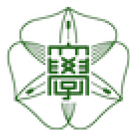
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The molecular mechanisms of the transcriptional control related to embryonal carcinoma cell differentiation: BOX DNA, EC cell specific enhancer, and its binding proteins

Fumiko Kihara-Negishi

①

DOCTORAL THESIS

The molecular mechanisms of the transcriptional control related to embryonal carcinoma cell differentiation:

BOX DNA, EC cell specific enhancer, and its binding proteins

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Sapporo 1994

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68 **Molecular mechanism of the transcription regulated by BOX DNA dependently pluripotency/differentiated states of cells.**

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ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
dNTP etc.	any deoxy ribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
Tris	tris(hydroxymethyl)aminomethane
EC	embryonal carcinoma
RA	retinotic acid
SV40	simian virus 40
HSV	herpes simplex virus
TK	thymidine kinase
CAT	chloramphenicol acetyltransferase
CMV	cytomegarovirus
BOX factor	proteins binding specificaly to BOX DNA

ABSTRACT

BOX DNA was previously isolated from the DNA sequence inserted in the enhancer B domain of the mutant polyomavirus DNA (fPyF9). We also reported that BOX DNA functioned negatively on DNA replication and transcription of another polyomavirus mutant (PyhrN2) in F9-28 cells, a subclone of mouse F9 embryonal carcinoma (EC) cells expressing the polyomavirus large T antigen. In this study, I demonstrate that BOX DNA enhances transcription from the thymidine kinase (TK) promoter in various EC cells. One or three copies of BOX DNA, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the herpes simplex virus (HSV) TK promoter, activated the promoter activity in F9, P19 and ECA2 cells. The band shift assays using BOX DNA as a probe revealed that specific binding protein(s) were present in all the EC cells examined: The patterns of BOX DNA-protein complexes were the same among them. A mutation introduced within BOX DNA abolished the enhancer activity as well as the formation of the specific DNA-protein complexes. In non-EC cells including L and Balb3T3 cells, the enhancer activity of BOX DNA on the TK promoter was not observed, although binding proteins specific to the sequence exist. In band shift assays, the patterns of the DNA-protein complexes of either L or Balb3T3 cells were different from those of EC cells. Furthermore, the enhancer activity of BOX DNA decreased upon differentiation induction in all the EC cells examined, of different origins and distinct differentiation ability. In parallel with the loss of enhancer activity, the binding proteins specific for BOX DNA decreased in these cells. Moreover, I cloned a genomic DNA of F9, termed BOXF1, containing BOX DNA sequence approximately 400 bp upstream from the RNA start site of the gene. BOXF1, containing a TATA-like motif and the binding elements for Sp1 and Oct in addition to BOX DNA, possessed promoter activity deduced by a BOXF1-CAT construct. Deletion analyses of the construct revealed that the transcription of BOXF1 gene is regulated by BOX DNA, preferentially in undifferentiated EC cells than differentiated cells. Hence, BOX DNA is probably a novel transcriptional element related to EC cell differentiation.

Furthermore, I cloned a cDNA corresponding to BOXF1. The gene expressed highly in undifferentiated EC cells. Its expression decreased after differentiation induction. It is thus thougt that the gene may be a target gene of BOX DNA binding proteins. Moreover, I purified BOX DNA binding proteins of P19, EC cells, and Raji, non-EC cells having no pluripotency. BOX DNA had no enhancer activity in Raji cells, where specific binding proteins were present. A 44kDa protein was purified from P19 cells.as the binding protein specific to BOX DNA 100/60 kDa proteins were mainly purified in Raji cells, although the 44kDa protein was slightly co-purified. Thus, it is speculated that BOX DNA enhancer activity is positively or repressively regulated by complex mechanisms under the influence of pluripotency and differentiated states of the cells.

CHAPTER 1

BOX DNA has enhancer activity specific for undifferentiated EC cells.

1.1 INTRODUCTION

Embryonal carcinoma (EC) cells are suitable materials to study molecular mechanisms of early stages of embryogenesis (41). EC cells are the stem cells of teratocarcinoma, and can be induced to differentiate into a variety of cells by aggregate formation and/or treatment with chemicals, notably retinoic acid (RA) (41, 54). EC cell differentiation is dircted by a lot of gene cascades and protein-protein interactions. It is also considered that differentiation proceeds by reduction of expression of genes involved in maintenance of undifferentiated states (17,36). It has been reported to date that the expression of a number of transcription factors alters during EC cell differentiation in stage specific manners. E1A-like transcriptional factors (DRTF) (31, 32), AP-1 (19), Oct-3 (47), Oct-4, 5 (51), Rex-1 (23) and PEA3 (61) decrease in amount during EC cell differentiation process, while AP-2 (39), HOX-2 (53), HOX-2.5 (29) and PEBP2, 3 increase. Other HOX families (9, 11, 33, 46), the c-*jun* product (12) and Oct-6 (43, 55) transiently increase and are followingly downregulated in the process. It is thougt selective expression of specific sets of genes is primarily controlled at the level of transcription. It thus suggest sthat transcription factors play important roles in embryogenesis cascades by switching on or off various genes involved in differentiation. Biological roles of these factors, however, have not yet been well clarified, although their functions have been speculated from the cDNA sequences encoding them.

Previously we have isolated a mutant of polyomavirus fPyF9, which can replicate in mouse EC cells (2). Differing from other polyomavirus EC mutants, fPyF9 could persist episomally in F9 cells. fPyF9 contained mutations in the enhancer B domain of wild-type polyomaviral DNA: The sequences of enhancer B were extremely rearranged in fPyF9, as in various mutants so far reported, and were inserted by three exogenous sequences. These inserted sequences were homologous to one another, and the sequence 5'GCATTCCATTGTTGTCAAAAG3' (designated 'BOX') was considered as the prototype (Fig.1). Moreover, we have shown that BOX DNA functioned negatively on viral DNA replication and transcription in a system where a polyomavirus hostrange mutant PyhrN2 was tested in F9-28 cells. F9-28 is an F9 clone expressing the

polyomavirus large T antigen (3). These results suggest BOX DNA will have EC cell specific activity influenced by differentiation.

In chapter 1, we examined transcriptional activity of BOX DNA in various EC cells of different developmental stages, in non-EC cells, and in EC cells after differentiation induction, to clarify the relationship between BOX DNA activity and EC cell differentiation. The results showed that BOX DNA activates transcription under the control of the HSV TK promoter specifically in EC cells at undifferentiated states. The BOX DNA-protein (s) complexes were different in EC cells and non-EC cells. Moreovere, the decrease of the transcriptional activation during differentiation was accompanied by the decrease in the proteins specifically bound to BOX DNA.

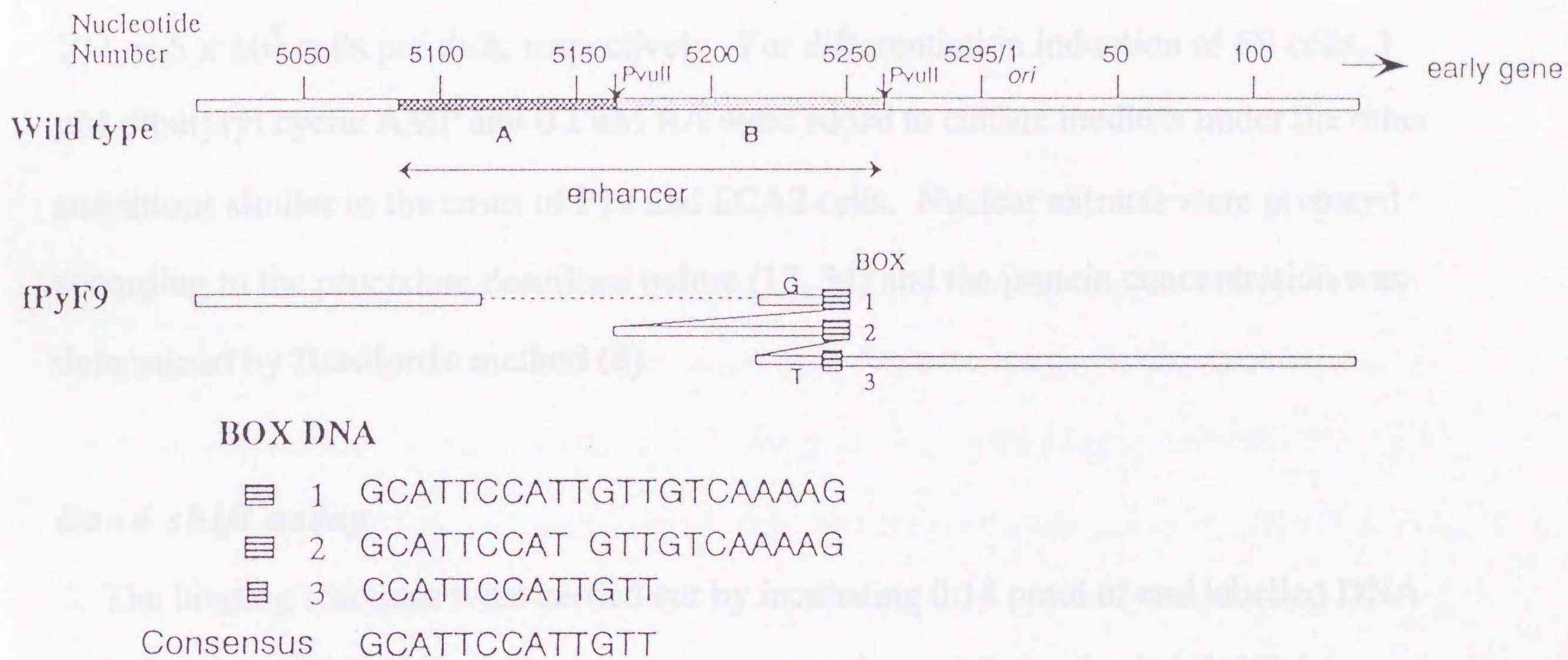


Fig.1 Enhancer regions of a wild type polyoma virus and fPyF9, a mutant polyoma virus

1.2 MATERIALS AND METHODS

Cell culture and preparation of nuclear extracts

Mouse L and Balb3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % calf serum. Mouse embryonal carcinoma cell lines P19, ECA2 and F9 were cultured in DMEM supplemented with 10 % fetal calf serum. P19 and ECA2 cells were induced to differentiate by the addition of 1 μM RA to the culture medium immediately after replating in a 100 or 150 mm dish at a density of 2 x 10^5 or 5 x 10^5 cells per dish, respectively. For differentiation induction of F9 cells, 1 μM dibutyryl cyclic AMP and 0.1 nM RA were added to culture medium under the other conditions similar to the cases of P19 and ECA2 cells. Nuclear extracts were prepared according to the procedure described before (13, 34) and the protein concentration was determined by Bradford's method (8).

Band shift assay

The binding reactions were carried out by incubating 0.14 pmol of end labelled DNA (5,000 cpm) with 7 - 15 μg of nuclear extract proteins and 2 μg of poly(dI-dC) in a buffer containing 15 mM Hepes (pH 7.9), 15 % glycerol, 2 % polyvinylalcohol, 36 mM NaCl and 0.4 mM DTT at room temperature for 10 min in a final volume of 15 μl. The reaction mixtures were then electrophoresed through a 4 % polyacrylamide (29:1) gel in 0.25 x TBE buffer [89 mM Tris-borate (pH 8.3), 25 mM EDTA] at 10 V/cm. For competition experiments, 2.8 or 14 pmol (10 or 200 fold amounts of labelled probe) of double stranded oligonucleotides were added to the reaction mixtures prior to addition of the nuclear extracts (15, 16).

Plasmid construction

pBLCAT2 is a plasmid carrying the CAT gene linked to the HSV TK promoter (37). pBLCAT2 was renamed as pTKCAT for transfection assays in the present study. The procedure of pBOXTKCAT and pMuBOXTKCAT construction was as follows: briefly, BOX and MuBOX oligonucleotides were synthesized according to the prototype

sequence inserted in fPyF9 and its mutant (see Fig.2A). The oligonucleotides were phosphorylated at the 5' ends with T4 polynucleotide kinase and cloned into the BamHI site in pUC19. The plasmids containing a few tandem repeats of the oligonucleotides were chosen. The SmaI site of pBLCAT2 was changed to HindIII site by linker ligation; the TKCAT fragment obtained by the digestion of the plasmid with HindIII and XbaI was cloned into the plasmids carrying a few BOX or MuBOX oligonucleotides. These plasmids were named p1BOXTKCAT, p3BOXTKCAT, p1MuBOXTKCAT and p2MuBOXTKCAT, respectively (Fig. 2B).

CAT assay

Two to 4 x10^5 of L, Balb3T3, or undifferentiated EC cells were plated on a 100 mm dish a day before transfection. Twenty μg of each CAT construct were transfected to the cells, together with 5 μg of the plasmid carrying the β-galactosidase gene linked to the cytomegarovirus (CMV) TK promoter, by the calcium phosphate method (20). The cells were harvested 24 or 48 hr after transfection. In the experiments of cell differentiation, 2 x 10^5 EC cells were plated on a 100 mm dish in the medium containing inducing reagents, which was renewed after 2 days. Next day (3 days after plating), the cells were transfected as above and were harvested 24 hr after transfection. Cell extracts were prepared as described previously (2) and assayed for β-galactosidase activity to normalize transfection efficiency. CAT assays were performed as described previously (18). The conversion of chloramphenicol to acetylated forms was measured using a densitometer PD-110 (Molecular Dynamic Lab.) or a bioimage analyzer BAS2000 (Fuji Film Co.) The same experiment was repeated at least 3 times and typical results were presented in the figures.

1.3 RESULTS

BOX DNA has enhancer activity specific for EC cells.

P19, ECA2 and F9 are EC cells widely used in the experiments to analyze undifferentiated stages of mouse embryogenesis. To examine the transcriptional activity of BOX DNA in EC cells, several TKCAT plasmids containing BOX or MuBOX oligonucleotides were constructed (Fig. 2A, B) and transfected to these cells. The HSV TK promoter was chosen in these constructs, because the TK promoter has been reported to be more susceptible to transcriptional regulating elements than the SV40 promoter in the octamer motif characterization (51). Five nucleotides within BOX DNA were substituted in the mutant oligonucleotide MuBOX (Fig. 2A). As shown in Fig. 2C, one copy of BOX DNA (1BOX) slightly stimulated the TK promoter activity. A strong activation of CAT expression was observed with the construct carrying three tandem repeats of BOX DNA (3BOX) in all the EC cells examined, although the stimulating efficiencies were different among the cells. Neither one nor two copies of MuBOX, on the other hand, did activate transcription in any of the cells. These results suggest BOX DNA functioned as a sequence-specific enhancer to the TK promoter in EC cells.

To investigate whether BOX DNA is an enhancer specific for EC cells or also active in other cells, the CAT assays were carried out using non-EC cells. The same CAT constructs as in Fig. 2 experiments were transfected to L and Balb3T3 cells, as well as pSV2CAT containing the SV40 enhancer/promoter region as a positive control. Both the wild type and the mutant BOX DNA scarcely activated transcription in either cell, whereas the SV40 enhancer/promoter functioned well (Fig. 3). BOX DNA is thus considered to be an enhancer which functions specifically in EC cells, but hardly in non-EC cells.

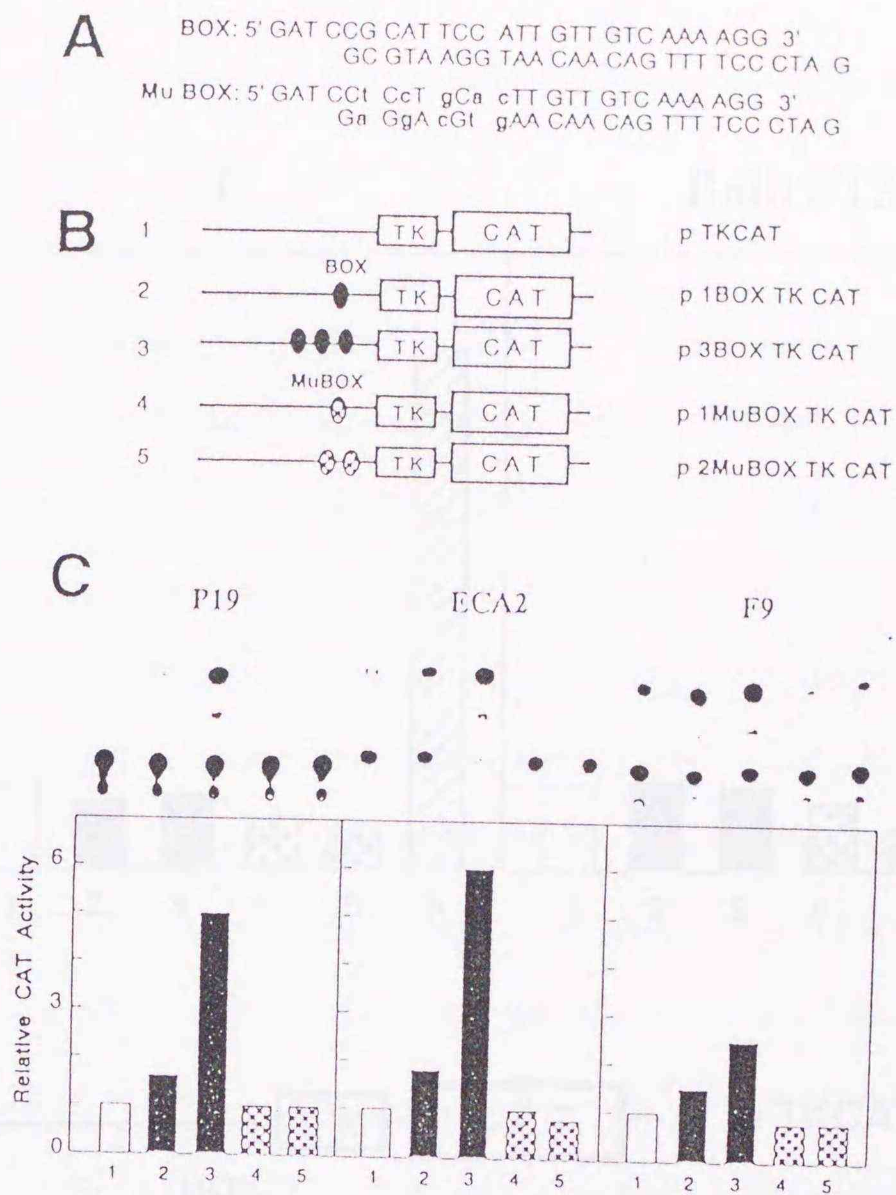
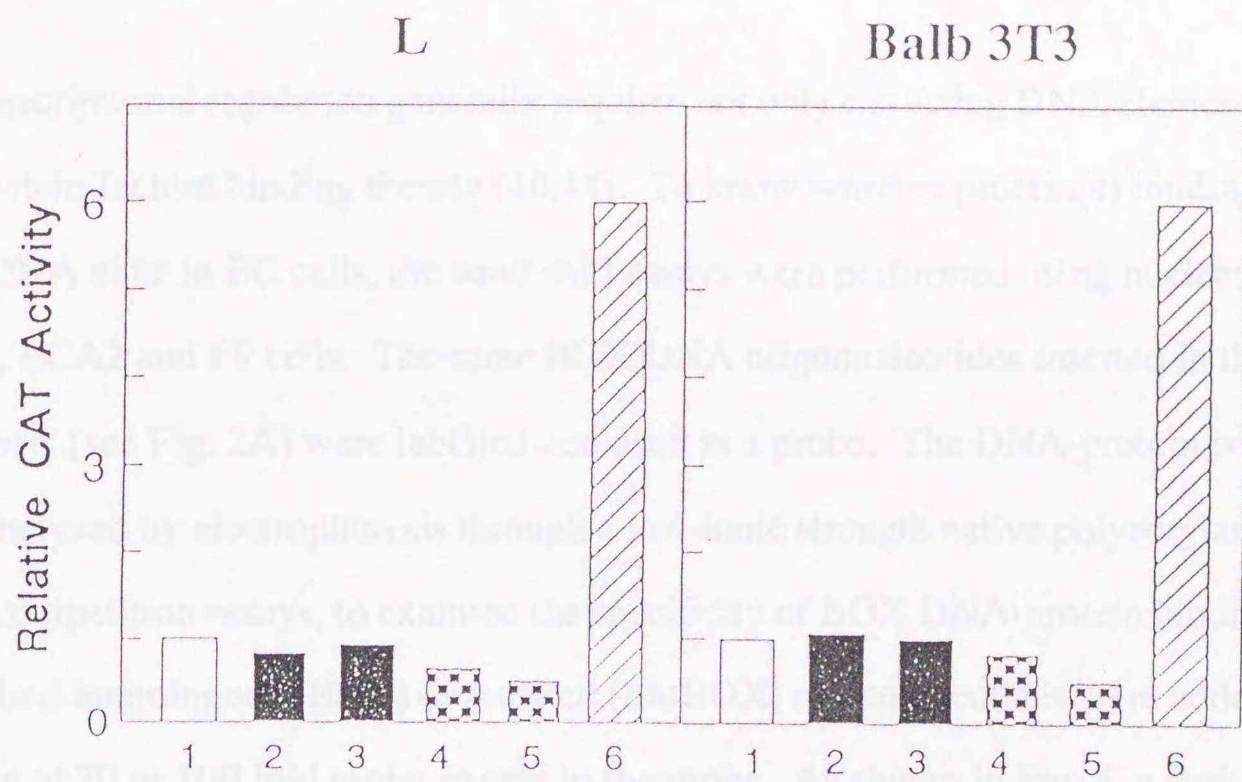
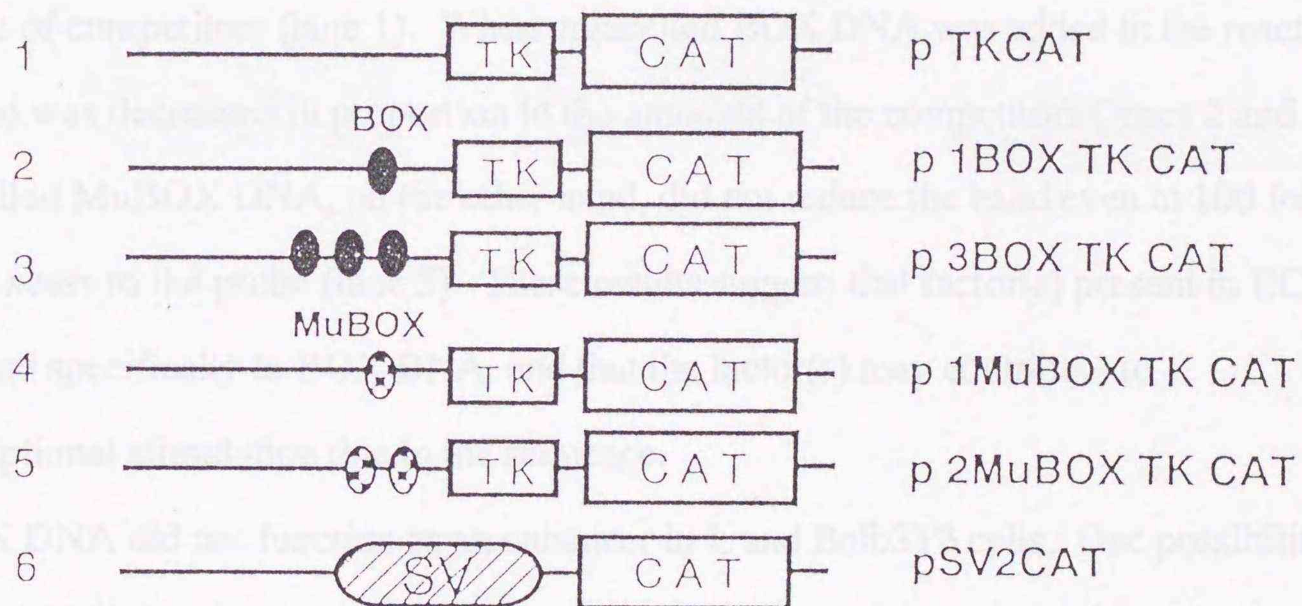


FIG. 2. Transcriptional activity of BOX DNA in various EC cells. (A) The nucleotide sequences of the BOX and MuBOX oligonucleotides are shown. Within MuBOX, 5 bases substituted for those in intact BOX are indicated in small letters. (B) The CAT constructs used are schematically indicated. p1BOXTKCAT and p3BOXTKCAT contain 1 or 3 copies of the BOX oligonucleotide inserted upstream of the HSV TK promoter followed by the CAT gene, respectively. p1MuBOXTKCAT and p2MuBOXTKCAT similarly carry 1 or 2 copies of the MuBOX oligonucleotide adjacent to the TKCAT sequences. (C) Several CAT constructs were cotransfected with pCMVß-galactosidase into P19, ECA2 and F9 cells. The cells were harvested 40 - 48 hr after transfection and CAT assays were performed. The same experiments were repeated at least 3 times, and typical data are shown. The autoradiographs of the CAT assays are displayed at the top. The column numbers (1 - 5) correspond to the plasmid numbers in (B). Relative CAT activities were calculated on the basis of pTKCAT; its activity was set as 1.





pFIG. 3. Transcriptional activity of BOX DNA in non-EC cells. The CAT assays were carried out using L and Balb3T3 cells transfected with various CAT constructs (1 - 6). Relative CAT activities were calculated as described in FIG. 2.

The BOX DNA-protein(s) complexes are different in EC and non-EC cells.

Transcriptional regulation generally requires not only *cis*-acting DNA element, but also protein factors binding thereto (40,44). To know whether protein(s) binding to BOX DNA exist in EC cells, the band shift assays were performed using nuclear extracts of P19, ECA2 and F9 cells. The same BOX DNA oligonucleotides inserted in the CAT constructs (see Fig. 2A) were labelled and used as a probe. The DNA-protein complexes were analyzed by electrophoresis through a low-ionic strength native polyacrylamide gel. In the competition assays, to examine the specificity of BOX DNA-protein binding, unlabelled homologous (BOX) or mutated (MuBOX) oligonucleotides were added to the reaction at 20 or 100 fold molar excess to the probe. As shown in Fig. 4, a major band of BOX DNA-protein(s) complexes was observed with all the EC cell extracts, in absence of competitors (lane 1). When unlabelled BOX DNA was added to the reaction, the band was decreased in proportion to the amounts of the competitors (lanes 2 and 3). Unlabelled MuBOX DNA, on the other hand, did not reduce the band even at 100 fold molar excess to the probe (lane 5). These results suggest that factor(s) present in EC cells bind specifically to BOX DNA, and that the factor(s) may contribute to transcriptional stimulation due to the sequence.

BOX DNA did not function as an enhancer in L and Balb3T3 cells. One possibility is that the BOX DNA binding protein(s) may be absent in these cells, while another is that they are present but in inactive forms. To examine these possibilities, band shift assays on BOX DNA using nuclear extracts from L and Balb3T3 cells were carried out. The same protein amounts of the extracts of L, Balb3T3 and EC cells were incubated with the probe in parallel and the patterns of the DNA-protein complexes were compared. The results after short run of gel electrophoresis are shown in Fig. 5A, and those after long run are in Fig. 5A', where the free probe ran out of the gel. Several complexes on BOX DNA were also observed in L and Balb3T3 cells. The mobilities of these complexes, however, were distinct from that of the EC-BOX DNA complex, and also different from

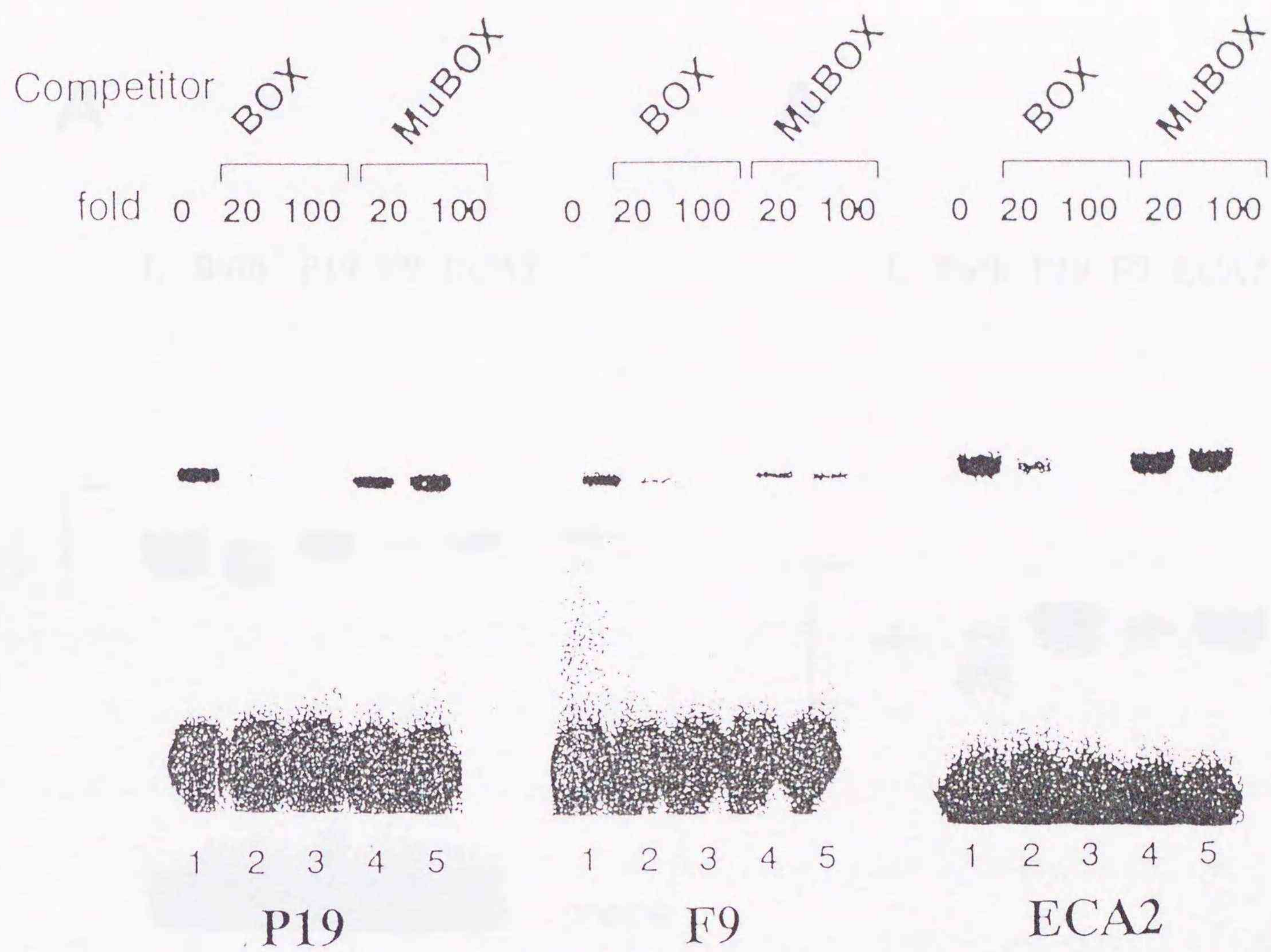


FIG. 4. The BOX DNA binding proteins in various EC cells. The band shift assays were performed using BOX DNA as a probe with nuclear extracts prepared from P19, F9 and ECA2 cells. Different amounts of unlabelled BOX DNA or MuBOX DNA were added to some reactions as competitors to assess specificity of binding. Lane 1, no competitors; lanes 2 and 3, with unlabelled BOX DNA at 20 or 100 fold molar excess to the probe, respectively; lanes 4 and 5, with unlabelled MuBOX DNA at 20 or 100 fold molar excess, respectively.

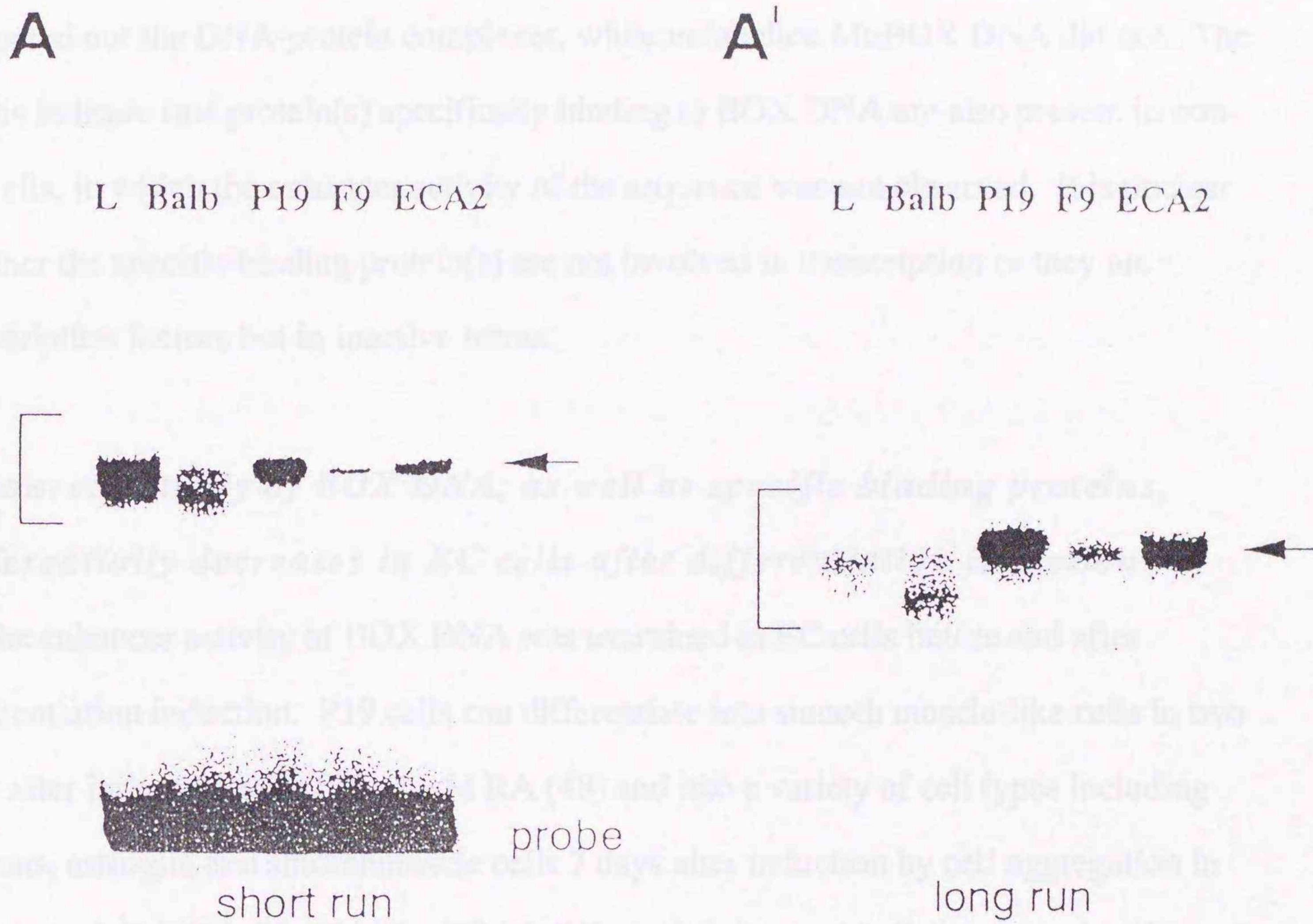


FIG. 5 Comparison of the BOX DNA binding proteins between EC and non-EC cells. The same protein amounts of nuclear extracts from L, Balb3T3, P19, F9 and ECA2 cells were reacted with the BOX DNA probe and separated in the same gel. The patterns after a short (A) and a long (A') run of electrophoresis are shown. The positions of complexes observed only in non-EC cells are indicated on the left, and an arrow on the right shows the band specific to EC cells. In A', the free probe had run out of the gel.

one another between L and Balb3T3 cells. The patterns of the DNA-protein complexes were thus different between EC and non-EC cells.

Further experiments using BOX or MuBOX DNA as a competitor were performed with L and Balb3T3 nuclear extracts (Fig. 6). Excess amounts of unlabelled BOX DNA competed out the DNA-protein complexes, while unlabelled MuBOX DNA did not. The results indicate that protein(s) specifically binding to BOX DNA are also present in non-EC cells, in which the enhancer activity of the sequence was not observed. It is unclear whether the specific binding protein(s) are not involved in transcription or they are transcription factors but in inactive forms.

Enhancer activity of BOX DNA, as well as specific binding proteins, preferentially decreases in EC cells after differentiation induction.

The enhancer activity of BOX DNA was examined in EC cells before and after differentiation induction. P19 cells can differentiate into smooth muscle-like cells in two days after induction with 0.5 - 1 μM RA (48) and into a variety of cell types including neurons, astroglia and smoothmuscle cells 7 days after induction by cell aggregation in presence of RA (24, 25, 42, 49). ECA2 differentiate into epithelial, neuronal and myoblast-like cells in 7 days after induction with 1 μM RA (56). F9 cells can differentiate into parietal endoderm-like cells at 5 days after induction with 0.1 μM RA and 1 μM dibutyryl cAMP. pTKCAT, p3BOXTKCAT, p2MuBOXTKCAT and pSV2CAT were transfected to these EC cells before and 3 days after differentiation induction. The cells were harvested 24 hr after transfection and CAT assays were performed. As shown in Fig. 7, the enhancer activity of BOX DNA, as compared with the SV40 enhancer/promoter, preferentially decreased after differentiation induction in all the cells examined (lanes 2 and 4). Mutated BOX DNA (MuBOX), which the BOX DNA binding protein(s) could not recognize in vitro, did not stimulate the TK promoter activity in either undifferentiated or differentiated cells (lane 3). Similar results were obtained when transfection was carried out with the EC cells 1 day after differentiation induction, and also with P19 cells induced by aggregation and 1 μM RA into alternative

differentiated state (data not shown). These results suggest that BOX DNA is a transcriptional regulatory element related to the differentiation states of EC cells.

It has been reported that the amounts of the factors binding to several transcriptional elements specific for undifferentiated EC cells fluctuate during differentiation. We therefore investigated the state of the BOX DNA-protein complexes during differentiation of P19, ECA2 and F9 cells. Nuclear extracts were prepared from the cells at 0, 2 and 4 days after differentiation induction and band shift assays were performed using BOX DNA or the Sp1 motif as a probe. The Sp1 motif was used as a control, since its binding protein is known to be expressed constitutively during differentiation (reviewed in 14). As shown in Fig. 8, the amounts of BOX DNA-protein complexes decreased during differentiation in all the EC cells tested, while Sp1 complexes were not affected by differentiation induction. It is thus considered that the BOX DNA binding protein(s) may be transcription factor(s) involved in the differentiation process of EC cells.

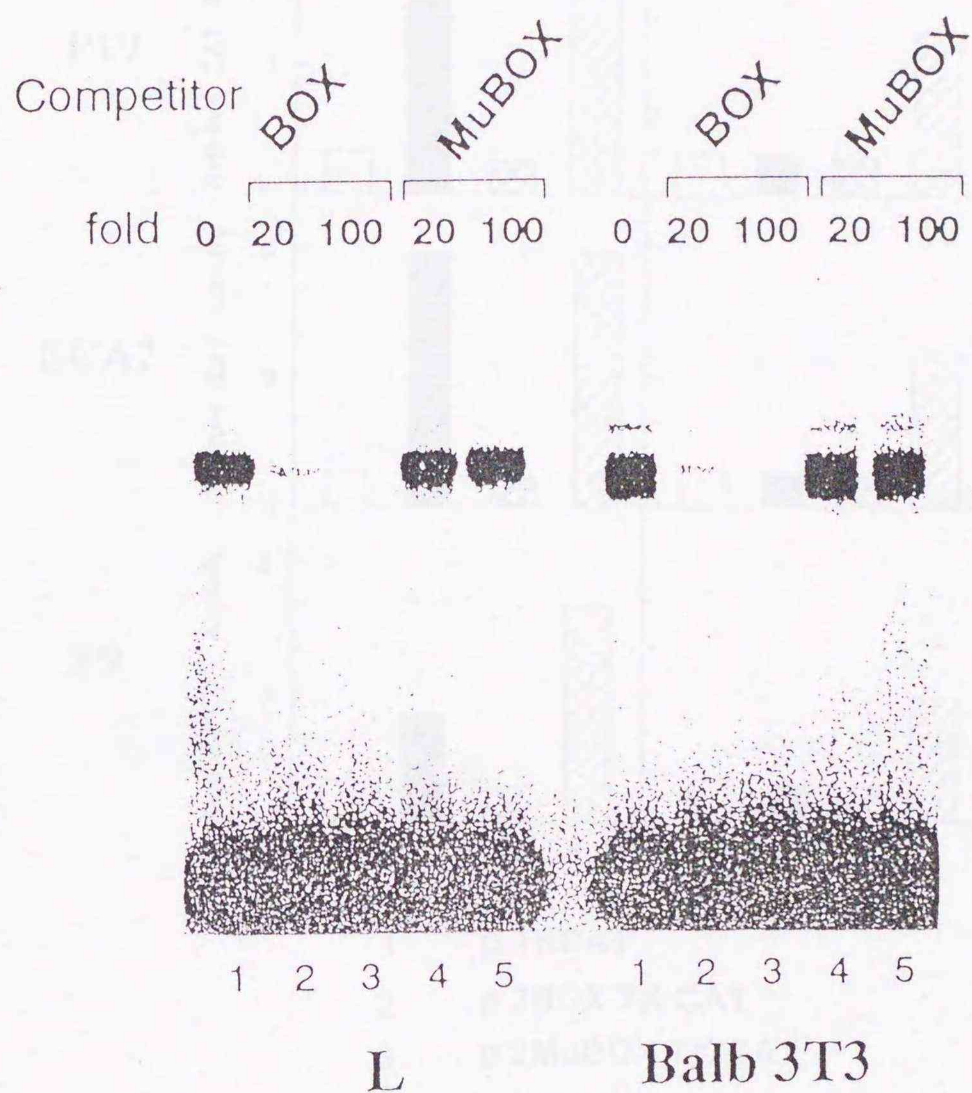


FIG. 6. The BOX DNA-protein complexes in non-EC cells. The band shift assays using the nuclear extracts prepared from L and Balb3T3 cells were carried out as described in FIG. 4.

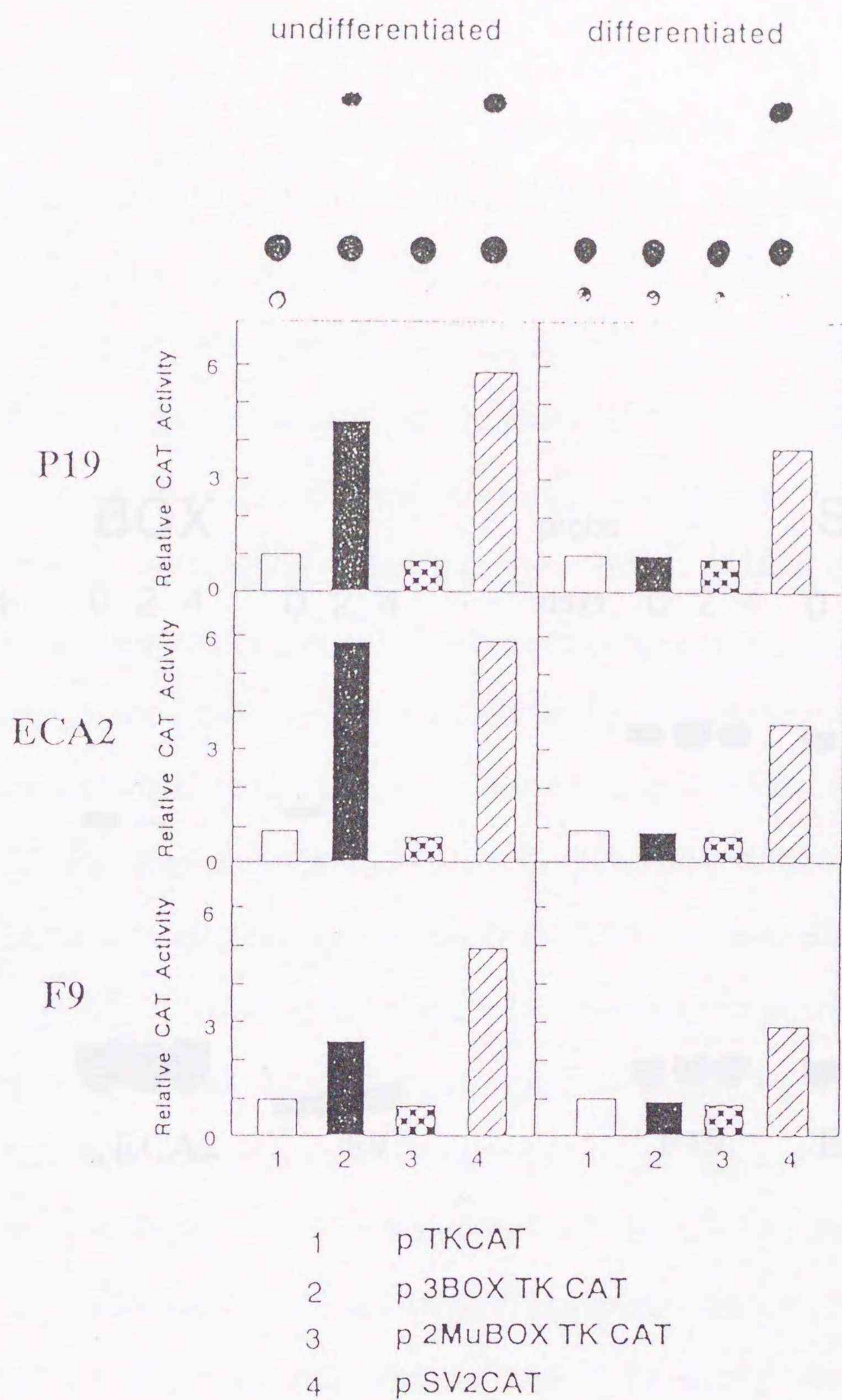


FIG. 7. Decrease of the enhancer activity of BOX DNA in differentiated EC cells. Several CAT constructs were transfected together with pCMVß-galactosidase to various EC cells before and after differentiation induction as described in 'MATERIALS AND METHODS'. Typical autoradiograms of the results for P19 cells are shown at the top. The CAT activities were calculated on the basis of pTKCAT in each state. Column 1, pTKCAT; column 2, p3BOXTKCAT; column 3, p2MuBOXTKCAT; 4, pSV2CAT (see Fig. 2, lower panel).

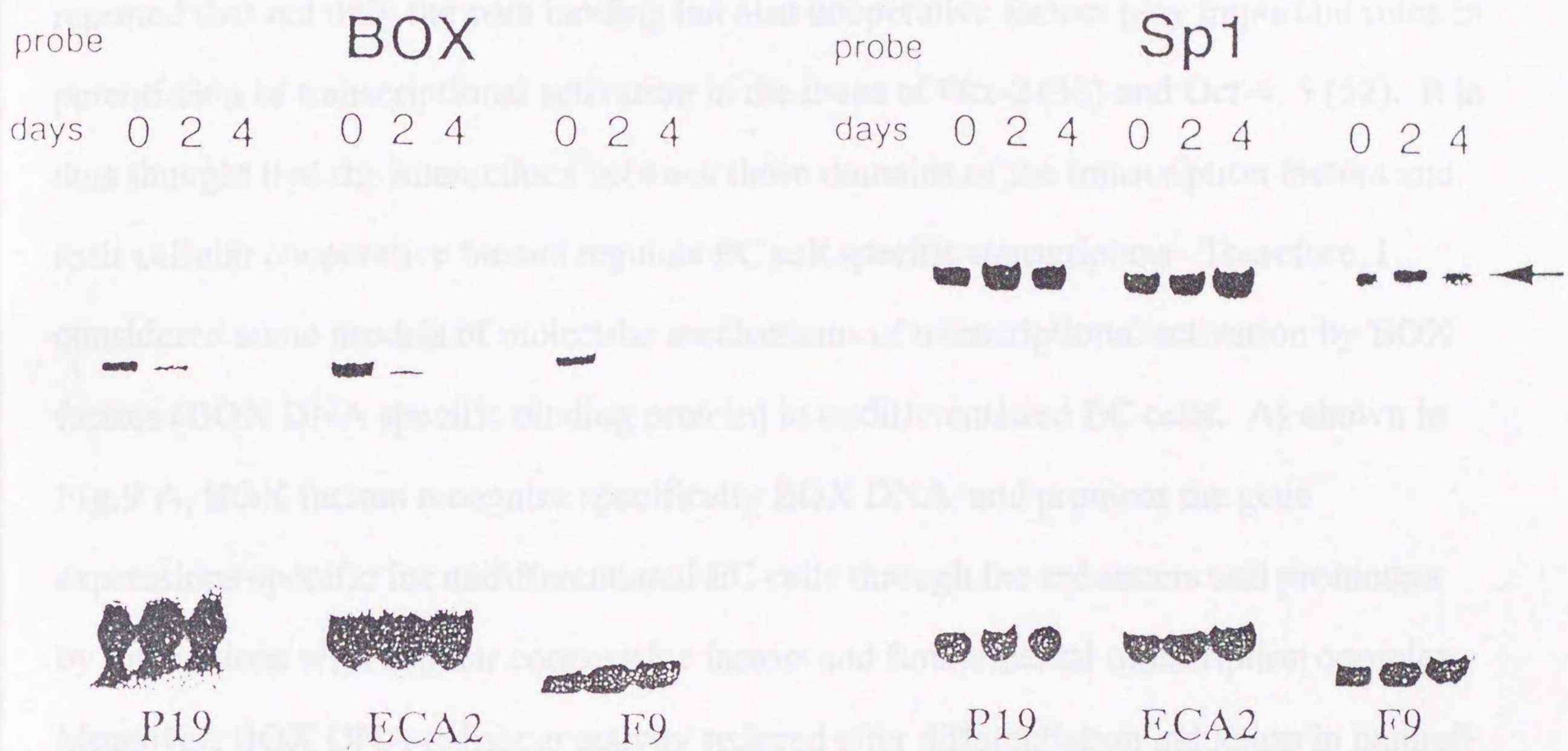


FIG. 8. Reduction of the BOX DNA-protein complexes during EC cell differentiation. Nuclear extracts were prepared from various EC cells before (0 day) and 2 or 4 days after differentiation induction. Equal protein amounts of each extract were reacted with labelled BOX DNA or Sp1 probe.

1.4 DISCUSSION

BOX DNA functioned as an enhancer specificaly in undifferentiated EC cells of different origins and distinct differentiation (P19, ECA2, and F9 cells). BOX DNA specific binding proteins, contributing to its enhancer activity, were present in all the EC cells examined. Oct-3, EC cell specific transcription factor, consists of DNA binding domains, cooperative interaction domains and transcriptional activation domains. It is reported that not only the core binding but also cooperative factors play important roles in potentiation of transcriptional activation in the cases of Oct-2 (38) and Oct-4, 5 (52). It is thus thought that the interactions between those domains of the transcription factors and their cellular cooperative factors regulate EC cell specific transcription Therefore, I considered some models of molecular mechanisms of transcriptional activation by BOX factors (BOX DNA specific binding protein) in undifferentiated EC cells. As shown in Fig.9 A, BOX factors recognize specifically BOX DNA and promote the gene expressions specific for undifferentiated EC cells through the enhancers and promoters by interactions with cellular cooperative factors and fundarmental transcription complex. Moreover, BOX DNA enhancer activity reduced after differentiation induction in pararell with the decrease of binding of BOX factors to BOX DNA. Thus, the models shown in Fig 9B are thought for the loss of BOX DNA enhancer activity after differentiation induction. a) It is known that the activities of transcription factors are generally regulated by various modifications (phosphorylation, dephosphorylation, et al.)(17). BOX factors may be inactivated by some modifications or the activities may be supressed by reduce of any modifications activating BOX factors, although BOX factors express almost equally to that of undifferentiated cells. b) It is reported that the expression of Oct-3, EC cell specific transcription factor, decreases after differentiation induction. The expression of BOX factors may also decrease and it may result in decrease of binding of BOX factors to BOX DNA and the loss of enhancer activity. c) Cooperative factors interacting with BOX factors may be inactivated or decrease.

Interestingly, BOX DNA specific binding proteins were also present in L and Balb 3T3 cells, where BOX DNA did not activate transcription. In band shift assays, the

mobility of the BOX DNA-protein complexes (non- EC BOX factors) in these non-EC cells were different from those in EC cells. These results suggest the cooperative/transcriptional transactivation domains of non-EC cells will be nonfunctional /different from those of EC cells although their DNA binding domains are functional. Thus, several possibilities are thougt for the inactivity of BOX as an enhancer in non-EC cells (Fig.10). a) Cooperative factor(s) required for displaying the activity may be absent although BOX factors are active forms. b) Cooperative domains of non- EC BOX factors may be different from active EC-BOX factors and so they cannot interact with the cellular cooperative factors. c) Non-EC BOX factors may be inactivated by some inhibitory factors or the modifying enzymes activating them may be lacking. d) Affinity of transcriptional transactivation domain of non-EC cells may be different from those of EC cells. et al. To clarify the molecular mechanisms of positive and repressive transcriptional regulation in undifferentiated EC, differentiated EC, and non-EC cells, identification of BOX factors, cooperative factors, and other cellular factors should be required.

I first carried out Southwestern analyses to identify the BOX factors. However, I could not recognize the bands due to the specific protein(s) bound to BOX DNA. Possibly, the BOX factors cannot be renatured during the transfer process from the SDS-polyacrylamide gel to the nitrocellulose filter. To analyze the properties and functions of BOX DNA, purification of BOX factors followed by cloning of the cDNA encoding them should be performed.

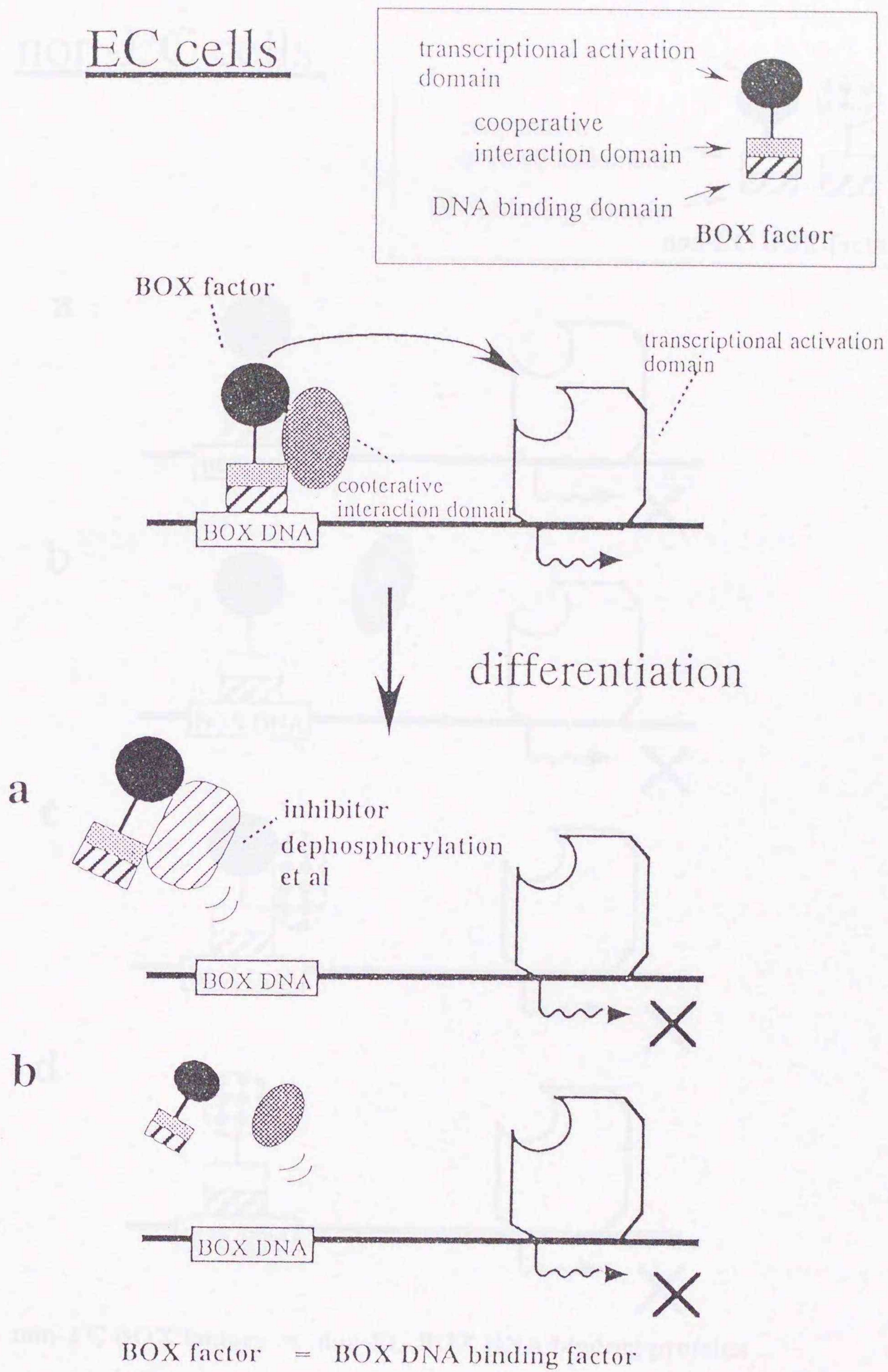
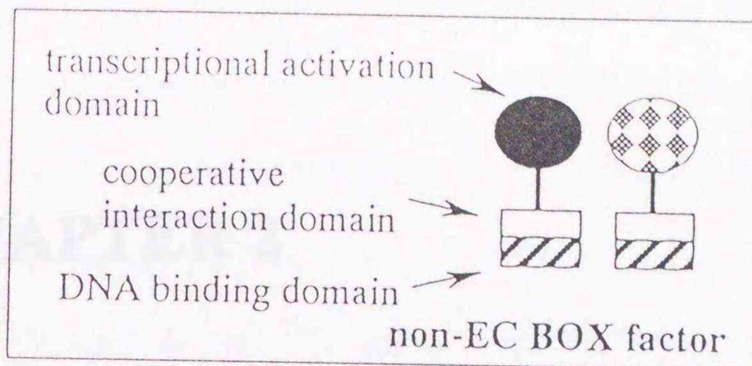
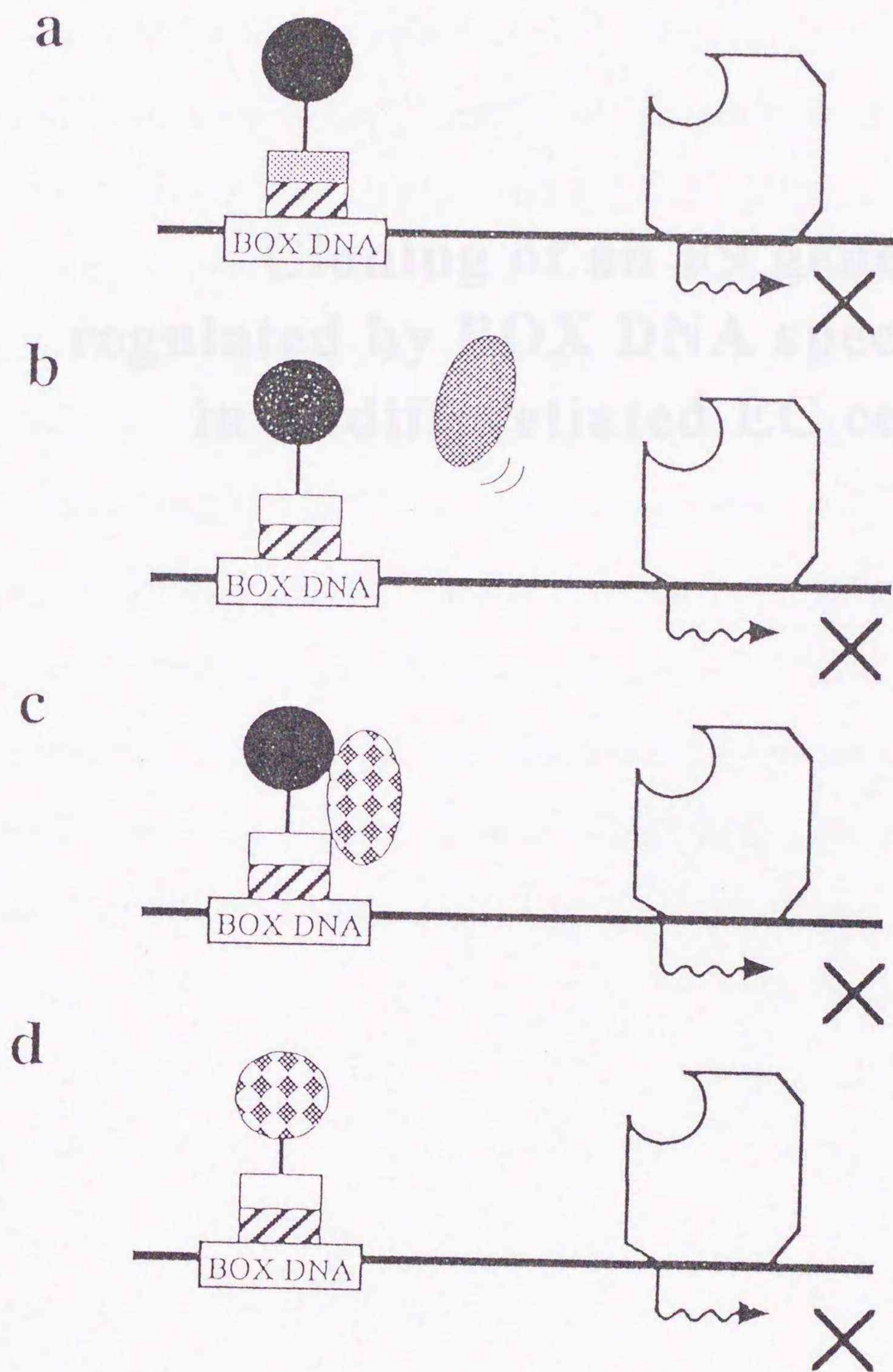


Fig.9 Models for molecular mechanisms of the transcriptional activation and inactivation of BOX DNA in EC cells before and after differentiation induction.



non-EC cells



non-EC BOX factors = non-EC BOX DNA binding proteins

Fig.10 Models for molecular mechanisms of the transcriptional repression of BOX DNA in non-EC cells

CHAPTER 2

Cloning of an F9 gene regulated by BOX DNA specifically in undifferetiated EC cells.

2.1 INTRTODUNTION

BOX DNA was found as the insertion sequence into enhancer B domain of fPyF9, a polyoma virus mutant. It has been unknown where BOX was derived from, although it was speculated to be of a cellular DNA. Molonyleukemia virus LTR (56) , PEA3 (61) , and E2A (6, 32) are EC specific enhancer/silencer motifs derived from virus. A number of studies concerning for these motifs have been performed and each transcription factor binding to the motif has been clarified. Nevertheless, the cellular target genes of those transcription factors have not been identified yet. To understand functions of a transcription factor in vivo, its cellular target genes should be clarified. To know the cellular target genes of BOX factors, I thus carried out cloning of an F9 genomic DNA containing the BOX DNA sequences speculated to be a regulatory region of its target gene. BOXF1, one of the cloned DNA had transcriptional promoter activity and a BOX DNA sequence in BOXF1 was a regulatory element dominant to the promoter in undifferentiated EC cells, but less effective in differentiated EC cells. Furthermore, I cloned BOXF1 cDNA, a gene coded downstream from BOXF1. Its RNA expressed well in undifferentiated EC cells and decreased after differentiation. These results suggest that BOXF1 is a cellular target gene of BOX factors and it may be a gene involved in EC cell differentiation .

2.2 MATERIAL AND METHOD

Cloning of F9 genomic DNAs containing BOXDNA sequences

Genomic DNA was extracted from F9 cells as described (41) and digested with HindIII. A half μg of the genomic DNA was amplified by PCR with Taq DNA polymerase (Perkin-Elmer-Cetus) using 0.5 μg of HindIII linker and 100 pmol of BOXDNA oligonucleotide 1 or 2 as primers. The template DNA was denatured at 94°C for 1 min and annealed with the primers at 55°C for 2 min. The extension reaction was carried out in a buffer containing 10 mM Tris -HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (W/V) gelatin, and 125 μM of the four dNTPs at 72°C for 3 min. These reactions were repeated for 40 cycles in a termal cycler (Perkin-Elmer-Cetus). The amplified DNAs were purified and separated in a 1.2% agarose gel.

PCR amplified DNAs were treated with Klenow fragment (DNA pol I) and cloned into HincII/HindIII site in pUC19, after phosphorylation followed by digestion with HindIII. Clones thus obtained were named pBOXF1, pBOXF5 and pBOXF6, respectively. Presence of BOXDNA in these clones was confirmed by nucleotide sequencing. To study the promoter activity, the inserts were linked to the CAT gene in both orientations, and the constructs were designated pF1(B)CAT, pF1(H)CAT, pF5(B)CAT, pF5(H)CAT, pF16(B)CAT, and pF16(H)CAT (see Fig.3). The deletion mutants of pF1(H)CAT were constructed as follows: pF1(H)CAT was digested with KpnI and SacI, followingly treated with exonuclease III and mung bean nuclease, and then with Klenow fragment (DNA pol I). Self-ligation of the fragments gave rise to pF1delBCAT and pF1delBSCAT (see Fig.5). pF1E1ECAT is a pUC derivative containing the 100 bp fragment obtained by EcoRI digestion of pBOXF1 (Fig.3), cloned into the EcoRI site, and the CAT gene inserted into the HindIII site (Fig.5). The other EcoRI fragment of 2900 bp was self-ligated, resulting in pF1E2HCAT (Fig.5).

Preparation of cytoplasmic RNA

F9 or P19 cells were washed twice with TBS, suspended with the lysis buffer (10 mM Tris-HCl buffer (pH 8.6) containing 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40 and

10 mM vanadryl ribonucleotide complexes), and kept on ice for 5 min. The cell lysate was centrifuged, and 40 μl of 10 mg/ml proteinase K and 10 μl of 10 % SDS were added to the supernatant and incubated at 60 °C for 5 min. After three times of phenol extraction, cytoplasmic RNA was precipitated by ethanol, dissolved in water, and used for S1 mapping or Northern blot hybridization .

S1 mapping

pF1BOX was digested with HindIII, dephosphorylated, and end-labelled with ^{32}P-γ-ATP. After digestion with SpeI, the 322bp fragment was purified and used as a probe for S1 mapping. Fifty-six μg of the cytoplasmic RNA and 10^4 cpm of the probe were hybridized at 41 °C for more than 12 hrs in a buffer containing 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, 400 mM NaCl, and treated with S1 nuclease, and analyzed in a 10% polyacrylamide gel containing 8 M urea.

Cloning of BOXF1 cDNA from F9 λ zap II cDNA library

Phage DNA was extracted from F9 λ zap II cDNA library as described in (62). A few hundred μg of Phage DNA was amplified by PCR with Taq DNA polymerase using 50 ng of T3 primer and T7 primer as primers. The extension reactions were likewise described as materials and methods in chapter 1. The PCR amplified DNAs were cloned using of TA cloning kit (*Invitrogen*). They were screened by southern hybridization. F1RNA oligonucleotide, a homologous sequence of 30 bp upstream of 3'end was used as a probe in screening

2.3 Results

An F9 genomic DNA containing BOX DNA sequence has transcriptional activity, which is regulated by BOX DNA specifically in undifferentiated EC cells.

BOX DNA was first found as the insertion sequence in a polyomavirus mutant (2). It has been unknown where BOX DNA was derived from, although it was speculated to be of a cellular DNA. To identify target genes of BOX DNA binding protein(s), we performed cloning of F9 genomic DNA containing the BOX DNA sequence and examined whether transcription of the genes is regulated by BOX DNA. The cloning was carried out by the PCR method described in Fig. 1. Briefly, total DNA from F9 cells was digested with HindIII and the fragments were subjected to PCR amplification using BOX DNA and HindIII linker as primers. The amplified DNAs were analyzed by agarose gel electrophoresis (Fig. 2). Several bands were observed in the reactions with both the combinations of primers (BOX DNA 1 / HindIII linker, and BOX DNA 2 / HindIII linker). Southern hybridization using BOX DNA as a probe revealed that all the amplified DNA fragments contained the BOX DNA sequence (data not shown). The results suggest that BOX DNA sequences exist at several copies in F9 genomic DNA.

The DNA fragments thus amplified were cloned into pUC19. Three clones described in Fig. 3, upper left panel were obtained (pBOXF1, pBOXF5 and pBOXF16). Sequence analyses revealed that one or two copies of BOX DNA were present in each clone. The results of southern hybridization of the F9 genomic DNA with the fragments inserted in the three clones indicate that the fragments, respectively, exist in F9 cells at one copy per haploid genome (data not shown).

BOXF1, BOXF5 and BOXF16, the F9-derived fragments inserted in pBOXF1, pBOXF5 and pBOXF16, respectively, were linked to the CAT gene in either orientation and examined for transcriptional activity (Fig.3, upper right panel). pUCCAT, carrying the enhancer/promoter-less CAT gene alone, was used as a negative control. The constructs were transfected into undifferentiated F9 cells and CAT assay was carried out. As shown in Fig.3, lower panel, only pF1(H)CAT, in which the HindIII end of BOXF1

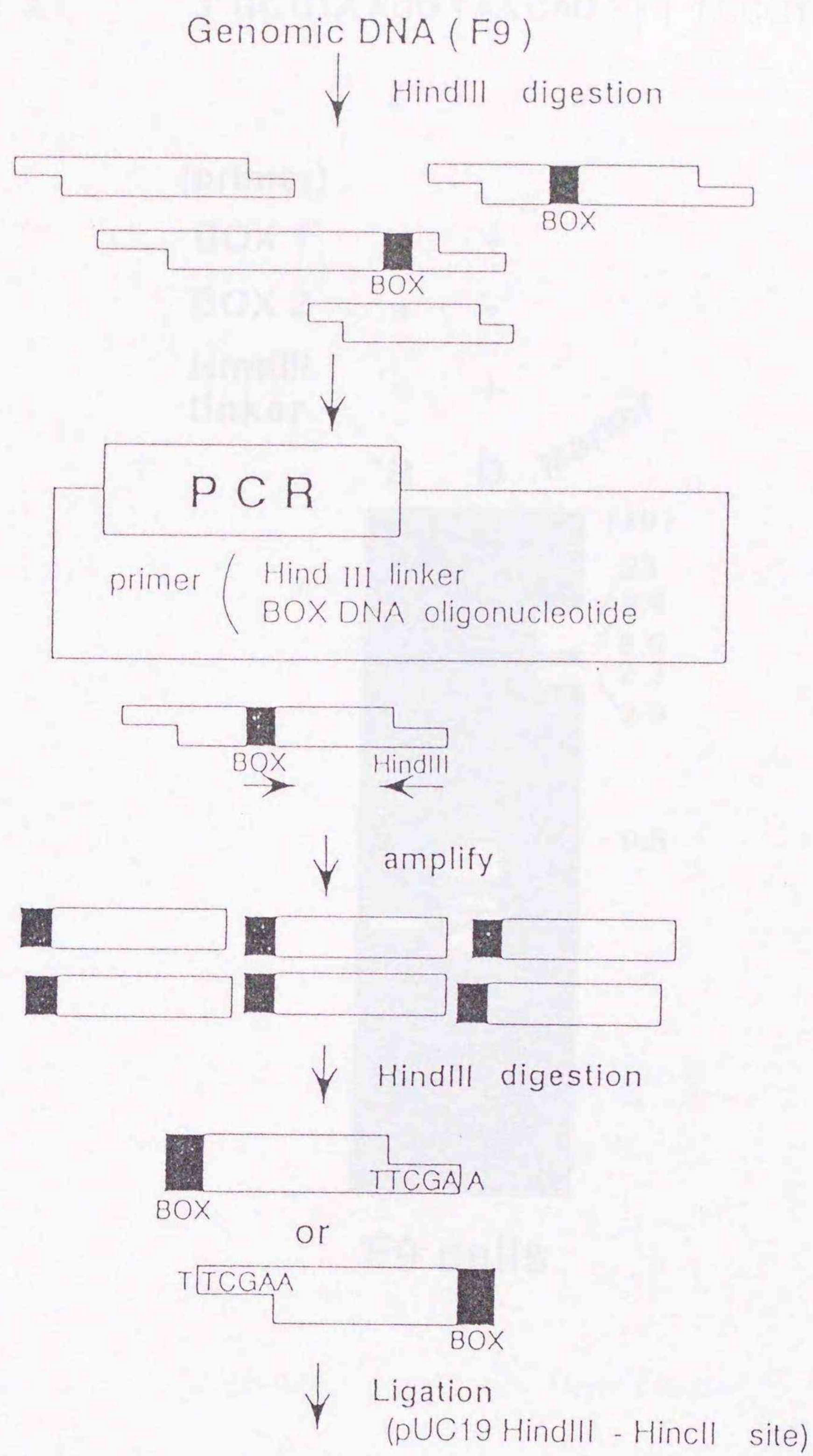
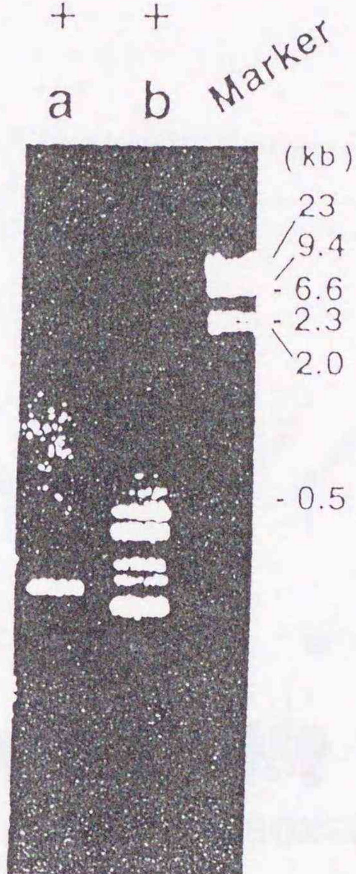


FIG. 1 Cloning of F9 genomic DNA containing the BOX DNA sequence. The PCR-mediated procedure of cloning genomic DNA containing BOX DNA in F9 cells are schematically shown.

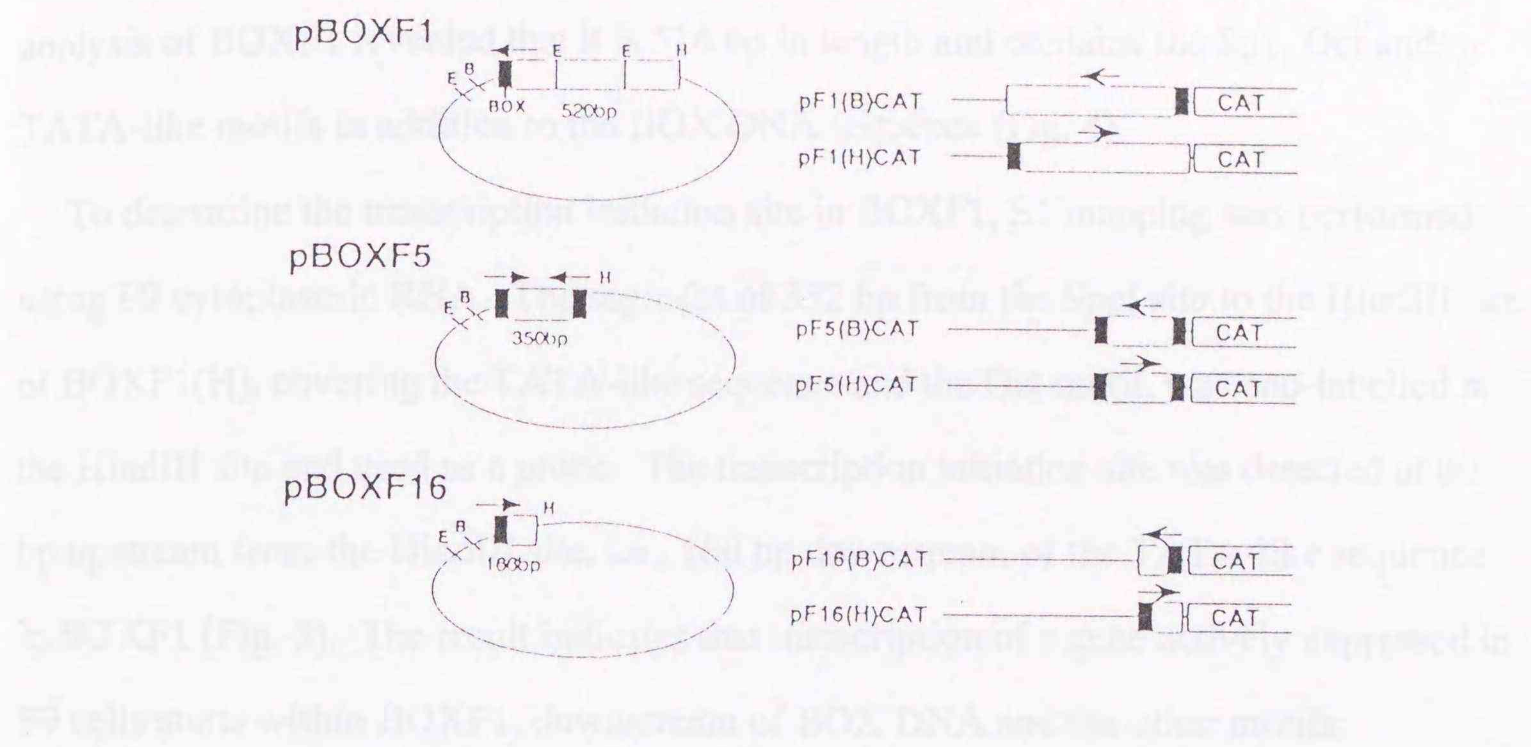
BOX 1 : 5' GAT CCG CAT TCC ATT GTC AAA AGG 3'
BOX 2 : 3' GC GTA AGG TAA CAG TTT TCCCTA5'

(primer)		
BOX 1	-	+
BOX 2	+	-
HindIII linker	+	+



F9 cells

FIG. 2. Detection of genomic DNAs containing the BOX DNA sequence in F9 cells. DNA fragments containing the BOX DNA sequence were amplified among the HindIII-digested F9 genomic DNAs by PCR using either pair of BOX 1/HindIII linkers or BOX 2/HindIII linkers as primers. The amplified DNAs were analyzed by agarose gel electrophoresis. Marker indicates HindIII-digested λ phage DNA, and the sizes (kb) are shown on the right.



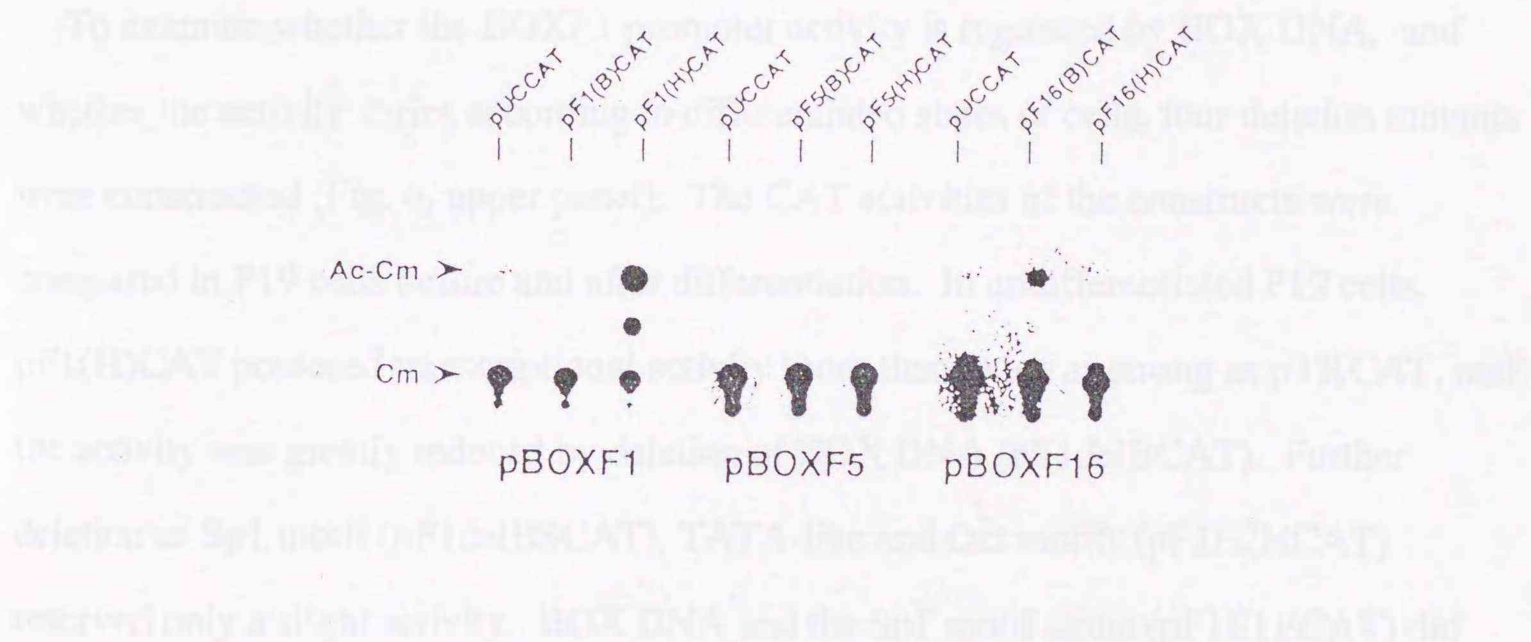


FIG. 3. Promoter activities of the F9 DNA fragments containing BOX DNA. The fragments of F9 genomic DNA containing the BOX DNA sequence (BOXF1, BOXF5, and BOXF16) were linked to the CAT gene in either orientation (pF1(B)CAT, pF(1H)CAT, pF5(B)CAT, pF5(H)CAT, pF16(B)CAT, and pF16(H)CAT). The constructs were transfected into F9 cells and CAT assays were carried out. Upper panel represents the schematic drawing of the pUC19 clones of the F9 DNA fragments containing BOX DNA (left) and of the CAT constructs (right). B, BamHI; E, EcoRI; H, HindIII. Lower panel shows the autoradiographs of the CAT assays. Cm, chloramphenicol; Ac-Cm, acethylated chloramphenicol.

is adjacent to the CAT gene, showed the transcriptional activity. It was hence suggested that a promoter is located downstream of BOX DNA in the BOXF1 fragment. Sequences analysis of BOXF1 revealed that it is 516 bp in length and contains the Sp1, Oct and TATA-like motifs in addition to the BOX DNA sequence (Fig. 4).

To determine the transcription initiation site in BOXF1, S1 mapping was performed using F9 cytoplasmic RNA. The segment of 332 bp from the SpeI site to the HindIII site of BOXF1(H), covering the TATA-like sequence and the Oct motif, was end-labelled at the HindIII site and used as a probe. The transcription initiation site was detected at 80 bp upstream from the HindIII site, i.e., 180 bp downstream of the TATA-like sequence in BOXF1 (Fig. 5). The result indicates that transcription of a gene actively expressed in F9 cells starts within BOXF1, downstream of BOX DNA and the other motifs.

To examine whether the BOXF1 promoter activity is regulated by BOX DNA, and whether the activity varies according to differentiated states of cells, four deletion mutants were constructed (Fig. 6, upper panel). The CAT activities of the constructs were compared in P19 cells before and after differentiation. In undifferentiated P19 cells, pF1(H)CAT possesed transcriptional activity more than twice as strong as pTKCAT, and the activity was greatly reduced by deletion of BOX DNA (pF1delBCAT). Further deletion of Sp1 motif (pF1delBSCAT), TATA-like and Oct motifs (pF1E2HCAT) reserved only a slight activity. BOX DNA and the Sp1 motif alone (pF1E1ECAT) did not yield a significant activity, compared with pUCCAT. A promoter required for a basal expression is thereby suggested to exist downstream of the Oct motif. In differentiated P19 cells, pF1(H)CAT, as well as the four deletion mutants showed a transcriptional activity much weaker than pTKCAT, and the activity was little affected by deleting BOX DNA (compare between columns 1 and 2). The results thus suggest that BOX DNA is a dominant regulatory element of an enhancer/promoter located in the BOXF1 fragment in undifferentiated cells, but less effective in differentiated cells. The gene coded downstream from BOXF1 may be one of the target genes of BOX factors.

BOXF1 Sequence

```
1   GATCCCTTTT GACAACAATG GAATGCGTGG ATAAGTGTGT
        BOX DNA
41  GAGGGCTGGG GCGGGACATG GACCAGACCA ACTCTCCACT
           Sp1-like
81  GAATTCCAAG TTCAGTAAGA CTCTGACTTA AGAAATAGAT
    EcoRI
121 GGAGAGCAAT CCAAGAGAGA CACACTTGGT TGAACTTGGG
161 CCTCCACACA TACAACATAC TAGTGTATGA GAACATGCAC
                           SpeI
201 ACACATGTGT GCACTGTACA GTAACTCAAT AACAATATCT
241 TTAATATAAA GCTAAACGGA AACAGATTAG GCTACATTTA
      TATA-like
281 GTATTTATCT GCTCCAAATT TGCAAATGAA TTCTATCAAG
                         Oct       EcoRI
321 ACTTCACTAA AACTCCTTTG TACTTAAACT AATGTTTTCT
361 TAAGAAAATG TGAACATTTT TTTGAGTTTC CATTGTCTTG
401 TCATTTTAGG AGTGCCAAAG TCGAATCTTT TACACACCAA
441 ATCAGTGAGA GGCCATAAAG ACTGCTTTGA AAAATACCAT
481 TTAATAGCAA ACCAGGATTG TTCTCGTTCC AAGCTT
                                     HindIII
```

FIG. 4. Nucleotide sequence of BOXF1. BOXF1 consists of 511 bp nucleotides. The BOX DNA sequence is boxed. Bold or plain underlines indicate the transcriptional regulatory motifs (Sp1-like, TATA-like, and Oct motifs) or the restriction sites, respectively. A dotted underline and an arrow show the initiation site and the direction of transcription deduced by S1 mapping (FIG. 4).

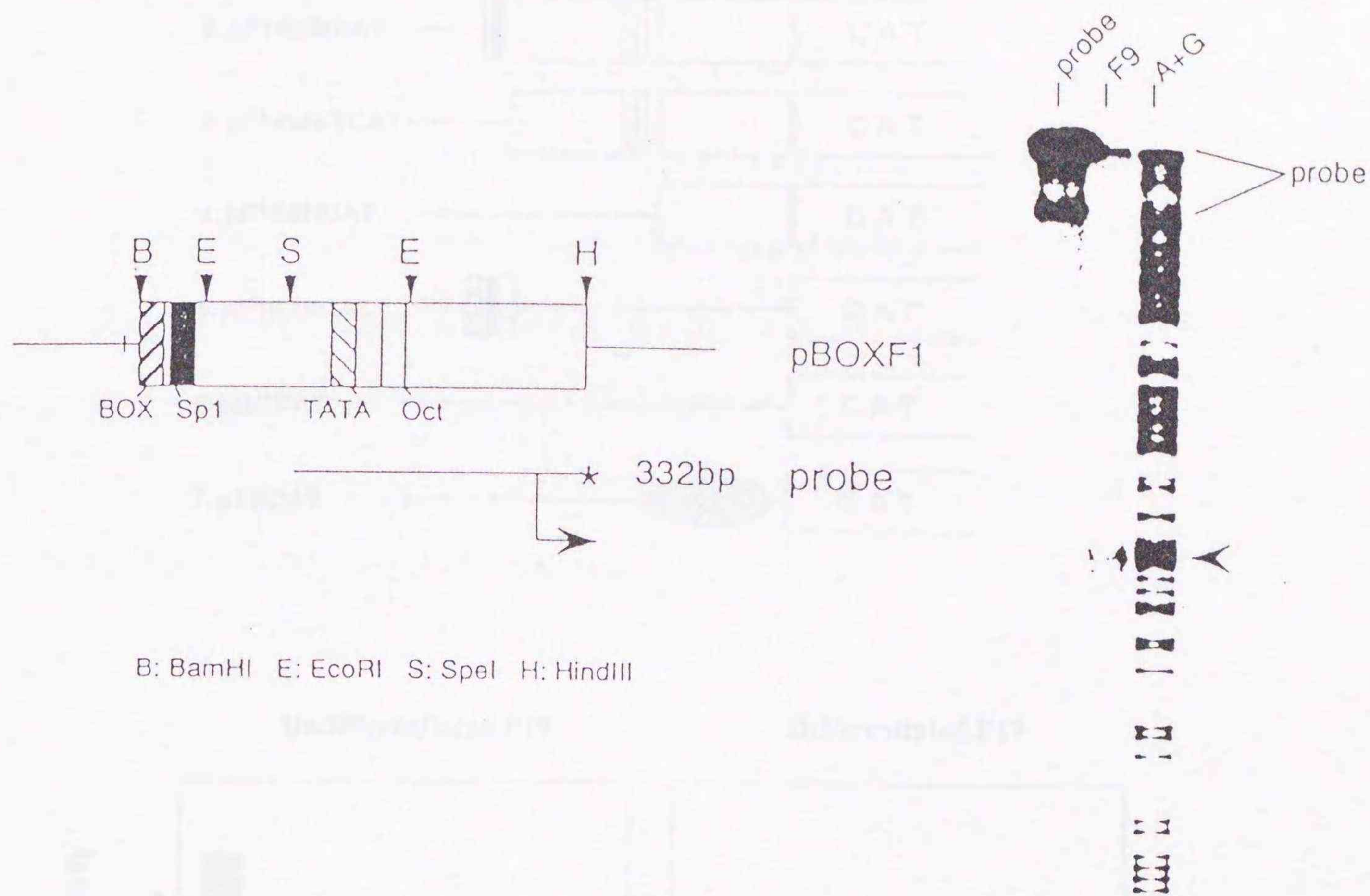
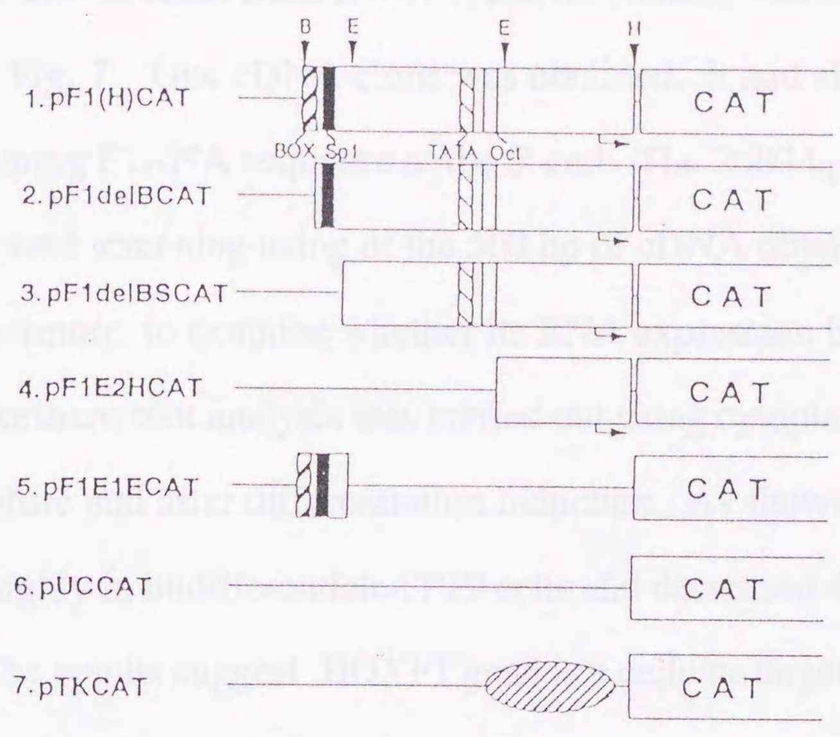


FIG. 5. Determination of the transcription initiation site in BOXF1 by S1 mapping analysis. Left panel: The structure of BOXF1 is schematically drawn. Putative sequences for transcriptional regulation, including BOX DNA, are shown. Triangles above indicate recognition sites for restriction enzymes. The SpeI-HindIII fragment of 332 bp (underlined) was labelled at the HindIII site (asterisked) and used as a probe. Right panel: The result of S1 mapping is shown. Cytoplasmic RNAs from F9 cells were subjected to the mapping as described in 'MATERIALS AND METHODS'. Lanes 'probe', 'F9' and 'A+G' contained the probe alone (without RNA), F9 RNA and the probe, or Maxam-Gilbert A+G reaction of the probe, respectively. 'Probe' and an arrow on the right indicate the positions of the input probe and the S1-protected band.



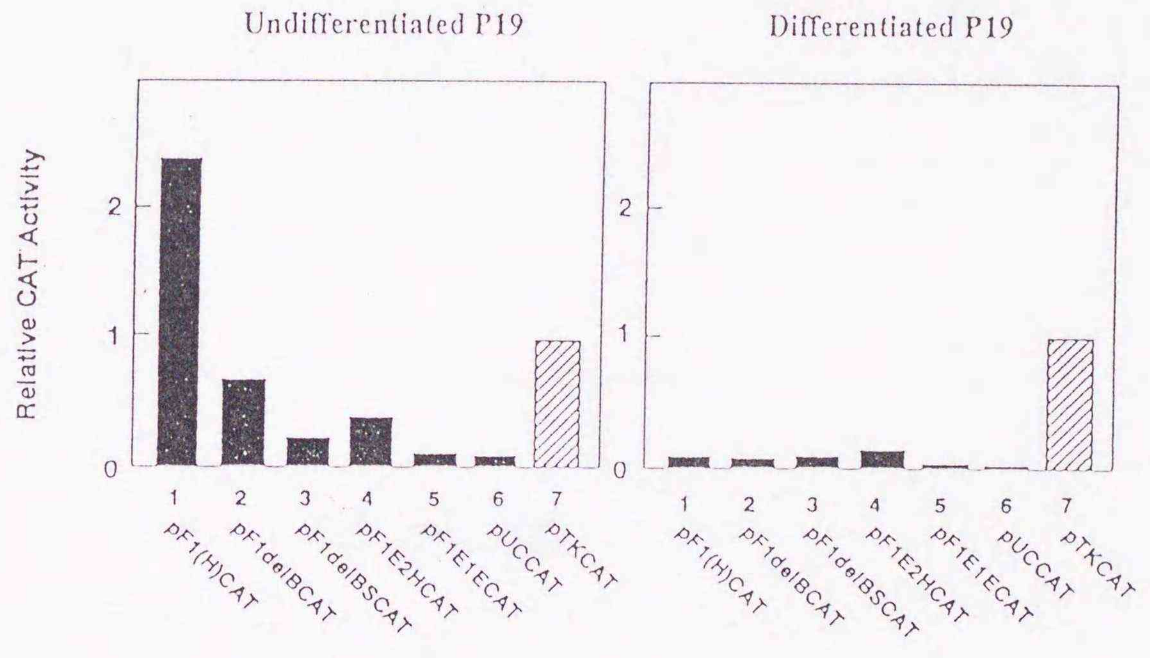
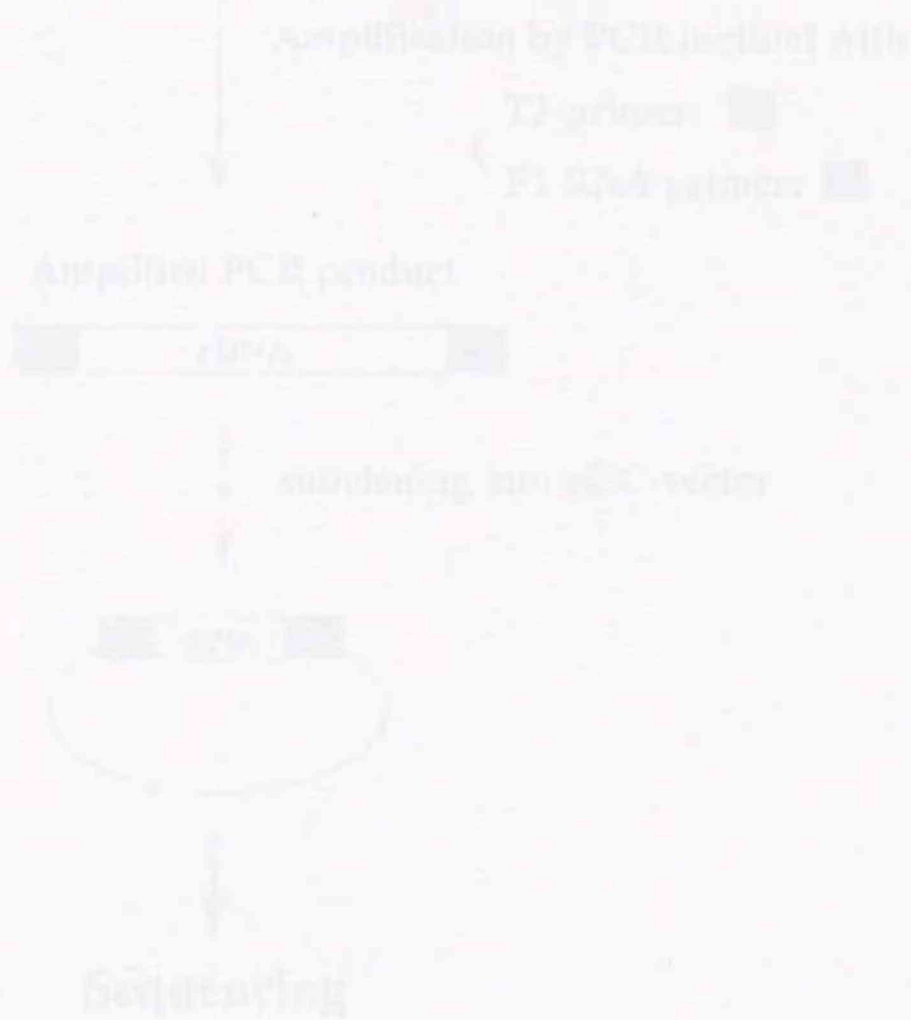


FIG. 6. Effect of BOX DNA on the BOXF1 promoter in EC cells before and after differentiation. Various deletion mutants of pF1(H)CAT (schematically shown in upper panel) were transfected into undifferentiated or differentiated P19 cells (at 2 days after differentiation induction), and CAT assays were performed as in Fig. 1 of chapter 1. Relative CAT activities standardized by that of pTKCAT are shown in lower panel.

Cloning of BOXF1 cDNA

To know a gene downstream from BOXF1, cDNA cloning was performed by the PCR method shown in Fig. 7 One cDNA clone was obtained. It had about 500 bp nucleotides containing F1RNA sequence at the 5' end. The 2600 bp of complete cDNA was cloned by second screening using of the 500 bp of cDNA obtained in first screening as a probe. Furthermore, to examine whether its RNA expression is influenced by differentiation, Northern blot analysis was carried out using cytoplasmic RNAs prepared from P19 cells before and after differentiation induction. As shown in Fig.8, BOXF1 RNA expressed highly in undifferentiated P19 cells and decreased 4 days after differentiation. The results suggest BOXF1 gene is a cellular target gene of BOX factors and will be involved in EC cell differentiation.



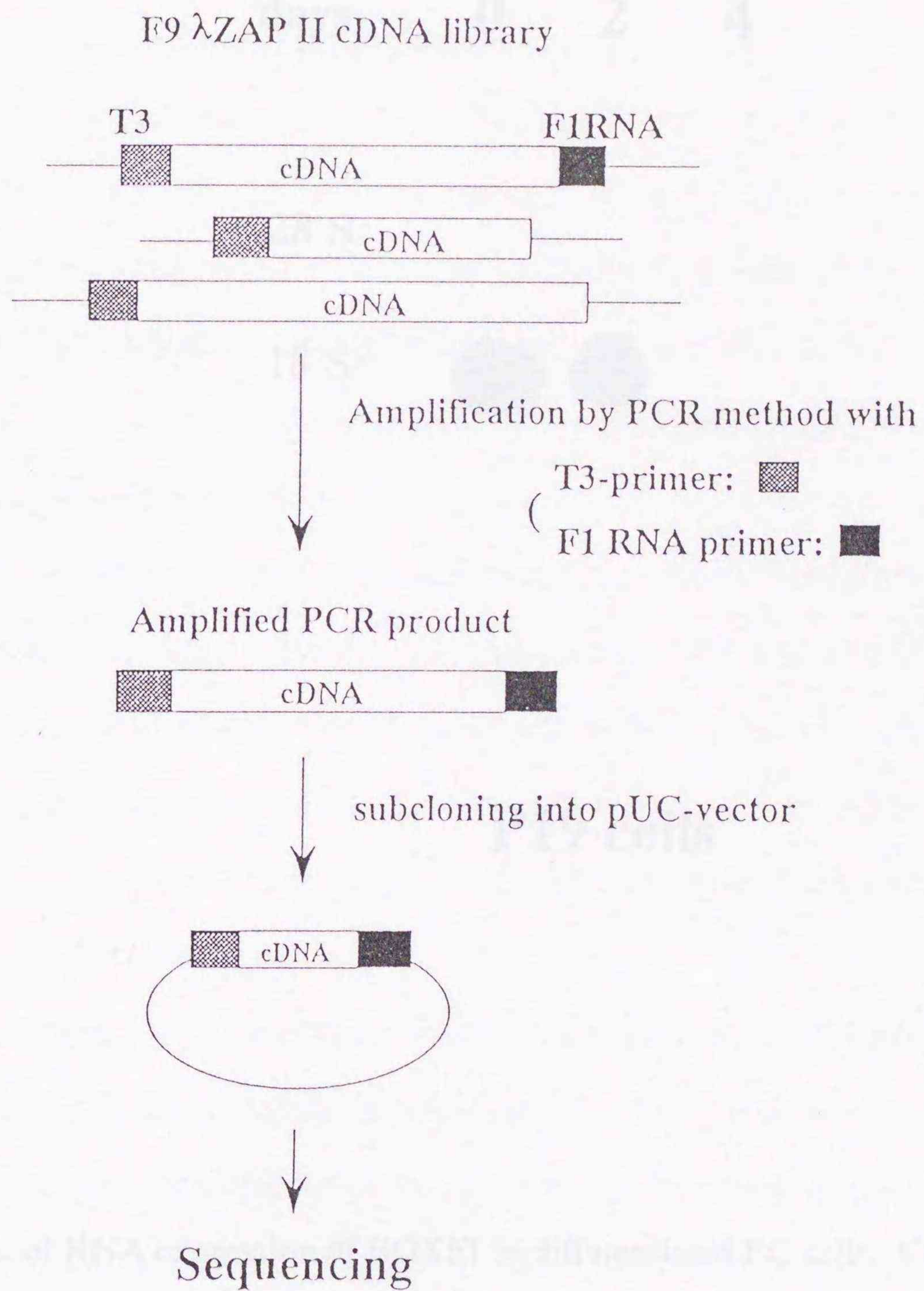
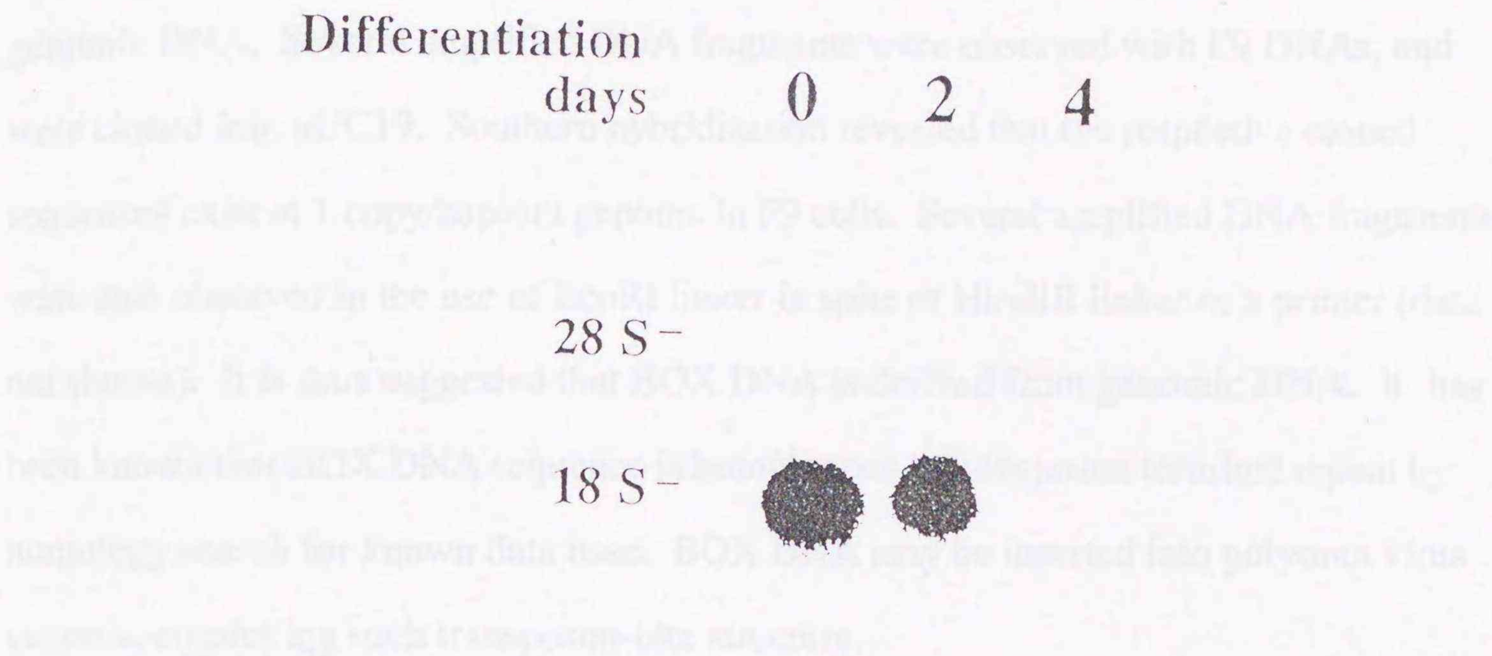


Fig.7 Cloning of BOXF1 cDNA from F9 λ Zap II cDNA library. The PCR-mediated procedure of cloning BOXF1 cDNA are schematically shown.



P19 cells

Fig.8 Decrease of RNA expression of BOXF1 in differentiated EC cells. Cytoplasmic RNAs were prepared from P19 cells 0days, 2days, and 4days after differentiation induction. Northern blot analysis were performed with these RNAs using of BOXF1 cDNA as a probe.

2.4 DISCUSSION

What genes are the cellular targets of the BOX factors? BOX DNA was first found as the motif inserted into the enhancer B domain of the mutant polyomavirus DNA fPyF9 (1). By the PCR-mediated method using the BOX DNA oligonucleotide and HindIII linker as primers, we searched for the genes containing BOX DNA sequences in the genomic DNA. Several amplified DNA fragments were observed with F9 DNAs, and were cloned into pUC19. Southern hybridization revealed that the respective cloned sequences exist at 1 copy/haploid genome in F9 cells. Several amplified DNA fragments were also observed in the use of EcoRI linker in spite of HindIII linker as a primer (data not shown). It is thus suggested that BOX DNA is derived from genomic DNA. It has been known that BOX DNA sequence is homologous to transposon terminal repeat by homology search for known data base. BOX DNA may be inserted into polyoma virus genome, employing such transposon-like structure.

One of the cloned DNAs, BOXF1, possessed promoter activity downstream from BOX DNA, and other transcriptional elements including the Sp1, TATA-like and Oct motifs. Expression due to BOXF1 was dominantly regulated by BOX DNA in undifferentiated P19 cells, but not in differentiated cells. These results indicate that BOXF1 belongs to the enhancer/promoter region of a target gene for BOX factors and the gene may be involved in EC cell differentiation.

Furthermore, I cloned BOXF1 cDNA. It highly expressed in undifferentiated P19 cells and decreased according to differentiation stats of cells. It is thus considered that BOXF1 gene is a target one of BOX factors. What a role does the gene play in EC cell differentiation ? It will be clarified by characterization of the gene through analysis of its genomic DNA, gene targeting, and over expression in different types of undifferentiated and differentiated cells et al.

CHAPTER 3

BOX DNA activity varies according to differentiation states of lymphoid cells.

3.1 INTRODUCTION

Many of the transcription factors, Oct (57), GATA(5), et al. are a part of protein family. Oct-1, Oct-2, and Oct-3 are involved in ubiquitous , B-cell specific, and EC cell specific transcriptions, respectively. These protein constitutions are very homologous each other among the transcription factor family. It is thougt that they employ a wide variety of tissue-specific transcriptions by interacting with cellular cooperative factors. I thus studied what activity BOX DNA has in lymphoid cell differentiations and how it regulates them. I examined the transcriptional activity of BOX DNA and BOX factors in human promyelotic leukemia cells, HL60, having pluripotency similar to EC cells. Moreover, I examined the BOX DNA activity in human T-cell lymphoma cells, Jurkat and human Barkit lymphoma cells, Raji, which cells have no pluripotency. BOX DNA binding proteins were observed in all the cells examined, independently of pluripotency. Those proteins bound specifically to BOX DNA in both of HL60 and Raji cells. Furthermore, BOX factors (BOX DNA specific binding proteins) of HL60 cells decreased after differentiation induction. The results suggest BOX DNA is also involved in lymphoid cell differentiation.

3.2 MATERIALS AND METHODS

Cell culture and preparation of nuclear extracts

P19 cells were cultured as described in chapter 1. Human promyelotic leukemia cells, HL60, human T-cell lymphoma leukemia cells, Jurkat, and human Burkitt lymphoma cells, Raji were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum. Differentiation induction of HL60 cells was performed by treatment of 3×10^6 cells with 10^{-5} M retinoic acid. Nuclear extracts were prepared according to the procedure described as chapter 1.

CAT assay

Eight μg of each CAT construct and 2 μg of CMV β-galactosidase gene were transfected into 2×10^7 of Raji cells, by the DEAE dextrane method (1) with a little modification. Other procedures were carried out as chapter 1.

3.2RESULTS

BOX DNA binding proteins were also present in lymphoid cells independently of pluripotency

To know whether BOX DNA is involved in lymphoid cell differentiation or not, I first examined presence of BOX DNA binding proteins in lymphoid cells with or without pluripotency. HL60 cells have pluripotency and can be induced to differentiate in vitro by several inducers . Both of Jurkat and Raji cells,on the other hands, have no pluripotency and can not be induced to differentiate. I performed the band shift assays with the nuclear extracts of these cells using of BOX DNA as a probe. As shown in Fig.1, several BOX DNA-protein complexes were observed in all the lympoid cells examined. These complexes were not the same among them. The data indicate that BOX factors (BOX DNA specific binding proteins) are also present in lymphoid cells independently of pluripotency.

BOX factors of HL60 cells decreased after differentiation induction.

To investigate whether BOX DNA binding proteins of lympoid cells recognize specifically to BOX DNA, band shift competition assays were carried out with the nuclear extracts of HL60 cells. BOX DNA-proteins complexes of HL60 cells were clearly decreased by addition of BOX DNA as a competitor, while MuBOX DNA did not reduce them (Fig. 2). The results suggest that BOX factors (BOX DNA specific binding proteins) will function as transcription factors in undifferentiated lymphoid cells with pluripotency. Moreovere, to know whether HL60 BOX factors function in lymphoid cell differentiation, band shift assays were performed with the nuclear extracts of HL60 cells before and after differentiation induction. 3×10^6 of HL60 cells were induced to differentiate with treatment of 10^{-5} M RA. The cells were harvested at 40, 60, and 80 hours after induction, and the nuclear extracts were prepared. As shown in Fig. 3, HL60 BOX factors decreased after differentiation induction. It is thus considered that HL60 BOX factors are involved in maintenance of undifferentiated states of the cells as transcription factors .

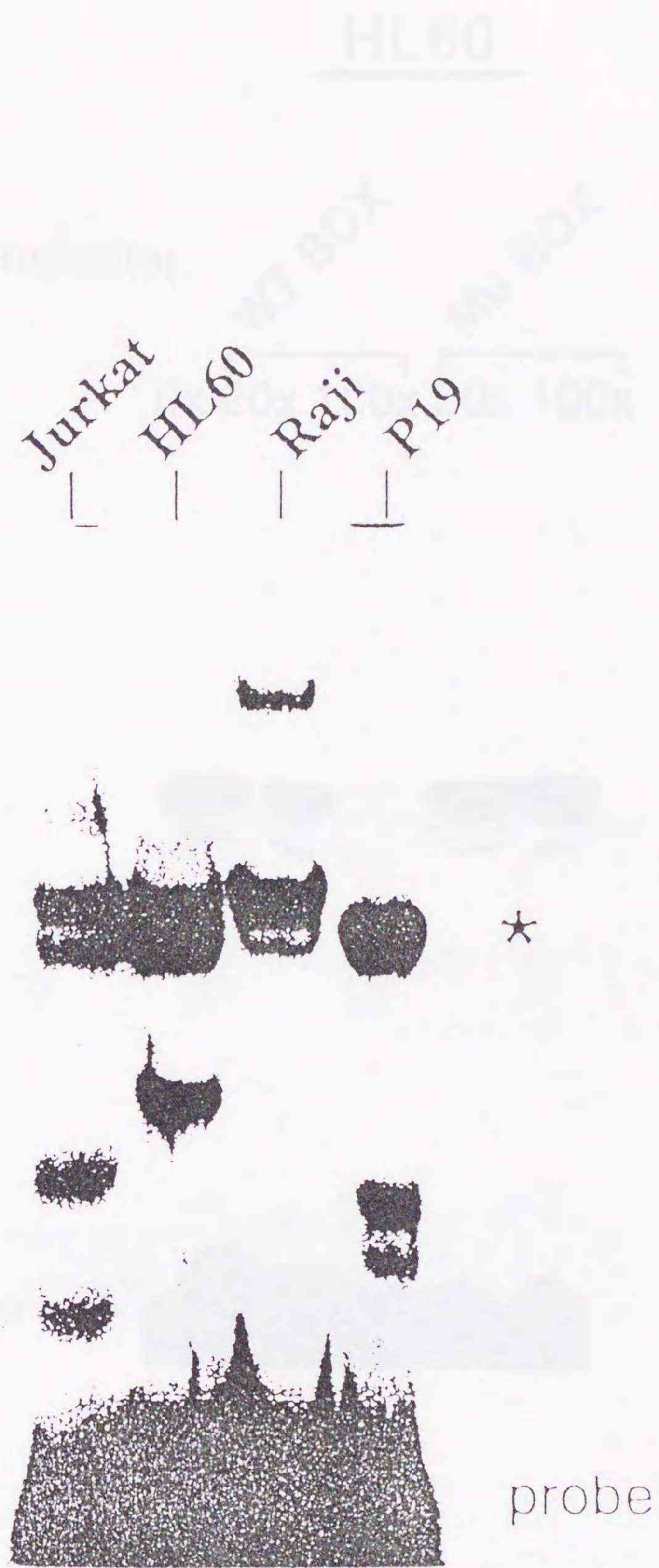


Fig.1 The BOX DNA binding proteins in various lymphoid cells. The band shift assays were carried out using BOX DNA as a probe with nuclear extracts from Jurkat,HL60, Raji cells, and P19 cells. An asterisk in the right of the panel indicates the protein complexes of P19 cells specifically binding to BOX DNA.

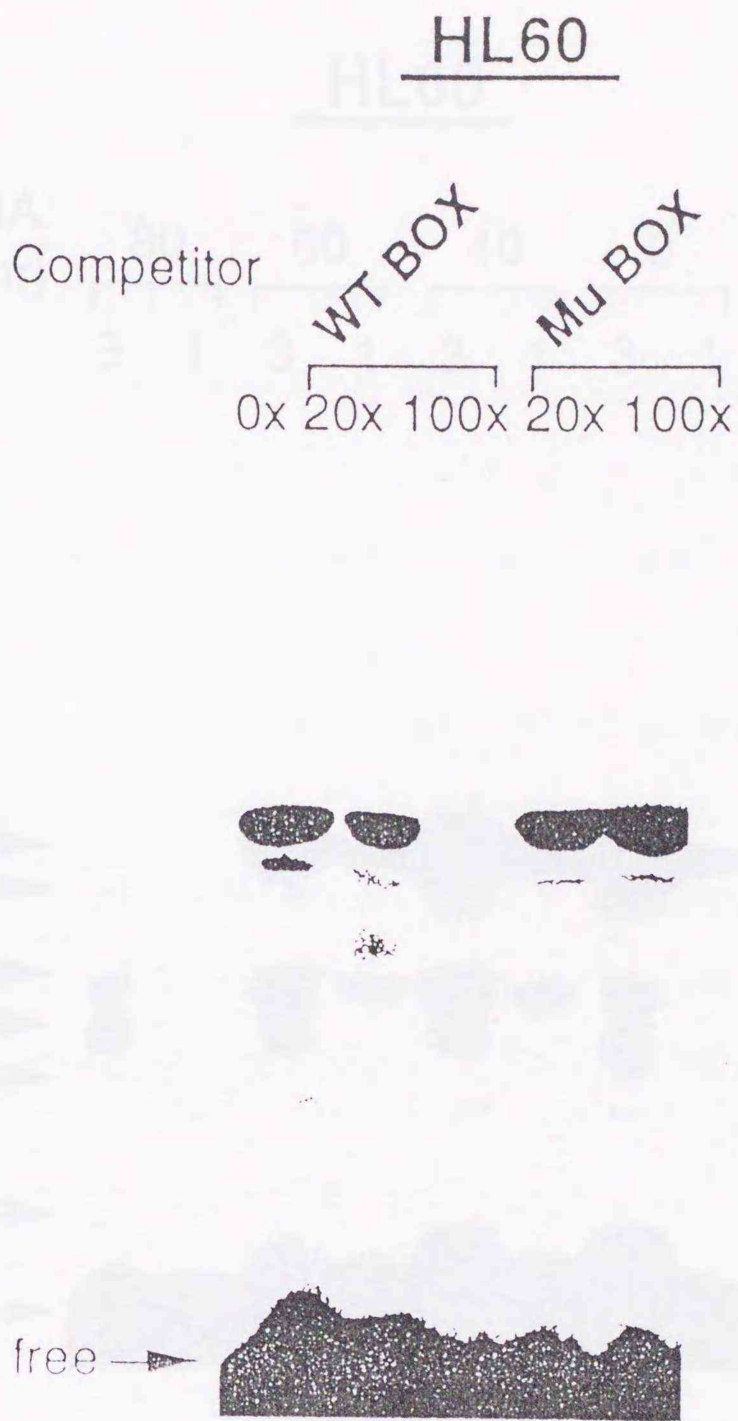


Fig.2 The competition analysis for binding to BOX DNA in HL60 cells. The band shift assays were carried out using BOX DNA as a probe with the nuclear extracts of HL60 cells. Different amounts of unlabelled BOX DNA or MuBOX DNA were added as competitors to assess specificity of binding. Lane 1., no competitors; lanes 2 and 3, with unlabelled BOX DNA at 20 or 100 fold moral excess to the probe, respectively; lanes 4 and 5, with unlabelled MuBOX DNA ar 20 or 100 fold molar excess, respectively.

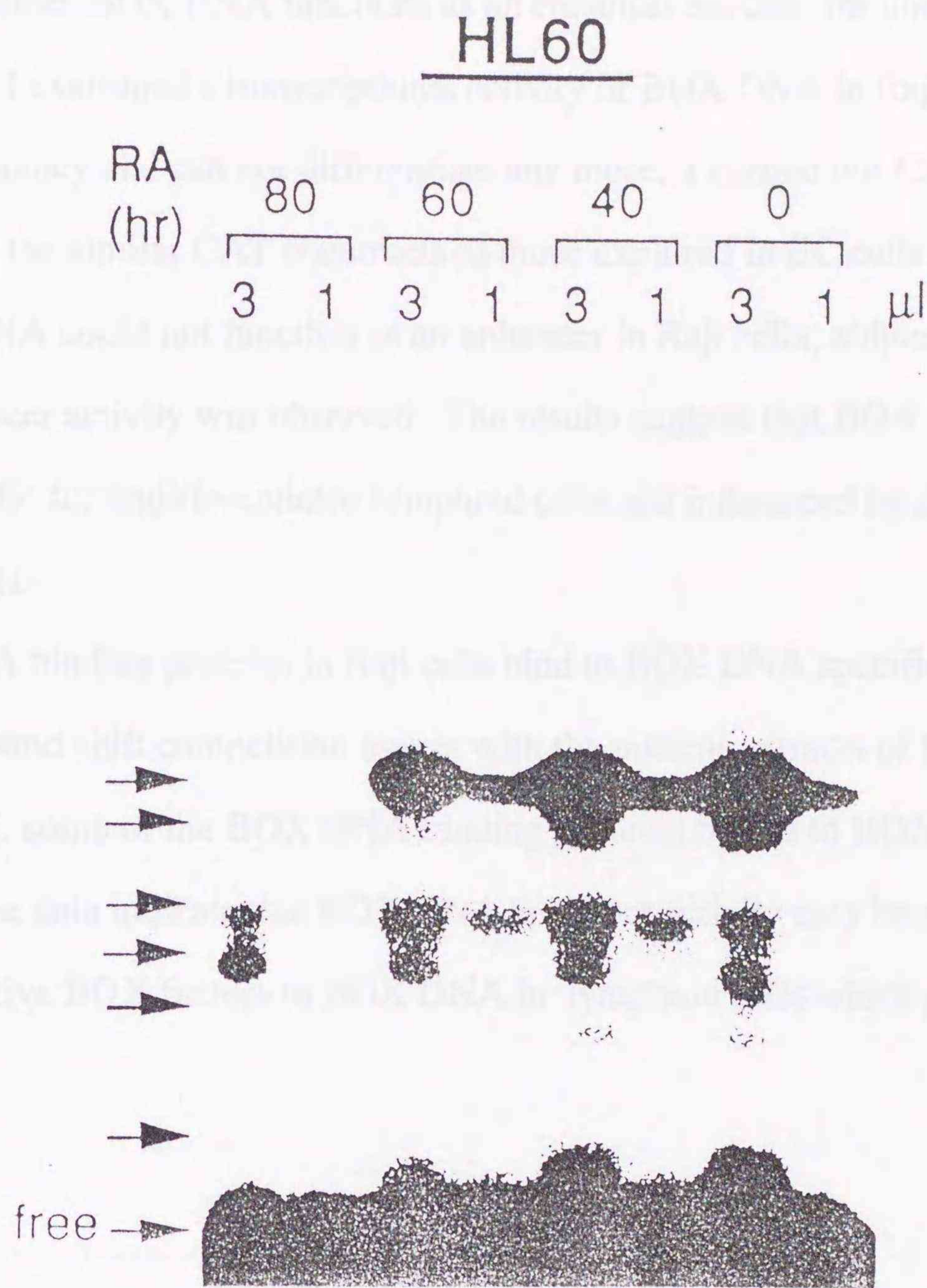
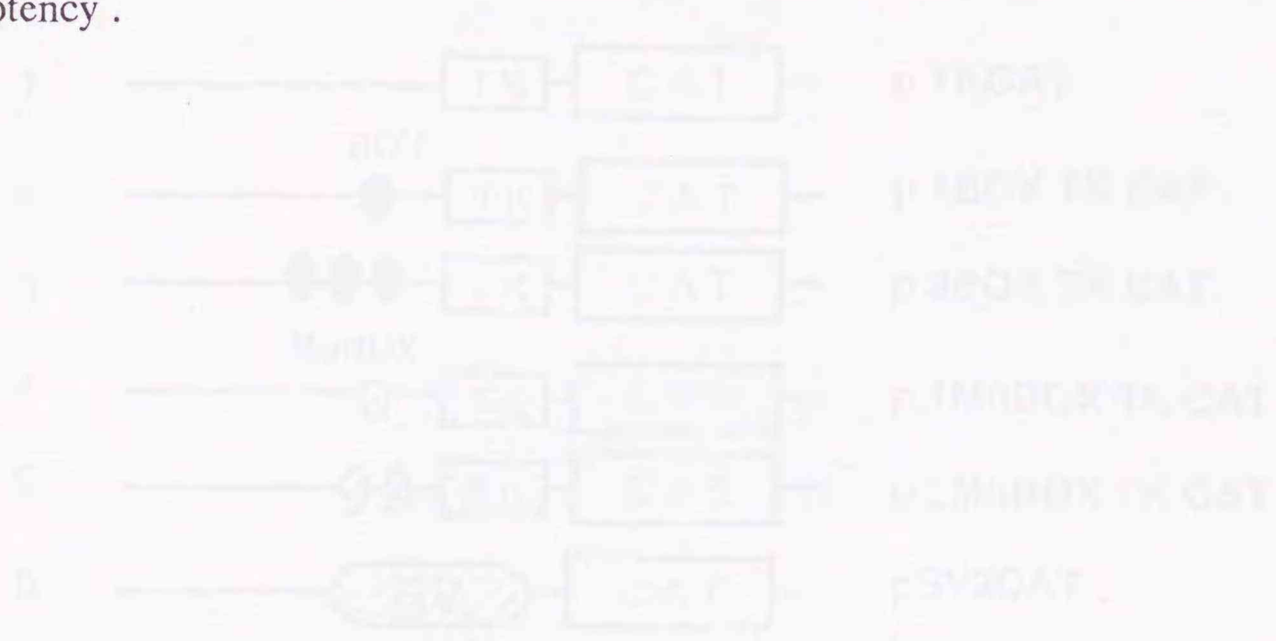


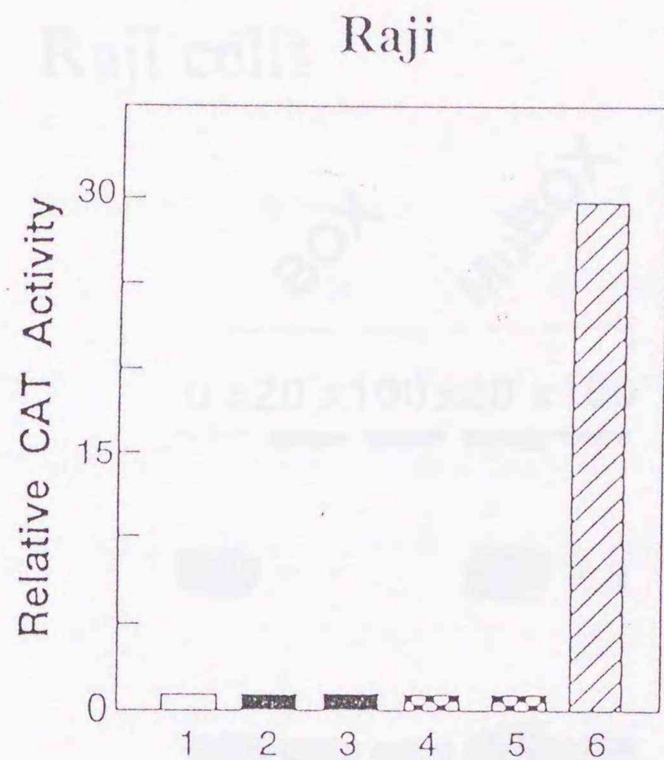
Fig.3 Decrease of BOX factors during HL60 cell differentiation. Radiolabelled BOX DNA was incubated with 1 μg or 3 μg of the nuclear extracts prepared at restrictive time after differentiation induction (shown at the top of the figure). Reactions were preformed as described as in materials and methods in chapter 1. Arrows indicate the BOX DNA binding proteins altering during differentiation.

BOX DNA does not function as an enhancer in Raji cells.

To know whether BOX DNA functions as an enhancer specific for undifferentiated lymphoid cells, I examined a transcriptional activity of BOX DNA in Raji cells. Raji cells have no pluripotency and can not differentiate any more. I carried out CAT assays with Raji cells using the similar CAT constructs to those explored in EC cells. As shown in Fig. 4, BOX DNA could not function as an enhancer in Raji cells, although SV40 promoter/enhancer activity was observed. The results suggest that BOX DNA enhancer activity is specific for undifferentiated lymphoid cells and influenced by differentiation states of the cells

Do BOX DNA binding proteins in Raji cells bind to BOX DNA specifically ? I performed the band shift competition assays with the nuclear extracts of Raji cells. As shown in Fig. 5, some of the BOX DNA binding proteins bound to BOX DNA specifically. The data indicate that BOX DNA enhancer activity may be repressed by binding of inactive BOX factors to BOX DNA in lymphoid cells which possess no pluripotency .





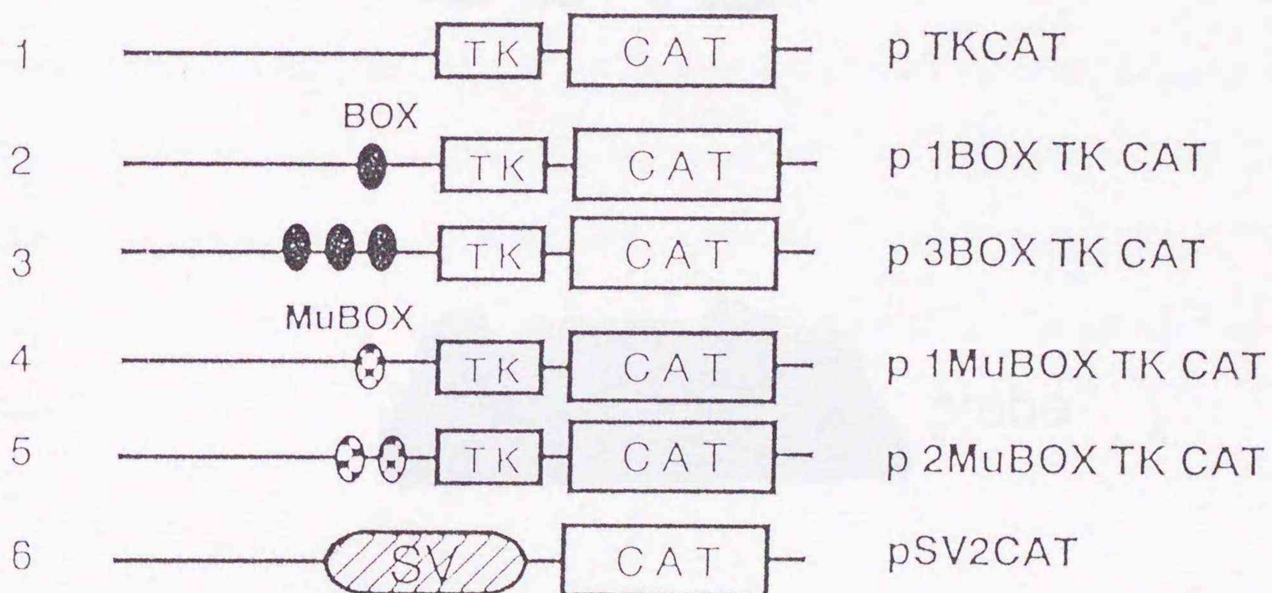


Fig.4 Transcriptional activity of BOX DNA in Raji cells. The CAT assays were performed using Raji cells transfected with various CAT constracts (1-6). Rerative CAT activities were calculated on the basis of pTKCAT; its activity was set as 1.

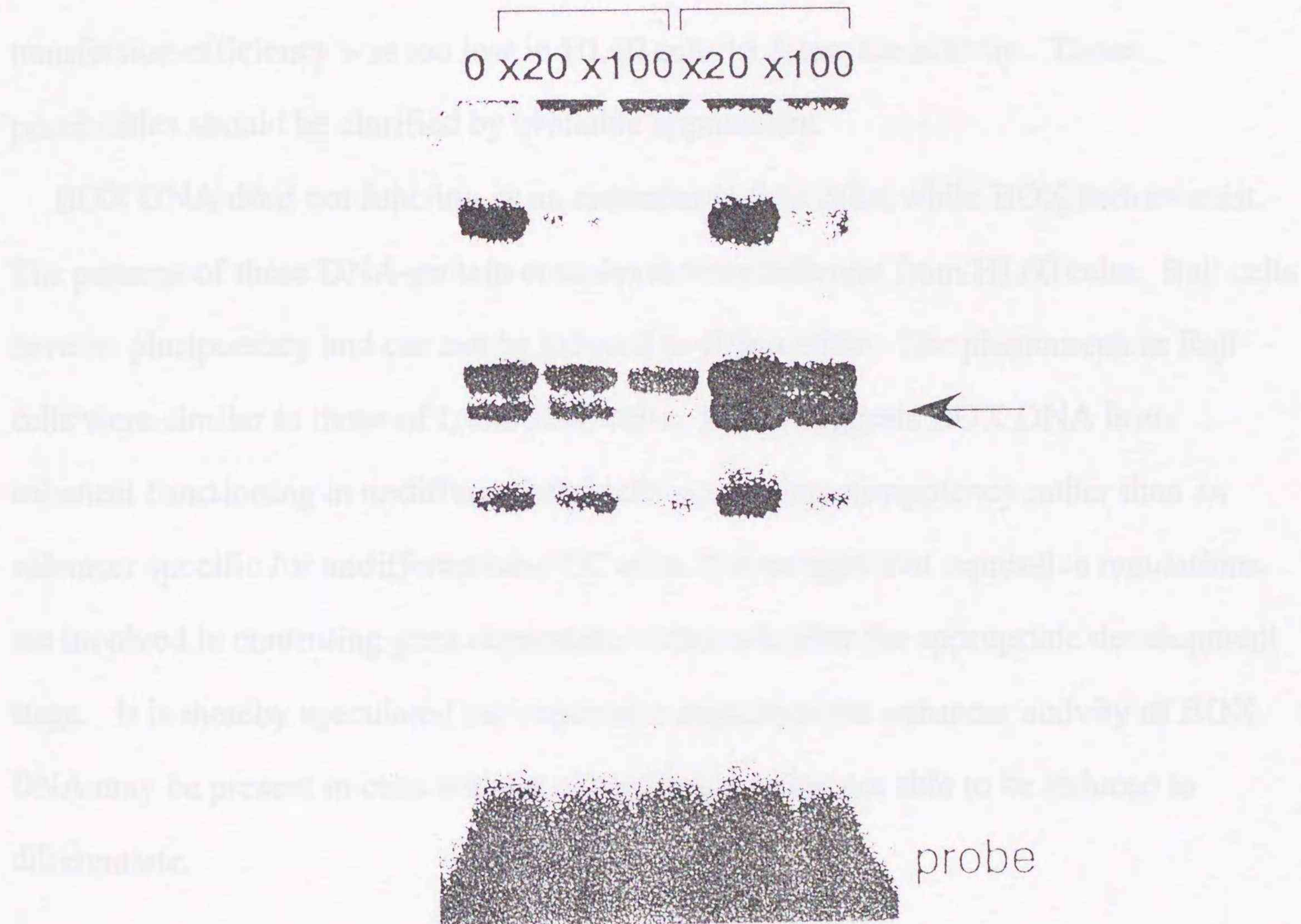


Fig.5 The competition analysis for binding to BOX DNA in Raji cells.The band shift assays were carried out with the nuclear extracts of Raji cells as described in Fig.1. An arrow indicates the proteins binding specifically to BOX DNA.

3.3 DISCUSSION

BOX factors were present in HL60, Jurkat, and Raji lymphoid cells, independently of pluripotency. HL60 BOX factors reduced after differentiation induction. It is thus thought that BOX factors may be part of families and play an important role in not only EC cell but also lymphoid cell differentiation. To clarify these possibilities, examination of the transcriptional activity of BOX DNA before and after differentiation induction in lymphoid cells with pluripotency should be required. I had examined the activities of BOX DNA in HL60 cells . Nevertheless, it was not detected. It was thougt that transfection efficiency was too low in HL60 cells to detect the activity. Those possibilities should be clarified by available approaches.

BOX DNA dose not function as an enhancer in Raji cells, while BOX factors exist. The patterns of these DNA-protein complexes were different from HL60 cells. Raji cells have no pluripotency and can not be induced to differentiate. The phenomena in Raji cells were similar to those of L and Balb cells. It thus suggests BOX DNA is an enhancer functioning in undifferentiated cells possessing pluripotency rather than an enhancer specific for undifferentiated EC cells. It is thought that repressive regulations are involved in controling gene expression before and after the appropriate development stage. It is thereby speculated that repressive regulation for enhancer activity of BOX DNA may be present in cells without pluripotency, being not able to be induced to differentiate.

CHAPTER 4

Purification of BOX factors from the cells with or without pluripotency.

4.1 INTRODUNTION

To understand the molecular mechanisms of controling gene expressions, it is considered that biochemical analysis or cDNA cloning of transcription factors are important. Purifications of transcription factors, Sp1(10), E2F(6), Oct-1(27), have enabled us to use various approaches such as in vitro reconstitution of transcription system or analysis using of those antibodies. On the other hand, cDNA of transcription factors, ets-1(21), ATF(22), Oct-2(45), C/EBP(58), have clarified those cooperative factors or their in vivo functions.

Thus, to clarify the functions and regulatory mechanisms of BOX DNA, I performed purification of BOX factors from P19, EC cells, using of BOX DNA affinity column. A 44 kDa protein was purified as a BOX factor from P19 cells, and it specifically bound to BOX DNA. Moreover, 100/60 kDa proteins were mainly purified from Raji cells with the co-purification of a trace amount of 44 kDa protein as from P19 cells. P19 cell have pluripotency and differentiate in vitro. Raji cells have no pluripotency and does not differentiate. It thus suggests BOX DNA is positively and repressively regulated by the actions of BOX factors and cellular cooperative factors, dependently of pluripotency.

4.2 MATERIALS AND METHODS

Cell culture and preparation of nuclear extracts

P19 cells were cultured as described in chapter 1. Human Burkitt lymphoma cells, Raji were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum. Nuclear extracts of 9 x 10^9 cells were prepared according to the procedure described in (60) and used for purification of BOX factors .

Purification of BOX factors from P19 cells.

Thirty-three mg of nuclear extracts were prepared from 9 x 10^9 P19 cells, precipitated with ammonium sulfate, and dialyzed against 50 mM NaCl-Buffer D [20 mM Hepes (pH 7.9), 20% glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT]. The crude extracts were loaded onto Heparin-sepharose column (Pharmacia) at 2 ml/30 min. After being washed with 50 mM NaCl-Buffer D, P19 BOX factors, having specific binding activity to BOX DNA, were eluted with a linear gradient from 50 mM to 600 mM NaCl in Buffer D. The peak of activity eluted at ~ 450 mM NaCl. The active fractions were pooled, dialized against 50 mM NaCl-Buffer D, and loaded onto BOX DNA affinity column at 2 ml/15 min. The DNA affinity ligand consisted of the concatenated BOX DNA was coupled with CNBr-activated sepharose as described by Kadonaga et al.(26). After washing with 100 mM NaCl-Buffer D, P19 BOX factors were eluted with a linear gradient from 100 mM to 1.2 M NaCl-Buffer D. The peak of activity was eluted ~ 400 mM NaCl. The active fractions of BOX DNA affinity column were pooled, dialyzed against 20 mM NaCl-Buffer D, and stored at -80°C. The binding activity of fractions of all elution steps was examined by band shift assays. The band shift assays were carried out as described in chapter 1 except for ^{32}P-labelled BOX DNA (70,000 cpm) and 1.4 μg of poly (dI-dC) in other procedures like as described in chapter 1. The protein concentration was measured by the Brad-ford assay, and estimates of molecular weight of purified BOX factors were performed with known amounts of molecular weight markers by silver-staining in sodium dodecyl sulfate (SDS) polyacrylamide gel.

Purification of BOX factors from Raji cells.

Twenty mg of crude nuclear extracts were prepared from 9 x 10^9 of Raji cells and loaded onto heparin-Sepharose column at 4 ml/30 min. After washed with 20 mM KCl-Buffer D, Raji BOX factors were eluted with a linear gradient from 50 mM to 500 mM KCl gradient. The peak activity eluted at ~ 250 mM KCl. The active fractions were pooled and dialyzed against 20 mM KCl-Buffer D. To exclude non-specific binding of proteins, these fractions were first loaded onto Alu DNA affinity column containing Alu core sequence (50) at 4 ml/30 min. After washed with 50 mM-KCl Buffer D, Raji BOX factors were eluted with a linear gradient from 50 mM to 500 mM KCl. The peak of BOX DNA specific binding activities eluted at 140 mM. The active fractions were pooled and then loaded onto BOX DNA affinity column. Raji BOX factors were eluted with a 50 mM to 300 mM KCl gradient. The peak of activities eluted at ~ 240 mM KCl. The active fractions were pooled, dialyzed with a buffer containing 25 mM Tris-HCl (pH 7.5), 1mM EDTA, 1mM 2-mercaptoethanol, 50 % glycerol, 0.1 M KCl and 0.01% NP-40, and stored -80°C. Identification of the binding activity of fractions and estimation of molecular weight of the purified proteins were carried out as above.

4.1 RESULTS

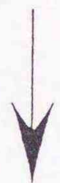
Purification of BOX factors from cells with pluripotency.

To identify BOX factors of the cells with pluripotency, BOX factors were purified from P19 cells. Purification of proteins was performed according to the procedures described in materials and methods and shown conveniently in Fig.1 . Fourty-seven μg of the BOX factors were prepared from 33 mg of the crude nuclear extracts. Fig.2A showed the results of band shift assays with the crude nuclear extract, the active fraction of heparin-Sepharose chromatography, and the active fraction of BOX DNA affinity chromatography, using BOX DNA as a probe. It is known that BOX DNA binding activities were concentrated by these procedures. Fig. 2B showed the results of the competition band shift assays with the active fraction of BOX DNA affinity chromatography, using of BOX DNA or MuBOXDNA (Fig. 2 in chapter 1) as a competitor. BOX DNA binding activities in the fractions were diminished by BOX DNA, but not by MuBOXDNA. The data indicates that P19 BOX factors are purified. To know the purity and estimate the molecular weights of isolated P19 BOX factors, the purified proteins were silver-stained in SDS-PAGE (Fig. 3). The result reveals that a 44 kDa protein was concentrated during these procedures, and suggests the 44 kDa protein is one of P19 BOX factors.

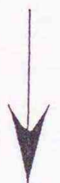
Purification of BOX DNA binding proteins

9×10^9 of P19 or Raji cells

Nuclear extract



Ammonium sulfate precipitation



Heparin - Sepharose column

bound fraction to BOX DNA

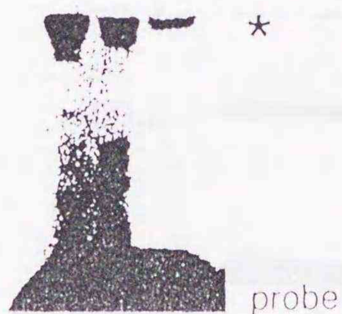
BOX DNA affinity column

bound fraction

Fig.1 Purification of BOX factors of P19 cells. Flow chart for purification of P19 BOX factors are summarized.

P19 cells

A



B

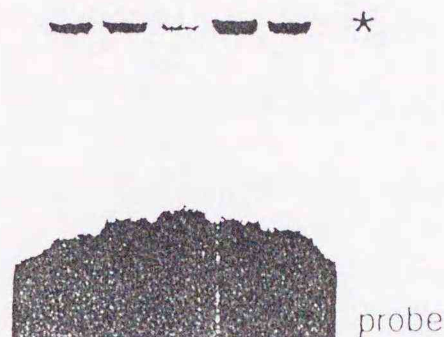


Fig.2 Analysis of binding activity of the purified P19 BOX factors. (A) The band shift assays were performed using BOX DNA as a probe with the crude nuclear extracts, the active fraction of heparin-Sepharose chromatography, and the active fraction of BOX DNA affinity chromatography. (B) Competition analysis with the purified P19 BOX factors; the band shift assays were carried out using of BOX DNA as a probe with an aliquot of the active fraction of BOX DNA affinity. Two or 5 fold of unlabelled BOX DNA or MuBOX DNA were added to some reactions as competitors to determine specificity of binding. An asterisk indicates the protein complexes specifically binding to BOX DNA.

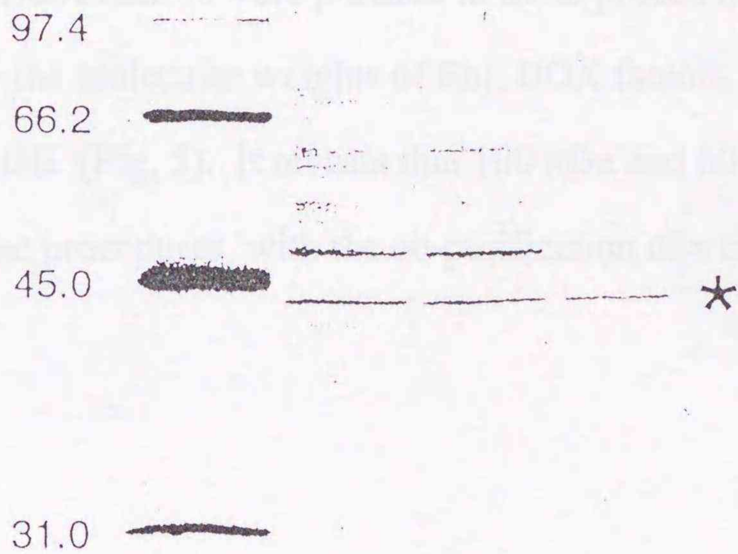


Fig.3 Silver-staining of the purified P19 BOX factors in SDS-PAGE. The crude nuclear extracts, the active fractions of heparin-Sepharose chromatography and BOX DNA affinity chromatography were silver-stained in SDS-PAGE. An asterisk indicates the protein complexes specifically binding to BOX DNA.

Purification of BOX factors from cells without pluripotency.

To know what characters BOX factors of cells without pluripotency have, I carried out purification of BOX factors from Raji cells. Purification of Raji BOX factors was done by the same procedures as those of P19 cells with a small modification. Band shift assays were carried out with the crude nuclear extract, the active fraction of heparin-sepharose chromatography, and the active fraction of BOX DNA affinity chromatography, using BOX DNA as a probe (Fig. 4A). Fig. 4B showed the results of the competition band shift assays with the active fraction of BOX DNA affinity chromatography, using of BOX DNA or MuBOXDNA as a competitor. BOX DNA binding activities in the fractions decreased by BOX DNA, not by MuBOXDNA, showing that Raji BOX factors were purified in these procedures. To investigate the purity and estimate the molecular weights of Raji BOX factors, proteins were silver-stained in SDS-PAGE (Fig. 5). It reveals that 100 kDa and 60 kDa proteins were mainly purified during these procedures, with the co-purification of a trace amount of a 44 kDa protein.

Raji cells

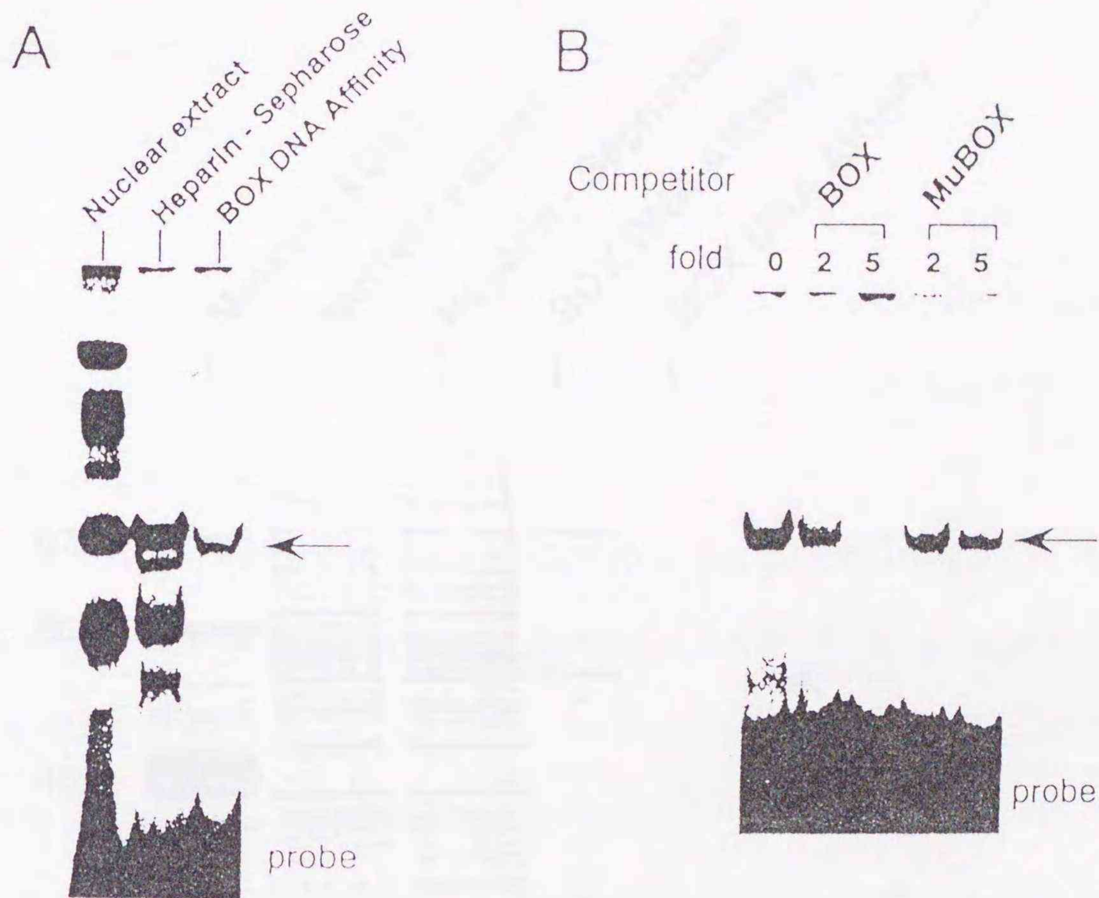


Fig.4 Analysis of binding activity of the purified Raji BOX factors. (A) The band shift assays were performed using BOX DNA as a probe with the crude nuclear extracts, the active fraction of heparin-Sepharose chromatography, and the active fraction of BOX DNA affinity chromatography. (B) Competition analysis with the purified Raji BOX factors; the band shift assays were carried out using of BOX DNA as a probe with an aliquot of the active fraction of BOX DNA affinity. Two or 5 fold of unlabelled BOX DNA or MuBOX DNA were added to some reactions as competitors to determine specificity of binding. An arrow indicates the protein complexes specifically binding to BOX DNA.

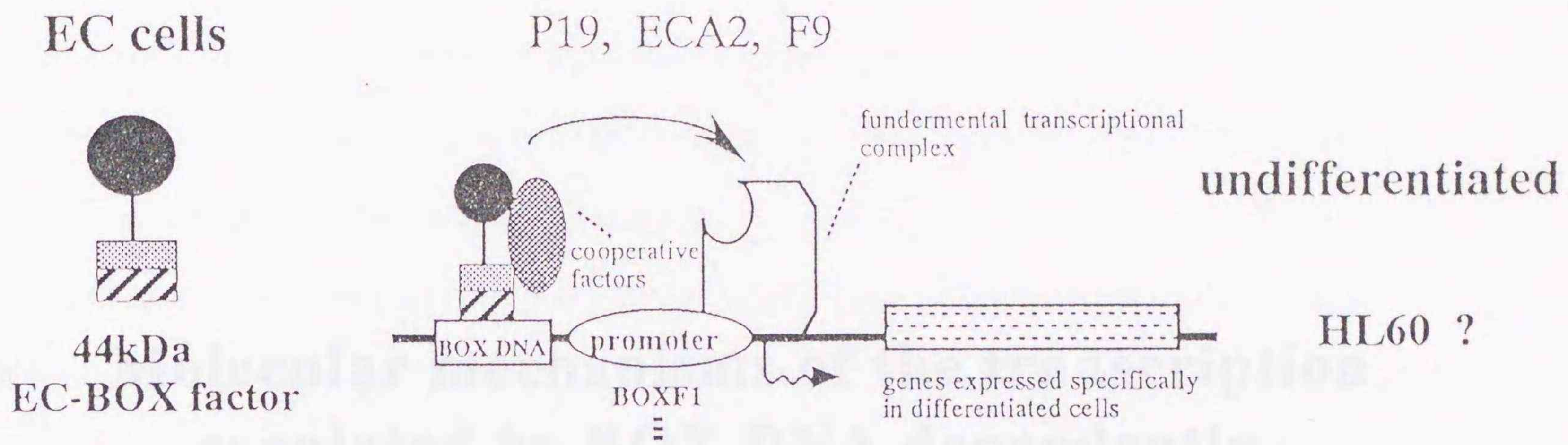
Fig.5 Silver-staining of the purified Raji BOX factors in SDS-PAGE. The crude nuclear extracts, the active fractions of Heparin-sepharose chromatography and BOX DNA affinity chromatography were silver-stained in SDS-PAGE. An arrow and an asterisk indicates the protein complexes specifically binding to BOX DNA.of Raji cells and P19 cells, respectively.

4.1 DISCUSSION

BOX DNA acts as an enhancer in P19 cells. A 44 kDa protein was partially purified as a BOX factor from P19 cells. I will clarify the character of the 44 kDa protein through determination of amino acid sequence and examination of transcriptional activity in vitro transcription system. It is considered that differentiation proceeds by reduction of expression of genes involved in maintenance of undifferentiated states (17,36). Thus, the possible molecular mechanisms of transcriptional activation by P19 BOX factors are shown in Fig.6A. These factors such as the 44 kDa protein will bind specifically to BOX DNA in enhancer/promoter region (for example, BOXF1) and promote the transcription of genes required for maintenance of undifferentiated states/pluripotency by interacting with cellular cooperative factors.

On the other hands, BOX DNA can not act as an enhancer in Raji cells. The 100/60 kDa proteins were purified as BOX factors of Raji cells, with a 44 kDa protein. The 44kDa protein is speculated to be the same protein observed in P19 cells. From these results, a suppression model of BOX DNA activity supposed is showed in Fig. 6B. The 44 kDa protein may be expressed too little to display the activity, or the modifying enzymes to activate them may be lacking. The 100/60 kDa proteins more preferentially will bind to BOX DNA than the 44 kDa protein. Nevertheless, BOX DNA is not activated, because the 100/60 kDa proteins can not interact with the cooperative factors/the fundermental transcriptional complexes. Alternatively, cooperative factors required for the BOX DNA activity may be absent or inactive in the cells without pluripotency. Thus, the gene expressions involved in pluripotency/maintenance of undifferentiated states will be repressed in the cells without pluripotency, such as Raji, L, and Balb cells.

(A)



(B)

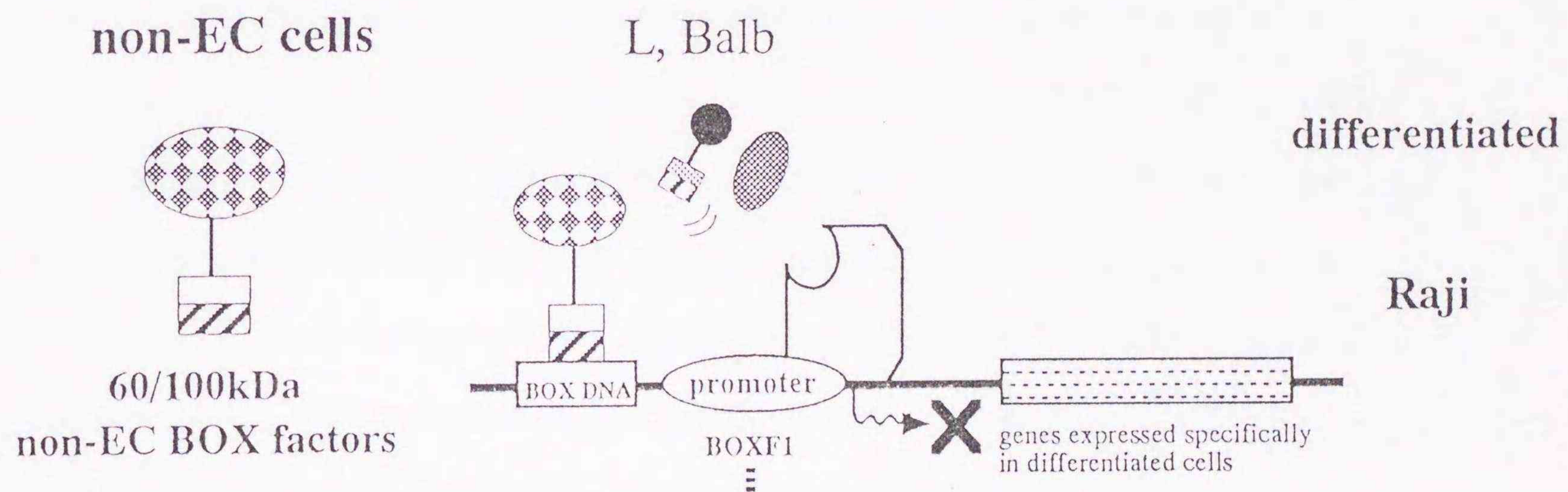


Fig. 6 Models for molecular mechanisms of the transcriptional regulation of genes required for maintenance of undifferentiated states/pluripotency. (A) Promotion of the gene expressions in undifferentiated cells having pluripotency. (B) Repression of the gene expressions in differentiated cells, cells without pluripotency, or cells being not able to differentiate any more.

SUMMARY

Molecular mechanisms of the transcription regulated by BOX DNA dependently of pluripotency/differentiation states of cells.

5.1 DISCUSSION

EC cell differentiation is correctly controlled by a complicated network of various genes in definite stages of the process. These genes' expressions are regulated by various transcription factors, including their own products. To understand EC cell differentiation, it is important to clarify the functions of these transcription factors. It has been reported that the activities of several transcription factors alter during the EC cell differentiation. Although changes in their expression levels are well studied, their roles in EC cell differentiation are little understood.

In chapter 1, I reported about BOX DNA, whose binding protein(s) would be novel transcription factors concerning EC cell differentiation. BOX DNA functioned as a specific enhancer to the TK promoter in undifferentiated P19, ECA2 and F9 cells, but the activity was repressed in the cells after differentiation. BOX DNA also functioned as an enhancer for the SV40 promoter in these EC cells (data not shown). Previously we reported BOX DNA as a silencer on transcription as well as on viral DNA replication. The silencer activity was observed upon transcription from the SV40 or polyomavirus promoter in F9-28, an F9 clone expressing polyomavirus large T antigen (3, 4). In the same cells, however, BOX DNA activated transcription from the TK promoter (data not shown). On the other hand, BOX DNA functioned as an enhancer upon transcription due to both TK and SV40 promoters in other EC cells, P19, ECA2 and F9, examined in this report. It is unclear what brought about the opposite effects of BOX DNA on different promoters in F9-28. Viral T antigen expressed in this cell line might be responsible for the differences.

Recently, it has been reported that several transcription factors function positively or negatively in cell type- or promoter-dependent manners (36): For instance, positive and negative activities of the Oct motif to different promoters are observed in F9 cells. It functions as an enhancer to the HSV TK promoter, but as a silencer to the c-*fos* promoter in the cells (35). E6, a B cell specific enhancer element, also has both positive and negative activities. Transcriptional activation by E6 was observed with the conalbumin promoter in ARH77 B cells, but not in HeLa cells. On the other hand, transcription

repression was observed in HeLa cells, but not in ARH77, when E6 was linked to the SV40 enhancer (59). Similarly, BOX DNA may be +/- bifunctional in different situations.

As for BOX DNA-protein complex(es), one specific band was detected at the same mobility by band shift assays in all EC cells including F9-28 (data not shown). The band of the BOX DNA-protein complex(es) preferentially decreased upon differentiation induction, in parallel with loss of enhancer activity. Considering that other differentiation-related transcription factors behave similarly and that the BOX DNA binding proteins are observed in various EC cells in different developmental stages, the BOX DNA binding proteins may be factors required for the maintenance of the undifferentiated state. The loss or decrease of their activity may therefore result upon promotion of EC cell differentiation. Combining the results with those in non-EC cells (discussed below), it is suggested that the DNA-protein complex(es) observed in undifferentiated EC cells are required, but not sufficient (as in the case of F9-28), for the enhancer activity of BOX DNA.

What genes are the cellular targets of BOX factors? In chapter 2, I showed the cellular target gene of BOX DNA. BOX DNA was first found as the motif inserted into the enhancer B domain of the mutant polyomavirus DNA fPyF9 (2). By the PCR-mediated method using the BOX DNA oligonucleotide and HindIII linker as primers, we searched for the genes containing BOX DNA sequences in the genomic DNA. Several amplified DNA fragments were observed with F9 DNAs, and were cloned into pUC19. Southern hybridization revealed that the respective cloned sequences exist at 1 copy/haploid genome in F9 cells . BOX DNA is hence suggested to be derived from genomic DNA. One of the cloned sequences, BOXF1, possessed promoter activity downstream from BOX DNA, and other transcriptional elements including the Sp1, TATA-like and Oct motifs. Expression due to BOXF1 was dominantly regulated by BOX DNA in undifferentiated P19 cells, but not in differentiated cells. Moreover, I isolated BOXF1 cDNA . BOXF1 cDNA expressed highly in undifferentiated P19 cells and reduced after differentiation. These results indicate that BOXF1 belongs to the

enhancer/promoter region of a target gene for BOX DNA binding proteins and the gene may be involved in EC cell differentiation. Characterization of the gene, as well as of cDNAs encoding BOX factors would provide a new approach for analyzing the mechanisms of cell differentiation.

Are BOX factors a part of families and involved in other cell differentiations ? In the chapter 3, I showed the study about BOX DNA activity in lymphoid cells in notice of pluripotency and cell differentiation. BOX DNA activity was also influenced by differentiation states of cells in lymphoid cell. BOX DNA could not function in Raji cells without pluripotency being not able to be induced to differentiate, while BOX factors were present in the cells. On the other hands, BOX factors of HL60 cells decreased after differentiation induction. Both of P19 and HL60 cells have pluripotency, although the origins derived from and the directions of differentiation are distinct each other. These results suggest BOX DNA is a regulatory element not only specific for EC cells but also functioning in a wide type of cells with pluripotency. BOX DNA may be in promoter/enhancer regions of the gene (for example, BOXF1 gene) required for maintenance of pluripotency. It is considered that reduction of such genes triggers proceeding of differentiation. Therefore, reduction/inactivation of BOX factors may cause differentiation by some molecular mechanisms (see Fig. 9 in chapter 1)

What mechanisms do BOX factors regulate a gene expression according to differentiated states of the cells by ? In chapter 4, I showed purification of BOX factors from cells with or without pluripotency. A 44kDa protein was purified from P19 cells with pluripotency. 100/60 kDa proteins were mainly purified from Raji cells without pluripotency, with co-purification of a 44 kDa protein (28). I thus considered the models for molecular mechanisms of positive and repressive regulation of BOX DNA enhancer activity by various BOX factors and these cellular cooperative factors (see Fig.6 in chapter 4). They will be clarified by more detailed analysis through identification and characterization of each factor. I hope these works will contribute to development of studies of cell differentiation.

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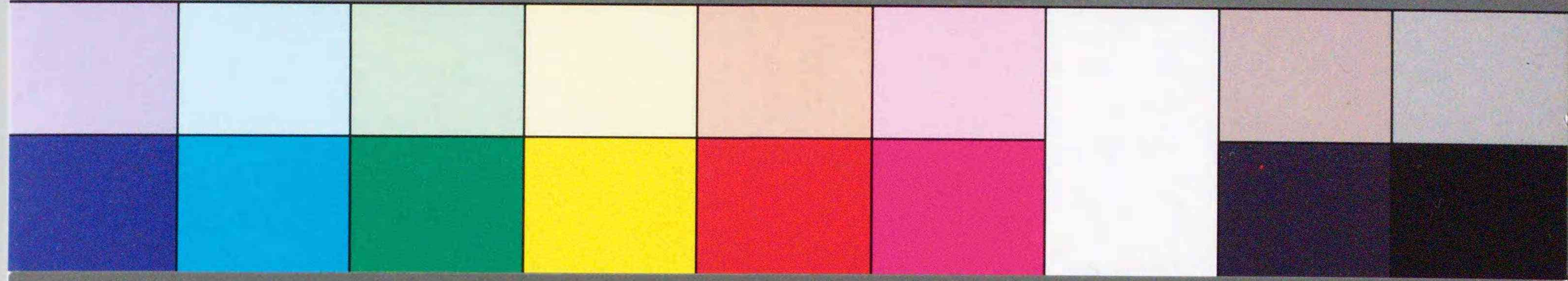
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