and Table 1. The sequence denoted "EV-1" was obtained from strain EV-1 cultured *in vitro* in choroid plexus cells, while "EV-1 (7)" was obtained from proviral DNA recovered from cells in efferent lymph collected from an MVV-infected sheep, i.e., *ex vivo* (Sargan *et al.,* 1995). Specific AP-1 (denoted AP-1 (control)) and OCT-1 oligonucleotides were generated using sequences from the human collagenase promoter (Lee *et al.,* 1987) and the immunoglobulin k gene promoter (Parslow *et al.,* 1984), respectively. The AP-1 oligonucleotide sequence was chosen because it is a well-characterized AP-1 site. All nucleotides possessed *Sal*I overhangs for cloning into plasmids.

Reporter constructs

For transfection analysis, double-stranded oligonucleotides were cloned into the Δ56 vector comprising the basal c-*fos* promoter upstream of a CAT gene (Gilman *et al.,* 1986), kindly supplied by Dr. J. Quinn, Medical Research Council, Brain Metabolism Unit, Edinburgh. The oligonucleotides were inserted upstream of the basal c-*fos* promoter using a *Sal*I site. Plasmids containing inserts were sequenced in order to determine the number and orientation of inserted sequences.

Gel retardation assays

For gel retardations, oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract (5 μg) was preincubated for 5 min with sonicated and annealed poly(dI–dC)–poly(dI–dC) (5 μg; Sigma) as a nonspecific competitor prior to the addition of the radiolabeled probe in 5× retardation buffer (20% glycerol, 50 m*M* HEPES, pH 7.6, 5 m*M* $MgCl_2$, 2.5 m*M* EDTA, 2.5 m*M* DTT). Competitor oligonucleotides were added to give a 500- or a 1000-fold molar excess and the mixture was incubated for 5 min. Thereafter, 1 μl of radiolabeled probe DNA was added and mixed, and the samples were incubated for a further 10 min. All incubations were carried out on ice. Oligonucleotide complexes were separated on 4% nondenaturing polyacrylamide gels and visualized by autoradiography after 24 hr. The same amount of probe was used on each lane of the gel.

For supershift experiments, complexes were formed and incubated with radiolabeled probe as described, and then antibody was added and the samples were further incubated on ice for 30 min before electrophoresis. A rabbit anti-jun antibody specific for a peptide sequence from murine c-Jun and reactive against all Jun family members was obtained from Santa Cruz Biotech, (San Diego, CA). Rabbit antisera specific for peptides derived from acute myeloid leukemia proteins AML-1, -2, and -3 were a kind gift from Dr. Scott Hiebert. The anti-AML-1 antiserum is weakly cross-reactive with mouse and rat AML-2, while the anti-AML-2 and anti-AML-3 antisera are completely specific to their cognate AML family members in mouse and rat (see Meyers *et al.,* 1996).

Statistics

The results of the transfection experiments were analysed using Student's two-sample *T* test.

RESULTS

AP-1 binding to the EV-1 and 1514 LTRs

The LTR sequences of four MVV isolates and CAEV are aligned in Fig. 1. The LTRs of the four MVV isolates share a common organization with regard to potential transcription factor binding sites, and the LTR from the closely related CAEV shows a similar structure. However, base changes between the sequences affect putative transcription factor sites. In both SA-OMVV and EV-1, base changes have mutated the consensus AP-1 site present in the 1514 virus (position 88). Although the 1514 virus contains a number of degenerate AP-1 sites, only this TATA box proximal consensus site has been shown to bind AP-1 *in vitro* (Gabuzda *et al.,* 1989; Shih *et al.,* 1992). In the 1772 virus, a derivative of 1514 (Andresson *et al.,* 1993), a consensus AP-1 site is still present, though its position is altered (Fig. 1) due to a point mutation (position 66) in one of the degenerate AP-1 sites present within the LTR. The EV-1 LTR contains the same G-to-A transition within the TATA box proximal AP-1 site, but unlike the 1772 LTR there is no second site mutation elsewhere in the LTR to regenerate a consensus site (Fig. 1). Indeed, from previous sequencing studies, the upstream AP-1 sites in the *ex vivo* EV-1 LTRs, e.g., EV-1(7), are further from consensus than those in 1514, with the exception of the potential site at position 24. The G-to-A transition in the TATA box proximal AP-1 site is found in all sequenced EV-1 LTRs (Sargan *et al.,* 1995) regardless of whether they are obtained from virus passaged *in vitro* or directly from infected animals (*ex vivo*). Studies on LTR variants suggested the binding of a transcriptional activator to this region because its duplication led to a twofold elevation in LTR activity (Sargan *et al.,* 1995).

The significance of this base change in EV-1 was analyzed by gel retardation assay using short oligonucleotides as well as longer oligonucleotides incorporating flanking sequences (Table 1). The results of assaying the long oligonucleotides are shown in Fig. 2. A control AP-1 sequence (Table 1) was used to confirm that any shifts observed with the MVV probes were due to AP-1 binding and not an additional transcription factor in this region.

Distinct transcription factor binding patterns were observed with the three long oligonucleotides. The long 1514 sequence bound both AP-1 and a second non-AP-1 factor (Fig. 2A, lane 6). This second protein was competed for by all three MVV-derived long sequences (lanes 7, 8, and 9), but by neither AP-1 (control) (lane 10) nor OCT-1 (not shown) consensus oligonucleotides. The two long EV-1 sequences did not bind AP-1 (lanes 1 and 11), even after longer autoradiography exposure times or varying the buffer conditions and salt concentrations (data not shown). This was also the case when the short oligonucleotides were used (data not shown). The long oligonucleotides consistently showed different affinities for the second factor, with long EV-1(7) (lanes 11 and 15) showing higher binding than the long EV-1 oligonucleotide (lanes 1 and 5), possibly due to the G-to-A transition at position 100 (Fig. 1; Table 1). This factor was also seen to be distinct from AP-1 due to its failure to interact with an anti-Jun antibody (Fig. 2B, lane 7), whereas the AP-1 factor was supershifted by the antibody (Fig. 2B, lane 3).

A second factor interacting with the MVV LTR is also a transcriptional activator

Comparison of the three LTR sequences suggested that the site for this factor was located downstream of

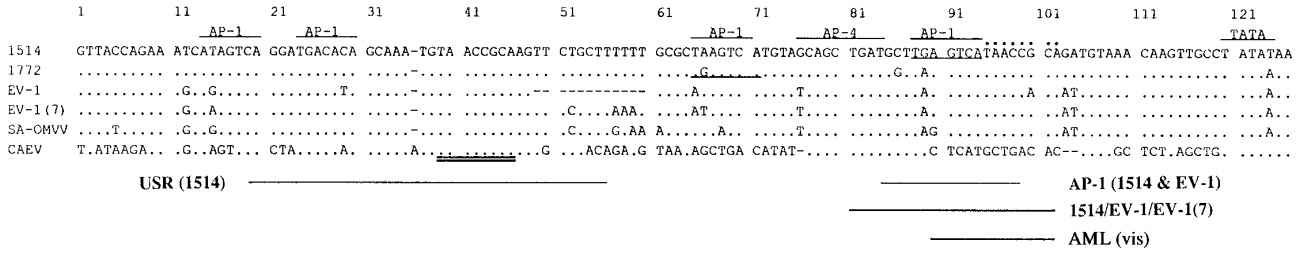

FIG. 1. Comparison of the LTR sequences of different MVV isolates and CAEV. Sequences are aligned against the 1514 sequence. First nucleotide corresponds to position 8857 for 1514 (Sonigo *et al.,* 1985), 8966 for 1772 (Andresson *et al.,* 1993), 8998 for the EV-1 sequences (Sargan *et al.,* 1991), 8904 for SA-OMVV (Querat *et al.,* 1990), and 8837 for CAEV (Saltarelli *et al.,* 1990). Potential AP-1 sites in the 1514 sequence are indicated at the top of the figure. The consensus AP-1 sites in 1514 and 1772 are underlined in each sequence. The sequence EV-1 is that of the published EV-1 viral sequence (Sargan *et al.,* 1991). EV-1(7) is a representative sequence of LTR sequences observed *in vivo* (Sargan *et al.,* 1995). The double-underlined sequence represents a conserved binding site for AML (vis) in both MVV and CAEV. The MVV LTRs carry a second site for this factor adjacent to the TATA box proximal AP-1 site. The AML (vis) consensus sequence at this site is indicated by dots above the bases. Sequences of oligonucleotides used in gel retardation assays and the LTR sequences from which they are derived are shown beneath the LTR sequence and in Table 1.

the AP-1 site. This site possessed the consensus sequence for the AML/PEBP2/CBF transcriptional factor family. This second factor was therefore tentatively designated AML (vis). An oligonucleotide derived from 1514 carrying the site for AML (vis) (Fig. 1; Table 1) was tested in gel retardation assays (Fig. 3A). This sequence gave a specific shift (lane 1) that was not competed by AP-1 (control) (lane 4) or OCT-1 (lane 5) sequences, but was competed by the long 1514 sequence (lane 3) as well as the homologous oligonucleotide (lane 2). This AML (vis) oligonucleotide does not contain the full AP-1 site, indicating that the binding site is distinct from that for AP-1. Figure 3B demonstrates that in 1514 the binding of AP-1 and this second factor is not codependent and they may be competed independently by AP-1 (control) (lane 4) and AML (vis) (lane 5). In no experiment was a third band observed that would correspond to a complex containing both AP-1 and AML (vis) bound to the 1514 sequence.

The LTRs of all the MVV isolates and CAEV contain a second, upstream copy of the AML (vis) site (position 39, Fig. 1). An oligonucleotide corresponding to the 1514 sequence and containing this sequence (designated USR in Fig. 1 and Table 1) was used in gel retardation assays. This sequence also contains a degenerate AP-1 site and a consensus E-box (CANNTG; Fisher and Goding, 1992). Figure 4 shows that the USR oligonucleotide generates a single specific shift by binding AML (vis) (lane 1). The degenerate AP-1 site and E-box do not appear to be functional as the AP-1 (control) oligonucleotide does not compete (lane 6) while the USR, AML (vis), long EV-1(7) and long 1514 oligonucleotides do (lanes

TABLE 1

Synthetic Oligonucleotides Used in the Study

AP-1 (control)	TTCCGGCTGACTCATCAAGCG
	AAGGCCGACTGAGTAGTTCGC
AP-1 (1514)	TGCTTGAGTCATAACC
	ACGAACTCAGTATTGG
AP-1 (EV-1)	TGCTTAAGTCATAACC
	ACGAATTCAGTATTGG
Long (1514)	TGATGCTTGAGTCATAACCGCA
	ACTACGAACTCAGTATTGGCGT
Long (EV-1 (7))	TGATGCTTAAGTCATAACCGCA
	ACTACGAATTCAGTATTGGCGT
Long (EV-1)	TGATGCTAAGTCATAACCACA
	ACTACGAATTCAGTATTGGTGT
AML (vis)	GAGTCA*TAACCGCA*
	CTCAGT*ATTGGCGA*
AML (consensus)	GGATCC*TAACCGCA*AAGTCGAC
	CCTAGG*ATTGGCGT*TTCAGCTG
OCT	TGTCGAATGCAAATCACTAGAA
	ACAGCTTACGTTTAGTGATCTT
Upstream region (USR)	TCAGGATGACACAGCAAATGTAACCGCAAGTTCTGCTT
	AGTCCTACTGTGTCGTTTACATTGGCGTTCAAGACGAA

Note. Bases underlined are those which differ between the EV-1/EV-1(7) and the 1514 sequences. Sequences shown in boldface are consensus or putative AP-1 sites. Sequence shown in boldface italics is the consensus sequence for AML-1/PEBP2/CBF (Kamaci *et al.,* 1990; Melnikova *et al.,* 1993; Wang and Speck, 1992; Thornell *et al.,* 1988).

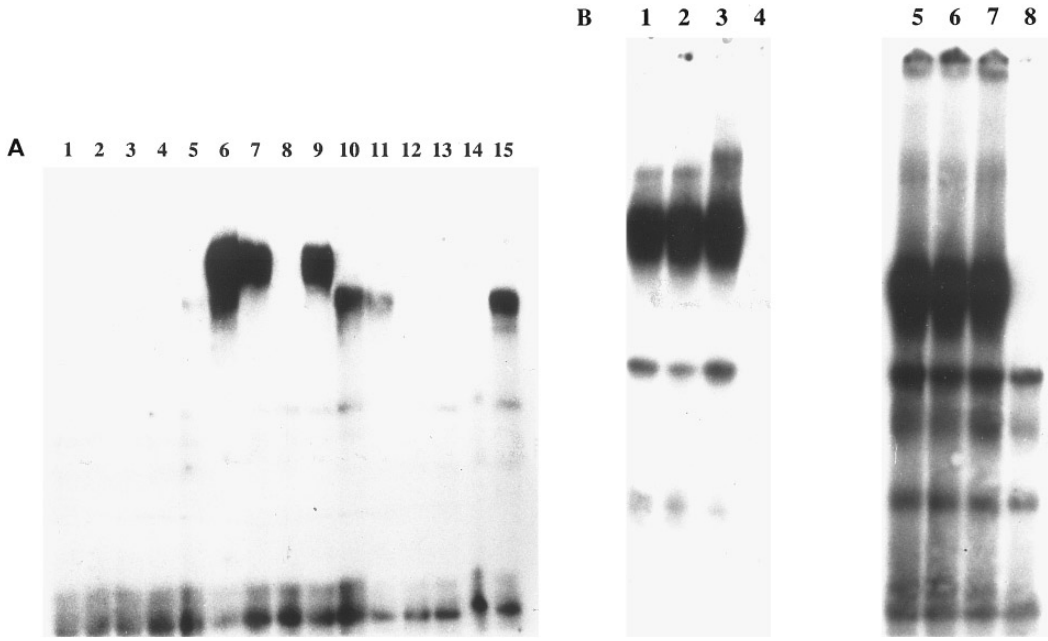

FIG. 2. Gel retardation and AP-1 supershift assays. (A) Comparison of the binding of ovine chondrocyte nuclear extracts to radiolabeled oligonucleotide analogues of EV-1, 1514, and an *ex vivo* derived sequence EV-1(7). The radiolabeled probes used were as follows: long EV-1, lanes 1–5; long 1514, lanes 6–10; long EV-1(7), lanes 11–15. The competitors used were as follows: none, lanes 1, 6, and 11; long EV-1, lanes 2, 7, and 12; long 1514, lanes 3, 8, and 13; long EV-1(7), lanes 4, 9, and 14; AP-1 control, lanes 5, 10, and 15. (B) AP-1 supershift assay. The radiolabeled short AP-1 1514 and AML (vis) probes were in lanes 1–4 and 5–8, respectively. The competitors used were as follows: none, lanes 1–3 and 5–7; control AP-1, lane 4; AML (vis), lane 8. Rabbit IgG antibodies were added to assay mixtures as follows: none, lanes 1 and 5; control IgG, lanes 2 and 6; IgG anti-Jun antibody, lanes 3 and 7.

2–5), although the long 1514 nucleotides do not contain E-box sequences.

This variation in factor binding in the gel shift assay was tested further in transfection experiments where these sequences were inserted upstream of the Δ56 basal promoter (Fig. 5). The data showed that the 1514 sequence was a more potent *cis*-activator than either EV-1 sequence (Fig. 5A). This result is consistent with the observations from the gel retardation experiments where there was no detectable binding of AP-1 to the long EV-1 sequences. The ability of the EV-1 sequences to *cis*-activate a heterologous promoter suggests that this factor is a transcriptional activator. However, neither serum nor PMA induced further activation of the Δ56 basal promoter over and above that of the vector without insert (data not shown), possibly due to the chondrocytes being maximally activated already *in vitro.*

In order to determine if AML (vis) is a transcriptional activator, multimerized sites were placed upstream of the c-fos basal promoter in the vector Δ56. The results showed that the factor does function as a *trans*-activator of transcription following incubation with 5% FCS or PMA (Fig. 5B). Again the factor appeared to be maximally induced in chondrocytes in *in vitro* culture in the absence of serum or PMA (data not shown).

The USR sequence is also capable of *cis*-activating the Δ56 heterologous promoter as shown in Fig. 5C. Comparison of other single copies of sequences containing the AML (vis) site suggests that the context of the upstream site confers an increased *cis*-activating activity. It is unclear whether this is due to the context of the AML (vis) site or to the presence of other degenerate transcription factor sites in the USR sequence. The USR oligonucleotide also contains a copy of the degenerate AP-1 site (position 24, Fig. 1), which is conserved between the 1514 and the EV-1 LTRs. This sequence did not generate a specific retarded complex in gel shift assays (Fig. 4).

The relationship between the AML (vis) site in the visna LTRs and the runt domain family of transcription factors was studied further using gel shift and competition assays with an oligonucleotide containing the consensus AML site (Table 1; Thornell *et al.,* 1988), and with antisera directed against peptides in each of the known AML family members. In these assays, extracts from ovine alveolar chondrocytes (Fig. 6), macrophages (Fig. 7), and spleen (Fig. 8) were used. In these experiments, the shift patterns seen differed in detail for each extract, but were the same whether AML (vis) or the AML (consensus) site was used. However, in each case a higher proportion of the oligonucleotide was shifted when the AML (consensus) site was used than when the AML (vis) site was used. When cold competitor oligonucleotides were added to the binding reactions, the AML (consensus) and the AML (vis) both reduced binding to each other.

In chondrocytes, the AML (consensus) oligonucleotide gave an additional major shift (Fig. 6, lane 1, bottom

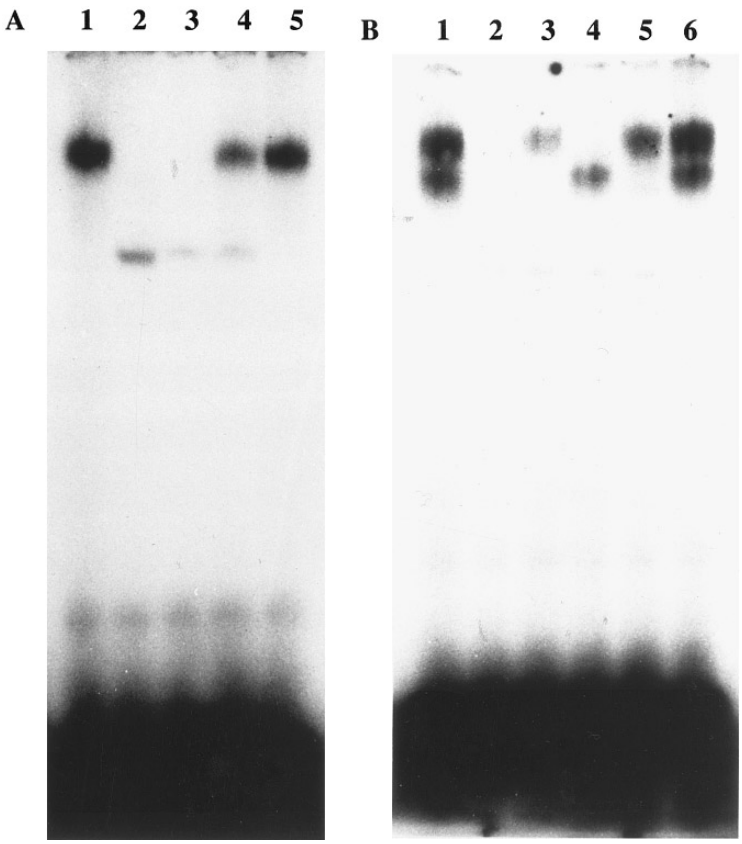

FIG. 3. Comparison of the AML (vis) with long 1514 and long EV-1 sequences (Table 1) in gel retardation assays. The assays were performed with radiolabeled oligonucleotide probes and ovine chondrocyte nuclear extracts. (A) The radiolabeled AML (vis) probe was used in lanes 1–5. The competitors used were as follows: none, lane 1; AML (vis), lane 2; long 1514, lane 3; AP-1 control, lane 4; OCT-1, lane 5. (B) The radiolabeled long 1514 probe was used in lanes 1–5. The competitors used were as follows: none, lane 1; long 1514, lane 2; long EV-1(7), lane 3; AP-1 control, lane 4; AML (vis), lane 5; OCT-1, lane 6.

arrow) with chondrocyte extracts. The major shifts observed were inhibitable by excess cold AML (vis) (Fig. 6, lane 2) or AML (consensus) oligonucleotide (Fig. 6, lane 3). In the converse experiment, gel shifts were obtained with labeled AML (vis) oligonucleotide (Fig. 6, lanes 4–6). The major shift (Fig. 6, lane 4, arrow) was inhibitable with unlabeled AML (vis) (Fig. 6; lane 5) or AML (consensus) oligonucleotide (Fig. 6, lane 6), though the AML (consensus) oligonucleotide gave better inhibition than the AML (vis) oligonucleotide.

In macrophages (Fig. 7), the AML (consensus) was able to abolish detectable binding to the labeled AML (vis) when present in a 100-fold excess (Fig. 7A, lane 9 versus lane 1), while in binding to labeled AML (consensus), a 2000-fold excess of AML (vis) (Fig. 7B, lane 8 versus lane 1) or a 100-fold excess of AML (consensus) (Fig. 7B, lane 9 versus lane 1) competed similarly, but not completely. A 2000-fold excess of AML (consensus) did, however, abolish binding (data not shown). In all reactions, poly(dI–dC) was present in $>$10,000-fold excess.

Anti-AML antisera were able to cause supershifts with macrophage extracts, and once again the results were similar whether the AML (consensus) or the AML (vis) sites were used. Supershifts were seen with antibodies directed against AML-1 (Fig. 7A, lane 2 versus lane 1) or AML-2 (Fig. 7A, lane 3 versus lane 1). The latter antiserum qualitatively supershifted the top shift band in this extract without affecting the bottom band. No supershifting was seen with the anti-AML-3 antiserum (Fig. 7A, lane 4). Similar results were obtained with the labeled AML (consensus) oligonucleotide (Fig. 7B, lanes 2 and 3, respectively), though the shifted bands were more prominent. A faint supershift was obtained with the anti-AML-3 antiserum (Fig. 7B, lane 4). The anti-AML-2 shifted band was inhibited by a 100-fold excess of cold AML (consensus) oligonucleotide (Fig. 7B, lane 5 versus lane 3).

The single major shift seen with labeled AML (vis) in chondrocytes (Fig. 6, lane 1) was supershifted weakly by the anti-AML-1 antiserum, but not supershifted at all with the anti-AML-2 or anti-AML-3 antisera (data not shown). A similar result was obtained when labeled AML (consensus) oligonucleotides were used, though the anti-AML-1 supershift was more marked than when labeled AML (vis) was used (data not shown). Spleen gave a much broader shift band of complex structure. In this extract, anti-AML-1 gave two weak supershift complexes with labeled AML (vis) (Fig. 8, lane 2) while anti-AML-2

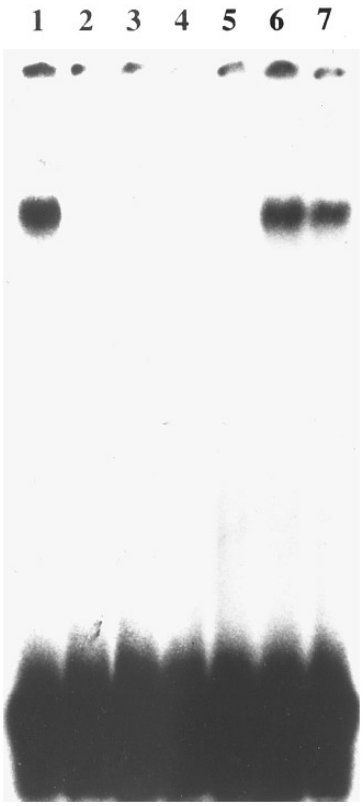

FIG. 4. Gel retardation analysis of the 1514 USR sequence. Autoradiograph of a gel retardation assay using chondrocyte extracts and radiolabeled USR oligonucleotide as probe (see Fig. 1). The competitors used were as follows: none, lane 1; USR, lane 2; AML (vis), lane 3; long EV-1(7), lane 4; long 1514, lane 5; AP-1 control, lane 6; OCT-1, lane 7.

gave a single strong supershift (Fig. 8, lane 3). Anti-AML-3 did not produce any supershifts (Fig. 8, lane 4). The supershift obtained with anti-AML-2 was inhibited completely with a 100-fold excess of cold AML (consensus) oligonucleotide (Fig. 8, lanes 5 and 6). The anti-AML-1 antiserum gave two distinct supershifted bands with the labeled AML (consensus) oligonucleotide (Fig. 8, lane 7), while both anti-AML-2 and anti-AML-3 antisera produced strong single supershifts (Fig. 8, lanes 8 and 9, respectively).

DISCUSSION

In the present study, some of the effects of LTR sequence variation between different MVV isolates on transcriptional factor binding are assessed. Differences were observed in the transcription factors interacting with the LTRs of the 1514 and EV-1 viruses. No direct evidence was found of AP-1 interaction with the EV-1 LTR. This may, in part, explain our previous observation that EV-1 LTR variants show lower transcriptional activity than the 1514 LTR (Sargan *et al.,* 1995). In contrast, a second factor was seen to bind both 1514 and EV-1 LTRs. The binding site possessed the consensus sequence for the AML/PEBP2/CBF family of transcription factors, and the factor was tentatively designated AML (vis). Unlike AP-1, the binding sites for AML (vis) are conserved between all MVV isolates sequenced to date (Sonigo *et al.,* 1985; Saltarelli *et al.,* 1990; Querat *et al.,* 1990; Sargan *et al.,* 1991; Andresson *et al.,* 1993), suggesting that this transcription factor plays a key role in the regulation of MVV replication. In contrast, the presence of a consensus AP-1 site appears to be restricted to the 1514 virus and its derivatives (Sonigo *et al.,* 1985; Andresson *et al.,* 1993). Both the 1514 and the 1772 LTRs carry a consensus AP-1 site. In contrast, no consensus site is found in either the EV-1 or the SA-OMVV LTRs (Fig. 1).

In the 1514 LTR, the TATA box proximal AP-1 site has been described as a key regulator of transcription (Gabuzda *et al.,* 1989; Hess *et al.,* 1989). It has been reported to be important for both inducible and tat activated transcription from the 1514 LTR. The failure to observe interaction of AP-1 with the EV-1 LTR sequence does not directly rule out the possibility that the degenerate AP-1 site at this position has an affinity too low to allow binding to be detected in the gel retardation assay. Degenerate AP-1 sites that do not bind *in vitro,* but that are active in the context of a basal promoter, have been described (Jain *et al.,* 1992). However, when this region from the 1514 site and that from the EV-1 site are compared with regard to their ability to drive transcription, it is clear that the sequence variation results in an altered *cis* regulatory function. The difference in activity argues strongly against an interaction of AP-1 with this site in this cell system. Rather, the *cis* regulatory activity of this region may be fully accounted for by the AML (vis) site.

A second, upstream copy of the AML (vis) site also lies in close proximity to a degenerate AP-1 site in both 1514 and EV-1. A single oligonucleotide copy was found to be as active as the promoter proximal AP-1/AML (vis) element of virus 1514. No AP-1-specific shift was found in gel retardation assays, suggesting that the functional activity was due to the AML (vis) site. However, as for the promoter proximal site, a role for the degenerate AP-1 site *in vivo* cannot be totally excluded (Jain *et al.,* 1992). The relation to consensus is also not predictive of AP-1 binding (Ryseck and Bravo, 1991) as both the sequences in the core "consensus" site and the flanking sequences may determine site affinity. In addition, the composition of the AP-1 complex affects the affinity of binding to a given sequence (Ryseck and Bravo, 1991). This raises the question as to whether or not the degenerate AP-1 site within the EV-1 LTR discriminates between AP-1 complexes of different compositions. This could be tested by using *in vitro* translated proteins to determine if certain combinations of Jun and Fos/Fra proteins are able to interact preferentially with the 1514 and EV-1 LTRs.

One interesting area of speculation is the effect of altering the position of the consensus AP-1 site in the 1772 virus compared to 1514. The movement of sites within the promoter region may alter the topology of the DNA/protein complex formed and such alterations can

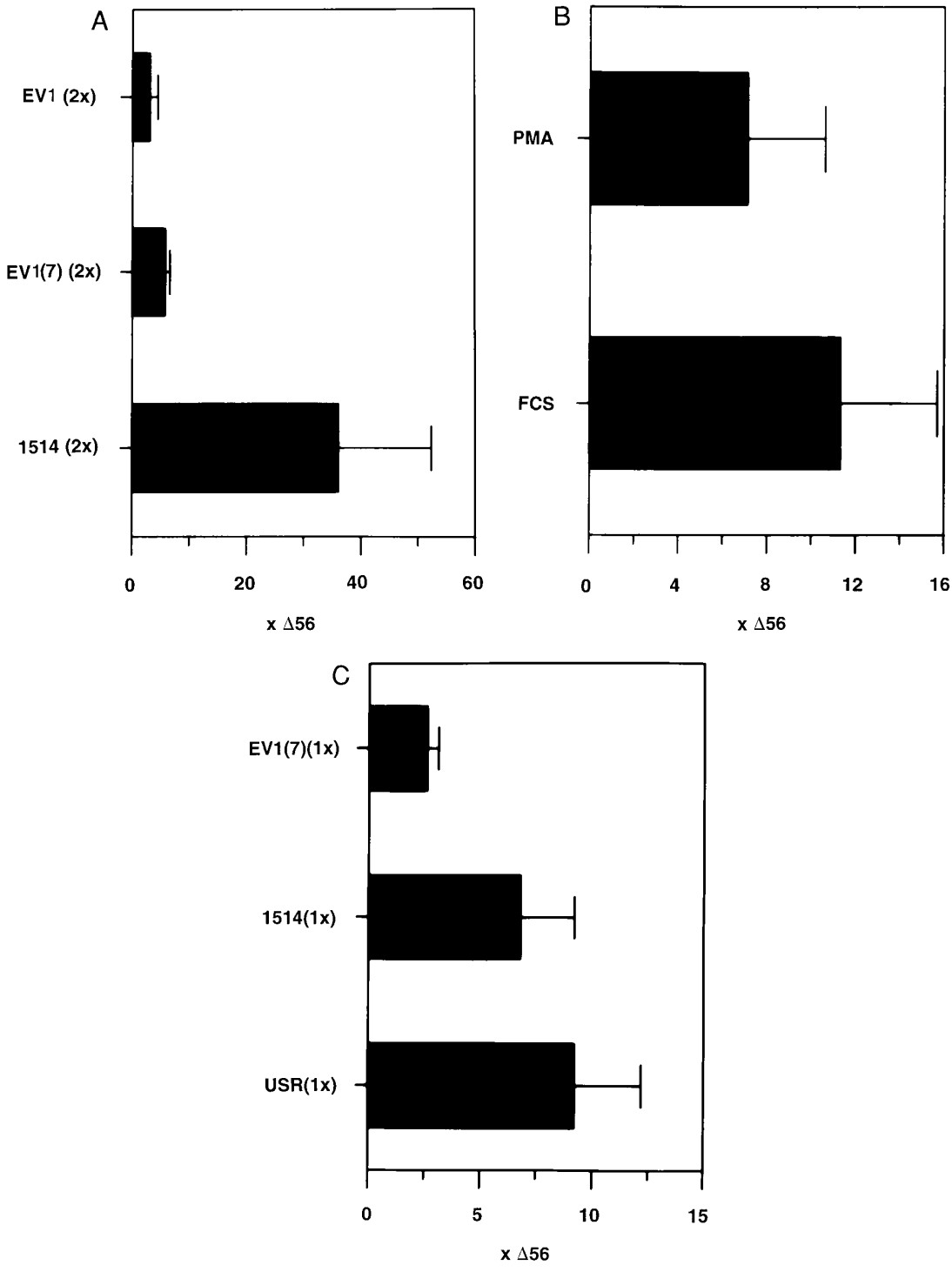

FIG. 5. (A) Transfection of long 1514, long EV-1(7), and long EV-1 sequences inserted in the Δ56 c-fos basal promoter vector into chondrocytes. The results are expressed as the fold increase in CAT expression over the Δ56 vector without any insert, and they are the mean + SD of 4 (1514; EV-1) or 5 (EV-1(7)) independent transfections. (B) Transfection of AML (vis) sequence inserted in the Δ56 c-fos basal promoter vector into chondrocytes which were subsequently incubated with either 5% FCS or PMA (100 ng/ml). The results are expressed as the fold increase in CAT expression over the Δ56 vector without any insert, and are the mean + SD of 5 (FCS) or 4 (PMA) independent transfections. (C) Transfection of the USR sequence in the Δ56 c-fos basal promoter vector into chondrocytes which were subsequently incubated with 5% FCS. The results are expressed as the fold increase in CAT expression over the Δ56 vector without any insert, and are the mean + SD of eight USR or four long EV-1(7) independent transfections.

have a considerable impact on promoter function and activity (Giese *et al.,* 1995; Cohen *et al.,* 1994; Tjuan and Maniatis, 1994; Du *et al.,* 1993; Natesan and Gilman, 1993; Thanos and Maniatis, 1992).

The conservation of the consensus AP-1 sites in 1514-like viruses compared to EV-1 and SA-OMVV raises questions as to whether these two sets of viruses have fine-tuned their transcriptional regulation. Previous compari-

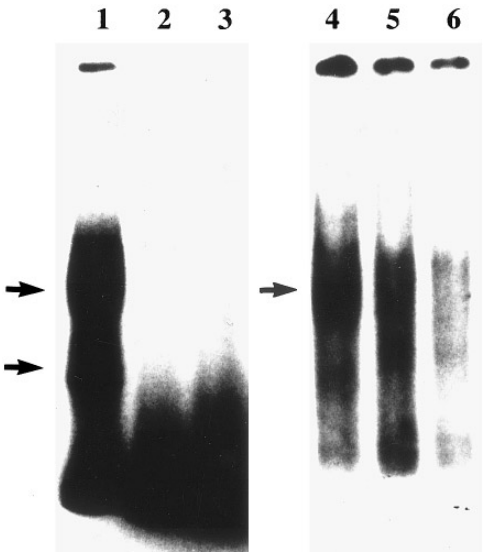

FIG. 6. Autoradiograph showing gel retardation assays using radiolabeled AML (consensus) or radiolabeled AML (vis) and nuclear extracts from chondrocytes. AML (consensus) (lanes 1–3); AML (vis) (lanes 4–6). The competitors used in 500-fold excess were as follows: none (lanes 1 and 4); AML (vis) (lanes 2 and 5); AML (consensus) (lanes 3 and 6).

son of the transcriptional activity of EV-1 LTR variants to 1514 indicated that these different LTR structures do give rise to different promoter activities (Sargan *et al.,* 1995). Whether this alteration in transcriptional regulation has any effect on the course of viral infection *in vivo* is unknown. However, it is clear that these differences in the LTR structure between the 1514 and EV-1 viruses persist during infection *in vivo.*

The sequence EV-1(7) corresponds to that prevalent *in vivo* in blood or lymph of EV-1 infected animals (Sargan *et al.,* 1995). This previous study indicated that the presence of a consensus AP-1 site within the LTR was not a requirement for viral persistence *in vivo.* None of the LTR sequences obtained from EV-1 infected animals contained such a site. In contrast, all EV-1 *ex vivo* LTRs (Sargan *et al.,* 1991, 1995) contained putative AML (vis) binding sites with 39/54 containing the high-affinity consensus site. It is important to note that the upstream AML (vis) site is in a region highly conserved between all MVV isolates and CAEV. The conservation of sites for this factor suggests that it may play an important role in controlling transcription in these two viruses. It is interesting to speculate that differences in usage of AP-1 or AML (vis) between 1514 and EV-1 may play a role in determining whether the CNS or the pulmonary system becomes clinically affected.

Transcription factors that bind AML (consensus) sites have been described under a variety of names in several systems. First identified as SL3-3 virus enhancer factor 1 (SEF-1; Thornell *et al.,* 1988), this factor has also been called polyoma enhancer-binding protein 2 (PEBP2; Kamaci *et al.,* 1990), AKV core-binding factor (CBF; Boral *et al.,* 1989), or acute myeloid leukemia 1 gene product (AML1; Miyoshi *et al.,* 1991). The site is also found in the enhancers of many genes abundantly expressed in cells of the myeloid lineages including T cell receptor γ, δ, and β; CD-3 ϵ and δ; immunoglobulin μ; the colony-stimulating factors MCSF and GMCSF; a number of interleukins, and TNFα and TNFβ. Site-directed mutation studies have shown that the site is essential for function of at least the first two of these genes (Hsiang *et al.,* 1993; Redondo *et al.,* 1991). Multiple polypeptides have been shown to bind this site, but the characterized transcription factors are heterodimers in which ubiquitously expressed β subunits, which are products of a single gene although subject to alternative splicing (Wang *et al.,* 1993), bind one of at least three tissue-specific and DNA binding α subunits with homology to the *Drosophila* runt gene (Wang and Speck, 1992; Bae *et al.,* 1994; Ogawa *et al.,* 1993; Levanon *et al.,* 1994). Further heterogeneity is also generated in the α subunits by alternate splicing (Levanon *et al.,* 1996). Interactions of different members

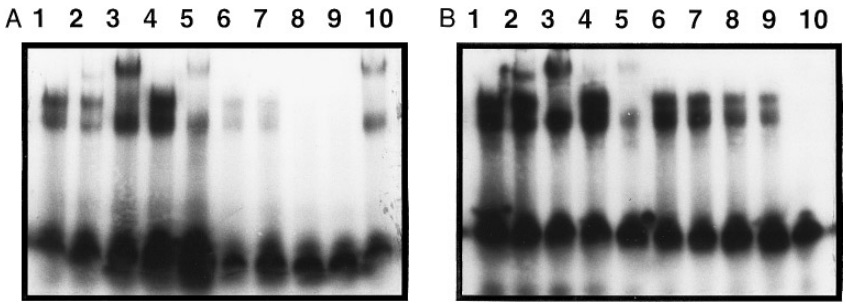

FIG. 7. Gel retardation assay of interactions between AML oligonucleotides and alveolar macrophage nuclear extract. (A) Autoradiography of a gel retardation assay using radiolabeled AML (vis) oligonucleotide (lanes 1–9) or AML (consensus) oligonucleotide (lane 10) as probe. Rabbit polyclonal antisera and unlabeled competitor oligonucleotides were added as follows: none, lane 1; anti-AML-1, lane 2; anti-AML-2, lane 3; anti-AML -3, lane 4; anti-AML-2 + AML (consensus) (×100 excess), lane 5; AML (consensus) (×1 excess), lane 6; AML (consensus) (×10 excess), lane 7; AML (consensus) (×100 excess), lane 8; AML (vis) (×2000 excess), lane 9; anti-AML-2, lane 10. (B) Autoradiography of a gel retardation assay using radiolabeled AML (consensus) oligonucleotide (lanes 1–10) as probe with macrophage extract (lanes 1–9) or no extract (lane 10). Rabbit polyclonal antisera and unlabeled competitor oligonucleotides were added as follows: none, lane 1; anti-AML-1, lane 2; anti-AML-2, lane 3; anti-AML -3, lane 4; anti-AML-2 + AML (consensus) (×100 excess), lane 5; AML (vis) (×20 excess), lane 6; AML (vis) (×200 excess), lane 7; AML (vis) (×2000 excess), lane 8; AML (consensus) (×100 excess), lane 9; anti-AML-2, lane 10.

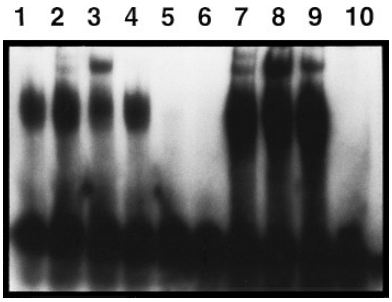

FIG. 8. Autoradiograph showing gel retardation assays using radiolabeled AML (vis) or radiolabeled AML (consensus) and nuclear extracts from spleen. AML (vis), lanes 1–6; AML (consensus) (lanes 7–10). Rabbit antisera and competitors were added as follows: normal rabbit serum, lanes 1 and 10; anti-AML-1, lanes 2 and 7; anti-AML-2, lanes 3, 5, and 8; anti-AML-3, lanes 4 and 9; AML (consensus) (100-fold excess), lanes 5 and 6. Lane 10 had no extract.

of the AML/PEBP2/CBF family with a promoter may either upregulate or block transcription (Bae *et al.,* 1994; Takahashi *et al.,* 1995).

The AML (vis) site (5′-TAACC$^{G}/_{A}$CA-3′) bears a striking resemblance to the consensus site for AML/PEBP2/CBF transcriptional factors. The results of our competition gel shift experiments showed that oligonucleotides containing the consensus sequence for AML (consensus) or AML (vis) cross-competed for specific shifts induced by extracts from chondrocytes, alveolar macrophages, and spleen cells. These shifts could be supershifted partly with antisera-specific AML factors. Thus members of the AML family can be identified in extracts from cell types in which the virus is transcriptionally active and can interact with sequences in the viral promoter. However, some of the interactions seen with the AML site in gel shift assays do not appear to give rise to supershifts with polyclonal antisera directed to the currently known AML family members, so that other proteins may also be involved in interactions with this LTR element. The results suggest that AML (vis) may indeed belong to the AML/PEBP2/CBF family of transcription factors and that it plays a central role in controlling ruminant lentivirus replication. Future studies will be directed toward a fuller characterization of AML (vis) in sheep cells and its role in controlling MVV replication.

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