JOURNAL OF VIROLOGY, Apr. 1991, p. 1991–1999
0022-538X/91/041991-09$02.00/0

Vol. 65, No. 4

Nuclear Factor 1 Activates the Feline Leukemia Virus Long Terminal Repeat but Is Posttranscriptionally Down-Regulated in Leukemia Cell Lines

M. PLUMB,[1] R. FULTON,[1] L. BREIMER,[2] M. STEWART,[1] K. WILLISON,[2] AND J. C. NEIL[1]*

Beatson Institute for Cancer Research, Bearsden, Glasgow G61 1BD, Scotland,[1] and Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, England[2]

Received 1 October 1990/Accepted 14 January 1991

A recombinant feline leukemia virus (FeLV) proviral clone (T17T-22) with a long terminal repeat (LTR) which differs from prototype FeLV by a point mutation within a conserved nuclear factor 1 (NF1)-binding motif in the LTR enhancer domain was found to be poorly expressed after DNA transfection. The NF1 point mutation reduced in vitro protein binding as assessed by gel shift analysis and reduced promoter activity significantly (2- to 10-fold). However, the degree of promoter impairment due to the NF1 site mutation varied according to cell type and was least severe in a feline leukemia cell line (T3) which had low levels of nuclear NF1 DNA-binding activity. Low NF1 DNA-binding activity was observed in three FeLV-induced leukemia cell lines (T3, T17, and FL74) and in murine F9 embryonal carcinoma cells. While similar levels of NF1 gene mRNA transcripts were detected in all cell lines, Western immunoblot analysis of F9, T17, and FL74 but not T3 nuclear extracts revealed very low levels of nuclear NF1 protein. These results indicate that NF1 activity is down-regulated in FeLV-induced leukemia cells by diverse posttranscriptional mechanisms. We suggest that NF1 down-regulation may be an important characteristic of target cells susceptible to FeLV transformation in vivo and may provide the selective pressure which favors duplication of the LTR core enhancer sequence in T-cell leukemogenic FeLV variants.

Leukemogenesis by type C retroviruses has revealed several characteristic mechanisms by which cellular gene expression can be deregulated, including transduction, in which host gene-coding sequences are incorporated into the retroviral genome (3), and insertional mutagenesis, in which a viral promoter or enhancer activates a cellular proto-oncogene (19, 39). Viral transcriptional control elements are crucial in all of these mechanisms, since in each case viral sequences supplant or override normal cellular transcriptional controls. Genetic studies of type C retroviruses have shown that these processes are governed by promoter and enhancer elements residing in the U3 domain of the long terminal repeat (LTR) (6, 11, 29, 48, 51, 54).

Molecular analysis of the U3 domains of mammalian type C retroviral LTRs has shown that these are modular structures containing multiple transcription factor-binding sites. For example, the enhancer domain of the Moloney murine leukemia virus (Moloney MuLV) LTR has binding sites for nuclear factor 1 (NF1), the simian virus 40 core enhancer-binding protein, leukemia virus factors a, b, and c, and a glucocorticoid response element (50, 51), and the order and spacing of most of these protein binding sites are conserved in murine, feline, and primate type C retrovirus LTRs (13, 15). Naturally occurring or directed mutations which abolish or decrease transcription factor binding to LTR sequences have been shown to severely reduce LTR function in vivo (4, 37, 51). However, most of the protein activities which bind to these sequences are not obviously cell specific, implying that subtle cell-specific quantitative differences, differential posttranslational modifications, or protein-protein interactions may underlie target cell preference in retroviral transcription and oncogenesis.

This investigation arose from experiments in which recombinant feline leukemia virus (FeLV) proviruses carrying a T-cell antigen receptor gene were introduced into cells by DNA transfection as a prelude to assessment of their oncogenic potential. We observed that a clone which had a naturally occurring mutation in the NF1 motif in the LTR enhancer (T17T-22) was also poorly expressed after transfection. We now report that the NF1 mutation confers reduced FeLV LTR activity in various cell types. However, while NF1 binding potentiates the FeLV LTR, binding activity is very low in several FeLV-derived T-cell leukemia lines. NF1 was first discovered as a cellular factor required for the initiation of adenovirus DNA replication (35) but is now known to represent a family of transcriptional regulatory proteins with binding sites in many cellular and viral promoters (reviewed in reference 20). We find that NF1 down-regulation in feline leukemia cell lines is a posttranscriptional phenomenon reflecting either the T-cell differentiation state or the leukemic phenotype of the tumor cells.

MATERIALS AND METHODS

Cell lines. AH297 is an immortal feline cell line of fibroblastic origin (42). T3, T17, and FL74, are FeLV-positive lymphoid tumor cell lines established from naturally occurring thymic lymphosarcomas (12, 23, 36). The T3 cell line contains a replication-competent FeLV helper and recombinant FeLV proviruses with a v-*myc* oncogene (36), while the T17 cell line contains defective FeLV-A, v-*myc*, and v-*tcr* recombinant proviruses which carry a transduced T-cell antigen receptor β-chain gene (12). The 3201B cell line was

* Corresponding author.

derived from an FeLV-free thymic lymphosarcoma (49). F9 is a murine embryonal carcinoma cell line (2).

FeLV LTRs. The T17T-22 provirus was cloned from tumor DNA from the primary T17 thymic lymphosarcoma. It represents one of a series of integrants which contained a transduced T-cell antigen receptor β-chain gene and was hence of interest with regard to its oncogenic potential. T17T-31, which yielded the deleted form T17T-31d, was an integrant from the same primary tumor DNA library as T17T-22 (12). The prototype weakly oncogenic FeLV LTR (G1) was derived from an infectious proviral clone (pFGA-5) of FeLV-A/Glasgow-1 (52), a virus which can induce a broad spectrum of tumors, but usually after a protracted latent period.

Northern (RNA) blot analysis. Total cellular RNA was prepared by using standard protocols with the RNAzol method (8), resolved by denaturing 1.2% agarose-formaldehyde gel electrophoresis, and transferred to nitrocellulose filters. After prehybridization at 42°C, the blots were hybridized to a rat liver NF1 cDNA probe (kind gift of R. Cortese). This was a 623-bp *Pst*I fragment corresponding to the sequence coding for the highly conserved DNA binding domain (nucleotides 98 to 711; amino acids 30 to 234 [38]) (14, 33, 38, 45). Blots were washed at a highest stringency of 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 22°C and resolved by autoradiography.

Oligonucleotides. The sequences of the double-stranded oligonucleotides containing NF1 consensus binding sites used are shown in Fig. 2A. NF1-Ad is based on the NF1-binding site at the adenovirus replication origin (35) but is altered by a single-base-pair change to make a perfect inverted repeat and has previously been shown to compete for the FeLV LTR NF1-binding site in footprint experiments (13). NF1(b), which contains two NF1-binding sites, CG CATTTGGCAGCTTGCCAAGGATCCTTGGCAGCTTGC CAAG (16), was used in the antiserum gel shift assays.

αP2a contains the mouse α-globin gene promoter CCAAT sequence (αP2A; GATCCAAACCAGCCAATGAGAACT GCTCCA) which binds the ubiquitous CCAAT-binding protein (CBP) (40).

Plasmids. *Pst*I-*Kpn*I restriction fragments containing FeLV LTR sequences derived from FeLV-A/Glasgow-1 (G1) (13) and pT17T-22 were subcloned into pIC20H (31). Plasmids were linearized by digestion with *Hin*dIII, treated with calf intestinal phosphatase, and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The insert was isolated for footprint experiments after secondary digestion with *Eco*RI (13, 40, 41).

For functional analysis, the FeLV LTR enhancer and promoter fragments were subcloned upstream of the human growth hormone gene (pOGH) (46). This was achieved by cloning *Pst*I-*Kpn*I fragments into the pIC20H vector, and the inserts were then excised with *Hin*dIII and cloned into the *Hin*dIII site of the pOGH plasmid.

Site-directed mutagenesis. A commercially available site-directed mutagenesis kit (SDM; BioRad Laboratories) was used. The method involves a phagemid vector system with positive selection for non-uracil containing mutant strands (26). The mutagenic primer employed was 5′-CTCAAGC TGGGGCCCAG-3′.

Growth hormone assay. DNA comprising nonspecific carrier (100 μg), the appropriate growth hormone construct (5 to 50 μg), and β-galactosidase plasmid (pHSVβGAL [18]) (20 μg) was transfected into cells by electroporation in cold growth medium (4°C) at 250 V, 960 μF. The cells were then reseeded at a density of 10^6 cells per ml and grown for five days, at which time 100 μl of culture supernatant was harvested and assayed by using the Allegro dual growth hormone-specific monoclonal antibody system essentially as recommended by the suppliers (Biogenesis Ltd.). Growth hormone levels were calculated by means of a standard curve using control human growth hormone following the deduction of background counts (normal serum only). β-galactosidase activity was monitored in transfected cell lysates (18) and used to correct for variations in transfection efficiency.

Footprint analysis. Nuclear protein extracts were prepared and footprint protection assays were performed as described previously (13, 40, 41). Briefly, 0 to 80 μl of nuclear extract (0 to 600 μg of protein) was incubated with 2 ng of 5′ end-labeled probe in the presence of 1 μg of poly(dI)-poly(dC) and in the presence or absence of 100 ng of double-stranded competitor oligonucleotide. After limited DNase I digestion, nucleic acids were purified and resolved by denaturing polyacrylamide gel electrophoresis and autoradiography. Markers were prepared from the same fragment by the G+A chemical sequencing reaction (32).

NF1 antiserum. A highly specific rabbit polyvalent antiserum was raised against an epitope of mouse NF1 (4b). Briefly, a CPDAGQQAGQVGFLNP amide peptide was synthesized by Multiple Peptide Systems (San Diego, Calif.) and conjugated to keyhole limpet hemocyanin by treatment with glutaraldehyde. The conjugate was emulsified with Freund's adjuvant, and a rabbit was immunized by subcutaneous injection.

Gel shift analyses. Double-stranded oligonucleotides were 5′ end labeled and gel purified essentially as previously described (40). Two protocols were employed. (i) Labeled oligonucleotide (100 to 500 pg) was incubated on ice for 1 h with 0 to 3 μl of nuclear extracts in the presence of 6 μg of poly(dI)-poly(dC) and 0 to 200 ng of competitor oligonucleotide in a final volume of 20 μl of storage buffer (40). (ii) Labeled oligonucleotide (100 pg), 0 to 2 μl of nuclear extract, and 5 μg of poly(dI)-poly(dC) in 20 μl of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9)–5 mM $MgCl_2$–0.1 mM EDTA–4% Ficoll–60 mM NaCl were preincubated at room temperature for 20 min. Anti-NF1 antiserum, preimmune serum, or antiserum which had been preincubated with peptide was added and incubated for a further 60 min at room temperature.

Nucleoprotein complexes were resolved by nondenaturing 5% polyacrylamide/0.25 to 0.5× Tris-borate-EDTA gel electrophoresis for 2 h on ice at 150 V, and the gel was dried for autoradiography.

Western immunoblot analyses. Nuclear extracts (50 μl, 100 to 200 μg) were resolved by sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis and transferred to nitrocellulose filters by semidry electroblotting (Startoblot II-S; Sartorius).

Filters were pretreated for 30 min with 100 ml of Blotto solution (phosphate-buffered saline containing 0.1% Nonidet P-40 and 25 g of powdered milk per liter [Marvel]). The filter was then incubated with the primary antibody (10 μl in 10 ml of Blotto) for 4 h, washed three times with 100 ml of Blotto, and incubated with the secondary antibody (Promega anti-rabbit immunoglobulin G conjugated to alkaline phosphatase; 3 μl in 10 ml of Blotto) for 60 min. The filter was washed three times with 100 ml of Blotto and twice with AP buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 5 mM $MgCl_2$) and developed in 50 ml of AP buffer containing 8 mg of 5-bromo-4-chloro-3 indolyl phosphate (Sigma) and 16 mg of Nitro Blue Tetrazolium (Sigma).

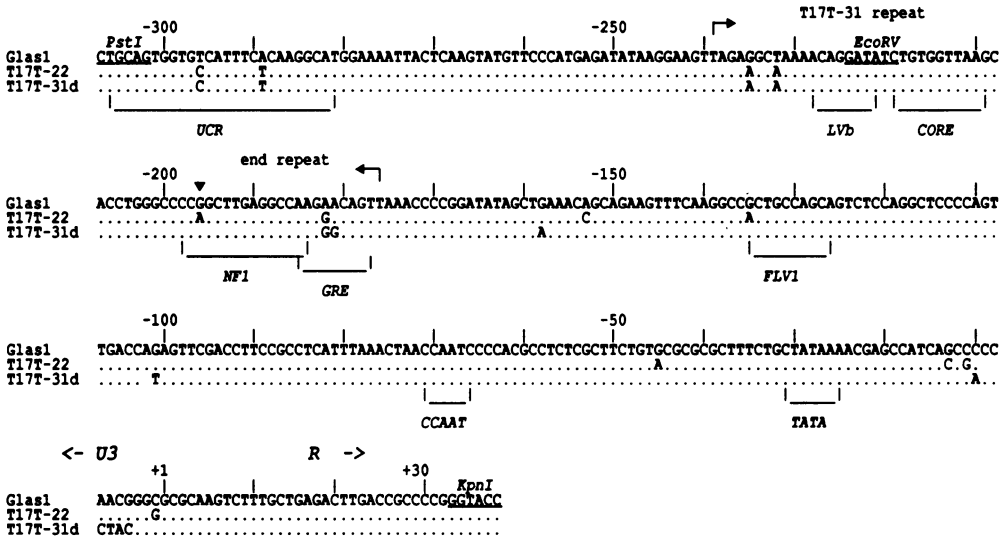

FIG. 1. Sequences of FeLV LTR fragments. The sequences shown are from the infectious pFGA-5 proviral molecular clone of FeLV-A/Glasgow-1 (G1) (12) and two T-cell tumor-derived proviruses, T17T-22 and T17T-31 (12). The T17T-31d sequence was derived by deletion of a direct repeat by using the conserved *Eco*RV site shown above the sequence. Also shown under the sequence are binding sites designated CORE, NF1, and FLV1, which have been described previously (13), and several additional motifs which may be relevant for the regulation of LTR activity: UCR (upstream control region), LVb (leukemia virus factor b) and GRE (glucocorticoid response element) (50). The mutation within the NF1 motif is indicated by an inverted arrowhead above the sequence. The G1M site-directed mutant LTR sequence differs from G1 only by the G→A change at this position.

RESULTS

A natural point mutation in the FeLV enhancer NF1 motif reduces factor binding in vitro. The plasmid pT17T-22 was the first FeLV proviral clone containing a transduced T-cell antigen receptor β-chain gene to be characterized (12). However, stable cotransfection experiments using feline fibroblast (AH927) cells showed this clone to be poorly expressed relative to other apparently identical integrated proviral structures cloned from the same tumor (51a). DNA sequence analysis of these integrants revealed that the LTRs were heterogeneous (13). Unlike its sister clones, pT17T-22 had only a single copy of the enhancer element and carried a G→A point mutation in a conserved NF1-binding motif (Fig. 1) which has been identified in the closely related MuLV LTR as a possible NF1 protein-DNA contact point by methylation interference analysis (50).

The consequence of the G→A NF1 site mutation in the T17T-22 LTR was addressed by using synthetic double-stranded oligonucleotides (Fig. 2A) as competitors in gel shift assays. Labeled NF1-Ad was bound to 3201B cell crude nuclear extract in the presence or absence of competitor oligonucleotides containing either the homologous NF1-Ad sequence or the LTR NF1-binding sites of a prototype FeLV-A/Glasgow-1 LTR (NF1-G1), T17T-22 (NF1-22), or oligo NF1-G1M, which corresponds to the NF1-G1 sequence containing a single-base-pair G→A change. A broad smear of nucleoprotein complexes seen on nondenaturing gel electrophoresis is inhibited by either a 20- to 60-fold molar excess of NF1-Ad or a 100-fold molar excess of NF1-G1 competitor (Fig. 2B). Bands which are not completely competed out by NF1-Ad competitor (labeled A, Fig. 2A, 4, and 6A) are thereby defined as nonspecific. The specific nucleoprotein complex migrates as a characteristic smear, which presumably reflects the multiple forms of NF1 protein translated from alternatively spliced NF1 gene transcripts (5, 17, 21, 33, 38, 45).

NF1-G1 is slightly less effective in competing for the labeled NF1-Ad probe than the cold homologous NF1-Ad competitor, although both completely abolish NF1 binding at less than a 100-fold molar excess (Fig. 2B). However, neither NF1-22 nor NF1-G1M competes at a 200- to 400-fold molar excess. Since the only difference between NF1-G1 and NF1-G1M is a single G→A base pair change which is also observed in NF1-22, this demonstrates that the single-base-pair substitution is sufficient to reduce NF1 protein binding significantly. NF1-22 also has an A→G base pair change at the 3′ end of the sequence (Fig. 1 and 2A), but as this occurs in the T17T-31 LTR which binds NF1 in vitro (13), the 5′ G→A base pair change appears to be the critical change which reduces NF1 protein binding.

The T17T-22 NF1 mutation confers markedly reduced LTR promoter activity, but in a cell-type-dependent manner. To examine the functional consequences of the NF1 mutation, we generated constructs in which LTR promoter and enhancer elements (*Pst*I-*Kpn*I) direct the expression of the human growth hormone reporter gene (45). We first compared the LTR of the NF1 mutant T17T-22 clone with the T17T-31d LTR. The T17T-31d LTR is a deletion mutant of the highly active T17T-31 LTR, in which one copy of the enhancer repeat has been removed, yielding a primary sequence very similar to that of T17T-22 apart from the NF1 mutation (Fig. 1). Deletion of the extra enhancer repeat made a 1.6- to 2-fold difference in promoter activity, an observation which is in accord with functional analyses of similarly deleted MuLV LTRs (30). However, as shown in Table 1, the T17T-22 LTR enhancer-promoter fragment is markedly less active than that of T17T-31d.

To measure the functional effect of the NF1 mutation alone, a mutant of the prototype FeLV-A/Glasgow-1 LTR (G1) was constructed which differed only by a G→A mutation at the NF1 site (G1M; Fig. 1 and 2A). The activity of the G1M LTR was also impaired relative to its wild-type control,

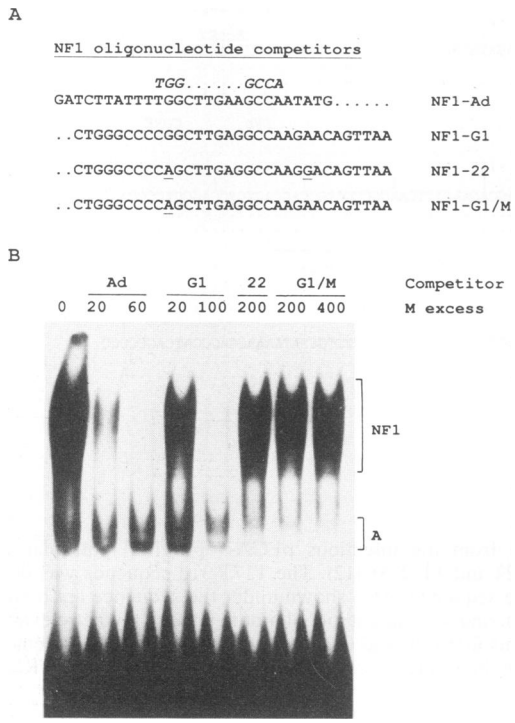

FIG. 2. Gel shift analysis of variant NF1 sequences. End-labeled NF1-Ad oligonucleotide probe (500 pg) was incubated with 1 μl of 3201B cell nuclear extract–6 μg of poly(dI)-poly(dC) in a final volume of 20 μl of storage buffer in the presence or absence of a 20- to 400-fold molar excess (10 to 200 ng) of cold competitor oligonucleotide NF1-Ad, NF1-G1, NF1-22, or NF1-G1M (A). NF1-Ad is an adenovirus NF1-binding site and includes a match to the consensus (TGG-N_{6-7}-GCCA). The other oligonucleotides were based on the FeLV LTR binding sites shown in Fig. 1. Nucleoprotein complexes were resolved by nondenaturing gel electrophoresis and autoradiography (B). The specific NF1 nucleoprotein complexes (labeled NF1) and the nonspecific artifact bands (labeled A) are shown.

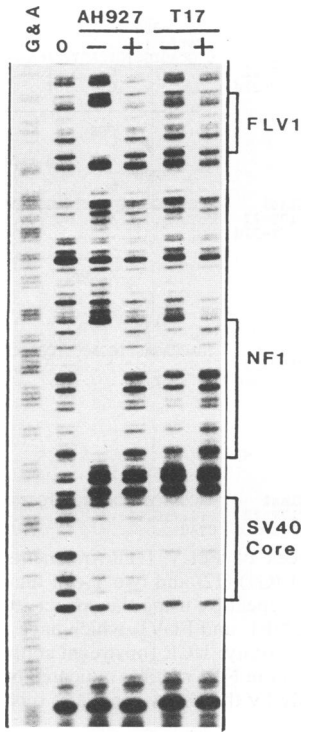

FIG. 3. Footprint analysis of the G1 LTR enhancer sequence. 5′ end-labeled G1 probe was incubated with 1 μg of poly(dI)-poly(dC) and 0 or 80 μl of nuclear extract from either T17 or AH927 cells and in the presence (+) or absence (−) of 100 ng of cold competitor oligonucleotide NF1-Ad. After limited DNase I digestion, nucleic acids were resolved by denaturing gel electrophoresis and autoradiography. The simian virus 40 (SV40) core enhancer- and NF1 and FLV1 protein-binding sites, identified in previous studies (13), are shown. Size markers are the G+A chemical sequencing (32) reaction products of the same probe.

although this was more marked in some cell types than in others (Table 1). Impairment was greatest in 3201B cells (10-fold), least in T3 cells (less than 2-fold), and intermediate in AH927 cells (almost 4-fold). In general, the impairment of T17T-22 relative to T17T-31d followed a cell-specific pattern similar to that of G1/G1M. Presumably, some of the other mutational differences between T17T-22 and T17T-31d account for the lack of complete correlation. In this respect, it was also interesting to note that the activity of the NF1 mutant T17T-22 LTR was almost as high as that of NF1 wild-type G1. Experiments are underway to define which change(s) compensates for the T17T-22 NF1 mutation.

TABLE 1. LTR-driven growth hormone expression in feline T-cell lines and fibroblast cell lines[a]

LTR	Growth hormone levels		
	3201B	T3	AH927
T-31	5.00	2.86	2.33
T-31d	3.65	1.54	1.26
T-22	0.65	0.91	0.77
G1	1.00	1.00	1.00
G1M	0.10	0.54	0.28

[a] LTR/growth hormone gene chimeric constructs were expressed in three cell lines as indicated. Growth hormone gene expression is expressed relative to the G1 (FeLV-A/Glasgow-1) prototype FeLV LTR. Transfection efficiencies were normalized relative to β-galactosidase activity (see Materials and Methods). Values represent the mean of three independent experiments.

NF1 DNA-binding activity is down-regulated in FeLV-derived T-cell lymphoma cells. The cell-type specific effect of the NF1 mutation implied that some difference existed in the nature and/or abundance of factors interacting with the NF1 site. Furthermore, footprint analyses with T-cell nuclear extracts indicated that NF1 DNA-binding activity was low compared with that in the fibroblastic cell line (AH927). This is illustrated in Fig. 3 in which, as in previous analyses (13), the AH927 nuclear extract identified three major protein-binding sites: the simian virus 40 core enhancer-binding site, the NF1-binding site, and FLV1, a sequence which is homologous to the PEA2 binding site of the polyomavirus enhancer. Both the NF1 and FLV1 footprints are inhibited by cold competitor NF1-Ad double-stranded oligonucleotide (Fig. 2A) (13) which contains a high-affinity NF1-binding site from the adenovirus origin of replication (35). Although the T17 nuclear extract protects the core site, little or no binding to either the NF1 or FLV1 site is detected, suggesting that these transcription factors are in a low abundance in the T17 extract.

Levels of NF1 DNA-binding activity were analyzed further by gel shift analysis (Fig. 4A) using labeled NF1-Ad

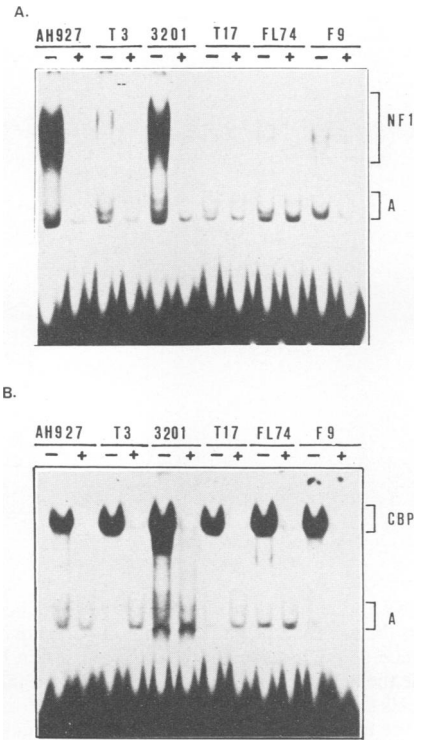

FIG. 4. Gel shift analysis of NF1 and CBP in the feline extracts. Nuclear extracts and 6 μg of poly(dI)-poly(dC) were incubated with 100 pg of 5′ end-labeled oligonucleotide NF1-Ad (A) (Fig. 2A) or oligonucleotide αP2a (B) in the presence (+) or absence (−) of cold competitor oligonucleotide, and nucleoprotein complexes were resolved by nondenaturing gel electrophoresis and autoradiography. The specific CBP and NF1 complexes are shown. Bands marked "A" are nonspecific artifacts. The amounts of nuclear extract used were as follows (in microliters): AH927, 1; T3, 1.5; 3201B, 1; T17, 2.5; FL74, 1; and F9, 1.5.

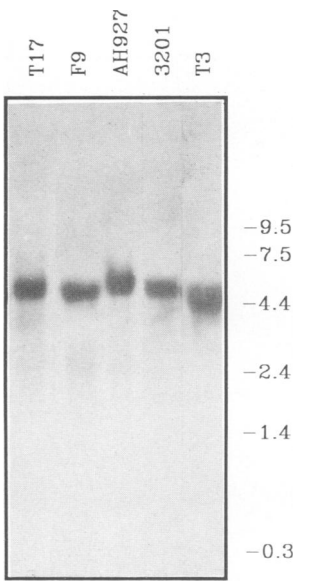

FIG. 5. Northern blot analysis of NF1 mRNA transcripts. Total cellular RNA (20 μg) from various cell lines (labeled at top) was resolved by denaturing agarose gel electrophoresis and transferred to nitrocellulose filters. The blot was probed with the 623-bp *Pst*I restriction fragment of the rat liver NF1 cDNA (38). The molecular size marker was an ethidium bromide-stained RNA ladder from Bethesda Research Laboratories. Apparent differences in mobility were not reproducibly observed.

probe bound to crude nuclear extracts prepared from three FeLV-containing leukemia cell lines (T3, T17, and FL74) and from a control feline fibroblastic cell line (AH927) and a FeLV-negative lymphoma cell line (3201B).

Comparison of the levels of nuclear extract NF1 DNA-binding activities revealed high levels of NF1 activity in the AH927 and FeLV-negative T-cell tumor (3201B) lines, whereas NF1 levels were approximately 10-fold lower in the FeLV-positive T3, T17, and FL74 tumor cell lines (Fig. 4A). Furthermore, a similar low level of NF1 activity was observed in the F9 murine embryonal carcinoma cell line, confirming results described by others (5, 17, 50). Mouse erythroleukemia cells transformed by the Friend spleen focus-forming type C retrovirus (SFFVa) have high levels of NF1 (data not shown) (5, 17). The modular organization of the SFFVa LTR is similar to that of the FeLV LTR and other MuLV LTRs (15), and the LTR contains a similar conserved NF1-binding site, indicating that low NF1 DNA-binding activity is neither an invariant feature of retrovirus-transformed cells per se nor a prerequisite for transformation.

To allow quantitative comparisons of transcription factor DNA-binding activities in different nuclear extracts we used three criteria. First, all gel shift experiments were performed with nuclear extracts on the basis of approximately the same numbers of cell nuclei. Second, nuclear extracts were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining, and there was less than a twofold difference in the protein concentrations in the various extracts and no obvious evidence of proteolytic degradation (data not shown). Third, we monitored the levels of the CBP (45) in gel shift experiments by using a double-stranded oligonucleotide containing the mouse α-globin gene promoter CCAAT sequence. CBP is present in essentially equivalent amounts in nuclear extracts from a variety of cell lines and tissues from different species (40). Levels of CBP binding activity were comparable in all the extracts examined and were specifically inhibited by excess CCAAT competitor oligonucleotide (Fig. 4B). Similarly, footprint analyses of the FeLV LTR core site suggest that binding activity at this site is not significantly different (at least quantitatively) in the various extracts (Fig. 3) (13) (data not shown). The low levels of NF1 in the T3, T17, FL74, and F9 nuclear extracts do not therefore reflect a general loss of transcription factors.

Although we cannot exclude the possibility that down-regulation differentially affects some minor subpopulation(s) of NF1 mRNA or protein, the use of a high-affinity binding site in the gel shift assays ensures that the bulk of NF1 DNA-binding activity was detected.

Down-regulation of NF1 in feline leukemia cells and F9 embryonal carcinoma cells is posttranscriptional. To determine whether low levels of NF1 DNA-binding activity were reflected in low levels of mRNA available for translation, steady-state NF1 mRNA levels in total cellular RNA were assayed on Northern blots by using a 623-bp restriction fragment probe encoding the conserved DNA-binding domain of a rat liver NF1 gene cDNA clone (38). A major transcript of 5 kb (Fig. 5) was detected in approximately equivalent amounts in all cell lines tested. Multiple minor

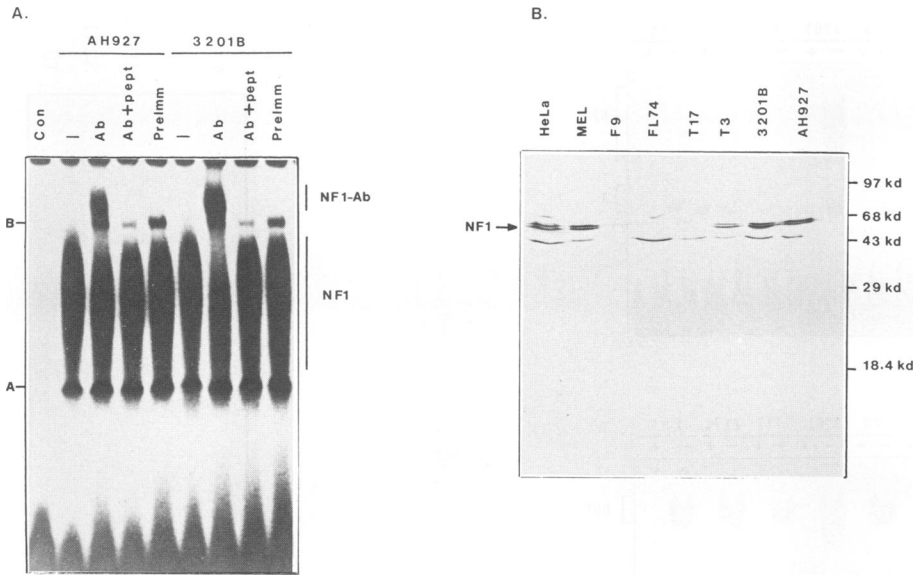

FIG. 6. Western blot analysis of nuclear NF1. (A) Antiserum raised to a synthetic peptide based on the NF1 gene sequence was tested in a gel shift assay. 5′ end-labeled oligonucleotide NF1(b) (100 pg) was incubated in the absence of nuclear extract (Con) or with nuclear extract (1 μl) from AH927 or 3201B cells and 5 μg of poly(dI)-poly(dC) in the absence (−) or presence of 1 μl of antiserum (Ab), 1 μl of antiserum which had been preincubated with 1 μg of the synthetic peptide used to raise the antiserum (Ab+pept), or 1 μl of preimmune serum (PreImm). Nucleoprotein complexes were resolved by nondenaturing gel electrophoresis and autoradiography. The NF1 nucleoprotein (NF1) and specific NF1-antibody (NF1-Ab) complexes are shown. Bands marked A and B are nonspecific artifact bands. (B) Nuclear extracts (50 μl) were resolved by gel electrophoresis, transferred to nitrocellulose, and probed with the anti-NF1 serum. The blot was then incubated with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and developed as described in Materials and Methods. Molecular size markers are prestained markers from GIBCO/BRL.

RNA species were also observed, especially at reduced hybridization and washing stringencies. This is consistent with the expression and differential splicing of the NF1 gene family (38, 44, 45), but again no obvious differences in the levels of NF1 mRNA transcripts were detected among cell types. The down-regulation of NF1 DNA-binding activity therefore appears to be predominantly posttranscriptional.

Nuclear extracts were next analyzed for total NF1 protein by Western blotting. The antiserum used was a rabbit antipeptide serum raised against a 16-amino-acid synthetic peptide containing an epitope (CPDAGQQAGQVGFLNP) which is conserved in the mouse, chicken, and rat NF1 proteins (38, 44, 45) (4a). Gel shift analysis (Fig. 6A) showed that the antiserum bound to and shifted the electrophoretic mobility of the feline AH927 and 3201 cell NF1 nucleoprotein complexes. The specificity of interaction was shown by the lack of binding with preimmune serum and by the inhibition of binding when the antiserum was preabsorbed with the synthetic peptide used to raise the antiserum. The nucleoprotein complex B (Fig. 6A) is nonspecific, as it only occurs in the presence of serum. Similar results have been obtained with mouse and chicken NF1 protein (4a).

Western blot analysis of nuclear extracts of the feline T-cell lines (3201B, T3, T17, and FL74), the feline fibroblast cell line (AH927), the murine F9 and erythroleukemia cell line (MEL), and human HeLa cells using the antiserum is shown in Fig. 6B. Three major polypeptides (43, 65, and 68 kDa) were detected in the nuclear extracts. Similar analyses with preimmune serum and antiserum blocked by preincubation with synthetic peptides indicated that the 43-kDa protein was detected nonspecifically. The 65- to 68-kDa polypeptides have apparent molecular sizes similar to those of NF1 proteins detected elsewhere (17, 21, 45).

The 65- to 68-kDa proteins were readily detected in the nuclear extracts of the AH297, 3201B, T3, HeLa, and MEL nuclear extracts but not in the T17, FL74, and F9 extracts (Fig. 6B). Therefore, except for the T3 nuclear extract, the lack of detectable 65- to 68-kDa proteins correlates with the loss of NF1 DNA-binding activity. NF1 proteins were not detectable in whole-cell extracts, presumably because the molar representation of NF1 protein is at least 10- to 20-fold lower than in the comparatively enriched nuclear extracts.

We therefore cannot distinguish whether the down-regulation of NF1 activity arises from a high turnover rate of protein, a translational block, or a failure of nuclear transport. The T3 nuclear extract has measurable levels of NF1 protein, but although T3 NF1 DNA-binding activity is somewhat above the levels seen in T17, FL74, and F9 cells (13) (Fig. 4), its specific activity of binding is clearly much lower than in AH927 and 3201 cells. It is possible that the FeLV-derived T-cell lines and F9 cell lines down-regulate NF1 DNA-binding activity by more than one mechanism.

DISCUSSION

A naturally occurring point mutation in the FeLV LTR (T17T-22) was shown to impair nuclear factor binding and in vitro promoter function, demonstrating that the conserved FeLV LTR enhancer binding site is necessary for maximal promoter activity. Paradoxically, NF1 DNA-binding activity is posttranscriptionally down-regulated in three FeLV-positive T-cell leukemia lines.

In vitro protein binding and functional studies on the modular MuLV (37, 50, 51, 53) and FeLV (13) LTRs have revealed a limited number of nuclear protein-binding sites. In FeLV (12), these binding sites include a simian virus 40

core enhancer consensus sequence (core site), an NF1-binding site, and the FLV1 site which is similar to the PEA2-binding site of the polyomavirus enhancer (5, 25). We have found that nuclear extracts from tumor cells are depleted in NF1 and FLV1 DNA-binding activities but retain high levels of core binding and CBP activities. Several distinct factors have been shown to bind to the core motif in the highly leukemogenic MuLV SL3-3, which is essentially identical to that of FeLV (9, 22, 27), but the predominant binding activity in murine lymphoid cells appears to be a distinct factor called SEF-1 (53) or S/CBF (4). It is presumably the feline homolog of this factor which accounts for the strong core-binding activity in the feline tumor cell lines.

A natural mutation in a highly conserved NF1-binding site in the FeLV LTR which abolishes factor binding in vitro is associated with significantly decreased promoter activity when introduced to a prototype FeLV LTR. Our results therefore indicate that NF1 can act as a positive regulator of FeLV transcription and concur with studies on the analogous site in murine retroviral LTRs (37, 51). The cell type-dependent effects of the LTR NF1 mutation presumably reflect the relative activities of NF1 and other transcription factors which interact with LTR sequences in the recipient cell lines. The severity of the NF1 mutant phenotype appears to be inversely proportional to the abundance of NF1 DNA-binding activity in the cells tested, albeit to a very rough approximation. The least significant impairment was in the T3 tumor cell line which of the three transfectable cell lines (3201B, AH927, and T3) has the lowest levels of NF1 DNA-binding activity. Conversely, mutation of the analogous Moloney MuLV NF1 motif has been noted to be particularly deleterious in 3T3 fibroblast cell lines (51), which are reported to have high levels of NF1-binding activity (17).

The posttranscriptional regulation of transcription factors has been documented and embraces a number of distinct levels of modulation, including nuclear transport (1, 47), phosphorylation (7, 34, 55), and heat induction (20, 24, 28). Although the low level of NF1 DNA-binding activity in embryonal carcinoma cells has been recorded by others (5, 17, 50), its posttranscriptional regulation has not to our knowledge been described before. Other transcription factors, such as AP1, are also down-regulated in embryonal carcinoma cells (25), but in contrast, this appears to operate at the level of c-*fos* and c-*jun* mRNA abundance (10). To extend the parallel between F9 embryonal carcinoma cells and the feline leukemia tumor lines, it is possible that low NF1 levels reflect the fixation of an immature but essentially normal cell phenotype. However, it is possible that two different mechanisms conferring low nuclear NF1 DNA-binding activity are operative here: (i) low levels of total NF1 protein were observed in T17, FL74, and F9 cells; we cannot as yet determine whether this represents a block to translation, NF1 protein stability, or nuclear transport; and (ii) high levels of nuclear NF1 protein were detected in T3 cells despite low levels of NF1 DNA-binding activity, suggesting that the NF1 protein in T3 cells is of low activity. The suggestion that two different mechanisms can depress NF1 activity raises the suspicion that this is an aberrant tumor phenotype. In this respect, it is interesting to note that an NF1-binding site mediates the response of a type I collagen gene promoter to the growth regulator TGFβ (43). Although this is an isolated example as yet, it is tempting to speculate that loss of NF1-binding activity in feline leukemia cells diminishes responsiveness to growth-limiting or differentiation-promoting signals.

Two of the three feline tumor cell lines (T3 and T17) with low NF1-binding activity also harbor FeLV proviruses with duplications of the enhancer domain. Such LTR enhancer duplications are frequently found in primary T-cell tumors induced by FeLV; the duplications are unique to each isolate but have a common 35-bp domain including the core-binding site. The NF1 site is often but not invariably part of the repeat unit (13). Similar duplications of the core site have been observed in T-cell leukemogenic isolates of MuLV, while in contrast viruses which transform other cell lineages show preferential duplication of the NF1 site (e.g., FBJ-murine sarcoma virus (15). We suggest that tandem duplications of specific protein-binding sites in the LTR may be selected on the basis of relative abundance of positive regulators of viral transcription in the target tissue. In feline leukemia cell lines, reduced levels of NF1 activity would then favor a compensating duplication of the adjacent core-binding site. A virus carrying such duplications may therefore replicate more efficiently in the leukemogenic target cell or perhaps favor cell growth by increased enhancer activation of cellular proto-oncogenes. These alternatives may be distinguishable by further functional analyses of the variant FeLV LTRs.

ACKNOWLEDGMENTS

This work was funded by the Cancer Research Campaign.

We thank Robert MacFarlane for technical assistance, B. Ozanne for helpful discussions, and R. Cortese for the gift of the rat NF1 cDNA clone.

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kB transcription factor. Cell **53**:211–217.
2. **Bernstine, E. G., M. L. Hooper, S. Grandchamp, and B. Ephrussi.** 1973. Alkaline phosphatase activity in mouse teratoma. Proc. Natl. Acad. Sci. USA **70**:3899–3903.
3. **Bishop, J. M., and H. Varmus.** 1985. Function and origins of retroviral transforming genes, p. 249–356. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **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.

4a. **Breimer, L.** Unpublished data.

4b. **Breimer, L., and K. Willison.** Unpublished data.

5. **Caruso, M., C. Iacobini, C. Passananti, A. Felsani, and P. Amati.** 1990. Protein recognition sites in polyomavirus enhancer: formation of a novel site for NF-1 factor in an enhancer mutant and characterization of a site in the enhancer D domain. EMBO J. **9**:947–955.
6. **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.
7. **Cherry, J. R., T. R. Johnson, C. Dollard, J. R. Schuster, and C. L. Denis.** 1989. Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. Cell **56**:409–419.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
9. **Clark, L., R. M. Pollock, and R. T. Hay.** 1988. Identification and purification of EBP1: a HeLa cell protein that binds to a region overlapping the 'core' of the SV40 enhancer. Genes Dev. **2**:991–1002.
10. **deGroot, R. P., J. Schoorlemmer, S. T. vanGenesen, and W. Kruijer.** 1990. Differential expression of jun and fos genes during differentiation of mouse F9 embryonal carcinoma cells. Nucleic Acids Res. **18**:3195–3202.

11. **DesGroseillers, L., and P. Jolicoeur.** 1984. Mapping the viral sequences conferring leukemogenicity and disease specificity in Moloney and amphotropic murine leukemia viruses. J. Virol. **52**:448–456.
12. **Fulton, R., D. Forrest, R. McFarlane, D. Onions, and J. C. Neil.** 1987. Retroviral transduction of T-cell antigen receptor β-chain and myc genes. Nature (London) **326**:190–194.
13. **Fulton, R., M. Plumb, L. Shield, and J. C. Neil.** 1990. Structural diversity and nuclear protein binding sites in the long terminal repeats of feline leukemia virus. J. Virol. **64**:1675–1682.
14. **Gil, G., J. R. Smith, J. L. Goldstein, K. Orth, M. S. Brown, and T. F. Osborne.** 1988. Multiple genes encode nuclear factor 1-like proteins that bind to the promoter for 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Proc. Natl. Acad. Sci. USA **85**: 8963–8967.
15. **Golemis, E. A., 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.
16. **Goodwin, G. H.** 1988. Identification of three sequence-specific DNA-binding proteins which interact with the Rous sarcoma virus enhancer and upstream promoter elements. J. Virol. **62**:2186–2190.
17. **Goyal, N., J. Knox, and R. M. Gronostajski.** 1990. Analysis of multiple forms of nuclear factor I in human and murine cell lines. Mol. Cell. Biol. **10**:1041–1048.
18. **Hall, C. V., P. Ellen Jacob, G. M. Ringold, and F. Lee.** 1983. Expression and regulation of Escherichia coli lacZ gene fusions in mammalian cells. J. Mol. Appl. Genet. **2**:101–109.
19. **Hayward, W. S., B. G. Neel, and S. M. Astrin.** 1981. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–480.
20. **Johnson, P. F., and S. L. McKnight.** 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58**:799–839.
21. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA binding protein that activates eukaryotic transcription and DNA replication. Cell **48**:79–89.
22. **Jones, N. C., P. W. J. Rigby, and E. B. Ziff.** 1988. Trans-acting protein factors and the regulation of eukaryotic transcription. Genes Dev. **2**:267–281.
23. **Kawakami, T. G., G. H. Theilen, D. L. Dungworth, R. J. Munn, and S. G. Beall.** 1967. C-type viral particles in plasma of cats with feline leukemia. Science **158**:1049.
24. **Kingston, R. E., T. J. Schuetz, and Z. Larin.** 1987. Heat-inducible human factor that binds to a human hsp70 promoter. Mol. Cell. Biol. **7**:1530–1534.
25. **Kryszke, M.-H., J. Piette, and M. Yaniv.** 1987. Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature (London) **328**:254–256.
26. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
27. **Landschultz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2**:786–800.
28. **Larson, J. S., T. J. Schuetz, and R. E. Kingston.** 1988. Activation in vitro of sequence-specific DNA binding by a human regulatory factor. Nature (London) **335**:372–375.
29. **Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins.** 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61**:693–700.
30. **LoSardo, J. E., L. A. Cupelli, M. K. Short, J. W. Berman, and J. Lenz.** 1989. Differences in activities of murine retroviral long terminal repeats in cytotoxic T lymphocytes and T-lymphoma cells. J. Virol. **63**:1087–1094.
31. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
32. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavage. Methods Enzymol. **65**:499–560.
33. **Mermod, N., E. A. O'Neill, T. J. Kelly, and R. Tjian.** 1989. The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. Cell **58**:741–753.
34. **Mylin, L. M., J. P. Bhat, and J. E. Hopper.** 1989. Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator. Genes Dev. **3**:1157–1165.
35. **Nagata, K., R. A. Guggenheimer, and J. Hurwitz.** 1983. Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA **80**:6177–6181.
36. **Neil, J. C., D. Hughes, R. McFarlane, N. M. Wilkie, D. E. Onions, G. Lees, and O. Jarrett.** 1984. Transduction and rearrangement of the myc gene by feline leukaemia virus in naturally occurring T-cell leukaemias. Nature (London) **308**:814–820.
37. **Nilsson, P., B. Hallberg, A. Thornell, and T. Grundstrom.** 1989. Mutant analysis of protein interactions with a nuclear factor I binding site in the SL3-3 virus. Nucleic Acids Res. **17**:4061–4075.
38. **Paonessa, G., F. Gounari, R. Frank, and R. Cortese.** 1988. Purification of a NF1-like DNA binding-protein from rat liver and cloning of the corresponding cDNA. EMBO J. **7**:3115–3123.
39. **Payne, G. S., J. M. Bishop, and H. E. Varmus.** 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature (London) **295**:209–214.
40. **Plumb, M., J. Frampton, H. Wainwright, M. Walker, K. Macleod, G. Goodwin, and P. Harrison.** 1989. GATAAG; a cis-control region binding an erythroid-specific nuclear factor with a role in globin and non-globin gene expression. Nucleic Acids Res. **17**:73–92.
41. **Plumb, M. A., and G. H. Goodwin.** 1988. Detection of sequence-specific DNA-protein interactions by the DNA footprinting technique, p. 139–164. *In* J. M. Walker (ed.), Methods in molecular biology. Humana Press, Clifton, N.J.
42. **Rasheed, S., and M. B. Gardner.** 1980. Characterization of cat cell cultures for expression of retrovirus, FOCMA and endogenous sarc genes, p. 393–400. *In* M. Essex and A. J. McClelland (ed.), Proceedings of the Third International Feline Leukemia Virus Meeting. Elsevier/North-Holland Publishing Co., Amsterdam.
43. **Rossi, P., G. Karsenty, A. B. Roberts, N. S. Roche, M. B. Sporn, and B. deCrombrugghe.** 1988. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen gene by transforming growth factor-beta. Cell **52**:405–414.
44. **Rupp, R. A. W., U. Kruse, G. Multhaup, U. Gobel, K. Beyreuther, and A. E. Sippel.** 1990. Chicken NFI/TGGCA proteins are encoded by at least three independent genes: NFI-A, NFI-B and NFI-C with homologues in mammalian genomes. Nucleic Acids Res. **18**:2607–2616.
45. **Santoro, C., N. Mermod, P. C. Andrews, and R. Tjian.** 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature (London) **334**:218–224.
46. **Selden, R. F., K. Burke-Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore.** 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol. Cell. Biol. **6**:3173–3179.
47. **Shirakawa, F., and S. B. Mizel.** 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. **9**:2424–2430.
48. **Short, M. K., S. A. Okenquist, and J. Lenz.** 1987. Correlation of leukemogenic potential of murine retroviruses with transcriptional tissue preference of the viral long terminal repeats. J. Virol. **61**:1067–1072.
49. **Snyder, H. W., Jr., W. D. Hardy, Jr., E. E. Zuckerman, and E. Fleissner.** 1978. Characterization of a tumor-specific antigen on the surface of feline lymphosarcoma cells. Nature (London) **275**:656–658.
50. **Speck, N. A., and D. Baltimore.** 1987. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney leukemia virus enhancer. Mol. Cell. Biol. **7**:1101–1110.
51. **Speck, N. A., B. Renjifo, and N. Hopkins.** 1990. Point mutations in the Moloney murine leukemia virus enhancer identify a

lymphoid-specific viral core motif and 1,3-phorbol myristate acetate-inducible element. J. Virol. **64**:543–550.

51a. **Stewart, M.** Unpublished data.

52. **Stewart, M. A., M. Warnock, A. Wheeler, N. Wilkie, J. I. Mullins, D. E. Onions, and J. C. Neil.** 1986. Nucleotide sequences of a feline leukemia virus subgroup A envelope gene and long terminal repeat and evidence for the recombinational origin of subgroup B viruses. J. Virol. **58**:825–834.

53. **Thornell, A., B. Halberg, 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.

54. **Tsichlis, P. N., and J. M. Coffin.** 1980. Recombinants between endogenous and exogenous avian tumour viruses: role of the C region and other portions of the genome in the control of replication and transformation. J. Virol. **33**:238–249.

55. **Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs III, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334**:494–498.