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An Embryonic DNA-Binding Protein Specific for the Promoter of the Retrovirus Long Terminal Repeat†

FREDERIC FLAMANT,‡* CHERYL C. GURIN, AND JOSEPH A. SORGE§

Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, California 92037

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Retrovirus expression is restricted in embryonal carcinoma (EC) cells but not in many differentiated cell lines. We used a very sensitive gel retardation assay to detect sequence-specific DNA-binding proteins in crude nuclear extracts obtained from EC and differentiated cells. Four binding sites were mapped in the noncoding sequences of the amphotropic murine leukemia virus. Strong binding to the CCAAT consensus sequence located in the promoter was specifically observed with EC nuclear extract. The binding protein is called EPBF (embryonal promoter-binding factor), and it is a candidate for the repressor of retrovirus transcription.

The major limitation to the use of retroviral vectors for embryonic gene transfer experiments is that preimplantation embryos do not permit retrovirus expression (13). In many respects, embryonal carcinoma (EC) cells are similar to early mouse embryo cells (25). The restriction of retrovirus expression in these cells is due, at least in part, to inefficient transcription from the promoter located in the upstream long terminal repeat (LTR). Despite numerous studies, the mechanism of repression remains elusive. DNA methylation occurs after provirus integration but seems to be an effect, not a cause, of inactivation (7, 17). Transient expression experiments have shown that enhancer sequences are probably involved in the repression mechanism. Competition experiments suggest that EC cells do not lack activating factors, but rather contain repressing factors (8). However, mutated enhancers that allow transient expression in embryonal cells generally are not able to restore proviral expression (15). In contrast, a point mutation occurring in the tRNA primer-binding site, which is located 400 nucleotides downstream from the enhancer, partially restores stable viral expression (2). Models relying only on enhancer repression are clearly unable to explain these observations.

With this in mind, we decided to search for DNA-binding proteins that would specifically interact with the retrovirus DNA sequences in both nonpermissive EC cells and permissive cell lines. Any systematic difference in the binding pattern might permit the identification of factors implicated in the repression mechanism. Since retroviral vectors which lack most of the retrovirus-coding sequences are also repressed in EC cells (18, 19, 24), such factors should bind to the noncoding sequences of the retroviruses used in these constructions, i.e., the Moloney murine leukemia virus (M-MuLV) and the amphotropic murine leukemia virus 4070A (A-MuLV).

We used a gel retardation assay and DNase I footprinting to identify sequence-specific DNA-binding proteins in crude nuclear extracts obtained from nonpermissive and permissive cells. Four binding sites were located on the retrovirus LTR. Strong binding that occurred in the promoter region was observed specifically with EC cell nuclear extracts.

MATERIALS AND METHODS

Mouse cell lines. F9 and PCC4 aza 1 (PCC4) are two nonpermissive EC cell lines. SSLC, a differentiated cell line that was obtained from PCC4 cells after induction of differentiation (22), is permissive for retrovirus expression. NIH 3T3 and NIH 3T6 are two permissive fibroblasts cell lines. All cells were grown as monolayers in Dulbecco modified Eagle medium plus penicillin, streptomycin, and 10% fetal bovine serum in dishes (100 mm in diameter).

Extreme care was taken to treat PCC4, F9, SSLC, NIH 3T3, and NIH 3T6 cells in the same way. Permissive and nonpermissive cells were incubated simultaneously in the same chamber with the same batch of culture medium. Nuclear extracts were prepared simultaneously with the same buffers.

Crude nuclear extracts. Each extract was prepared from 20 dishes of near-confluent cells. The extraction was usually prepared by the method of Strauss and Varshavsky (23). For some experiments (indicated in the text), the procedure of Dignam et al. (5) was followed. The protein concentration (1 to 10 μg/μl) was determined by the Bradford assay (3).

Probe preparation. The 1.1-kilobase *Hin*dIII-*Cla*I fragment of cistor (20) containing a complete LTR of A-MuLV was cloned in the plasmid pBluescript (Stratagene, San Diego, Calif.). The M-MuLV probes were obtained from p48, a plasmid containing a full M-MuLV provirus (1). In most experiments, the restriction-digested plasmid was end labeled with Klenow polymerase or polynucleotide kinase by the standard protocols (16). To obtain many different probes, we also used single-stranded DNA which was rescued after M13 infection. The synthesis of the second strand was initiated from a T7 oligonucleotide primer (hybridized upstream from the insert) by Klenow polymerase with both radioactive and nonradioactive nucleotides (50 μCi of dTTP, 400 μCi/mmol for 1 μg of DNA, and 0.2 mM each dATP, dCTP, and dGTP, 10 mM Tris hydrochloride [pH 8.0], 10 mM $MgCl_2$, and 50 mM NaCl; 2 h, 30°C). The synthesis was completed by adding cold dTTP (0.2 mM for 15 min), and the double-stranded molecules were restriction digested.

The labeled fragments were loaded on a 6% acrylamide gel in 0.5× TBE buffer (45 mM Tris-borate, 45 mM boric acid, 2 mM EDTA) and electrophoresed at 25 V/cm for 1 h. After 5 min of autoradiography, the fragments were located, cut out,

* Corresponding author.

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‡ Present address: INRA, Ecole Nationale Supérieure Agronomique, 35042 Rennes Cedex, France.

§ Present address: Stratagene, 11099 N. Torrey Pines Rd., La Jolla, CA 92037.

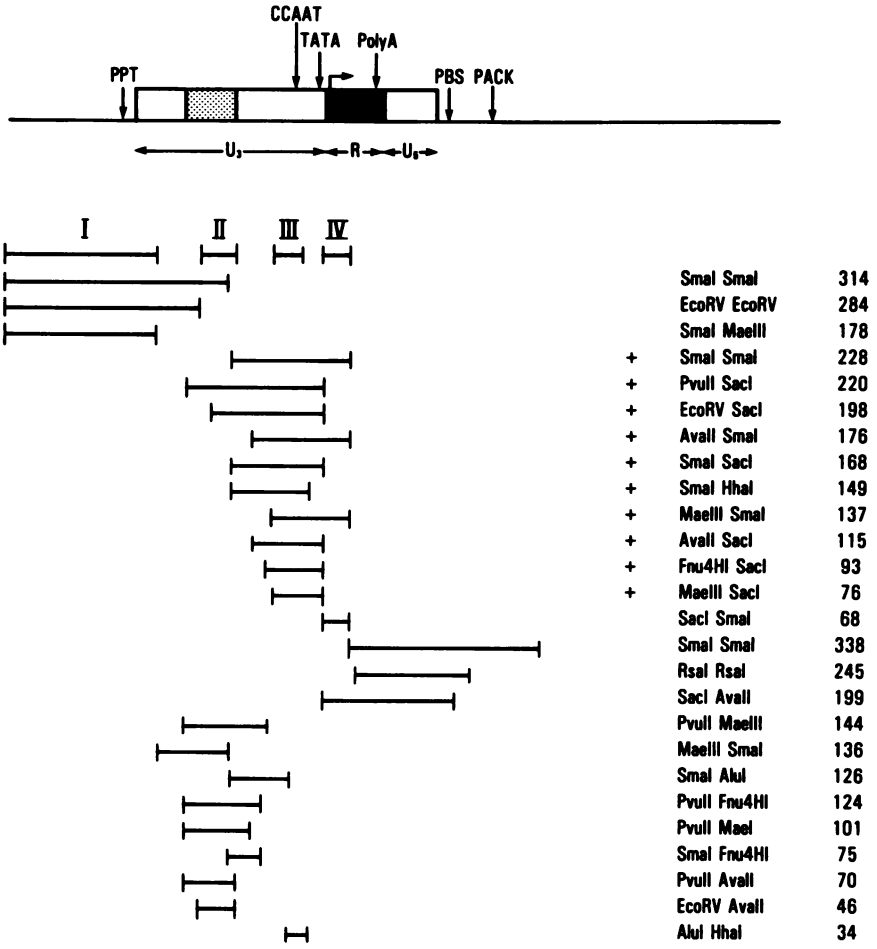

FIG. 1. Probes used to detect retrovirus DNA-binding protein. A simplified map of the A-MuLV noncoding sequences. The boxed part represents the LTR. Symbols: ▬, R region; ▭, enhancer sequences located in U_3; ↱, beginning of transcription (cap site). PPT, Polypurine tract (necessary for plus-strand DNA synthesis); CCAAT, consensus sequence located at position −80 (before the cap site); TATA, consensus sequence located at position −30; poly(A), AATAAA consensus sequence necessary for RNA polyadenylation; PBS, primer-binding site used in the first step of RNA reverse transcription; PACK, sequences necessary for efficient packaging of RNA in the virions. I, II, III, and IV are the four identified binding sites. Probes are listed in order according to their flanking restriction sites (with the 5′ site first) and their sizes (in nucleotides). One *Sma*I site and one *Eco*RV site belong to the vector polylinker. Positions of other flanking restriction sites are: *Alu*I, −73; *Ava*II, −183, −146, and +166; *Eco*RV, −229; *Fnu*4HI, −124; *Hha*I, −50; *Kpn*I, +36; *Mae*I, −150; *Mae*III, −335 and −107; *Pvu*II, −251; *Rsa*I, +34 and +279; *Sac*I, −31; and *Sma*I, −199 and +30. Binding was observed with probes overlapping with either region I, II, III, or IV. EPBF-binding probes are indicated (+). Positions of binding sites: I, between −513 and −335; II, between −229 and −183; III, between −107 and −50; and IV, between −31 and +30. Site III was limited to a shorter area by DNase I footprinting (Fig. 5).

and soaked overnight in 4 ml of 0.5 M NaCl–0.1 M Tris (pH 8.0)–10 mM EDTA. The buffer was filtered through a membrane (pore size, 0.45 μm). Ethanol (8 ml) was added, and the DNA was recovered by centrifugation (Beckman SW41 rotor, 35,000 rpm, 30 min). Pellets were suspended in 0.4 ml of the soaking buffer.

DNA was purified by one phenol-$CHCl_3$ (1/1) extraction, one $CHCl_3$ extraction, and ethanol precipitation. Pellets were suspended in 20 μl of 5 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA. When double digestion was necessary, giving numerous small size fragments, the first digestion was followed by a round of acrylamide gel purification.

Gel retardation assay. The gel shift assay was done as described previously (4), with some modifications. Ten microliters of diluted protein suspension (1 μg diluted in 0.35 M NaCl–10 mM sodium EDTA–10 mM Tris hydrochloride [pH 7.5]) of buffer D was added to 10 μl of a mixture containing 1 μg of poly(dI-dC) (Pharmacia, Inc.) as a nonspecific competitor and 200 to 2,000 Cerenkov cpm of DNA probe (1 ng) in 24 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9)–12 mM KCl–10 mM $MgCl_2$–8 mM Tris hydrochloride (pH 8.0)–1.2 mM EDTA–1.2 mM dithiothreitol–1.2 mM β-mercaptoethanol. The binding reaction was done for 30 min at 30°C.

The low-ionic-strength gel contained 4% acrylamide and 0.01% bisacrylamide in 6.7 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–3.3 mM sodium acetate. The gel dimensions were 14 by 15 by 0.1 cm. The prerun was done at 4°C under 150 V for at least 2 h, until stabilization of amperage was observed (15 mA). Loading was done under 250 V (2 min). The samples were electrophoresed for 2 to 3 h (depending on the probe size) under 150 V at 4°C, with the buffer being recirculated between the two compartments. Autoradiography was performed, usually overnight, at −70°C with an intensifying screen.

DNase I footprinting. A partially purified PCC4 nuclear

extract was prepared as follows. A Dignam extract was first loaded on a heparin agarose column (Sigma Chemical Co.). The column was then rinsed with 0.4 M TETG (50 mM Tris hydrochloride [pH 7.5], 10 mM EDTA, 0.1% Tween 20, 15% glycerol, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.4 M NaCl), and embryonal promoter-binding factor (EPBF) was eluted in 2 column volumes of 0.7 M TETG (the same buffer except that the NaCl was 0.7 M). This fraction was loaded directly on a gel filtration column (P150; Bio-Rad Laboratories) and eluted in 0.1 N TETG. The active fractions were pooled and used for the DNase I footprinting experiment, which was carried out as described elsewhere (14).

RESULTS

Retrovirus DNA-binding proteins in PCC4 and SSLC crude nuclear extracts. A-MuLV and the retroviral vectors containing the A-MuLV LTR have been previously shown to be repressed in the undifferentiated PCC4 EC cell line (19). SSLC is a differentiated subclone of the PCC4 cell line which is permissive for expression of retroviruses, including A-MuLV. We prepared nuclear extracts from these two cell lines and looked for factors that are able to bind to the A-MuLV LTR. Since SSLC and PCC4 cells are very similar, we suspected that differences in the nuclear protein binding pattern might be relevant to the repression mechanism. The different probes that we used to perform the gel retardation assay are shown in Fig. 1.

DNA-binding proteins found in both permissive and nonpermissive cell lines. Binding in three sites on the LTR was obtained with both SSLC and PCC4 nuclear extracts. The first binding site was mapped between positions −510 and −335 (before the cap site; site I in Fig. 1). This region contains the polypurine tract which is necessary for plus-strand synthesis and the beginning of the U_3 region of the LTR (Fig. 2A).

As our work was in progress, Speck and Baltimore described a number of factors that bind to the M-MuLV enhancer in different cell lines, including PCC4 (21). In the initial experiments in which we used the Strauss and Varshavsky protocol for nuclear extraction, we did not find evidence of binding to the A-MuLV or M-MuLV enhancer. However, by using extracts prepared by the Dignam procedure (also used by Speck and Baltimore), we obtained a binding with both SSLC and PCC4 protein extracts. The major binding was located between positions −220 and −183 (site II in Fig. 1 and 2B). Other bindings in the enhancer regions were found to be very weak and not easily reproducible.

A third binding site was located between positions −31 and +30 in a region containing the TATA consensus sequence (site IV in Fig. 1). Two weak bands were observed with the *Sac*I-*Sma*I61 probe with both SSLC and PCC4 extracts (Fig. 2C). The upper band was not observed in all our experiments. We did not determine whether these bands corresponded to two factors or to two forms of a single factor.

DNA-binding protein specific for the embryonal cells that binds to the CCAAT box. Binding activity in region III clearly distinguished PCC4 and SSLC nuclear extracts. With the *Ava*II-*Sac*I115 probe, strong binding was easily detectable with the PCC4 nuclear extract. In contrast, the SSLC nuclear extract provided very weak binding activity, which was detected only when a large amount of protein was used (Fig. 3A). The intensity of the binding signal was shown to be at least 10 times weaker by scintillation counting. This result was also observed with Dignam extracts (Fig. 3B). We called this binding factor, which is found mainly in EC cells, EPBF. Other faint bands were also detected with the same probe; a band was obtained only with the SSLC extract with both the Strauss-Varshavsky and Dignam extracts. These complexes might correspond to other binding factors or to different forms of EPBF (e.g., processed forms). Since the binding of EPBF was the major difference found between PCC4 and SSLC cells, we focused further experiments on this factor.

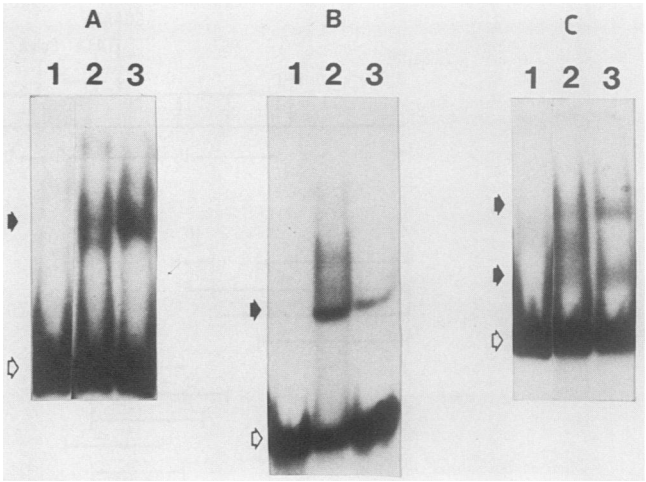

FIG. 2. Binding proteins (at least three) present in both SSLC and PCC4 extracts. A 1-μg sample of PCC4 (lane 3) or SSLC (lane 2) nuclear extract was incubated with the *Sma*I-*Mae*III178 (A), *Eco*RV-*Ava*II46 (B), or *Sac*I-*Sma*I68 (C) probe. White arrows indicate unbound probe. Black arrows indicate the positions of the different DNA protein complexes. Lanes 1 are controls with no nuclear extract. Panels A, B, and C correspond to binding sites I, II, and IV, respectively, which are depicted in Fig. 1.

Refined mapping of the EPBF-binding site. Numerous probes were used to map precisely the EPBF-binding site on the A-MuLV LTR (site III in Fig. 1). Binding was observed with both the *Sma*I-*Hha*I149 and *Mae*III-*Sma*I137 probes (not with the *Mae*III-*Sma*I136 probe). The binding site was then located between positions −107 (*Mae*III site) and −50 (*Hha*I site) (Fig. 4).

Since we did not observe any binding with the *Sma*I-*Alu*I126 and *Alu*I-*Hha*I34 probes, it seemed likely that the *Alu*I site (−73) was part of the binding site. This restriction site is located 3 nucleotides downstream from the CCAAT consensus sequence. EPBF also bound with the same efficiency to the M-MuLV promoter (results not shown). The sequence of this virus differs from the sequence of A-MuLV at position −72 (C-T); thus, this mutation did not affect EPBF binding. We also found that guanine methylation on either strand failed to interfere with EPBF binding (results not shown).

A more precise definition of the EPBF-binding site was obtained by DNase I footprinting (Fig. 5). The protected area is located between nucleotides −87 and −59 and contains the CCAAT consensus sequence which is necessary for promoter function (11) (Fig. 6). This footprint pattern is clearly different from the one described for CBP, another CCAAT-binding factor found in rat liver (11).

Control experiments. The fact that EPBF did not bind to probes without binding site III (Fig. 1) indicated that the binding was sequence specific (Fig. 4, lane 6, for example).

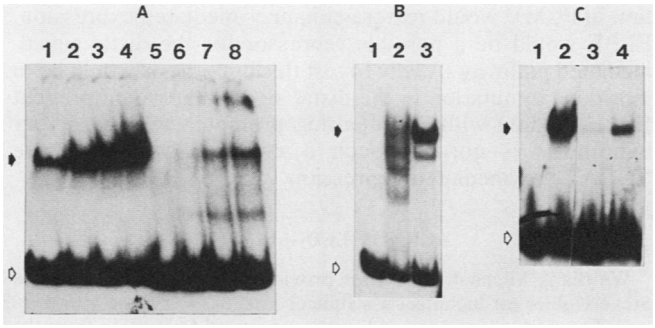

FIG. 3. EPBF binding specific for undifferentiated EC cells. Binding with the *Ava*II-*Sac*I115 probe. Black markers indicate the EPBF-probe complex. White markers indicate the unbound probe. (A) The probe was incubated with increasing amounts (1 to 4 μg) of PCC4 (lanes 1 to 4) or SSLC (lanes 5 to 8) extract, both of which were prepared by the method of Strauss and Varshavsky (23). (B) The probe was incubated with 2 μg of SSLC (lane 2) or PCC4 (lane 3) nuclear extract, both of which were prepared by the method of Dignam et al. (5). Lane 1 is a control with no nuclear extract. Overexposure was necessary to detect the other binding activities. (C) Competition experiment. The probe was incubated with 1 μg of PCC4 extract and various competitor DNAs. Lanes: 1, control with no nuclear extract; 2, 1 μg of poly(dI-dC); 3, 0.5 μg of poly(dI-dC) plus 0.5 μg of cistor (a plasmid containing two A-MuLV LTRs cloned in the bacterial vector pMLII) (20); 4, 0.5 μg of poly(dI-dC) plus 0.5 μg of pMLII. Binding was specifically prevented when the competitor DNA contained the retrovirus promoter.

We also performed competition experiments in which 0.5 μg of unlabeled plasmid was added to the binding reaction mixture. Binding was strongly reduced only when the added plasmid contained retroviral binding site III (Fig. 3C). Binding was totally prevented by pretreating the nuclear extract with proteinase K (100 μg/ml, 10 min, 50°C) but was unchanged when proteinase K was omitted or replaced by RNase A. These controls confirmed that EPBF is a sequence-specific DNA-binding protein.

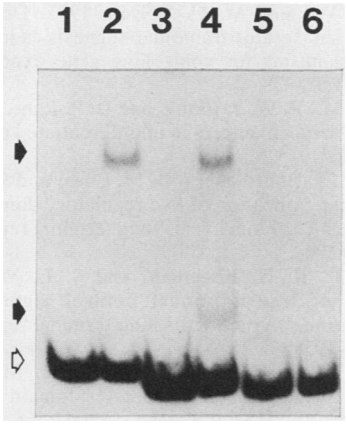

FIG. 4. Precise mapping of the EPBF-binding site. PCC4 nuclear extract (1 μg) was incubated with probes *Sma*I-*Hha*I149 (lane 2), *Mae*III-*Sma*I137 (lane 4), and *Sma*I-*Alu*I126 (lane 6). Lanes 1, 3, and 5 are controls with no nuclear extract. Binding with *Sma*I-*Hha*I149 and *Mae*III-*Sma*I137 (not *Mae*III-*Sma*I136) demonstrates that binding site II is located between the *Mae*III and *Hha*I restriction sites (site III in Fig. 1). The absence of binding with *Sma*I-*Alu*I126 indicates that the *Alu*I site is located either upstream to or within the EPBF-binding site. This was confirmed by DNase footprinting (Fig. 5). No binding was obtained with an *Alu*I-*Hha*I34 probe (results not shown). Therefore, *Alu*I is more likely located within the binding site. Since *Mae*III-*Sma*I137 overlaps with binding site IV (Fig. 1), a second complex is observed with that probe (lane 4). Black arrows indicate protein DNA complexes; white arrows indicate unbound probes.

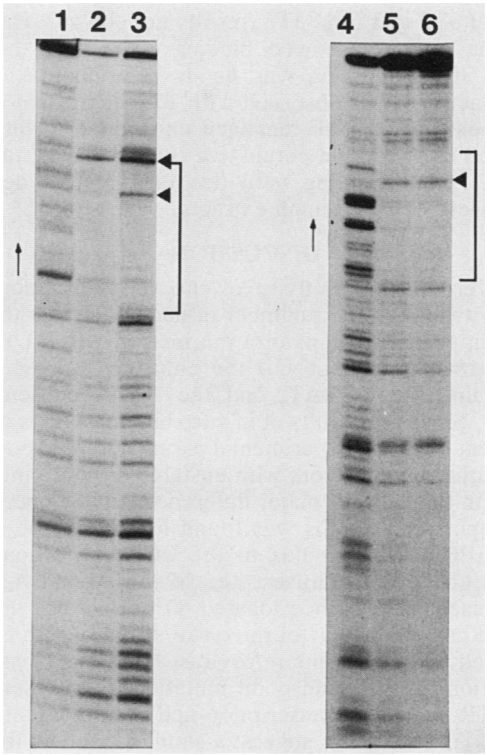

FIG. 5. DNase footprinting. The probe labeled at only one end of one strand was incubated with 0 (lanes 1 and 4), 3 (lanes 2 and 5), or 10 μg (lanes 3 and 6) of a partially purified PCC4 extract and then treated with DNase I (0.5 to 1.5 U for 30 s), purified, denatured, and loaded on an 8% polyacrylamide sequencing gel. Lanes 1 to 3, Probe *Ava*II-*Sac*I115 5′-end labeled at the *Ava*II site by polynucleotide kinase treatment (coding strand); lanes 4 to 6, probe *Ava*II-*Sac*I115 3′-end labeled at the *Ava*II site by Klenow polymerase treatment (noncoding strand). Brackets indicate the EPBF protection area. Symbols: ◀, position where EPBF binding results in increased DNase sensitivity; →, position of the CCAAT box.

Binding activity in the nonpermissive F9 EC cell was similar to that obtained with PCC4 cells. Only a weak binding to the *Ava*II-*Sac*I115 probe was found in two fibroblast cell lines that are permissive for retrovirus replication,

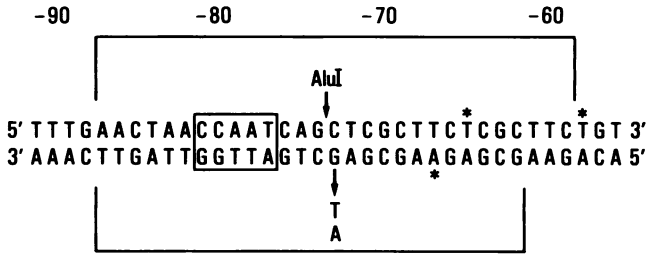

FIG. 6. EPBF-binding site. Brackets above and below the sequence designate the maximum nucleotide boundaries of the DNase I footprint protection. The nucleotides are numbered from the cap site. *Alu*I restriction digestion prevents EPBF binding. Symbols: →, position where A-MuLV differs from M-MuLV (this mutation does not affect EPBF binding); *, position where EPBF binding induces an increased sensitivity to DNase I. The CCAAT consensus sequence is boxed.

i.e., NIH 3T3 and NIH 3T6 (results not shown). However, the same extracts showed binding activity in the other regions. EPBF binding was highly reproducible. Similar binding activity was observed with 15 independent EC cell nuclear extracts. EPBF remained undetected (or at a very low level) in 10 distinct permissive cell nuclear extracts. By contrast, other binding activities (see results described above) were detected in all extracts.

DISCUSSION

The very high sensitivity of the gel retardation assay allowed us to identify a number of nuclear factors that bind in four important regions of a murine retrovirus LTR, i.e., the upstream region of U_3, the enhancer, the sequences surrounding the CCAAT, and the TATA consensus sequences. Since the results of in vitro binding assays are very dependent on many experimental parameters, the possibility remains that other factors with unstable or poor binding are present in the cells. A major difference between permissive and nonpermissive cells was found for one of the factors, called EPBF, which binds to the CCAAT consensus sequences of two retroviruses, i.e., M-MuLV and A-MuLV. This consensus sequence located 80 nucleotides upstream from the cap site is part of the distal signal, which is highly conserved among murine retroviruses and has been shown by deletion analysis and point mutation to be essential for both LTR-mediated transcription and retrovirus infectivity (10–12). These findings suggest a simplistic model for retrovirus inactivation in EC cells in which EPBF binding prevents the proper initiation of RNA transcription. This hypothesis seems to contradict the current idea that repression in EC cells acts mainly on the enhancer sequences. A careful examination of the published data shows, however, that the apparent contradiction should be examined more closely.

Transient expression of LTR-promoted genes in EC cells can be partially restored by deleting the enhancer sequences (8) and totally restored by replacing them with a mutant polyomavirus enhancer (15). However, none of these modifications restores the expression of the integrated provirus or permits virus infectivity in EC cells. Furthermore, a deletion of the CCAAT box greatly reduces virus infectivity but has only a limited effect on transient expression in the same cells. These observations clearly show that transient expression experiments and infection experiments are not directly comparable and that the effect of EPBF binding might not be detectable in a transient assay.

Mutant retroviruses that are able to replicate in EC cells have been isolated recently (6). One of these mutants, PCMV, is expressed (although at substantially reduced titers) in both F9 and PCC4 EC cells. The sequence mutations do not map in the distal signal but are more likely located in the enhancer sequences (Manuel Grez, personal communication). However, the infectivity of PCMV in PCC4 cells is still 100-fold lower than in NIH 3T3 fibroblasts. Thus, the enhancer mutation fails to restore a normal level of virus expression in PCC4 embryonal cells, suggesting that other sequences are involved in the repression mechanism. Finally, it has been shown that in certain situations a CCAAT box deletion has a dramatic effect on LTR-driven expression, whereas an enhancer deletion does not. Under other experimental conditions, however, the opposite result is obtained. These observations have led to the proposal that the enhancer sequences and the CCAAT box facilitate the LTR transcriptional activity by two pathways (9).

We propose that both of these pathways are involved with repression in embryonal cells. In this hypothesis, the mutation in PCMV would restore enhancer-mediated expression. EPBF would be a possible repressor for the distal signal-mediated pathway. A way to test this hypothesis would be to introduce a mutation in the distal signal that would prevent EPBF binding without impairing promoter activity. If our hypothesis is correct, such a mutation would restore CCAAT box-mediated expression.

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LITERATURE CITED

1. **Bacheler, L., and H. Fan.** 1981. Isolation of recombinant DNA clones carrying complete integrated proviruses of Moloney murine leukemia virus. J. Virol. **37**:181–190.
2. **Barklis, E., R. C. Mulligan, and R. Jaenisch.** 1986. Chromosomal position or virus mutation permits retrovirus expression in embryonal carcinoma cells. Cell **47**:391–399.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **86**:142–146.
4. **Carthew, R. W., L. A. Chodosh, and P. A. Sharp.** 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell **43**:439–448.
5. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
6. **Franz, T., F. Hilberg, B. Seliger, C. Stocking, and W. Ostertag.** 1986. Retroviral mutants efficiently expressed in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA **83**:3292–3296.
7. **Gautsch, J. W., and M. C. Wilson.** 1983. Delayed de novo methylation in teratocarcinoma suggests additional tissue-specific mechanisms for controlling gene expression. Nature (London) **301**:32–37.
8. **Gorman, C. M., P. W. J. Rigby, and D. P. Lane.** 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell **42**:519–526.
9. **Graves, B. J., S. P. Eisenberg, D. M. Coen, and S. L. McKnight.** 1985. Alternate utilization of two regulatory domains within the Moloney murine sarcoma virus long terminal repeat. Mol. Cell. Biol. **5**:1959–1968.
10. **Graves, B. J., R. N. Eisenman, and S. L. McKnight.** 1985. Delineation of transcriptional control signals within the Moloney murine sarcoma virus long terminal repeat. Mol. Cell. Biol. **5**:1948–1958.
11. **Graves, B. J., P. F. Johnson, and P. C. McKnight.** 1986. Homologous recognition of a promoter domain common to the MSV LTR and the HSV-TK gene. Cell **44**:565–576.
12. **Hanecak, R., S. Mittal, B. R. Davis, and H. Fan.** 1986. Generation of infectious Moloney murine leukemia viruses with deletions in the U3 portion of the long terminal repeat. Mol. Cell. Biol. **6**:4634–4640.
13. **Jahner, D., M. Stuhlmann, C. G. Stewart, K. Harbers, J. Lolher, I. Simon, and R. Jaenisch.** 1982. De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature (London) **298**:623–628.
14. **Jones, K. A., K. R. Yamamoto, and R. Tijan.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**:559–572.
15. **Linney, E., B. Davis, J. Overhauser, E. Chao, and H. Fan.** 1984. Nonfunction of a Moloney murine leukemia virus regulatory sequence in F9 embryonal carcinoma cells. Nature (London) **308**:470–472.

16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Niwa, O., Y. Yokota, H. Ishida, and T. Sughara.** 1983. Independent mechanisms involved in suppression of the Moloney leukemia virus genome during differentiation of murine teratocarcinoma cells. Cell **32**:1105–1113.
18. **Rubenstein, J. L. R., and F. Jacob.** 1984. Construction of a retrovirus capable of transducing and expression genes in multipotential embryonic cells. Proc. Natl. Acad. Sci. USA **81**:7137–7141.
19. **Sorge, J., V. Erdman, A. Cutting, and J. W. Gautsch.** 1984. Integration specific retrovirus expression in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA **81**:6627–6631.
20. **Sorge, J., D. Wright, V. D. Erdman, and A. E. Cutting.** 1984. Amphotropic retrovirus vector system for human cell gene transfer. Mol. Cell. Biol. **4**:1730–1737.
21. **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.
22. **Speers, W. C., J. W. Gautsch, and F. J. Dixon.** 1980. Silent infection of murine embryonal carcinoma cells by Moloney murine leukemia virus. Virology **105**:241–244.
23. **Strauss, F., and A. Varshavsky.** 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell **37**:889–901.
24. **Taketo, M., E. Gilboa, and M. L. Sherman.** 1985. Isolation of embryonal carcinoma cell lines that express integrated recombinant gene flanked by the Moloney murine leukemia virus long terminal repeats. Proc. Natl. Acad. Sci. USA **82**:2422–2426.
25. **Teich, N. M., R. A. Weiss, G. R. Martin, and D. R. Lowy.** 1977. Virus infection of murine teratocarcinoma stem cell lines. Cell **12**:973–982.