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Mechanism of Suppression of the Long Terminal Repeat of Moloney Leukemia Virus in Mouse Embryonal Carcinoma Cells

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Sequence-specific DNA-binding proteins that bind to the long terminal repeat (LTR) of Moloney leukemia virus in undifferentiated and differentiated mouse embryonal carcinoma (EC) cells were identified by gel retardation assay. The proteins that bind to the CCAAT box were present in both undifferentiated and differentiated EC cells. The amounts and the number of species of the proteins that bind to the enhancer and the GC-rich region were far lower in undifferentiated EC cells than in the differentiated counterparts. These proteins were supposed to be transcriptional activators. Proteins that bind upstream of the enhancer, namely, the −352 to −346 region and the −407 to −404 region, were identified. These proteins were designated the embryonic LTR-binding protein (ELP) and the LTR-binding protein, respectively. The ELP was present only in undifferentiated EC cell lines. The LTR-binding protein was detected in all cell lines tested. The mechanism of suppression of the LTR was investigated by the chloramphenicol acetyltransferase assay. The enhancer and the GC-rich region of the LTR functioned poorly in undifferentiated cells. When eight copies of ELP-binding sequences were inserted upstream of the enhancer region, expression of the chloramphenicol acetyltransferase gene was reduced about threefold in ECA2 cells. From these data, we concluded that two mechanisms, the shortage of activator proteins and the presence of a negative regulatory protein (ELP), are involved in the suppression of the LTR in undifferentiated EC cells.

Embryonal carcinoma (EC) cells are often used for the study of developmental biology, because they provide a useful model for undifferentiated preimplantation mouse embryo cells (14). In comparison with cells derived from somatic tissues such as fibroblasts, EC cells are peculiar with respect to the genes expressed. In undifferentiated EC cells, some genes, such as heat shock protein 70 (9) and the E2 gene of adenovirus (9), are constitutively activated. On the other hand, the promoter of the class I major histocompatibility antigen genes (17), the enhancer of polyomavirus (1), and the long terminal repeat (LTR) of Moloney murine leukemia virus (Mo-MuLV) (6, 11, 18) are suppressed in these cells.

In embryonal stem cells, all luxury genes should be suppressed in a reversible manner, because the stem cells differentiate into all types of tissues where these genes are expressed. Thus, suppression of luxury genes is essential in the maintenance of the undifferentiated state of embryonal stem cells. We have investigated the mechanism of suppression of Mo-MuLV in undifferentiated EC cells. Two mechanisms were shown to act on the viral genome during differentiation of the cells (5, 19). Immediately after infection, the cellular factor(s) acts on the viral genomes and suppresses them. For 1 to 2 weeks after infection, the integrated proviral genome becomes progressively methylated, possibly as a consequence of the suppression. The cellular factor(s) responsible for the suppression is thought to be *trans*-acting. Although exceptions exist (2, 23), the Mo-MuLV genomes integrated at random sites are generally repressed. The target of the suppression was shown to be the LTR (6, 18), but the mechanism is not yet clear.

In recent years, the gel retardation assay, a sensitive method for the detection of sequence-specific DNA-binding proteins, was developed. We applied this method and identified the proteins that bind to the LTR of Mo-MuLV in EC cells. We also utilized the chloramphenicol acetyltransferase (CAT) assay to study the function of protein-binding sites of the LTR. On the basis of the data in the present study, the mechanism of suppression of the LTR in undifferentiated EC cells is discussed.

MATERIALS AND METHODS

Cell cultures. ECA2 (a subline of PCC4 Aza1 cells [5]), PCC4, PCC3, and F9 cells are undifferentiated mouse EC cell lines. NEC8 cells (gift of S. Sekiya, Chiba University) are a human EC cell line. These cells, together with Ltk$^-$, NIH 3T3, and HeLa cells, were maintained in minimal essential medium alpha (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% fetal calf serum.

Differentiated ECA2 cells were obtained by treating the cells with 0.1 to 1 μM retinoic acid (Sigma Chemical Co., St. Louis, Mo.) for 1 week.

Preparation of nuclear extracts. Each extract was prepared by the method of Miskimins et al. (16). The protein concentration was 5 to 15 mg/ml, as determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Plasmids. p2XLTR, which carries two tandem copies of the LTR of Mo-MuLV, was provided by T. Seyama of our laboratory. The 268-base-pair (bp) *Nhe*I (−418)-to-*Xba*I (−150) fragment and the 180-bp *Xba*I (−150)-to-*Kpn*I (+31) fragment of Mo-MuLV LTR from p2XLTR was subcloned into pUC119. Numbering of nucleotides was as previously described (8). Subfragments of above regions were further cloned into pUC119 (Fig. 1).

The structures of the plasmids for the CAT assay are shown in Fig. 2. pMolXKCAT carries the promoter region [*Xba*I (−150) to *Kpn*I (+31)] upstream of the CAT gene. The regions including the enhancer, the GC-rich region, and the

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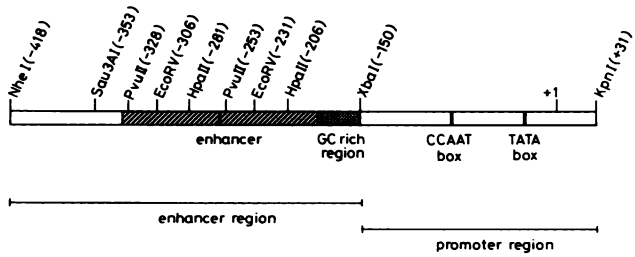

FIG. 1. Map of the restriction sites and the control signals of Mo-MuLV LTR. Designations for the enhancer and the promoter regions used in our experiments were as indicated in the map. Numbering of the nucleotides is as in a previous report (8).

promoter [*Pvu*II (−328) to *Kpn*I (+31)] were ligated to the CAT gene in pMolPKCAT. In the construct pSP8PKCAT, eight copies of the *Sau*3AI (−353)-to-*Pvu*II (−328) fragment, containing the binding site for the embryonic LTR-binding protein (ELP), were inserted 5′ to the enhancer of pMolPKCAT. pL4PKCAT carries four copies of the region from −418 to −388, which contain the binding site for the LTR-binding protein (LBP), in place of the ELP site in pSP8PKCAT.

Preparation of probes and competitors used for gel retardation assays. DNA fragments for probes in gel retardation assays were terminally labeled by the use of the Klenow fragment of *Escherichia coli* DNA polymerase I (Takara Shuzo Co., Kyoto, Japan), [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill. or Dupont, NEN Research Products, Boston, Mass.), and cold deoxynucleoside triphosphates. The *Nhe*I (−418)-to-*Xba*I (−150) fragment was used as a region carrying the Mo-MuLV enhancer sequence. The *Xba*I (−150)-to-Kpn (+31) fragment of Mo-MuLV LTR was used as the promoter region of Mo-MuLV. Each DNA fragment was size separated by an acrylamide gel and recovered by electroelution onto DEAE paper.

Gel retardation assays. In a typical experiment, 5 μg of nuclear extract was preincubated for 5 min at room temperature in 10 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–5% glycerol–1 mM dithiothreitol–1 mM EDTA–2 to 4 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia, Inc., Piscataway, N.J.). The probe (0.1 to 0.5 ng, 1×10^4 to 5×10^4 cpm) was then added, and the mixture was incubated for another 30 min at room temperature in a final volume of 20 μl. For competition experiments, a 200× molar excess of unlabeled DNA fragments was added to the reaction mixtures simultaneously with the probes. The binding mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris hydrochloride (pH 8.3), 89 mM boric acid, and 2 mM EDTA. The gel was dried and autoradiographed at −70°C.

DNaseI footprinting. Binding reactions for DNaseI footprinting were performed at a scale 10 times that of the usual gel retardation assays, except that EDTA was eliminated and calcium and magnesium were added to 0.5 and 1 mM, respectively. After the binding reactions, DNaseI (Sigma) was added to a final concentration of 2 to 3 μg/ml and incubated at 20°C for 90 to 120 s. The digestion was stopped by the addition of EDTA to 25 mM, and the reaction mixtures were loaded onto the native acrylamide gels. After electrophoresis, the complexed and the free fragments were excised and electroeluted onto DEAE paper. The recovered fragments were phenol extracted, ethanol precipitated, and analyzed by electrophoresis through sequencing gels. The probe DNA was chemically cleaved as previously described (15) and run in parallel as markers.

Dimethyl sulfate (DMS) protection assay. The DNA fragments were partially methylated at guanine residues as previously described (15), ethanol precipitated twice, and used as probes. Binding reactions were performed at a scale 10 times that of the usual gel retardation assays. After the binding reactions, the reaction mixtures were loaded onto the native acrylamide gels and exposed. The complexed and the free fragments were electroeluted as described above, phenol extracted, and precipitated twice by ethanol. The DNA fragments were digested by 1 M piperidine at 90°C for 20 min; digestion was followed by ethanol precipitation and lyophilization. They were analyzed by electrophoresis on sequencing gels. The markers were prepared as described above.

CAT assay. NIH 3T3 and ECA2 cells were seeded at a density of 5×10^5 per 10-cm plate 16 h before transfection. Ten micrograms each of CAT plasmid and pUC119 as carrier DNA was mixed and transfected onto a plate. Transfection and the CAT assay were done as described by Gorman et al. (6), except that 0.1 μCi of [^{14}C]chloramphenicol was used per reaction.

RESULTS

Detection of proteins in EC cells which bind specifically to the LTR of Mo-MuLV. Control elements within Mo-MuLV LTR have been previously mapped with fibroblasts (10), frog

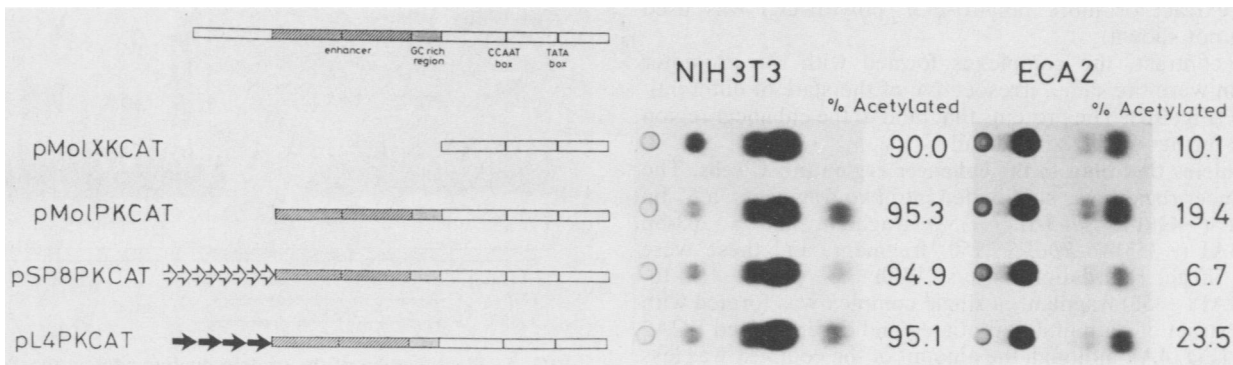

FIG. 2. Functional analysis of the protein-binding sequences in undifferentiated EC cells. Ten micrograms each of CAT plasmids and pUC119 as carrier DNA was transfected to NIH 3T3 and ECA2 cells. The open and closed arrows indicate the binding sequences for the ELP and the LBP, respectively. The numbers indicate percentages of the acetylated form of chloramphenicol. The structure of each plasmid is described in Materials and Methods.

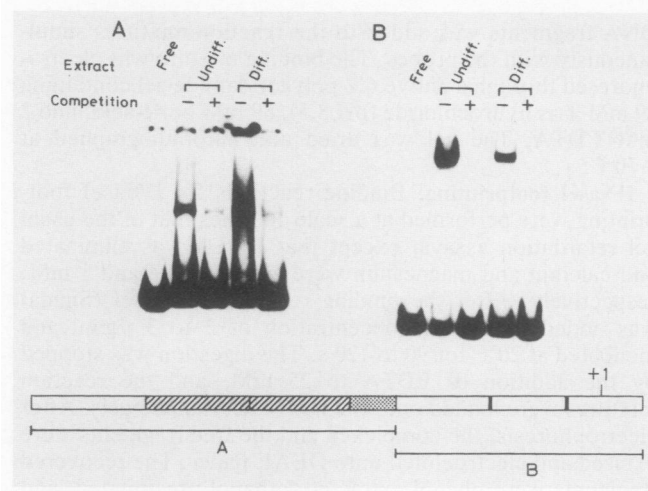

FIG. 3. Detection of proteins that bind to the LTR of Mo-MuLV in EC cells. The enhancer region (A) and the promoter region (B) were used as probes for gel retardation assays. Undiff. and Diff., undifferentiated and differentiated EC A2 cells, respectively. The regions of the fragments used as probes are indicated at the bottom of the figure. In Fig. 3 to 5, the hatched boxes indicate the enhancer and GC-rich regions, respectively. +1 indicates the transcription start site.

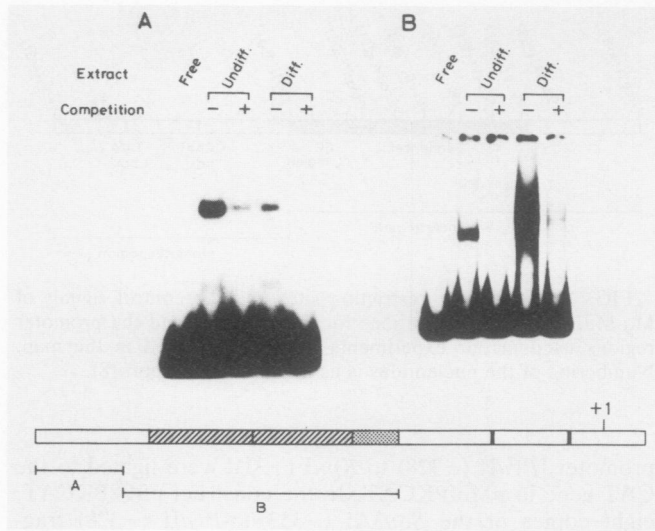

FIG. 4. Detection of proteins that bind to the enhancer region of Mo-MuLV LTR. The *Nhe*I (−418)-to-*Sau*3AI (−353) fragment (A) and the *Sau*3AI (−353)-to-*Xba*I (−150) fragment (B) were used as probes for gel retardation assays. The regions of the fragments used as probes are indicated at the bottom of the figure.

oocytes (7), and EC cells (6, 12). An enhancer composed of a 75-bp repeat, the GC-rich region, and the CCAAT box has been shown to participate in transcriptional activation (Fig. 1). We divided these regions into two fragments, namely, a 268-bp *Nhe*I (−418)-to-*Xba*I (−150) fragment designated the enhancer region and containing the 75-bp repeat and the GC-rich region, and a 180-bp *Xba*I (−150)-to-*Kpn*I (+31) fragment designated the promoter region and containing the CCAAT box, the TATA box, and the transcriptional initiation site.

In gel retardation assays, the complexes were formed with the enhancer and promoter regions by the extract of undifferentiated and differentiated ECA2 cells (Fig. 3). Formation of these complexes was inhibited by an excess amount of unlabeled fragments, indicating that the interaction was sequence specific. A single complex was formed with the enhancer fragment by the extract of undifferentiated ECA2 cells, and the smear pattern was obtained with the same fragment by the extract of differentiated ECA2 cells (Fig. 3A). The latter complex was not due to the overloading of the extract, because the same pattern was observed when less extract or more poly(dI-dC) · poly(dI-dC) was used (data not shown).

In contrast, the complexes formed with the promoter region were the same, irrespective of the state of differentiation (Fig. 3B). The proteins that bind to the enhancer region were further analyzed in detail.

Proteins that bind to the enhancer region in EC cells. The enhancer region was subdivided into two fragments, a 65-bp *Nhe*I (−418)-to-*Sau*3AI (−353) fragment and a 203-bp *Sau*3AI (−353)-to-*Xba*I (−150) fragment, and these were used in gel retardation assays. With the *Nhe*I (−418)-to-*Sau*3AI (−353) fragment, a single complex was formed with the extract of both undifferentiated and differentiated ECA2 cells (Fig. 4A), although the amount of the complex was less abundant in differentiated cells. The factor involved in formation of this complex was designated the LBP for convenience. The complexes with the *Sau*3AI (−353)-to-*Xba*I (−150) fragment differed depending on the state of differentiation (Fig. 4B). A single distinct complex was formed by the extract of undifferentiated ECA2 cells. The complex with the extract of differentiated ECA2 cells had a broad mobility upon electrophoresis. Since the *Sau*3AI (−353)-to-*Xba*I (−150) region exhibited a differential response in complex formation, the proteins that bind to this region were analyzed further.

Mapping of the protein-binding sites in the *Sau*3AI (−353)-to-*Xba*I (−150) fragment by competition experiments. The protein-binding sites in the *Sau*3AI (−353)-to-*Xba*I (−150) fragment were mapped by competition experiments. This fragment was digested with restriction enzyme *Pvu*II or *Eco*RV, and the resulting fragments were used for competition. The complex formed in the extract of undifferentiated

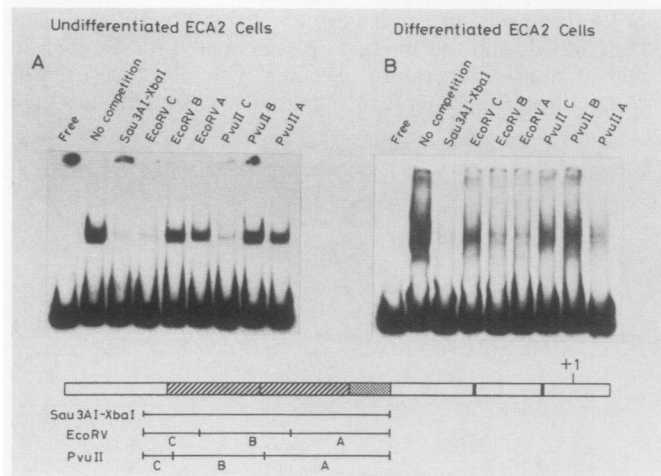

FIG. 5. Identification of the protein-binding sites in the *Sau*3AI (−353)-to-*Xba*I (−150) fragment by the competition experiment. The nuclear extracts from undifferentiated ECA2 cells (A) and differentiated ECA2 cells (B) were used. The regions of the fragments used as competitors are indicated at the bottom of the figure.

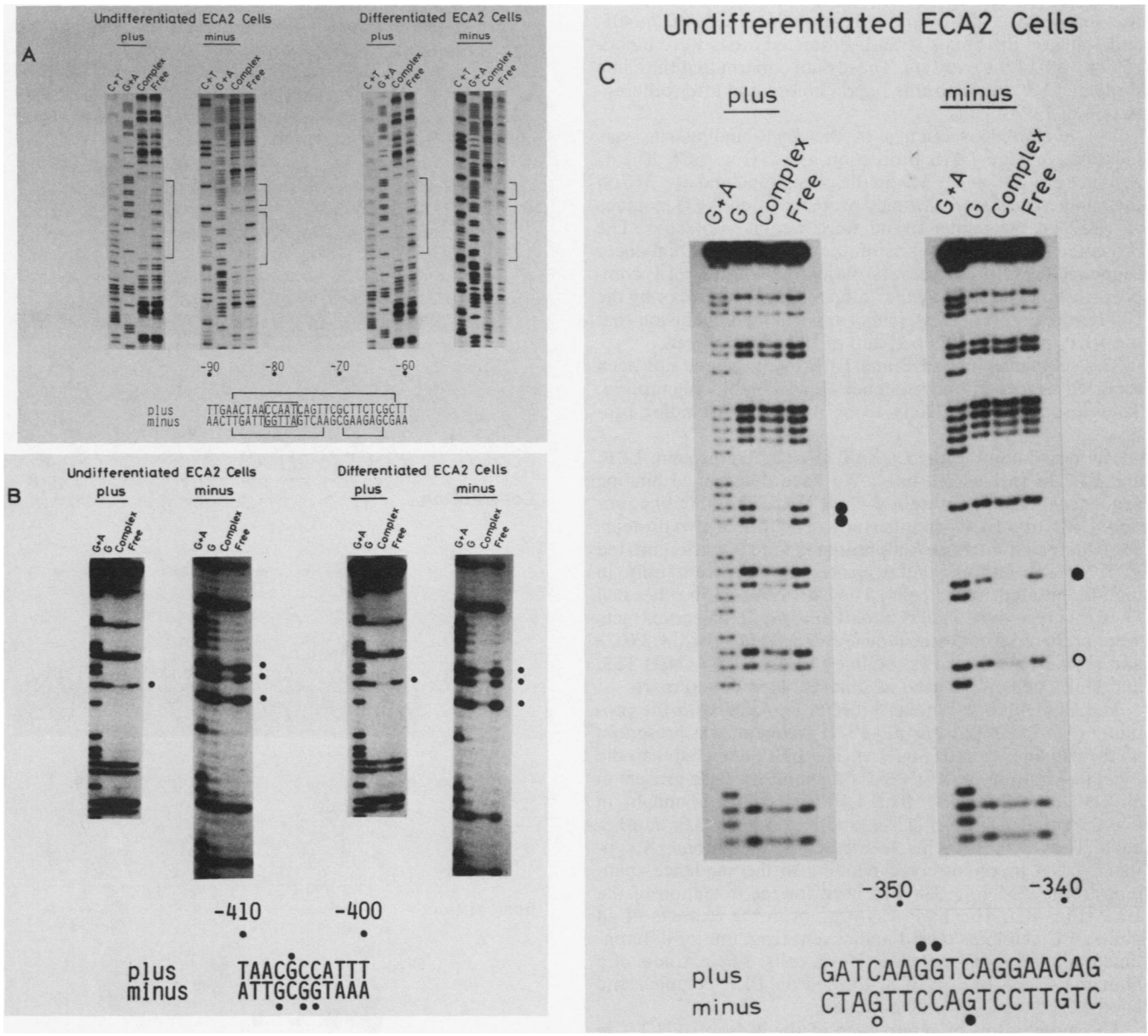

FIG. 6. Determination of the nucleotide sequence of the protein-binding sites. (A) DNaseI footprinting of the protein-binding sequence in the promoter region. The protected area is indicated by brackets. In the sequence at the bottom, the protected sequence is in brackets and the CCAAT box is in a box. (B) DMS protection assay of the LBP-binding sequence. Symbol: ●, protected guanine residues. The nucleotide sequence of the region is indicated below. (C) DMS protection assay of the ELP-binding sequence. Symbols: ●, strongly protected guanine residues; ○, weakly protected residue. The nucleotide sequence of each region is shown at the bottom of each panel.

ECA2 cells was inhibited only by the *Pvu*II C and *Eco*RV C fragments, which mapped at the left end of the probe (Fig. 5A). No competition was observed for the *Pvu*II A, *Pvu*II B, *Eco*RV A, and *Eco*RV B fragments, which carry the enhancer and the GC-rich region. Therefore, within the *Sau*3AI (−353)-to-*Xba*I (−150) region, protein binds only to the left end and not to the enhancer or the GC-rich region. In the case of the complexes with the extract of differentiated ECA2 cells, competition experiments yielded less clear results. Although every subfragment of *Pvu*II or *Eco*RV digestion showed the capacity for competition, none of them was as strong a competitor as the full-length *Sau*3AI (−353)-to-*Xba*I (−150) fragment. This led to the possibility that there are multiple binding sites in this region for factors in differentiated ECA2 cells. This conclusion was confirmed by the direct detection of proteins that bind to the subfragments of this region (data not shown).

Determination of nucleotide sequences of the protein-binding sites. The protein-binding sequence in the promoter region (*Xba*I [−150] to *Kpn*I [+31]) was determined by DNaseI footprinting. The protected sequences were indistinguishable for both of the extracts and spanned from −86 to −62 on the plus strand and from −86 to −64 on the minus strand (Fig. 6A). This region contained the CCAAT box. These data suggest that the same factor binds to this region, irrespective of the state of differentiation.

The binding sequence for the LBP was determined by a DMS protection assay (Fig. 6B). The protected G residue

was located at −406 on the plus strand and at −407, −405, and −404 on the minus strand. Protected areas were identical for both of the extracts. These data confirm that the same protein, LBP, binds to this region before and after differentiation of ECA2 cells.

The nucleotide sequence of the ELP-binding site was identified by the DMS protection assay (Fig. 6C). The G residues at −348 and −349 on the plus strand and at −346 on the minus strand were strongly protected, and the G residues at −352 on the minus strand were weakly protected. The protected site is upstream of the 75-bp repeat enhancer sequence (10). In the gel retardation assays, the ELP complex was not inhibited by the 75-bp repeat sequence or by the GC-rich region (Fig. 5A). This supports the conclusion that the ELP indeed binds upstream of the 75-bp repeat.

The sequences for LBP and ELP binding have not been reported as recognition sequences for DNA-binding protein. Therefore, these are likely to be novel DNA-binding proteins.

The distribution of the CCAAT box-binding protein, LBP, and ELP in various cell lines. We have determined binding sequences for three proteins—the CCAAT box-binding protein, LBP, and ELP—in this study. The former two proteins were detected in both differentiated and undifferentiated ECA2 cells, and the latter protein was present only in undifferentiated ECA2 cells. Their distribution in other cell lines was tested by a gel retardation assay. Nuclear extracts were prepared from three mouse EC cell lines (PCC4, PCC3 and F9) and a human EC cell line (NEC8). Ltk$^-$, NIH 3T3, and HeLa cells were used as differentiated counterparts.

The CCAAT box-binding protein, detected with the promoter (*Xba*I [−150]-to-*Kpn*I [+31]) fragment, was present in all the cell lines tested (Fig. 7A). The LBP, detected with the *Nhe*I (−418)-to-*Sau*3AI (−353) fragment, was also present in all cell lines (Fig. 7B). The LBP was more abundant in undifferentiated ECA2, PCC4, and NEC8 cells than in other cells. These two proteins seemed to be ubiquitous. A synthetic DNA fragment corresponding to the sequence spanning from −355 to −338 was used for the detection of the ELP (Fig. 7C). The ELP was present in the extracts of all mouse EC cell lines tested and absent from that of differentiated counterparts, including HeLa cells. The extracts of a human EC cell line, NEC8, formed an ELP complex and another sequence-specific complex.

The mechanisms of suppression of the Mo-MuLV LTR in undifferentiated EC cells. The CAT assay was employed to investigate the mechanisms of suppression of Mo-MuLV LTR in undifferentiated EC cells. pMolXKCAT carried the promoter region of the LTR upstream of the CAT gene, while pMolPKCAT had the enhancer and the GC-rich region together with the promoter region to 5′ of the gene. pSP8PKCAT and pL4PKCAT contained eight copies of the ELP-binding sequence and four copies of the LBP-binding sequence, respectively, upstream of the enhancer of pMolPKCAT. They were transfected to NIH 3T3 and ECA2 cells, and the CAT activity was assayed.

The enhancer and the GC-rich region were inactive in undifferentiated EC cells (Fig. 2). The low activity of the enhancer and the GC-rich region was likely due to the low amount of activator proteins in undifferentiated EC cells (Fig. 4). The transcriptional activation by the enhancer and the GC-rich regions in NIH 3T3 cells is not clear in Fig. 2, because the efficiency of transfection was too high. These regions were shown to increase the CAT activity about 20-fold in NIH 3T3 cells when smaller amounts of CAT plasmids were used (data not shown).

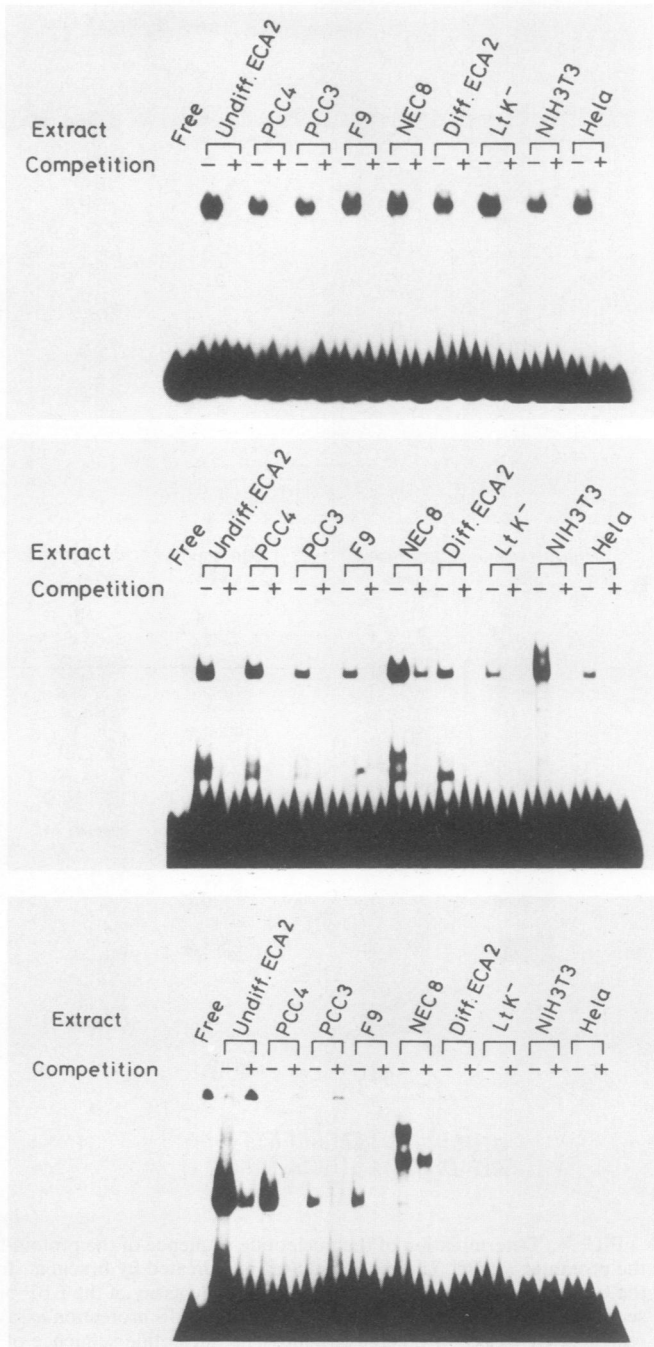

FIG. 7. The presence of the CCAAT box-binding protein, the LBP, and the ELP in various cell lines. The *Xba*I (−150)-to-*Kpn*I (+31) fragment (A), the *Nhe*I (−418)-to-*Sau*3AI (−353) fragment (B), and the synthetic DNA fragment spanning from −355 to −338 (C) were used for detection of the CCAAT box-binding protein, the LBP, and the ELP, respectively.

The insertion of eight copies of the ELP-binding sequence reproducibly resulted in the suppression of CAT expression about threefold in undifferentiated ECA2 cells. We also obtained essentially the same results with undifferentiated F9 cells (data not shown). These data suggested that the ELP is involved in suppression of the LTR of Mo-MuLV in undifferentiated EC cells. The insertion of the LBP-binding sites resulted in a slight increase in CAT activity in ECA2 cells. The significance of these data is under investigation.

DISCUSSION

Many animal viruses, including Mo-MuLV, do not propagate in undifferentiated EC cells (1, 22, 24). In the case of Mo-MuLV, the absorption and penetration of viruses and the integration of viral genomes into the chromosome of host cells take place normally. The restriction of viral propagation is at the level of transcription of proviral genomes (5, 19, 21, 24). This transcriptional repression is dependent on the LTR of the viral genome (18). Two models for the mechanisms of restriction have been proposed: the presence of a negative regulator(s) (6, 18) and the lack of an activator(s) (3, 11, 12, 20) acting on the LTR in undifferentiated EC cells. So far, both of the models still lack enough evidence to be verified.

In the present study, sequence-specific DNA-binding proteins that bind to the LTR of Mo-MuLV were identified in undifferentiated and differentiated EC cells. Among them, the ELP was thought to be a novel protein, as judged by its binding sequence and specific distribution in undifferentiated EC cells. The insertion of the binding sequence of this protein upstream of the enhancer region of the LTR resulted in specific suppression of gene expression in undifferentiated EC cells. These data lead us to conclude that the ELP is involved in the suppression of the LTR of Mo-MuLV in undifferentiated EC cells. It is noteworthy that the ELP-binding site coincides with a site of the mutation in a host range mutant, PCC4 cell-passage neomycin-resistant myeloproliferative sarcoma virus (8), which propagates in undifferentiated EC cells. We made a synthetic oligomer for the mutated ELP-binding site and observed that this mutation reduced the activity of binding to the ELP (data not shown).

The pattern of protein binding to the CCAAT box was indistinguishable in undifferentiated and differentiated cell lines by gel retardation assay and DNaseI footprinting analysis. This is consistent with the previous report that the promoter of Mo-MuLV LTR is functional in undifferentiated EC cells (12).

Recently, Flamant et al. (4) reported that a CCAAT box-binding protein is the candidate for the repressor of viral LTR in undifferentiated EC cells. Their conclusion was based on the point that this protein was more abundant in undifferentiated EC cells than in differentiated cells. We found no difference in the levels of the protein in both cell types, and this may be due to the different methods used to prepare the nuclear extract. It is our experience that the yield of DNA-binding proteins was higher in extracts prepared by the method of Miskimins et al. (16) than in those prepared by the method of Dignam, which was used in the study of Flamant et al. (data not shown).

The LBP was also present in all cell lines tested, though the amount of this protein varied among cell lines. The function of this protein is not clear in our present study. We are now preparing experiments to elucidate the function of this protein.

The amounts of proteins that bind to the enhancer and the GC-rich regions were drastically increased when EC cells were induced to differentiate. Since these proteins are thought to function as transcriptional activators, their shortage may cause the suppression of the LTR in undifferentiated EC cells. The functional assay confirmed this conclusion.

We could not detect the proteins that bind to the enhancer and the GC-rich region in the extract of undifferentiated EC cells with the *Sau*3AI (−353)-to-*Xba*I (−150) fragment as a probe (Fig. 4A). When the subfragments of this region were used as probes, however, the proteins that bind could be detected in the extract of undifferentiated EC cells, although the amounts and the number of species are lower than in differentiated cells (20; our unpublished results). A possible explanation for this is that the ELP may inhibit the binding of activator proteins. This hypothesis can be finally tested if the cDNAs for the ELP are expressed in fibroblasts, in which activator proteins are abundant, and if functional assay and gel retardation assay are performed with these cells. We are working on this project at present.

We propose here that two mechanisms, the lack of activator proteins and the presence of a negative regulatory protein (ELP) are operating for the suppression of the LTR of Mo-MuLV in undifferentiated EC cells.

Recent reports (2, 3, 12, 13) showed the presence of a intragenic domain that is involved in viral suppression in undifferentiated EC cells. Although little is known about them, these mechanisms of suppression are thought to regulate a variety of cellular genes to maintain the undifferentiated state of embryonic stem cells. Elucidation of the mechanisms of suppression of Mo-MuLV in undifferentiated EC cells will reveal what determines the undifferentiated phenotypes of EC cells.

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