JOURNAL OF VIROLOGY, Feb. 1990, p. 543–550
0022-538X/90/020543-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 64, No. 2

# Point Mutations in the Moloney Murine Leukemia Virus Enhancer Identify a Lymphoid-Specific Viral Core Motif and 1,3-Phorbol Myristate Acetate-Inducible Element

NANCY A. SPECK,† BORIS RENJIFO, AND NANCY HOPKINS*

*Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

Received 17 July 1989/Accepted 4 October 1989

**The transcriptional enhancer of the Moloney murine leukemia virus (MoMLV) is organized as a 75-base-pair repeat, and in each copy of the repeat there are multiple binding sites for nuclear factors. We have introduced point mutations into each of the known nuclear factor-binding sites in the MoMLV enhancer, in both copies of the direct repeat, and have analyzed the transcriptional activity conferred by the mutated enhancers by transient-expression assays in both hematopoietic and nonhematopoietic cell lines. Mutation of individual binding sites in the MoMLV enhancer has moderate effects (<2-fold to 20-fold) on transcription in six independent cell lines. Several mutations decreased transcription from the MoMLV enhancer ubiquitously (the leukemia virus factor b site and the glucocorticoid response element), whereas others affected transcription specifically in lymphoid cell lines (core motif) or, more significantly, in fibroblasts (nuclear factor 1 site). The transcriptional activity of the MoMLV enhancer can be induced 8- to 10-fold by 1,3-phorbol myristate acetate in Jurkat T cells. Mutations in any of three adjacent binding sites (leukemia virus factor b and c sites and the core motif) within a 28-base-pair region in the center of the direct repeat sequence of the MoMLV enhancer completely attenuate the response to 1,3-phorbol myristate acetate.**

The transcriptional enhancers of murine type C retroviruses are usually present as a direct repeat of a sequence roughly 50 to 100 base pairs (bp) in length and are located about 200 to 350 nucleotides 5′ of the cap site (27). Because enhancers play such an important role in viral gene expression, it is not surprising that these sequences encode determinants of many interesting viral phenotypes. Perhaps most interesting in the case of the nondefective murine type C viruses is the role of enhancers as determinants of viral pathogenesis. Naturally occurring murine type C viruses differ dramatically in their ability to cause leukemias, in the latency period of disease induction, and in the types of hematopoietic tumors they induce (61). The long terminal repeat and, in several cases, more specifically, the retroviral enhancer have been shown to specify these phenotypes, as well as the related properties of organ-tropic or cell-type-specific viral replication (4, 5, 9, 12, 21, 22, 26, 30–32, 46). To better understand how enhancer elements encode viral phenotypes, we and others have undertaken detailed genetic and biochemical analyses of these elements (3, 15, 22, 33, 54, 55).

In vitro biochemical analyses have revealed a complex organization of binding sites for nuclear factors on type C murine leukemia virus (MLV) enhancers (33, 54). The enhancer from Moloney MLV (MoMLV), for example, contains three glucocorticoid response elements (GRE), the binding site for hormone receptors, four binding sites for nuclear factor 1 (NF1), and two copies of a conserved viral core motif [TGTGG(A/T)(A/T)(A/T)G] also found in the simian virus 40 (SV40) and polyomavirus enhancers that is the binding site for both the CCAAT/enhancer-binding protein (C/EBP) and activating protein 3 (AP-3) (8, 24, 34, 35, 54, 60). In addition, there are binding sites for factors that were identified as binding activities in unfractionated nuclear extracts, originally named leukemia virus factors a, b, and c (LVa, LVb, and LVc), and the corresponding sites are called the LVa-, LVb-, and LVc-binding sites, respectively (54). It is now apparent, both by analogy to other systems and from further characterization of the MoMLV enhancer, that multiple proteins can bind to some of these sites in vitro, and in some cases both tissue-specific and ubiquitously expressed proteins bind to the same site (25, 33, 54; N. R. Manley, M. A. O'Connell, and N. Hopkins, unpublished results). The assembly of proteins on the intact enhancer in vivo, however, is presumably determined by the availability of proteins specific for each site in the nucleus of the infected cell, as well as by interactions with proteins binding to adjacent sites on the enhancer. It is supposedly this assembly of nuclear factors on the intact enhancer in vivo that, by some mechanism, confers a distinct disease phenotype to the virus.

To study the contribution of individual protein-binding sites to viral enhancer function, and with the goal of ultimately understanding the role of these proteins in specifying disease phenotypes, we have introduced point mutations at several nucleotides in each of the identified binding sites on the MoMLV enhancer. The specific nucleotides that were mutated were chosen from among those previously determined by methylation interference assays to be critical contacts for protein-DNA complex formation (54). In this report, we describe the effects of these mutations on both the basal level of transcriptional activity of the MoMLV enhancer in established cell lines and the inducibility of transcription by 1,3-phorbol myristate acetate (TPA) in Jurkat T cells. The effect of the mutations on the disease-inducing phenotype of MoMLV will be described elsewhere (N. A. Speck, B. Renjifo, E. Golemis, T. N. Fredrickson, J. W. Hartley, and N. Hopkins, Genes Dev., in press).

* Corresponding author.
† Present address: Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756.

## MATERIALS AND METHODS

**Cell lines.** The following cell lines were used in transfection experiments: EL4, a mouse T cell line; Rat-1, a rat embryonic fibroblast line; S194, a mouse B cell line; NIH 3T3, mouse fibroblasts; Jurkat, a human T cell lymphoma; and MEL, a mouse erythroleukemia line. EL4, Jurkat, MEL, and Rat-1 cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% inactivated fetal calf serum (IFCS) plus 100 U of penicillin per ml and 10 μg of streptomycin per ml. S194 was maintained in RPMI 1640 plus 5% IFCS. NIH 3T3 cells were grown in DMEM plus 10% newborn calf serum.

**Plasmids and mutations.** The 384-bp *Sau*3AI-*Kpn*I fragment of the MoMLV U3 region, containing the 75-bp repeat and extending 30 bp 3′ to the viral cap site was subcloned into the polylinker of pUCCAT to generate the plasmid pMoCAT (Fig. 1A). This plasmid was then used to generate gapped duplexes for mutagenesis. The mutagenic oligonucleotides (0.4 μg) were annealed to 1 μg of heteroduplex pMoCAT plasmid carrying a single-stranded gap covering the U3 region. Annealed products were repaired for 1.5 h at room temperature with Klenow DNA polymerase and T4 DNA ligase in 50 mM Tris hydrochloride (pH 7.6)–10 mM $MgCl_2$–10 mM dithiothreitol–1 mM spermidine–1 mM ATP–0.1 mg of bovine serum albumin per ml. The repaired products were transformed into bacteria, and mutants were selected by colony filter hybridization with the end-labeled mutagenic oligonucleotide probe (29, 64). Sequences from *Sau*3AI to *Kpn*I of the enhancer mutants were confirmed by dideoxy-chain termination (Sequenase) (48).

Enhancers containing mutations in both copies of the direct repeat were assembled from enhancers that were mutated in either the promoter-proximal or promoter-distal repeat by substituting the internal *Eco*RV-*Eco*RV fragment from an enhancer that contained a mutation in sequences flanking the internal *Eco*RV fragment, with the 75-bp *Eco*RV fragment from an enhancer with a mutation within the *Eco*RV restriction sites. For mutations at LVb, LVb/core, and LVb/LVc, which destroy the *Eco*RV site, enhancers were assembled from *Hin*dIII-*Pvu*II, *Pvu*II-*Pvu*II, and *Pvu*II-*Kpn*I fragments, cloned into the pUCCAT vector at *Hin*dIII-*Kpn*I. All plasmids used for transfection were banded two to four times by equilibrium density gradient centrifugation in CsCl. The migration of unrestricted plasmids in agarose gels, minus ethidium bromide, was analyzed to confirm that each plasmid had an approximately equivalent distribution of form I, II, and III DNA.

**Transfection and CAT assays.** Transient transfection of EL4, Jurkat, and S194 cells was performed by the DEAE-dextran method (20). Cells ($5 \times 10^6$ to $1 \times 10^7$) were suspended in 1 ml of TS (8 g of NaCl per liter, 0.38 g of KCl per liter, 0.1 g of $Na_2HPO_4 \cdot 7H_2O$ per liter, 3.0 g of Tris per liter, 0.1 g of $MgCl_2$ per liter, 0.1 g of $CaCl_2$ per liter) (pH 7.4) containing 2 μg of the appropriate plasmid plus 8 μg of sheared salmon sperm DNA, and 250 μg of DEAE-dextran ($M_r$ $5 \times 10^5$; Pharmacia, Inc.) for 20 min at room temperature. Medium (10 ml) containing 5% IFCS plus 100 μM chloroquine diphosphate (Sigma Chemical Co.) was then added. The cells were incubated for 40 min at 37°C in capped tubes. The cells were then pelleted, suspended in 10 ml of medium plus 10% IFCS and antibiotics, and incubated for 48 h at 37°C under 7.5% $CO_2$.

NIH 3T3 and Rat-1 cells were transfected by the $CaPO_4$ method (14). Cells ($5 \times 10^5$) were plated on 100-mm dishes 24 h before transfection. The cells were transfected with 2 μg of plasmid DNA plus 8 μg of sheared salmon sperm DNA. The $CaPO_4$ DNA precipitates were left on the cells for 4 h and then replaced with fresh media.

MEL cells were transfected by electroporation (42, 63). Cells ($1 \times 10^7$ in 0.7 ml of DMEM plus 10% IFCS) were mixed in a sterile cuvette (Bio-Rad Laboratories) along with 20 μg of plasmid DNA in 0.1 ml of 10 mM Tris–1 mM EDTA. Electroporation conditions were 960 F and 250 V. Following electroporation, cells were left in the cuvette for 5 to 10 min and then transferred to a flask containing 30 ml of DMEM–IFCS.

Inductions with TPA (Sigma) were conducted in DMEM containing 10% IFCS. TPA was added to the cells 24 h posttransfection, at a final concentration of $10^{-7}$ M (1 μl of a $10^{-3}$ M stock in dimethyl sulfoxide plus 10 ml of medium).

At 48 h later, all cells were pelleted, washed in phosphate-buffered saline, and suspended in 100 ml of 0.25 M Tris (pH 7.8). Cell lysates were prepared by three freeze-thaw cycles, and protein concentrations of the supernatants were determined (Bio-Rad Laboratories) (17). Assays were standardized to 10 to 100 μg of protein for each sample and incubated with 0.1 μCi of [$^{14}$C]chloramphenicol for 0.5 to 3 h at 37°C. The conversion of chloramphenicol to its mono- and diacetylated derivatives was monitored by thin-layer chromatography (17). Transfections were normalized by repeated transfections ($n$ = 2 to 6), with three to five parallel plates transfected for each mutant in each experiment.

## RESULTS

**Introduction of point mutations in the MoMLV enhancer.** A DNA fragment from the MoMLV U3 region, including sequences from the *Sau*3AI restriction site immediately 5′ to the direct repeat, to the *Kpn*I site 30 bp 3′ to the viral cap site at the 5′ boundary of the R region, was subcloned into a pUC13-based vector containing the gene encoding the bacterial enzyme chloramphenicol acetyltransferase, to generate the plasmid pMoCAT (Fig. 1A). This plasmid was then used to prepare gapped duplexes for site-directed mutagenesis by using synthetic oligonucleotide primers. The nucleotides that were mutated were chosen on the basis of results of methylation interference data that identified important bases for protein-DNA complex formation (54). In addition, the mutations introduced at the core site included a T → C transition that had been suggested by Clarke et al. to be an important determinant for transcriptional activity of MLV enhancers (7). A summary of the specific mutations that were introduced is shown in Fig. 1A.

We ultimately plan to correlate the effect of the enhancer mutations on gene expression with the effect on leukemogenicity. Since it had been directly demonstrated that removal of one copy of the MoMLV direct repeat significantly increased the latent period of disease onset by MoMLV, we opted to retain both copies of the direct repeat in all of the constructs (31). Therefore, we introduced identical point mutations into nuclear factor-binding sites in each copy of the repeat in pMoCAT. The binding sites that were mutated are indicated in parentheses [e.g., pMo(LVa)CAT contains mutations in both LVa sites]. The panel of mutated enhancers is illustrated schematically in Fig. 1B, with vertical lines indicating the two- or three-point mutations introduced into each binding site.

**Basal transcriptional activity conferred by the mutated MoMLV enhancers.** The transcriptional activity of the mutated MoMLV enhancers was analyzed by transient transfections of all of the constructs into a variety of cell lines,

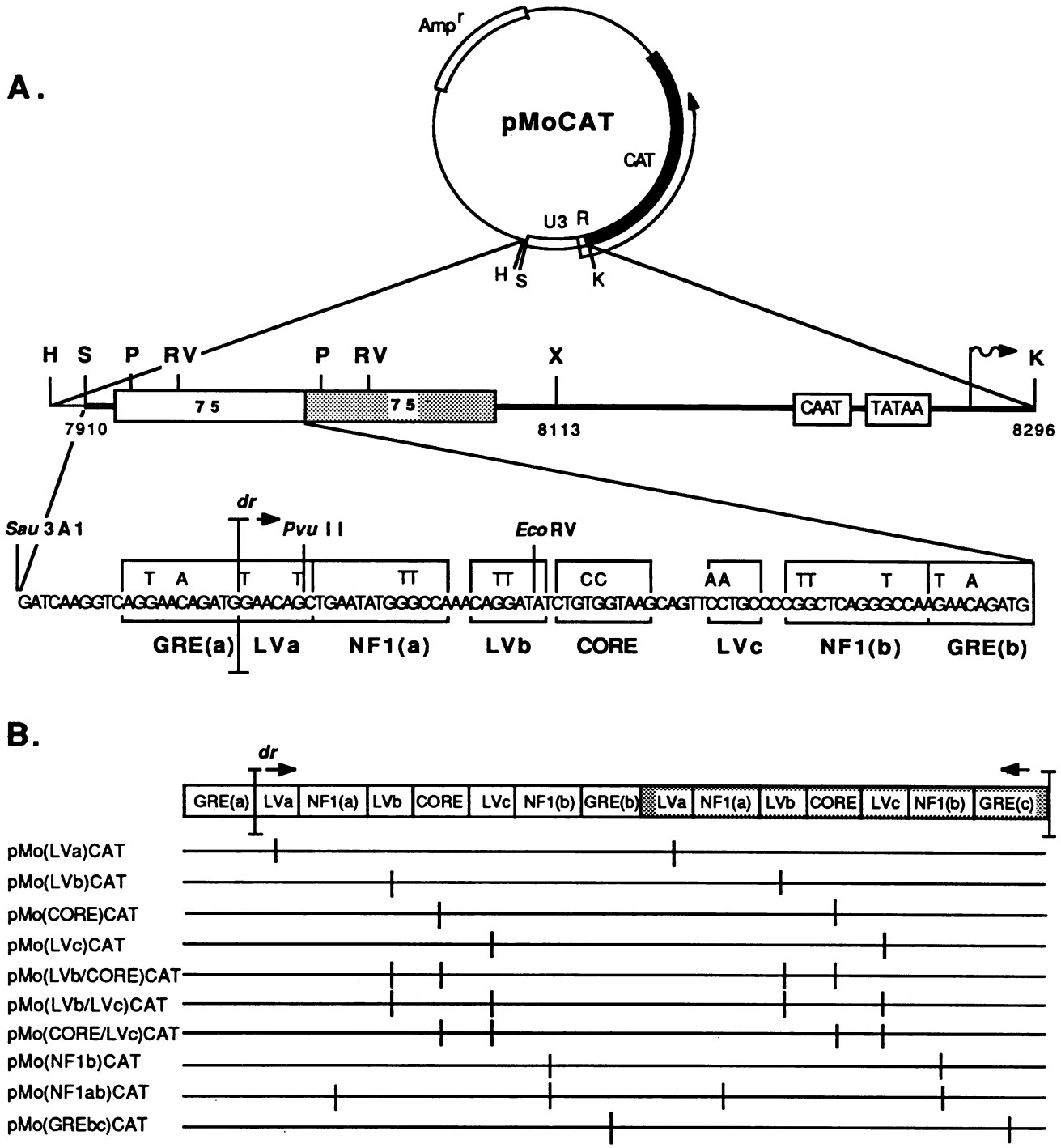


FIG. 1. Structure of the plasmids used for mutagenesis and transfections. (A) Sequences from *Sau*3AI-*Kpn*I (nucleotides 7910 to 8296, numbering from the 5′ end of the viral RNA genome [61]) encompassing a 384-bp region from the MoMLV U3/R region, including the 75-bp repeat and extending 30 bp 3′ to the viral cap site, were subcloned into the *Bam*HI-*Kpn*I site of the pUC18 polylinker in pUCCAT to generate the plasmid pMoCAT. A DNA fragment from *Xba*I at position 8113 to *Kpn*I at position 8296, containing the MoMLV promoter-proximal sequences minus the 75-bp repeat, was also subcloned into the *Xba*I-*Kpn*I site of pUCCAT to generate pMoPCAT (diagram not shown). Synthetic oligonucleotides containing base substitutions were annealed to a 400-bp single-stranded region of a gapped duplex prepared from pMoCAT, from *Hin*dIII in the pUC18 polylinker to *Kpn*I. The sequence at the bottom of this panel represents the promoter-distal copy of the direct repeat, from *Sau*3AI to the end of the first repeat. The binding sites for nuclear factors are boxed. The mutations are shown above the sequence; multiple base changes were introduced into each site. The notations a, b, or c (e.g., NF1b or NF1ab) refer to the location of these sites within the 75-bp repeat. Abbreviations: H, *Hin*dIII; S, *Sau*3AI; P, *Pvu*II; RV, *Eco*RV; X, *Xba*I; K, *Kpn*I; *dr*, direct repeat. (B) Diagram of the 75-bp repeat and the corresponding enhancer mutations. A vertical line is drawn to indicate the position of the mutation in these plasmids.

TABLE 1. Relative transcriptional activity of MoMLV enhancer mutations

| Mutant | % Wild-type activity[a] | | | | | |
|---|---|---|---|---|---|---|
| | Rat-1 | NIH 3T3 | EL4 | Jurkat | S194 | MEL[b] |
| pMoCAT | 100 | 100 | 100 | 100 | 100 | 100 |
| pMo(LVa)CAT | 175 | 177 | 117 | 125 | 139 | ND[c] |
| pMo(LVb)CAT | 33 | 30 | 37 | 14 | 21 | 74 |
| pMo(CORE)CAT | 133 | 94 | 34 | 12 | 43 | 104 |
| pMo(LVc)CAT | 152 | 150 | 80 | 87 | 37 | 145 |
| pMo(LVb/CORE)CAT | 9 | ND | 9 | ND | ND | ND |
| pMo(LVb/LVc)CAT | 39 | ND | 17 | ND | ND | ND |
| pMo(CORE/LVc)CAT | 46 | ND | 33 | ND | ND | ND |
| pMo(NF1b)CAT | 34 | 4 | 77 | 58 | 66 | 59 |
| pMo(NF1ab)CAT | 22 | ND | 51 | 78 | ND | ND |
| pMo(GREbc)CAT | 32 | 27 | 63 | 18 | 42 | 71 |
| pMoPCAT[d] | ND | 3 | <0.1 | <0.1 | 3 | ND |

[a] Values are represented as percent activity of pMoCAT for each cell line. Absolute percent conversions of chloramphenicol to acetylated forms for pMoCAT in each experiment are as follows: Rat-1, 48.5% ($n$ = 4), 18.5% ($n$ = 4), 26.0% ($n$ = 5), 40.7% ($n$ = 5), 27.6% ($n$ = 5), 11.0% ($n$ = 5); NIH 3T3, 38.8% ($n$ = 5), 18.5% ($n$ = 3), 27.9% ($n$ = 5); EL4, 15.6% ($n$ = 5), 30.6% ($n$ = 4), 30.3% ($n$ = 4), 12.0% ($n$ = 4), 59.8% ($n$ = 4); Jurkat, 3.8% ($n$ = 5), 6.2% ($n$ = 3), 14.8% ($n$ = 4), 5.4% ($n$ = 5), 20.2% ($n$ = 3); S194, 15.5% ($n$ = 5), 10.5% ($n$ = 4), 27.9% ($n$ = 5); MEL, 1.7% ($n$ = 3), 2.6% ($n$ = 3). Values in parentheses indicate the number of independent cultures transfected per construct for each experiment. $F$ ratios were determined for each experiment; in all but one case the null hypothesis was rejected at the $P < 0.01$ level of significance.

[b] Results of one experiment significant at $P < 0.05$.

[c] ND, Not done.

[d] pMoPCAT contains promoter-proximal sequences from MoMLV, minus the direct repeat (see the legend to Fig. 1 for a description).

followed by CAT enzyme analysis. Table 1 is a summary of data from transfections into Rat-1 embryonic fibroblasts, NIH 3T3 fibroblasts, Jurkat and EL4 T cells, S194 B cells, and MEL erythroid cells. The values in Table 1 represent the activity of the mutated enhancers normalized to that of the wild-type plasmid, pMoCAT.

Mutation of both copies of a particular binding site resulted in relatively small but reproducible differences in basal transcriptional activity relative to the wild-type MoMLV enhancer (pMoCAT). The magnitude of the effect is not surprising, given the multiplicity of binding sites on the enhancer, and is consistent with similar mutational analyses of the 72-bp repeat from SV40 (39, 62). Several of the MoMLV enhancer mutations altered the transcriptional activity relative to pMoCAT similarly in both hematopoietic and nonhematopoietic cell lines. Mutation of the binding site for LVa [pMo(LVa)CAT], for example, increased the transcriptional activity of the MoMLV enhancer in Rat-1, NIH 3T3, EL4, Jurkat, and S194 cell lines, indicating that the association of a protein to the LVa site mildly represses transcription from the enhancer. Mutation of the LVb site [pMo(LVb)CAT] decreases transcriptional activity in all cell lines, except MEL cells, relative to pMoCAT. This is consistent with previous in vivo analyses that localized important *cis*-acting sequences for the transcriptional activity of the closely related Moloney murine sarcoma virus enhancer at or several nucleotides immediately 5′ to the *Eco*RV sites in the 75-bp repeat, by both 5′ deletions and linker insertion into the *Eco*RV site (50).

Mutation of the core motif [pMo(CORE)CAT] had a differential effect on transcription in lymphoid and nonlymphoid cell lines; the transcriptional activity relative to pMoCAT was attenuated in EL4 and Jurkat T cells and S194 B cells, but was equivalent to or slightly greater than that of the wild-type enhancer in Rat-1 and NIH 3T3 fibroblasts and MEL cells. Mutation of the LVc site [pMo(LVc)CAT] decreased the transcriptional activity of the MoMLV enhancer in S194 B cells, did not significantly decrease transcription in T cells, and increased transcription in fibroblasts and MEL cells. Mutation in two or in all four of the binding sites for NF1 [pMo(NF1b)CAT and pMo(NF1ab)CAT, respectively] decreased the basal level of transcription in all cell lines, but most dramatically in Rat-1 and NIH 3T3 fibroblasts.

Mutation in the binding sites for hormone receptor [pMo(GREbc)CAT] also decreased the basal transcriptional activity of the enhancer in all cell lines. All experiments were performed in the absence of exogenous hormone; however, occasional impurities in phenol red preparations can have a mild steroid effect and could conceivably contribute to the basal level of transcription of constructs with wild-type GRE sites by activating the hormone receptor (1). To eliminate this possibility, we also determined the transcriptional activity of pMoCAT and pMo(GREbc)CAT in DMEM minus phenol red indicator. No difference in the relative transcription of pMoCAT or pMo(GREbc)CAT was seen in the presence or absence of phenol red, as analyzed in NIH 3T3 cells (data not shown). Since the nuclear translocation and hence the transcriptional activation by hormone receptors is dependent on the hormone ligand (23), we conclude that proteins distinct from hormone receptors may also bind to sequences in the GRE sites and contribute to the basal level of transcription from the MoMLV enhancer in the absence of exogenous hormone. In support of this conclusion, Grundstrom has identified multiple proteins specific for the GRE sites in the SL3-3 MLV enhancer. One of these proteins, called SL3-3 enhancer factor 2, was determined to be distinct from hormone receptors (T. Grundstrom, personal communication).

We also analyzed the transcriptional activity of enhancers containing mutations at two different binding sites, both LVb plus core, core plus LVc, or LVb plus LVc. An enhancer with mutations at both LVb plus core [pMo(LVb/CORE)CAT] showed a significant decrease in transcriptional activity compared with pMoCAT, pMo(LVb)CAT, or pMo(CORE)CAT in both EL4 and Rat-1 cells. Mutations at LVb plus LVc and core plus LVc [pMo(LVb/LVc)CAT and pMo(CORE/LVc)CAT] also attenuated transcription relative to pMoCAT in Rat-1 and EL4 cells. In Rat-1 cells, mutation of LVb plus core or core plus LVc decreased transcription more than would have been predicted from the effects of mutations in either site alone. For example, neither mutations in the core nor those in LVc attenuate transcription in Rat-1 cells, yet mutation in both sites decreases activity by a factor of 2. This effect was not seen in EL4 cells; thus, mutation of the core motif, either alone or in combination with mutations in either the adjacent LVb or LVc sites, had a differential effect on transcription in lymphoid and nonlymphoid cell lines. Together, these data provide genetic evidence that distinct proteins interact with the core sequence in the cell lines that were analyzed.

**Identification of a TPA-responsive element.** Elsholtz et al. reported that transcription from the MoMLV enhancer-promoter is inducible by TPA and by epidermal growth factor (11). We determined the effect of the mutations we had constructed on the inducibility of transcription of the MoMLV enhancer-promoter by TPA in Jurkat T cells. Figure 2A shows a representative experiment, and Fig. 2B is a summary of data from several experiments. Addition of $10^{-7}$ M TPA to Jurkat T cells cultured in 10% serum 24 h before the cells are harvested results in an 8- to 10-fold

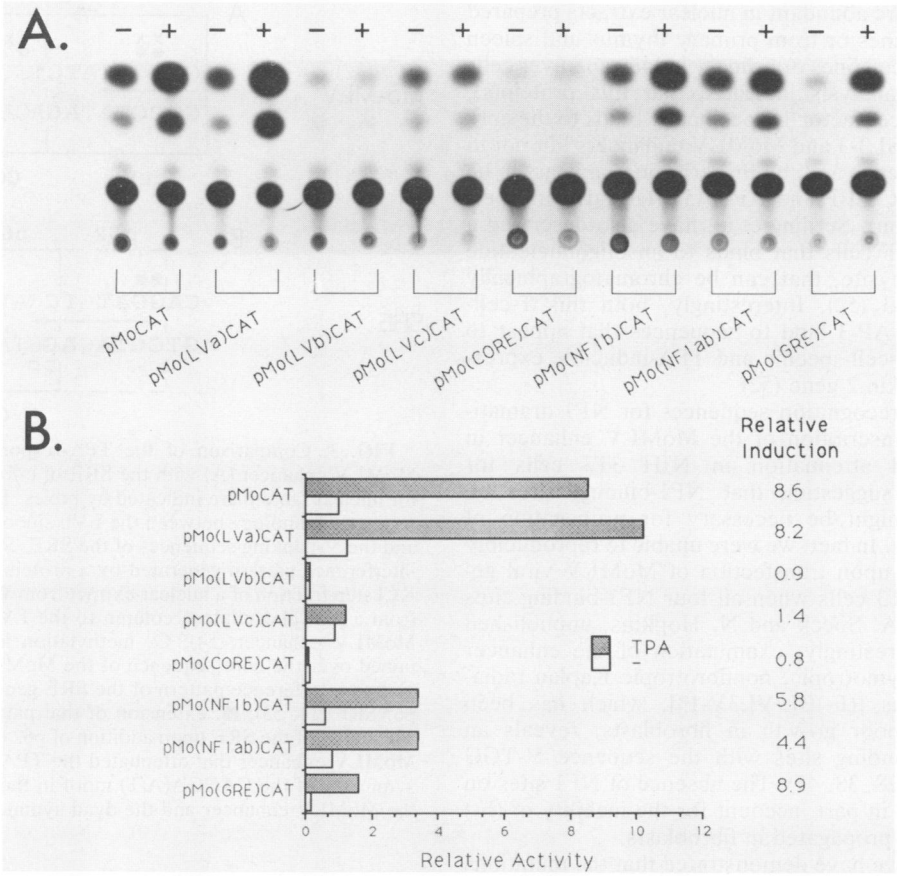


FIG. 2. Effect of mutations in the MoMLV enhancer on transcriptional activation by TPA. (A) Representative chloramphenicol acetyltransferase assay of transfections of MoMLV enhancer mutations into Jurkat T cells, following addition of TPA (+), versus unstimulated control cultures (−). (B) Summary of TPA induction experiments. Absolute percent conversions of chloramphenicol to acetylated forms for pMoCAT in noninduced cultures are the same as reported in Table 1. The bars represent the relative activity of each extract compared with the noninduced wild-type enhancer, pMoCAT. Values on the right-hand side indicate the induction by TPA of each construct relative to its basal activity. Results are significant at $P < 0.01$.

induction of chloramphenicol acetyltransferase activity from the wild-type enhancer, pMoCAT. Mutations in the LVa or GRE sites [pMo(LVa)CAT and pMo(GREbc)CAT] do not affect the induction by TPA. In contrast, mutations in the LVb-, core-, or LVc-binding sites eliminate the TPA response. Mutations in two or four of the NF1 sites [pMo(NF1b)CAT and pMo(NF1ab)CAT] reduce the TPA induction twofold relative to pMoCAT, suggesting that the association of NF1 is necessary for the maximal TPA response, or, alternatively, that the TPA-responsive element extends into the adjacent NF1-binding sites.

## DISCUSSION

Mutations introduced into previously identified binding sites for nuclear factors on the MoMLV enhancer have identified both positive and negative *cis*-acting regulatory sequences. Mutation of either the LVb or GRE sites attenuated transcription in most cell lines. Several mutations caused differential effects in different cell lines: mutation of the conserved core sequence decreased transcription in B and T cell lines, but not in fibroblasts or MEL cells; conversely, mutations in the binding sites for NF1 decreased transcription more significantly in fibroblasts than in hematopoietic cell lines.

The finding that the viral core sequence confers a transcriptional preference in T cells corroborates results obtained by Thornell et al. (55) and Boral et al. (3) for the analogous core sequence from the SL3-3 MLV enhancer. In vivo transcriptional analyses demonstrated that the SL3-3 core motif (TGTGGTTAA) conferred two- to fivefold more activity in T cells than did the core sequence from either Akv-MLV (TGTGGTCAA) or a mutated core sequence (TGTGTGGAA) (3, 55). Both investigators reported that the SL3-3 MLV core sequence was preferentially transcribed in T cells compared with B cells. Our results suggest that the transcriptional attenuation upon mutating the MoMLV core sequence is lymphoid specific (B plus T) rather than T-cell specific, as was seen for the core motif from SL3-3 MLV.

Two distinct proteins have been purified that can bind to the conserved core sites in both the SV40 and Moloney murine sarcoma virus enhancers. Neither of these proteins, AP-3 or C/EBP, appears to be specifically expressed in T cells; AP-3 was purified from an epithelial cell line (HeLa), and the expression of C/EBP appears to be restricted to cells that rapidly metabolize lipid and cholesterol-related compounds (2, 6, 24, 34, 36). Thornell et al., however, have identified a protein-DNA complex in unfractionated nuclear extracts that binds the core sequence from SL3-3 MLV and

that is relatively more abundant in nuclear extracts prepared either from T cell lines or from primary thymus and spleen cells than in non-T-cell lines or primary kidney and liver cells (55). Competition analysis indicated that this protein(s), called SL3-3 enhancer factor 1, specifically binds to the core sequence from the SL3-3 and MoMLV enhancers but not to the SV40 core sequence and, by this criterion, appears to be distinct from both C/EBP and AP-3 (55; Grundstrom, personal communication). Serfling et al. have also described a protein from EL4 T cells that binds to an oligonucleotide containing the core site, that can be chromatographically resolved from AP-3 (52). Interestingly, both this T-cell-specific factor and AP-3 bind to sequences that appear to contribute to the T-cell-specific and TPA-inducible expression of the interleukin-2 gene (52).

Mutation of the recognition sequences for NF1 dramatically decreased transcription of the MoMLV enhancer in fibroblasts [20-fold attenuation in NIH 3T3 cells for pMo(NF1b)CAT], suggesting that NF1-binding sites on MLV enhancers might be necessary for propagation of MLVs in fibroblasts. In fact, we were unable to reproducibly obtain viral stocks upon transfection of MoMLV viral genomes into NIH 3T3 cells when all four NF1-binding sites were mutated (N. A. Speck and N. Hopkins, unpublished observations). Interestingly, examination of the enhancer sequence of the thymotropic, nonfibrotropic Kaplan radiation leukemia virus [(F-)BL/VL3V-13], which has been shown to confer poor growth in fibroblasts, reveals an absence of NF1-binding sites with the sequence 5′-TGG $N_{6-7}$GCCA-3′ (10, 28, 38, 45). The absence of NF1 sites on this enhancer may, in part, account for the inability of (F-) BL/VL3V-13 to be propagated in fibroblasts.

In several cases we have demonstrated that the mutations we introduced disrupt the binding of nuclear factors to the enhancer. The mutations in the core site eliminate binding of the C/EBP (B. Graves and N. A. Speck, unpublished results); mutations in the GRE sites disrupt binding of purified glucocorticoid hormone receptor (N. A. Speck, unpublished results); and mutations in the LVb and LVc sites disrupt binding of a factor, detected in unfractionated T cell extracts and designated LVt, that binds to these sites but appears to be distinct from either the LVb or LVc factor (N. R. Manley, N. A. Speck, and N. Hopkins, unpublished results). We have not yet determined that the mutations affected the formation of the LVa, LVb, LVc, or NF-1 protein-DNA complexes as they were originally defined in mobility shift assays (54).

Mutation in sequences previously identified as three adjoining binding sites, LVb, core, and LVc, attenuated induction by TPA, thus defining a minimal 28-bp *cis*-acting sequence that confers the TPA response of the MoMLV enhancer. This organization of multiple binding sites in the TPA-responsive region from the MoMLV enhancer bears a striking resemblance to that of the serum response element (SRE) found in the c-*fos* enhancer. Figure 3 shows a comparison of the sequence of the TPA-responsive region of the MoMLV enhancer and the sequence of the SRE. The SRE contains a central motif, the CArG sequence (CCATATT AGG), that binds the p67 serum response factor (p67/SRF) (14, 18, 43, 44, 49, 57–59). Experiments by Shaw et al. (53) and Ryan et al. (47) indicate that additional proteins (p62) interact with the 5′-flanking sequences of the p67 binding site, either by binding the DNA directly or through the formation of a ternary complex with p67/SRF. At eight of nine nucleotides the 5′-flanking sequence of the SRE (CAG GATGTC) is homologous to the LVb sequence of the

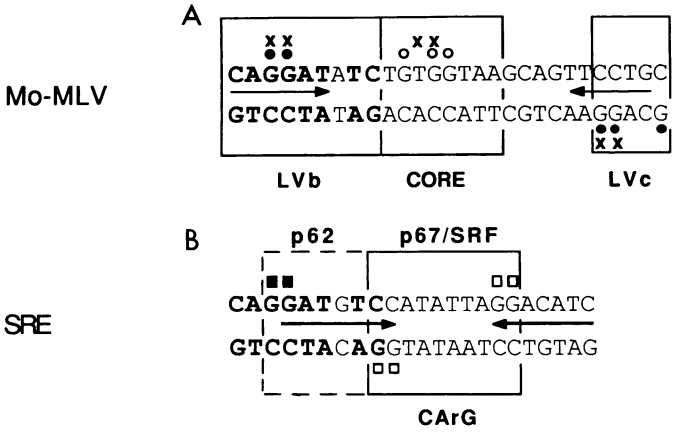


FIG. 3. Comparison of the TPA-responsive sequences of the MoMLV enhancer (A) with the SRE of c-*fos* (B). The binding sites for nuclear factors are indicated by boxes. Bold type represents the sequence homology between the LVb site of the MoMLV enhancer and the 5′-flanking sequences of the SRE. Symbols: ●, methylation interference pattern generated by a protein that elutes in a 0.25 M KCl step fraction of a nuclear extract from WEHI 231 mouse B cells from a phosphocellulose column to the LVb and LVc sites in the MoMLV enhancer (54); ○, methylation interference pattern obtained over the core sequence of the MoMLV enhancer; □, methylation interference pattern of the SRE generated by association of p67/SRF (14, 53); ■, extension of that pattern into the 5′-flanking sequences of the SRE upon addition of p62 (53); ×, mutations in the MoMLV enhancer that attenuated the TPA response; →, ←, dyad symmetry of the CAGGA(A/T) motif in the LVb and LVc sites on the MoMLV enhancer and the dyad symmetry in the SRE.

MoMLV enhancer (CAGGATATG), suggesting that a protein similar to p62 may interact with the LVb sequence. The sequence of the LVb-binding site is remarkably well conserved in the enhancers from exogenous type C murine, feline, and primate retroviruses, with 32 of 35 retroviruses maintaining the exact sequence. In all cases the LVb site is found immediately 5′ to a core element (16).

In MoMLV, the 5′ half of the LVb sequence [CAGGA (T/A)] is present as a dyad. The other half of the dyad appears in the same orientation on the noncoding strand in what was originally described as the LVc site; mutation of the two G nucleotides in either the LVb or the noncoding strand of the LVc site attenuates the TPA response. This sequence, CAGGA(T/A), is also found in the polyomavirus α domain, where it flanks a binding site for activating protein 1 (AP-1) (41). Thus, the CAGGA(T/A) sequence is juxtaposed to three different protein-binding sites (core, CArG, and AP-1) in three enhancers (type C MLVs, c-*fos*, and polyomavirus, respectively).

Transcription from the enhancer of human immunodeficiency virus is also activated in T cells and promonocytic cells by TPA through the nuclear factor κB (NF-κB)-binding site (19, 37, 51, 56). Transcription of human immunodeficiency virus is also activated in T cells, promonocytic cells, and macrophages by several cytokines, including granulocyte-macrophage colony-stimulating factor, interleukin-1, and tumor necrosis factor α, through NF-κB-dependent and independent pathways (13, 19, 40). It has been proposed that T cell or macrophage activation during the course of immunological responses would transiently activate the expression of HIV in latently infected cells and thus might contribute to pathogenesis by the virus. We have attempted to correlate the effects on transcription of mutations in the

MoMLV enhancer with their impact on disease induction (Speck et al., in press). As one might expect, our results indicate that there is an inverse correlation between the basal level of transcription of the MoMLV enhancer in T cells and the latent period of disease induction by MoMLV. In other words, enhancer mutations that attenuate transcription in T cell lines also increase the latent period of disease induction by MoMLV. Surprisingly, however, a virus that contains, in the LVc site, mutations that attenuate the TPA response but not the basal transcriptional activity of the MoMLV enhancer in T cells, has a latent period and disease specificity indistinguishable from those of the wild-type MoMLV. It appears, therefore, that TPA inducibility of viral gene expression may not be an important determinant of disease latency or specificity in MoMLV.

## ACKNOWLEDGMENTS

We thank Erica Golemis for providing the pMo(LVb)CAT plasmid, Michael Lenardo for helpful advice on mutagenesis experiments, and Deborah Galson for advice on electroporation. We especially thank Lucinia Pilapil for outstanding technical assistance. We also thank Ranjan Sen, Michael Gilman, and Nancy Manley for critically reading the manuscript. We thank Frank Mercurio for helpful discussions and Thomas Grundstrom and Barbara Graves for communicating unpublished results.

This work was supported by Public Health Service grant R01-CA19308 from the National Institutes of Health to N. Hopkins and partially by grant P01-CA42063 (Core) to P. A. Sharp. N. Speck was supported by a fellowship from the Medical Foundation, Inc.

## LITERATURE CITED

1. **Berthois, Y., J. A. Katzenellenbogen, and B. S. Katzenellenbogen.** 1986. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc. Natl. Acad. Sci. USA **83**:2496–2500.
2. **Birkenmeyer, E. H., B. Gwynn, S. Howard, J. Garry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue specific expression, developmental regulation, and genetic mapping of the gene encoding CCCAT/enhancer binding protein. Genes Dev. **3**:1146–1156.
3. **Boral, A. L., S. A. Okenquist, and J. Lenz.** 1989. Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. J. Virol. **63**:76–84.
4. **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.
5. **Chatis, P. A., C. A. Holland, J. E. Silver, T. N. Fredrickson, N. Hopkins, and J. W. Hartley.** 1984. A 3′ end fragment encompassing the transcriptional enhancer of nondefective Friend virus confers erythroleukemogenicity on Moloney murine leukemia virus. J. Virol. **52**:248–259.
6. **Chiu, R., M. Imagawa, R. Imbra, J. R. Bockoven, and M. Karin.** 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. Nature (London) **329**: 648–651.
7. **Clark, S. P., R. Kaufhold, A. Chan, and T. W. Mak.** 1985. Comparison of the transcriptional properties of Friend and Moloney retrovirus long terminal repeats: importance of tandem duplications and the core enhancer sequence. Virology **144**: 481–494.
8. **DeFranco, D., and K. 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.
9. **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.
10. **DeVries, E., W. van Driel, M. Tromp, J. H. van Boom, and P. C. van der Vliet.** 1985. Adenovirus DNA replication *in vitro*: site directed mutagenesis of the nuclear factor I binding site of the Ad2 origin. Nucleic Acids Res. **13**:4935–4952.
11. **Elsholtz, H. P., H. J. Mangalam, E. Potter, V. R. Albert, S. Supowit, R. M. Evans, and M. G. Rosenfeld.** 1986. Two different cis-active elements transfer the transcriptional effects of both EGF and phorbol esters. Science **234**:1552–1557.
12. **Evans, L., and J. Morrey.** 1987. Tissue-specific replication of Friend and Moloney murine leukemia viruses in infected mice. J. Virol. **61**:1350–1357.
13. **Folks, T. M., K. A. Clouse, J. Justement, A. Rabson, E. Duh, J. H. Kehrl, and A. S. Fauci.** 1989. Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. USA **86**:2365–2368.
14. **Gilman, M. Z., R. N. Wilson, and R. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6**:4305–4316.
15. **Golemis, E., Y. Li, T. N. Fredrickson, J. W. Hartley, and N. Hopkins.** 1989. Distinct segments in the enhancer region collaborate to specify the type of leukemia induced by nondefective Friend and Moloney viruses. J. Virol. **63**:328–337.
16. **Golemis, E., N. A. Speck, and N. Hopkins.** 1990. Alignment of U3 region sequences of mammalian type C viruses: identification of highly conserved motifs and implications for enhancer design. J. Virol. **64**:534–542.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Greenberg, M. E., Z. Seigfried, and E. B. Ziff.** 1987. Mutation of the c-*fos* gene dyad symmetry element inhibits serum inducibility of transcription in vivo and the nuclear regulatory factor binding in vitro. Mol. Cell. Biol. **7**:1217–1225.
19. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. Nabel.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. Nature (London) **339**: 70–73.
20. **Grosschedl, R., and D. Baltimore.** 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell **41**:885–897.
21. **Ishimoto, A., A. Adachi, K. Sakai, and M. Matsuyama.** 1985. Long terminal repeat of Friend-MCF virus contains the sequence responsible for erythroid leukemia. Virology **141**:30–42.
22. **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.
23. **Jensen, E. V., T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut, and E. R. DeSombre.** 1968. A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Natl. Acad. Sci. USA **59**:632–637.
24. **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.
25. **Johnson, P. F., and S. L. McKnight.** 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58**:799–839.
26. **Laimins, L., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
27. **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.
28. **Leegwater, P. A. J., P. C, van der Vliet, F. A. W. Rupp, J. Nowock, and A. Suppel.** 1986. Functional homology between the sequence-specific DNA-binding proteins nuclear factor 1 from HeLa cells and the TGGCA protein from chicken liver. EMBO J. **5**:381–386.
29. **Lenardo, M., J. W. Pierce, and D. Baltimore.** 1987. Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. Science **236**:1573–1577.
30. **Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine.** 1984. Determination of the

leukaemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature (London) **308**:467–470.
31. **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**:696–700.
32. **Lung, M. L., J. W. Hartley, W. P. Rowe, and N. H. Hopkins.** 1983. Large RNase T1-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.
33. **Manley, N. R., M. A. O'Connell, P. A. Sharp, and N. Hopkins.** 1989. Nuclear factors that bind to the enhancer region of nondefective Friend murine leukemia virus. J. Virol. **63**:4210–4223.
34. **Mercurio, F., and M. Karin.** 1989. Transcription factors AP-3 and AP-2 interact with the SV40 enhancer in a mutually exclusive manner. EMBO J. **8**:1455–1460.
35. **Miksicek, R., A. Heber, W. Schmid, U. Danesch, G. Posseckert, M. Beato, and G. Schutze.** 1986. Glucocorticoid responsiveness of the transcriptional enhancer of Moloney murine sarcoma virus. Cell **46**:283–290.
36. **Mitchell, P. H., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription *in vitro*: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
37. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
38. **Nagata, K., R. A. Guggenheimer, U. Inomoto, H. 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.
39. **Nomiyama, H., C. Fromenthal, J. H. Xiao, and P. Chambon.** 1987. Cell specific activity of the constituent elements of the simian virus 40 enhancer. Proc. Natl. Acad. Sci. USA **84**:7881–7885.
40. **Osborn, L., S. Kunkel, and G. Nabel.** 1989. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86**:2336–2340.
41. **Piette, J., and M. Yaniv.** 1987. Two different factors bind to the α-domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-*fos* enhancers. EMBO J. **6**:1331–1337.
42. **Potter, H., L. Weir, and P. Leder.** 1984. Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B cells by electroporation. Proc. Natl. Acad. Sci. USA **81**:7161–7165.
43. **Prywes, R., and R. G. Roeder.** 1986. Inducible binding of a factor to the c-*fos* enhancer. Cell **47**:777–784.
44. **Prywes, R., and R. G. Roeder.** 1987. Purification of the c-*fos* enhancer-binding protein. Mol. Cell. Biol. **7**:3482–3489.
45. **Rassart, E., Y. Paquette, and P. Jolicoeur.** 1988. Inability of Kaplan radiation leukemia virus to replicate on mouse fibroblasts is conferred by its long terminal repeat. J. Virol. **62**:3840–3848.
46. **Rosen, C., W. 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.
47. **Ryan, W. A., B. R. Franza, and M. Z. Gilman.** 1989. Two distinct cellular phosphoproteins bind to the c-*fos* serum response element. EMBO J. **8**:1785–1792.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
49. **Schroter, H., P. E. Shaw, and A. Nordheim.** 1987. Purification of intercalator-released p67, a polypeptide that interacts specifically with the c-*fos* serum response element. Nucleic Acids Res. **15**:10145–10158.
50. **Schulze, G., E. Boehnlein, and P. Gruss.** 1985. Mutational analysis of the Moloney murine sarcoma virus enhancer. DNA **4**:193–202.
51. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
52. **Serfling, E., R. Barthelmas, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio, and M. Karin.** 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. EMBO J. **8**:465–473.
53. **Shaw, P. E., H. Schroter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-*fos* promoter. Cell **56**:563–572.
54. **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.
55. **Thornell, A., B. Hallberg, and T. Grundstrom.** 1988. Differential protein binding in lymphocytes to a sequence in the enhancer of the mouse retrovirus SL3-3. Mol. Cell. Biol. **8**:1625–1637.
56. **Tong-Starksen, S. E., P. A. Luiciw, and B. M. Peterlin.** 1987. Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. Proc. Natl. Acad. Sci. USA **84**:6845–6849.
57. **Treisman, R.** 1985. Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5′ element and c-*fos* 3′ sequences. Cell **42**:889–902.
58. **Treisman, R.** 1986. Identification of a protein-binding site that mediated transcriptional response of the c-*fos* gene to serum factors. Cell **46**:567–574.
59. **Treisman, R.** 1987. Identification and purification of a polypeptide that binds to the c-*fos* serum response element. EMBO J. **6**:2711–2717.
60. **Weiher, H., M. Zonig, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**:626–631.
61. **Weiss, R., N. Teich, H. Varmus, and J. Coffin (ed.).** 1982. RNA tumor viruses, vol. 1 and 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
62. **Zenke, M., T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.
63. **Zimmerman, U., and J. Veinken.** 1982. Electric field-induced cell-to-cell fusion. J. Membr. Biol. **67**:165–182.
64. **Zoller, M., and M. Smith.** 1984. Oligonucleotide directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded DNA template. DNA **3**:479–488.