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Analysis of the Binding Proteins and Activity of the Long Terminal Repeat of Moloney Murine Leukemia Virus during Differentiation of Mouse Embryonal Carcinoma Cells

TOSHIO TSUKIYAMA, OHTSURA NIWA,* AND KENJIRO YOKORO

Department of Pathology, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734, Japan

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Mouse embryonal carcinoma (EC) cell lines were established which carry the stably integrated chloramphenicol acetyltransferase (CAT) gene under the control of the transcriptional elements of the long terminal repeat (LTR) of Moloney murine leukemia virus. The activity of three elements of the stably integrated LTR was analyzed in undifferentiated EC cells (stable CAT assay). Results of the study are summarized as follows. (i) In the stable assay, the promoter region of the LTR was inactive in undifferentiated ECA2 and F9 cells, and the level of the activity was 10^{-4} of that in NIH 3T3 cells. (ii) In contrast to the results of the transient assay, the enhancer was active in undifferentiated ECA2 cells and in F9 cells. It activated CAT activity more than 60-fold and about 8-fold in ECA2 cells and F9 cells, respectively. (iii) Suppression by ELP, the embryonal LTR-binding protein, was more pronounced in the stable assay than in the transient assay. These data suggest that, when compared with NIH 3T3 cells, a major factor for the inactivity of the LTR in EC cells is the inefficiency of the promoter in this assay. Transcriptional activity of the LTR was analyzed during the differentiation of EC cells. In the case of ECA2 cells, the magnitude of activation by the enhancer did not change during differentiation. The activity of the promoter increased about 10-fold, and the suppression by ELP became negligible 4 days after the induction of differentiation. Upon differentiation of F9 cells, the activity of the enhancer increased more than 300-fold, but the promoter remained inactive. The pattern of LTR-binding proteins also varied during the differentiation of EC cells. Our present data suggest that the activity of LTR elements as assayed by the stable assay differs from the activity as assayed by the transient assay. It also indicates that the activity of these elements exhibits cell-type-specific changes during the differentiation of EC cells.

Moloney murine leukemia virus (Mo-MuLV) propagates well in fibroblasts but fails to do so when infecting embryonal carcinoma (EC) cells (5, 15, 16, 19, 22, 25). Three mechanisms have been identified for the suppression of Mo-MuLV. These involve inactivation of the enhancer through the lack of activator proteins (4, 10, 11, 21, 26); suppression by ELP, the embryonal long terminal repeat (LTR)-binding protein (26); and suppression by the negative regulatory element in the 5′ noncoding region of the virus (4, 11, 27, 28).

Conclusions of previous works were reached mainly on the bases of the transient expression assay of the LTR-driven chloramphenicol acetyltransferase (CAT) gene (CAT assay) (4, 10, 26, 27) and of the stable expression of the LTR-driven neomycin resistance gene (Neo^r colony assay) in the forms of virus (28) or plasmids (11, 15). The transient CAT assay is somewhat artificial. The number of extrachromosomal copies of plasmids introduced by transfection is too large to be natural. In addition, the gene is transcribed from extrachromosomal closed circular plasmids. The stringency of gene regulation may differ between extrachromosomal and intrachromosomal sequences. In addition, one cannot compare the absolute levels of gene expression among cell lines in the transient assay because of the difference in the efficiency of transfection between cell lines. The Neo^r colony assay is semiquantitative because any cell expressing higher than a certain level of mRNA can survive and is scored as positive.

In this report, we established EC cell lines with stably integrated CAT genes under the control of the transcriptional elements of the LTR of Mo-MuLV. CAT activity in these cells was studied during differentiation. The analysis revealed a previously unidentified mechanism of suppression of the LTR in undifferentiated EC cells. The LTR-binding proteins were also analyzed.

MATERIALS AND METHODS

Cell culture. ECA2 cells, a subline of PCC4 Aza1 cells (5), and F9 cells are mouse EC cell lines. These cells and NIH 3T3 cells were maintained in α minimal essential medium supplemented with 8% fetal bovine serum. Differentiation of EC cells was induced by the addition of 1 μM retinoic acid (RA; Sigma Chemical Co., St. Louis, Mo.) (24) in growth medium.

Plasmids. Plasmid constructions are as shown in Fig. 1. pMolXKCAT and pMolPKCAT carry the promoter region (the *Xba*I-*Kpn*I fragment) and the enhancer-promoter region (the *Pvu*II-*Kpn*I fragment) of the Mo-MuLV LTR, respectively, placed upstream of the CAT gene. Eight copies of the binding site for ELP were inserted upstream of the enhancer region of pMolPKCAT, and the resulting plasmid was designated pSP8PKCAT.

Transfection. To establish cells with the stably integrated CAT genes, 2 μg of pRSVneo and 20 μg of CAT plasmids were cotransfected by the $CaPO_4$ method (7). The cultures

* Corresponding author.

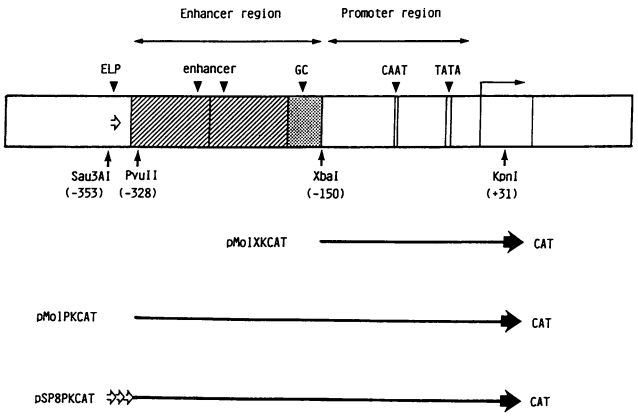

FIG. 1. Structure of CAT constructs. Details of the constructs are as previously described (26). Numbering of the nucleotides is as in a previous report (8).

were selected in the presence of G418 (GIBCO, Grand Island, N.Y.) at 200 μg/ml for ECA2 and F9 cells and at 400 μg/ml for NIH 3T3 cells. The resistant colonies were cultured in a mixture and designated according to the names of the cells and the transfecting plasmid. For example, ECA2/SP8 cells denote ECA2 cells transfected with pSP8PKCAT, and 3T3/XK cells denote NIH 3T3 cells transfected with pMolXKCAT. The mixtures of clones were frozen at early passages and used for analysis within several passages thereafter.

CAT assay. The cell extract for the CAT assay was prepared as described elsewhere (6). The quantity of extracts was adjusted so that the acetylated form of chloramphenicol in each assay was less than 40%. The condition of the reaction was as described previously (26).

Gel retardation assay. The preparation of the nuclear extracts (13) and the conditions of the gel retardation assay were as previously described (26).

RESULTS

Establishment of cells with stably integrated CAT plasmids. We established cell lines carrying CAT plasmids stably integrated into the cellular genome (stable CAT cell lines) to assay the function of the control elements of the LTR (stable CAT assay system). For this purpose, 20 μg of CAT plasmids and 2 μg of pRSVneo were cotransfected into ECA2, F9, and NIH 3T3 cells. We obtained 40 to 50 colonies for each of the EC lines and more than 200 colonies for NIH 3T3 cells after selection in G418 medium. These colonies were propagated as a mixture and used for the analysis.

The average copy number of CAT constructs was determined by Southern blot analysis. Total cellular DNA was digested with *Pst*I. This enzyme cleaves at two sites in each construct: at the multicloning site of pUC119 just upstream of the control element, and at a region downstream of the poly(A)$^+$ signal. The size of the *Pst*I fragments was 2.4 kb for pMolXKCAT and 2.6 kb for pMolPKCAT and pSP8 PKCAT. Hybridization was performed with probes for the CAT gene and the α-globin gene (Fig. 2). An estimation was made of the average copy number of CAT genes in each transfectant. Interestingly, the copy number of each CAT plasmid was almost the same within a particular cell line.

Activity of the transcriptional elements in the LTR as determined by the stable CAT assay. In our present study, the number of cells for each assay was adjusted so that the acetylated form of chloramphenicol was less than 40% (Fig. 3). The activity was corrected for the copy number of the CAT plasmids and for the number of cells used in the reaction (Table 1). Therefore, the normalized value represents an average of the CAT activity for one copy of the CAT gene (CAT activity per copy) in a cell. This enabled us to compare the activity of sequence elements among cell lines, regardless of the efficiency of transfection. In both of the EC cell lines, the activity of the promoter region was less than 10^{-4} of that in NIH 3T3 cells. This was the greatest difference between EC cells and fibroblasts in the activity of transcriptional elements tested here. Another unexpected result was in the function of the enhancer region. The enhancer activated CAT expression 65-fold in ECA2 cells and about 8-fold in F9 cells. In our previous study using the transient CAT assay, activation by the enhancer in EC cells was less than twofold. Both of these results were highly reproducible.

The suppression by the ELP binding site was more pronounced in the stable assay than in the transient assay and was about eightfold in ECA2 cells, less than threefold in F9

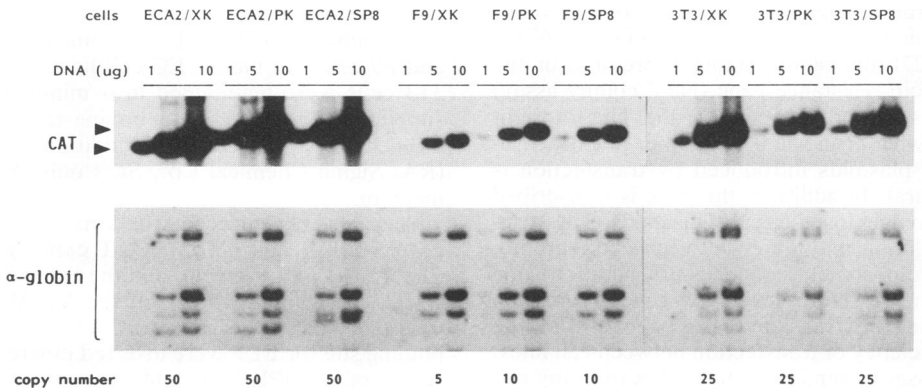

FIG. 2. Copy number estimation of CAT plasmids in stable CAT cell lines. Total cellular DNA from each stable CAT line was digested with *Pst*I. The quantity of DNA electrophoresed is indicated. The filters were prepared in duplicate, and one was hybridized with CAT probe and the other with α-globin probe. The former was exposed overnight, and the latter was exposed for 4 days. See text for the designation of the cells.

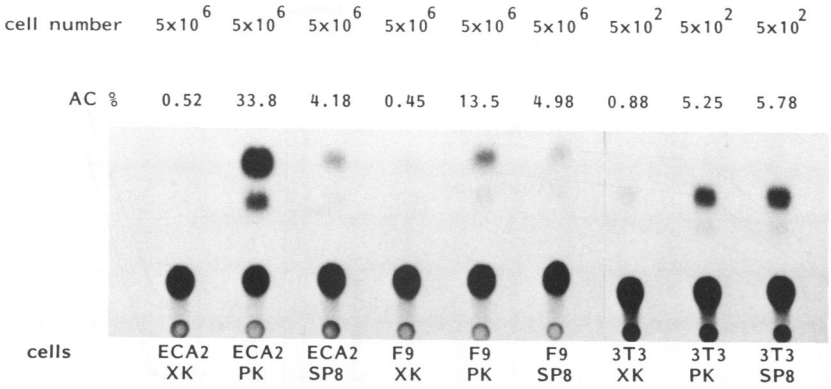

FIG. 3. Activity of transcriptional elements of the LTR in stable CAT assay. AC %, Percentage of acetylated chloramphenicol. The number of cells used for each reaction is indicated above. The data were normalized for cell number and for copy number of CAT plasmids, as indicated in Table 1.

cells, and absent in NIH 3T3 cells (Table 1). The difference in the level of suppression was in proportion to the amount of ELP in these cell lines (26).

Analysis of gene expression from the LTR during differentiation of EC cells. Stable CAT EC cell lines were induced to differentiate by RA, and CAT activities were assayed. The data were normalized as in Fig. 3. During the differentiation of ECA2/XK cells, the level of CAT expression driven by the LTR promoter increased about 10-fold (Fig. 4A). CAT activity in ECA2/PK cells increased on the first day of the RA treatment. The activity dropped transiently on the second day, followed by a steady increase up to the seventh day. The magnitude of the difference in CAT expression between ECA2/XK cells and ECA2/PK cells was more or less the same during differentiation (compare the upper and the lower curves in Fig. 4A). This suggests that the degree of activation by the enhancer was fairly constant during the differentiation of ECA2 cells. In undifferentiated ECA2 cells, CAT gene expression was suppressed about 10-fold when the ELP element was present (ECA2/SP8 cells). The suppression by the ELP element became negligible 4 days after the induction of differentiation. The suppression was marginal in differentiated ECA2 cells. The CAT activity in ECA2/SP8 cells increased more than 100-fold upon the differentiation of the cells. This was the result of the alleviation of ELP-mediated suppression and the induction of promoter function.

TABLE 1. Normalized CAT activity in stable CAT cell lines

Cell line	CAT activity/copy	Activation by enhancer[a]	Suppression by ELP[b]
ECA2/XK	2.1×10^{-9}		
ECA2/PK	1.4×10^{-7}	65.0	1/8.1
ECA2/SP8	1.7×10^{-8}		
F9/XK	1.8×10^{-8}		
F9/PK	1.3×10^{-7}	7.5	1/2.7
F9/SP8	5.0×10^{-8}		
3T3/XK	7.0×10^{-5}		
3T3/PK	4.2×10^{-4}	6.0	1/0.95
3T3/SP8	4.6×10^{-4}		

[a] CAT activity of PK cells/CAT activity of XK cells.
[b] CAT activity of SP8 cells/CAT activity of PK cells.

In F9 cells, the pattern of induction of CAT activity was markedly different from that in ECA2 cells (Fig. 4B). Although a similar decrease was observed on the second day, the increase in CAT activity of F9/PK cells was more prominent and it was over 300-fold 1 week after the induction of differentiation. In addition, the promoter function in F9/XK cells was not activated upon differentiation. Therefore, the CAT activity in F9/PKCAT increased solely through the activation of the enhancer element. In Fig. 4B, the activity of the enhancer in undifferentiated F9 cells seems to be less than that shown in Table 1. A gradual decrease of CAT activity in stable F9 cells was observed, and this may be due to DNA methylation. It is our experience that when CAT activity is low, larger deviation is associated with the results. The experiment whose results are presented in Fig. 4B was done later, and CAT activity was lower than that shown in Table 1. Seemingly inactive enhancer activity in Fig. 4B is within the range of deviation associated with the extremely low CAT activity.

The suppression by ELP was weak throughout the course of differentiation of F9 cells. The CAT activity of F9/PK cells and F9/SP8 cells decreased 14 days after induction. The loss of viability of differentiated F9 cells at this stage (data not shown) may account for the decrease. Although increased, the level of CAT gene expression in differentiated ECA2 cells and F9 cells was nevertheless lower than that in NIH 3T3 cells by about 10^2.

Changes in the LTR-binding proteins during differentiation of EC cells. In our previous report, LTR-binding proteins were examined only in undifferentiated and differentiated EC cells and in fibroblasts (26). Here, we analyzed DNA-binding proteins to the ELP element, the enhancer, and the promoter of the LTR during differentiation of ECA2 cells and F9 cells.

In ECA2 cells, the ELP complex increased slightly 6 h after the induction of differentiation, decreased afterward, and became undetectable after 2 days (Fig. 5A). After 4 days of RA treatment, a novel complex with a distinct mobility appeared, increased in amount after 7 days (indicated by arrow in Fig. 5A), and disappeared after 14 days of treatment. The binding site of the complex was exactly the same as that of authentic ELP in the dimethyl sulfate protection assay (25a). The function of this complex is unknown since

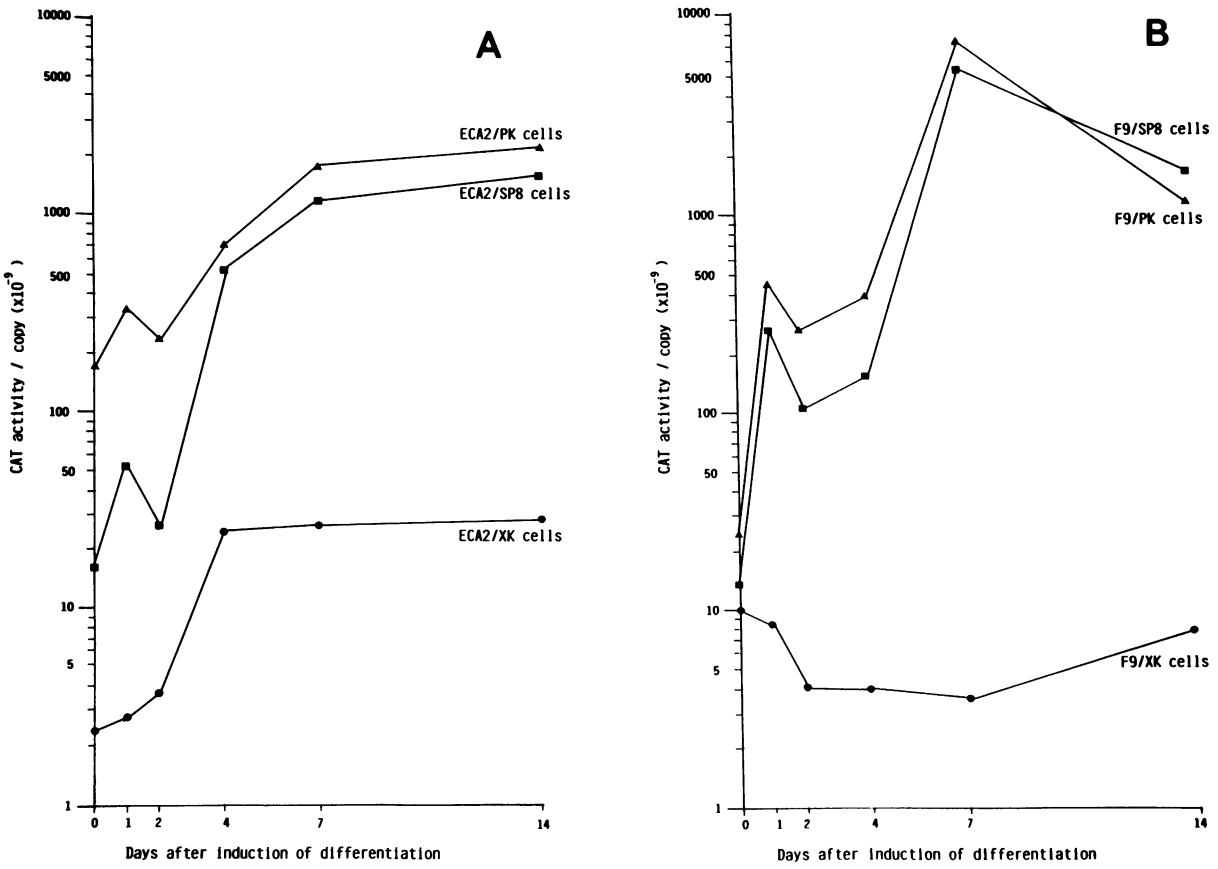

FIG. 4. Analysis of gene expression from the LTR during differentiation of EC cells. Stable CAT ECA2 cells (A) and stable CAT F9 cells (B) were induced to differentiate, and CAT activity was assayed on the days indicated. The data were normalized as in Table 1.

no change was detected in the CAT activity of ECA2/SP8 during this period.

The complexes on the enhancer region varied during differentiation (Fig. 5B). In undifferentiated ECA2 cells, a faint complex was present. This complex increased transiently and then disappeared 1 day after the induction of differentiation, and numerous complexes appeared thereafter. After 14 days of induction, the complex on the enhancer exhibited a broad mobility, indicating the binding of multiple proteins to this region. The probe used for the enhancer region was 178 bp in length, extending from the *Pvu*II (−328) to the *Xba*I (−150) sites. At least six sequence elements reside in this region (21). Present results only suggest that the enhancer-binding proteins increase both in species and quantity during differentiation. We do not know at present the relationship between these proteins and the activity of the enhancer in ECA2 cells.

A single complex was formed on the promoter region which remained constant in amount during differentiation (Fig. 5C). This was previously shown to bind to the CCAAT box of the promoter region (26).

In undifferentiated F9 cells, the amount of ELP was much less than in ECA2 cells. The fact that the complex on the ELP element was strongly induced 6 h after induction of differentiation was unexpected (Fig. 5D). The ELP complex decreased to the same level as in undifferentiated F9 cells on day 1 and became undetectable on day 2 of induction. The novel ELP complex found in ECA2 cells was not detected in F9 cells after 4 to 7 days of induction.

Faint complexes were formed on the enhancer region in undifferentiated F9 cells (Fig. 5E). The complexes were distinct from those in undifferentiated ECA2 cells and were strongly induced 6 h after RA treatment. This induction coincided with a rapid increase in CAT activity in F9/PK and F9/SP8 cells. The complexes disappeared after 1 to 2 days of differentiation in parallel with a decrease in CAT activity. The amount of enhancer-binding proteins decreased after 14 days of differentiation and was associated with decreased enhancer activity (Fig. 4B), which may be due to loss of cell viability at this stage.

The DNA binding protein to the promoter region also changed (Fig. 5F). After 6 h of RA treatment, a novel complex with a slower mobility appeared in addition to the major complex. This complex disappeared afterward. We were unable to assess its function.

DISCUSSION

Regulation of the LTR in undifferentiated EC cells. The results obtained by the stable CAT assay were different from those of previous reports. The results of the semiquantitative Neo^r colony assay suggested that the promoter region has the same activity in a myoblastic line as in EC lines (11). In addition, our previous work using the transient assay also

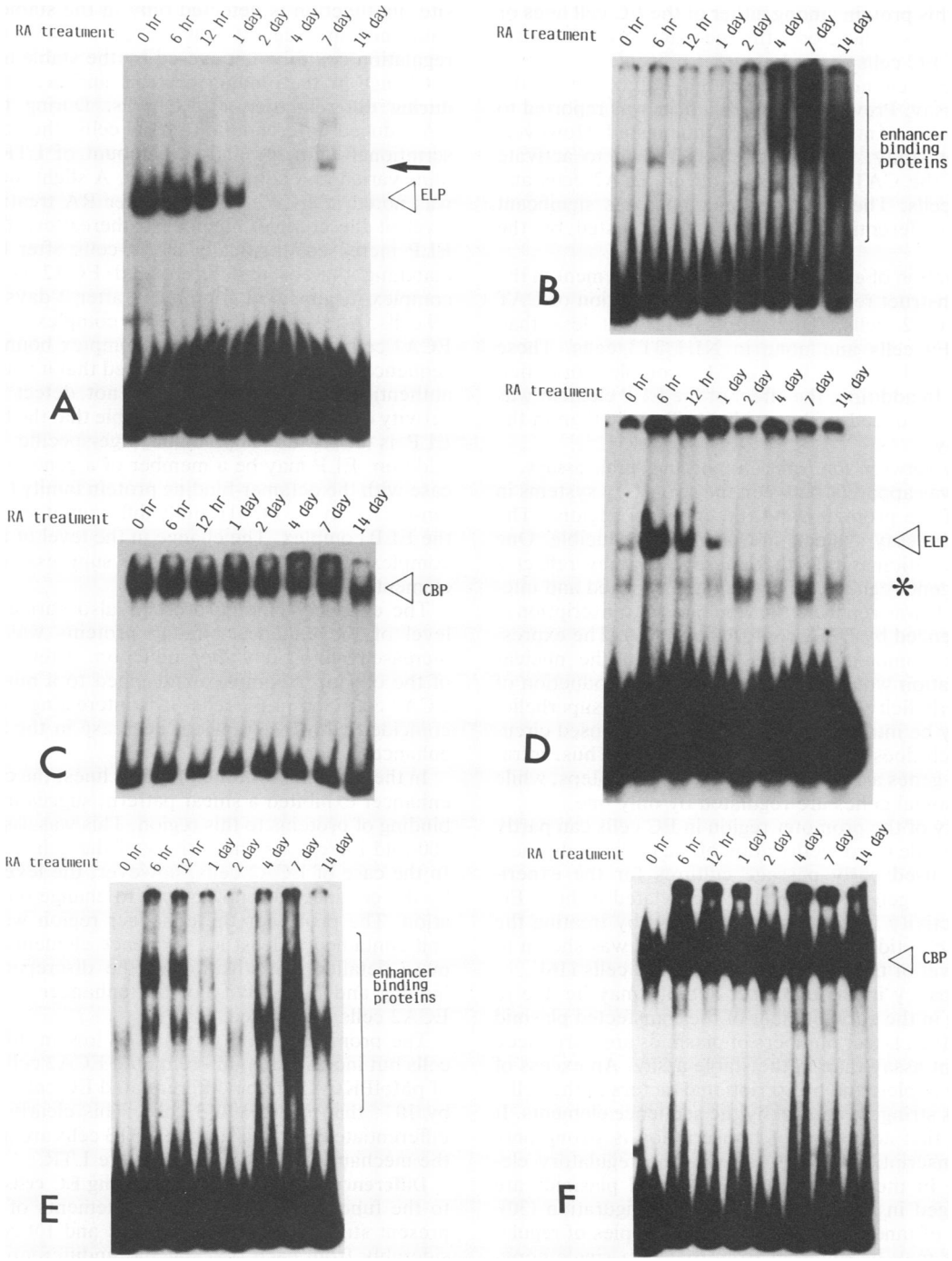

FIG. 5. The LTR-binding proteins during differentiation of EC cells. Nuclear extracts were prepared at the time after the induction of differentiation as indicated. A total of 5 μg of extract was used per lane. Panels A to C, ECA2 cells; panels D to F, F9 cells. CBP denotes the CCAAT box-binding protein. The probes were the *Sau*3AI (−353)-to-*Pvu*II (−328) fragment (panels A and D), the *Pvu*II (−328)-to-*Xba*I (−150) fragment (panels B and E), and the *Xba*I (−150)-to-*Kpn*I (+31) fragment (panels C and F) (see Fig. 1 for the restriction map). The asterisk in panel D indicates sequence nonspecific complex (data not shown). The solid arrow in panel A indicates a novel complex with a distinct mobility.

demonstrated that the promoter functions rather efficiently in ECA2 cells as well as in NIH 3T3 cells (26). However, our present study indicated that the promoter was inactive in undifferentiated EC lines when compared with NIH 3T3 cells. The magnitude of the suppression was on the order of 10^{-4}. These results suggest that, in the stable assay, the promoter plays a major part in the difference in the activity of the LTR between undifferentiated EC cells and NIH 3T3 cells. The CCAAT box-binding protein is the only protein detected to bind to this region, and no difference was

observed for this protein among either of the EC cell lines or for NIH 3T3 cells. Therefore, the mechanism of promoter disfunction in EC cells is not known at present.

The enhancer element also behaved differently in the stable CAT assay. Previously, the enhancer was reported to have poor activity, if any, in every EC line tested. However, in the present study, the element was shown to activate expression of the CAT gene by 65-fold in ECA2 cells and 8-fold in F9 cells. Therefore, the enhancer has significant activity in undifferentiated EC cells when tested by the stable assay.

The introduction of eight copies of the ELP element in the LTR-CAT construct resulted in a 10-fold reduction of CAT activity in ECA2 cells. The suppression was less than threefold in F9 cells and none in NIH 3T3 cells. These results match well with the level of ELP complex formation in these cells. In addition, the effect of the ELP element was again more pronounced in the stable CAT assay than in the transient assay.

Discrepancy between the transient and the stable assays. A discrepancy was apparent between the two assay systems in the actions of the promoter and the enhancer regions. The results of both assay systems are highly reproducible. One possible explanation is that the discrepancy may reflect a difference of gene regulation between unintegrated and integrated genes. It was reported that the rate of transcription is strongly influenced by DNA conformation (29). The expression of intrachromosomal genes depends on the nuclear matrix association which is required for the introduction of negative superhelicity (23). In contrast, negative superhelicity can readily be introduced into unintegrated closed circular DNA which does not have a free terminal. Thus, intrachromosomal genes are regulated by at least two steps, while extrachromosomal genes are regulated by only one.

Low activity of the promoter region in EC cells can partly be explained by de novo methylation of the integrated genes. Although we used early passage cultures for the experiments, the CAT genes were highly methylated in both EC lines. CAT activity increased about 20-fold by treating the cells with 5-azacytidine (25a). This treatment was shown to reduce the level of the methylcytocine in EC cells (16).

The discrepancy in the enhancer activity may be due to the difference in the copy number of the transfected plasmid in the cells. Much larger numbers of plasmids are introduced in the transient assay than in the stable assay. An excess of plasmids may deplete the transcriptional factors in the cells, leading to less stringent control by the sequence elements. It was reported that activation of transcription is strong only when the transcription factors saturate the regulatory elements (2, 9). In the stable assay, integrated plasmids are usually arranged in a linear head-to-tail configuration (30). The behavior of tandem arrays of multiple copies of regulatory elements may be different from that of a single copy, and this may bias the result of the stable assay.

It was reported that the transient assay mimics the natural state of transcription more than the stable assay does (18). The authors concluded that flanking cellular sequences influenced the expression of integrated genes. In our assay system, however, the position effect was minimized by using a mixture of clones. In addition, the level of suppression of the LTR in the stable assay was more similar to that observed in viral infection than in the transient assay (5, 16, 19, 22, 25, 28).

Recently, Loh et al. (12) reported that when the EC cell-specific negative element in the 5′ noncoding region of Mo-MuLV is placed upstream of the transcriptional start site, its function is detected only in the stable assay. Their data and ours suggest that the novel mechanisms of gene regulation could be uncovered by the stable assay.

Change in the binding proteins and activity of the LTR during differentiation of EC cells. During the course of RA-induced differentiation of EC cells, the activity of transcriptional elements and the amount of LTR-binding proteins varied in a complex manner. A slight increase in ELP was noted in ECA2 cells 6 h after RA treatment, and the level of the complex decreased thereafter. The amount of ELP increased drastically in F9 cells after 6 h, the level matching that of undifferentiated ECA2 cells. The ELP complex disappeared completely after 2 days in ECA2 and F9 cells. After 4 to 7 days, a novel complex appeared only in ECA2 cells. Although the new complex bound to the same sequence, tryptic cleavage indicated that it was distinct from authentic ELP (25a). We could not detect the biological activity of this complex. It is possible that the binding site for ELP is under developmental-stage-specific regulation. In addition, ELP may be a member of a gene family, as is the case with the octamer-binding protein family (17, 20) and the *jun-fos* family (3, 14, 31). Fully differentiated EC cells lacked the ELP complex. The change in the level of authentic ELP complex paralleled the degree of suppression by the ELP element.

The enhancer-binding proteins also varied. Initially, the level of the enhancer-binding proteins was low. It then increased 6 h to 1 day after induction. After 2 days, the level of the enhancer complexes dropped to a minimum both in ECA2 cells and in F9 cells. It is interesting to note that this coincided with the transient decrease in the activity of the enhancer.

In the fully differentiated EC cell lines, the complex on the enhancer exhibited a smear pattern, suggesting the multiple binding of proteins to this region. This was associated with a 300-fold increase in the activity of the enhancer in F9 cells. In the case of ECA2 cells, however, the level of activation by the enhancer did not appear to change during differentiation. The probe for the enhancer region was rather large and contained at least six sequence elements. Therefore, a more detailed investigation of the discrepancy in protein binding and the activity of the enhancer in differentiating ECA2 cells is required.

The promoter activity remained low in differentiated F9 cells but increased in differentiated ECA2 cells. The activity of pMolPKCAT in the differentiated EC cells was still lower by 10^{-2} than in NIH 3T3 cells. This clearly indicates that differentiated EC cells and NIH 3T3 cells are quite distinct in the mechanisms of regulation of the LTR.

Difference of gene regulation among EC cells. With respect to the function of the sequence elements of the LTR, the present study indicates that ECA2 and F9 cells differ considerably from each other at the undifferentiated state and during the course of differentiation. These differences are summarized in Table 2. It was shown that the host range mutants of polyomavirus (1) and Mo-MuLV (8) have different mutations depending on the EC lines in which they propagate well. The negative regulatory element in the 5′ noncoding region of the Mo-MuLV genome functions well in F9 cells but not so well in ECA2 cells (27). These data suggest that each EC line utilizes specific mechanisms of suppression of viruses.

The difference among EC cells may be a reflection of the capacity of differentiation of each line. F9 cells differentiate into parietal endoderm cells (22), and ECA2 cells differentiate into a variety of cells, the majority being epithelial cells,

TABLE 2. Differences between ECA2 cells and F9 cells in undifferentiated state and during differentiation

Cell type	Undifferentiated cells[a]			During differentiation		
	Activation by enhancer[b]	Suppression by ELP[c]	ELP binding	Increase in promoter activity	Increase in enhancer activity	Differentiated cell type
ECA2	+++ (60)	++ (1/8)	+++	+ (10)	−	Various types
F9	+ (8)	+ (1/3)	+	−	+++ (300)	Parietal endoderm

[a] +++, strong; ++, intermediate; +, weak; −, none.
[b] CAT activity of PK cells/CAT activity of XK cells.
[c] CAT activity of SP8 cells/CAT activity of PK cells.

neuronal cells, and myoblasts. F9 cells and ECA2 cells may represent the embryonal cells of different developmental stages. An elucidation of the embryonal stage corresponding to each EC cell line may help to reveal the gene regulation in cells of early embryos.

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