MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7747–7756
0270-7306/93/127747-10$02.00/0

Vol. 13, No. 12

BOX DNA: a Novel Regulatory Element Related to Embryonal Carcinoma Cell Differentiation

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Received 19 August 1993/Accepted 20 September 1993

BOX DNA was previously isolated from the DNA sequence inserted in the enhancer B domain of mutant polyomavirus (fPyF9) DNA. We also reported that BOX DNA functioned negatively on DNA replication and transcription of another polyomavirus mutant (Py*hr*N2) in F9-28 cells, a subclone of mouse F9 embryonal carcinoma (EC) cells expressing the polyomavirus large T antigen. In this study, we 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 gene under the control of the herpes simplex virus TK promoter, activated promoter activity in F9, P19, and ECA2 cells. Band shift assays using BOX DNA as a probe revealed that specific binding proteins were present in all EC cells examined; the patterns of BOX DNA-protein complexes were the same among them. A mutation introduced within BOX DNA abolished enhancer activity as well as the formation of specific DNA-protein complexes. In non-EC cells, including L and BALB/3T3 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 BALB/3T3 cells were different from those of EC cells. Furthermore, the enhancer activity of BOX DNA decreased upon differentiation induction in all 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, we 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-chloramphenicol acetyltransferase construct. Deletion analyses of the construct revealed that the transcription of BOXF1 gene is regulated by BOX DNA, preferentially in undifferentiated EC cells versus differentiated cells. Hence, BOX DNA is probably a novel transcriptional element related to EC cell differentiation.

Embryonal carcinoma (EC) cells are suitable for studies of the molecular mechanisms of the early stages of embryogenesis (28). 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) (28, 38). It has been reported that the expression of a number of transcription factors alters during EC cell differentiation in stage-specific manners. E1A-like transcriptional factors (DRTF) (20, 21), AP-1 (12), Oct-3 (32), Oct-4 and Oct-5 (35), Rex-1 (14), and PEA3 (42) decrease in amount during the EC cell differentiation process, while AP-2 (27), HOX-2 (37), HOX-2.5 (17), PEBP2 and PEBP3 increase. Other HOX families (5, 6, 19, 31), the c-*jun* product (7), and Oct-6 (30, 39) transiently increase and are then downregulated in the process. These observations suggest that transcription factors play important roles in embryogenesis cascades by switching on or off various genes involved in differentiation. The biological roles of these factors, however, have not been clarified, although their functions have been inferred from the cDNA sequences encoding them.

Previously we isolated a mutant of polyomavirus, fPyF9, which can replicate in mouse EC cells (1). Unlike other polyomavirus EC mutants, fPyF9 could persist episomally in F9 cells. fPyF9 contained mutations in the enhancer B domain of wild-type polyomavirus DNA: the sequences of enhancer B were highly 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′-GCATTCCATTGTTG TCAAAAG3′ (designated BOX) was considered the prototype. Moreover, we showed that BOX DNA functioned negatively in viral DNA replication and transcription in a system in which a polyomavirus host range mutant, Py*hr*N2, was tested in F9-28 cells. F9-28 is an F9 clone expressing the polyomavirus large T antigen (2).

In this study, we examined the 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 suggest that BOX DNA activates transcription under the control of the herpes simplex virus (HSV) thymidine kinase (TK) promoter specifically in EC cells at undifferentiated states. The decrease in transcriptional activation during differentiation was accompanied by a decrease in the proteins specifically bound to BOX DNA. Furthermore, we cloned an F9 genomic DNA containing the BOX DNA sequence. The DNA had transcriptional promoter activity, and the BOX DNA upstream was a regulatory element dominant to the promoter in undifferentiated EC cells. The data suggest that the DNA contains the enhancer/promoter region of a gene probably related to EC cell differentiation and that BOX DNA is a novel element involved in the regulation of gene expression during differentiation.

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MATERIALS AND METHODS

Cell culture and preparation of nuclear extracts. Mouse L and BALB/3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Mouse EC cell lines P19, ECA2, and F9 were cultured in Dulbecco's modified Eagle's medium 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-diameter dish at a density of 2×10^5 or 5×10^5 cells per dish, respectively. For differentiation induction of F9 cells, 1 μM dibutyrl cyclic AMP (cAMP) and 0.1 nM RA were added to culture medium under conditions similar to those used for P19 and ECA2 cells. Nuclear extracts were prepared as described before (22), and the protein concentration was determined by Bradford's method (4).

Band shift assay. Binding reactions were carried out by incubating 0.14 pmol of end-labelled DNA (5,000 cpm) with 7 to 15 μg of nuclear extract proteins and 2 μg of poly(dI-dC) in a buffer containing 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 15% glycerol, 2% polyvinyl alcohol, 36 mM NaCl, and 0.4 mM dithiothreitol 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× 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 (9, 10).

Plasmid construction. pBLCAT2 is a plasmid carrying the chloramphenicol acetyltransferase (CAT) gene linked to the HSV TK promoter (25). pBLCAT2 was renamed pTKCAT for transfection assays in this study. pBOXTKCAT and pMuBOXTKCAT were constructed as follows. Briefly, BOX and MuBOX oligonucleotides were synthesized according to the prototype sequence inserted in fPyF9 and its mutant (Fig. 1A). The oligonucleotides were phosphorylated at the 5' ends with T4 polynucleotide kinase and cloned into the *Bam*HI site in pUC19. The plasmids containing a few tandem repeats of the oligonucleotides were chosen. The *Sma*I site of pBLCAT2 was changed to a *Hin*dIII site by linker ligation; the TK-CAT fragment obtained by the digestion of the plasmid with *Hin*dIII and *Xba*I was cloned into the plasmids carrying a few BOX or MuBOX oligonucleotides. These plasmids were named p1BOXTKCAT, p3BOXTKCAT, p1MuBOXTKCAT, and p2MuBOXTKCAT, respectively (Fig. 1B).

Cloning of F9 genomic DNA containing BOX DNA sequences. Genomic DNA was extracted from F9 cells as described previously (41) and digested with *Hin*dIII. One-half microgram of the genomic DNA was amplified by polymerase chain reaction (PCR) with *Taq* DNA polymerase (Perkin-Elmer-Cetus), using 0.5 μg of *Hin*dIII linker and 100 pmol of BOX DNA 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 (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, and 125 μM of the four deoxynucleoside triphosphates at 72°C for 3 min. These reactions were repeated for 40 cycles in a thermal cycler (Perkin-Elmer-Cetus). The amplified DNAs were purified and separated in a 1.2% agarose gel.

PCR-amplified DNAs were treated with the Klenow fragment of DNA polymerase I and cloned into *Hin*cII-*Hin*dIII site in pUC19, after phosphorylation followed by digestion with *Hin*dIII. Clones thus obtained were named pBOXF1, pBOXF5, and pBOXF6, respectively. The presence of BOX DNA in these clones was confirmed by nucleotide sequencing. To study 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. 10). The deletion mutants of pF1(H)CAT were constructed as follows. pF1(H)CAT was digested with *Kpn*I and *Sac*I, treated with exonuclease III and mung bean nuclease, and then treated with the Klenow fragment of DNA polymerase I. Self-ligation of the fragments gave rise to pF1delBCAT and pF1delBSCAT (see Fig. 13). pF1E1ECAT is a pUC derivative containing the 100-bp fragment obtained by *Eco*RI

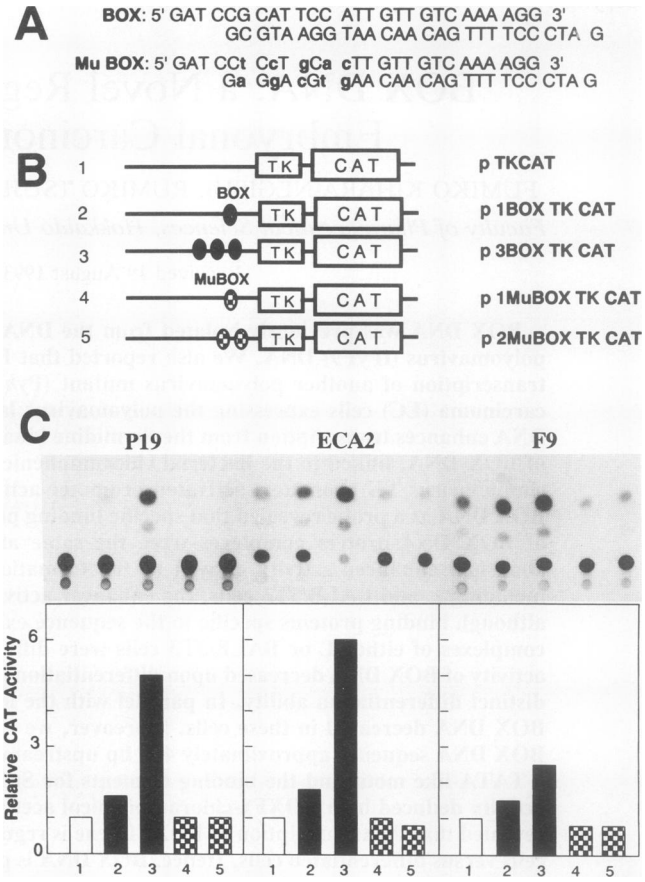


FIG. 1. Transcriptional activity of BOX DNA in various EC cells. (A) Nucleotide sequences of the BOX and MuBOX oligonucleotides. Within MuBOX, five bases substituted for those in intact BOX are indicated by lowercase letters. (B) CAT constructs used. p1BOXTKCAT and p3BOXTKCAT contain one and three copies,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

digestion of pBOXF1 (see Fig. 10), cloned into the *Eco*RI site, and the CAT gene inserted into the *Hin*dIII site (see Fig. 13). The other *Eco*RI fragment of 2,900 bp was self-ligated, resulting in pF1E2HCAT (see Fig. 13).

Preparation of cytoplasmic RNA. F9 cells were washed twice with Tris-buffered saline, suspended in the lysis buffer (10 mM Tris-HCl buffer [pH 8.6] containing 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, and 10 mM vanadyl ribonucleotide complexes), and kept on ice for 5 min. The cell lysate was centrifuged, 40 μl of 10-mg/ml proteinase K and 10 μl of 10% sodium dodecyl sulfate (SDS) were added to the supernatant, and the mixture was incubated at 60°C for 5 min. After phenol extraction three times, cytoplasmic RNA was precipitated by ethanol, dissolved in water, and used for S1 mapping.

S1 mapping. pF1BOX was digested with *Hin*dIII, dephosphorylated, and end labelled with [γ-^{32}P]ATP. After digestion with *Spe*I, the 322-bp fragment was purified and used as a probe for S1 mapping. Fifty-six micrograms of the cytoplasmic RNA and 10^4 cpm of the probe were hybridized at 41°C for more than 12 h in a buffer containing 80% formamide, 40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, and 400 mM NaCl, treated with S1 nuclease, and analyzed in a 10% polyacrylamide gel containing 8 M urea.

CAT assay. L, BALB/3T3, or undifferentiated EC cells (2×10^5 to 4×10^5) were plated on a 100-mm-diameter dish 1 day before transfection. Twenty micrograms of each CAT construct was transfected to the cells, together with 5 μg of the plasmid carrying the β-galactosidase gene linked to the cytomegalovirus TK promoter, by the calcium phosphate method (13). The cells were harvested 24 or 48 h after transfection. In cell differentiation experiments, 2×10^5 EC cells were plated on a 100-mm-diameter dish in medium containing inducing reagents, which was renewed after 2 days. On the next day (3 days after plating), the cells were transfected as described above and were harvested 24 h after transfection. Cell extracts were prepared as described previously (1) and assayed for β-galactosidase activity to normalize transfection efficiency. CAT assays were performed as described previously (11). The conversion of chloramphenicol to acetylated forms was measured with a densitometer (model PD-110; Molecular Dynamics) or a bioimage analyzer (model BAS2000; Fuji Film Co.). The experiment was repeated at least three times, and typical results are presented in the figures.

RESULTS

BOX DNA has enhancer activity specific for EC cells. P19, ECA2, and F9 are EC cells widely used in experiments to analyze undifferentiated stages of mouse embryogenesis. To examine the transcriptional activity of BOX DNA in EC cells, several TK-CAT plasmids containing BOX or MuBOX oligonucleotides were constructed (Fig. 1A and B) and transfected to these cells. The HSV TK promoter was chosen for these constructs because the TK promoter has been reported to be more susceptible to transcription-regulating elements than the simian virus 40 (SV40) promoter in the octamer motif characterization (35). Five nucleotides within BOX DNA were substituted in the mutant oligonucleotide MuBOX (Fig. 1A). As shown in Fig. 1C, 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 EC cells examined, although the

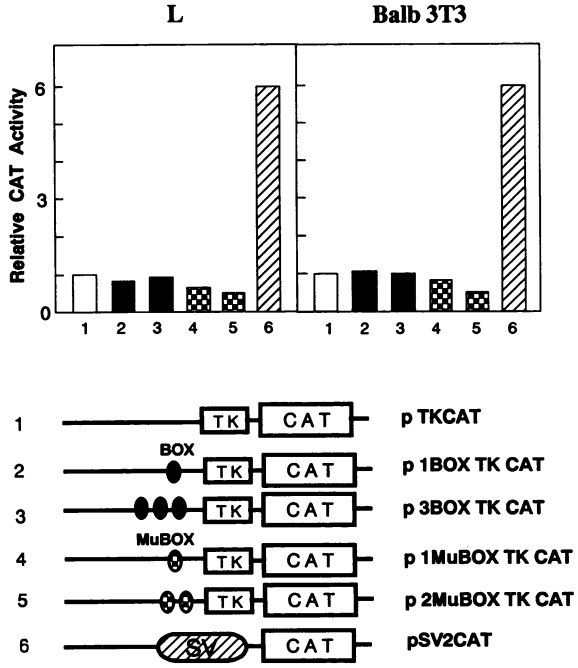


FIG. 2. Transcriptional activity of BOX DNA in non-EC cells. CAT assays were carried out with L and BALB/3T3 cells transfected with various CAT constructs (1 to 6). Relative CAT activities were calculated as described for Fig. 1.

stimulating efficiencies were different among the cells. Neither one nor two copies of MuBOX, on the other hand, activated transcription in any of the cells. These results suggest that BOX DNA functioned as a sequence-specific enhancer of 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 with non-EC cells. The CAT constructs used for Fig. 1 were transfected to L and BALB/3T3 cells; pSV2CAT containing the SV40 enhancer/promoter region was used as a positive control. Both the wild-type and the mutant BOX DNA scarcely activated transcription in either cell type, whereas the SV40 enhancer/promoter functioned well (Fig. 2). BOX DNA is thus considered to be an enhancer which functions specifically in EC cells and very little in non-EC cells.

The BOX DNA-protein complexes are different in EC and non-EC cells. Transcriptional regulation generally requires not only *cis*-acting DNA elements but also protein factors binding thereto. To determine whether proteins binding to BOX DNA exist in EC cells, band shift assays were performed with nuclear extracts of P19, ECA2, and F9 cells. The same BOX DNA oligonucleotides as inserted in the CAT constructs (see Fig. 1A) were labelled and used as probes. 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. 3, a major band of BOX DNA-protein complexes was observed with all of the EC cell extracts in absence of competitors (lane 1). When unlabelled BOX DNA was added to the reaction, the band was de-

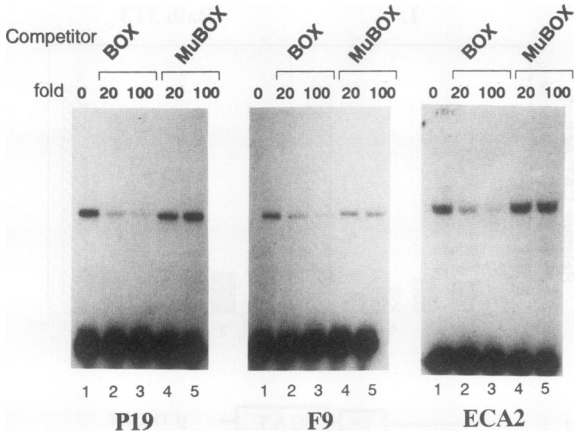


FIG. 3. The BOX DNA-binding proteins in various EC cells. Band shift assays were performed by using BOX DNA as a probe with nuclear extracts prepared from P19, F9, and ECA2 cells. Different amounts of unlabelled BOX DNA or MuBOX DNA were added to some reactions as competitors to assess specificity of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

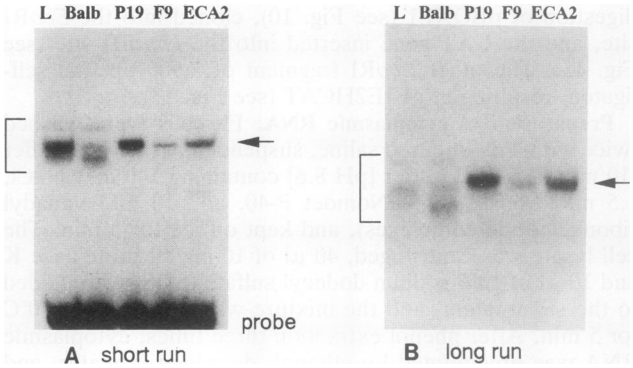


FIG. 4. Comparison of BOX DNA-binding proteins in EC and non-EC cells. The same protein amounts of nuclear extracts from L, BALB/3T3, P19, F9, and ECA2 cells were reacted with the BOX DNA probe and separated in the same gel. The patterns after a short (A) and a long (B) run of electrophoresis are shown. 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 panel B, the free probe had run out of the gel.

creased 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 factors present in EC cells bind specifically to BOX DNA and that the factors may contribute to transcriptional stimulation due to the sequence.

BOX DNA did not function as an enhancer in L and BALB/3T3 cells. One possibility is that the BOX DNA-binding proteins 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 were carried out with nuclear extracts from L and BALB/3T3. The same protein amounts of the extracts of L, BALB/3T3, and EC cells were incubated with the probe in parallel, and the patterns of the DNA-protein complexes were compared. The results after a short run of gel electrophoresis are shown in Fig. 4A, and those after a long run are shown in Fig. 4B, where the free probe ran out of the gel. Several complexes on BOX DNA were also observed in L and BALB/3T3 cells. The mobilities of these complexes, however, were distinct from that of the EC-BOX DNA complex and also different from those of complexes in L and BALB/3T3 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 BALB/3T3 nuclear extracts (Fig. 5). Excess amounts of unlabelled BOX DNA abolished the DNA-protein complexes, while unlabelled MuBOX DNA did not. The results indicate that proteins 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 proteins are not involved in transcription or 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 2 days after induction with 0.5 to 1 μM RA (34) and into a variety of cell types, including neurons, astroglia, and smooth muscle cells, 7 days after induction by cell aggregation in the presence of RA (15, 16, 29, 33). ECA2 cells differentiate into epithelial, neuronal, and myoblast-like cells in 7 days after induction with 1 μM RA (40). F9 cells can differentiate into parietal endoderm-like cells at 5 days after induction with 0.1 nM RA and 1 μM dibutyryl cAMP. pTK CAT, p3BOXTKCAT, p2MuBOXTKCAT, and pSV2CAT were transfected to these EC cells before and 3 days after differentiation induction. The cells were harvested 24 h after transfection, and CAT assays were performed. As shown in Fig. 6, the enhancer activity of BOX DNA, 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 proteins could not recognize in vitro, did not stimulate the TK promoter activity in either undifferentiated or differentiated cells (lane 3). Similar results were obtained

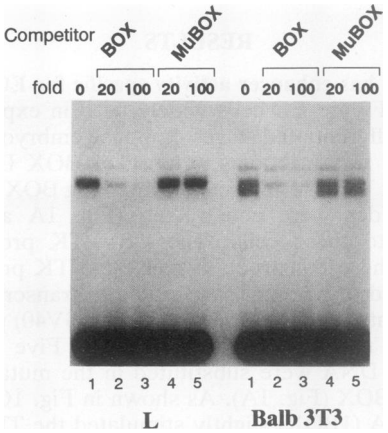


FIG. 5. BOX DNA-protein complexes in non-EC cells. Band shift assays using nuclear extracts prepared from L and BALB/3T3 cells were carried out as described for Fig. 3.

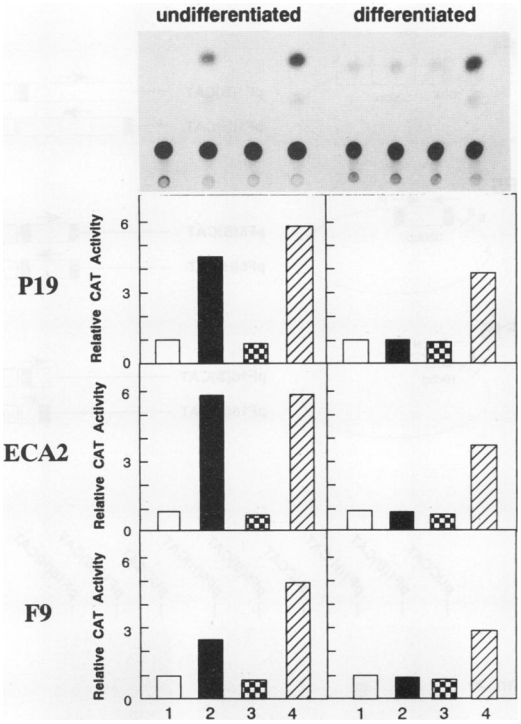


FIG. 6. Decrease of the enhancer activity of BOX DNA in differentiated EC cells. Several CAT constructs were transfected together with pCMVβ-galactosidase to various EC cells before and after differentiation induction as described in Materials and Methods. Typical autoradiograms of the results for P19 cells are shown at the top. CAT activities were calculated on the basis of pTKCAT in each state. Columns: 1, pTKCAT; 2, p3BOXTKCAT; 3, p2MuBOX TKCAT; 4, pSV2CAT (see diagrams in Fig. 2).

when transfection was carried out with EC cells 1 day after differentiation induction and also with P19 cells induced by aggregation and 1 μM RA into an 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 with 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 reference 8). As shown in Fig. 7, 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 a transcription factor involved in the differentiation process of EC cells.

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 (1). The origin of BOX DNA is unknown but has been speculated to be a cellular DNA. To identify target genes of

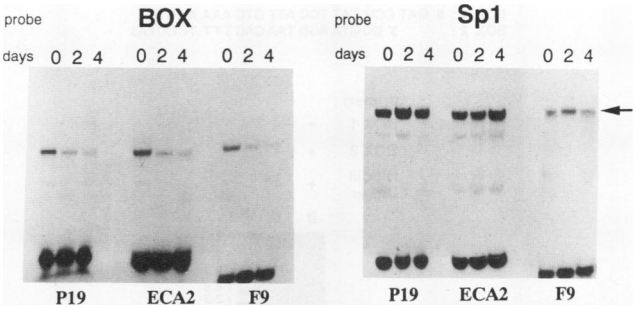


FIG. 7. Reduction of BOX DNA-protein complexes during EC cell differentiation. Nuclear extracts were prepared from various EC cells before (0 day) and 2 or 4 days after differentiation induction. Equal protein amounts of each extract were reacted with labelled BOX DNA or Sp1 probe.

the BOX DNA-binding protein(s), we cloned 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. 8. Briefly, total DNA from F9 cells was digested with *Hin*dIII, and the fragments were subjected to PCR amplification using BOX DNA and *Hin*dIII linker as primers. The amplified DNAs were analyzed by agarose gel electrophoresis (Fig. 9). Several bands were observed in the reactions with both combinations of primers (BOX DNA 1-*Hin*dIII linker and BOX DNA 2-*Hin*dIII linker). Southern hybridization using BOX DNA as a probe revealed that all of

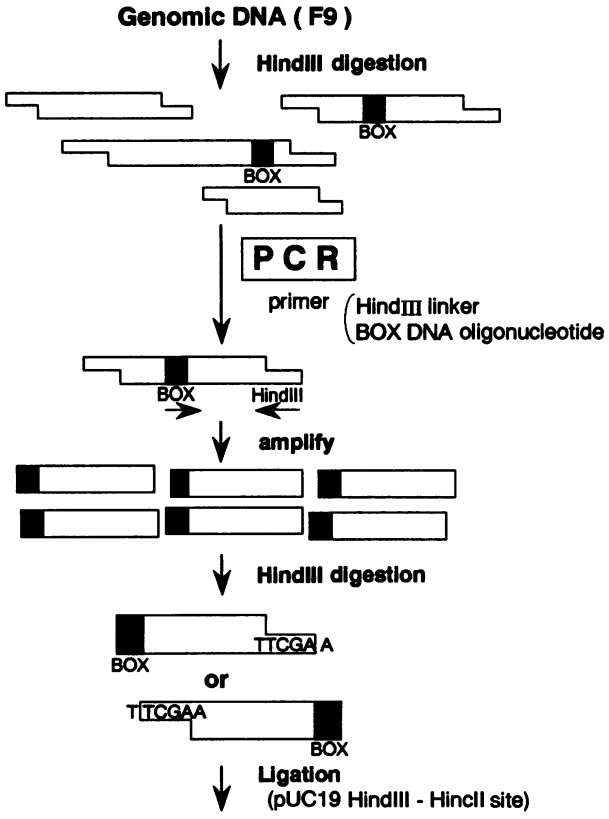


FIG. 8. The PCR-mediated procedure for cloning genomic DNA containing BOX DNA in F9 cells.

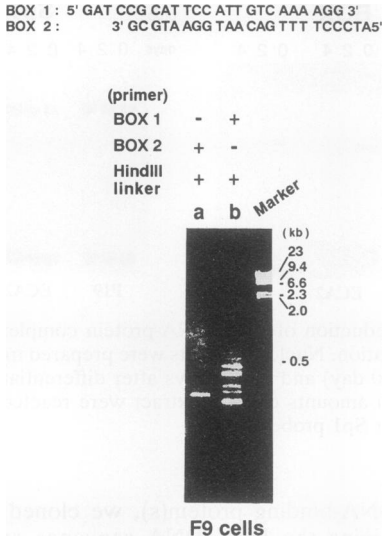


FIG. 9. Detection of genomic DNAs containing the BOX DNA sequence in F9 cells. DNA fragments containing the BOX DNA sequence were amplified among the *Hin*dIII-digested F9 genomic DNAs by PCR, using either BOX 1-*Hin*dIII linkers or BOX 2-*Hin*dIII linkers as primers. The amplified DNAs were analyzed by agarose gel electrophoresis. Marker indicates *Hin*dIII-digested λ phage DNA, and sizes are shown on the right.

the amplified DNA fragments contained the BOX DNA sequence (data not shown). The results suggest that the BOX DNA sequence exists in several copies in F9 genomic DNA.

The DNA fragments thus amplified were cloned into pUC19. Three clones described in Fig. 10 (pBOXF1, pBOXF5, and pBOXF16) were obtained. 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 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. 10A). pUCCAT, carrying the enhancer/promoter-less CAT gene alone, was used as a negative control. The constructs were transfected into undifferentiated F9 cells, and the CAT assay was carried out. As shown in Fig. 10, only pF1(H)CAT, in which the *Hin*dIII end of BOXF1 is adjacent to the CAT gene, showed 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. 11).

To determine the transcription initiation site in BOXF1, S1 mapping was performed with F9 cytoplasmic RNA. The segment of 332 bp from the *Spe*I site to the *Hin*dIII site of BOXF1(H), covering the TATA-like sequence and the Oct motif, was end labelled at the *Hin*dIII site and used as a probe. The transcription initiation site was detected at 80 bp upstream from the *Hin*dIII site, i.e., 180 bp downstream of the TATA-like sequence in BOXF1 (Fig. 12). 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 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. 13A). The CAT activities of the constructs were compared in P19 cells before and after differentiation. In undifferentiated P19 cells, pF1(H)CAT possessed transcriptional activity more than twice as strong as that of pTKCAT, and the activity was greatly reduced by deletion of BOX DNA (pF1delBCAT). Further deletion of the Sp1 motif (pF1delBSCAT) and the TATA-like and Oct motifs (pF1E2HCAT) resulted in only slight activity. BOX DNA and the Sp1 motif alone (pF1E1ECAT) did not yield significant activity compared with pUCCAT. A promoter required for 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 that of pTKCAT, and the activity was little affected by deleting BOX DNA (Fig. 13B; 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 is less effective in differentiated cells. The gene coded downstream from BOXF1 may be one of the target

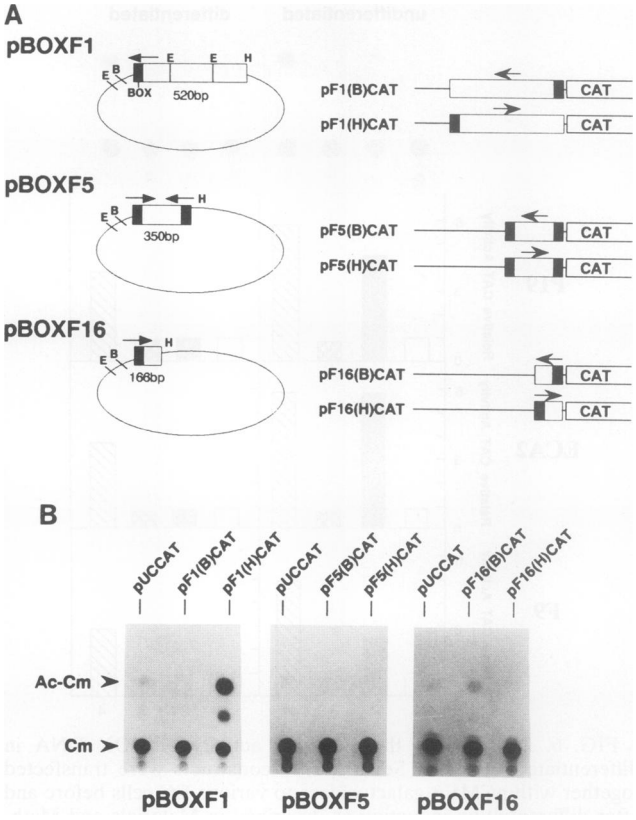


FIG. 10. Promoter activities of F9 DNA fragments containing BOX DNA. The fragments of F9 genomic DNA containing the BOX DNA sequence (BOXF1, BOXF5, and BOXF16) were linked to the CAT gene in either orientation [pF1(B)CAT, pF(1H)CAT, pF5 (B)CAT, pF5(H)CAT, pF16(B)CAT, and pF16(H)CAT]. The constructs were transfected into F9 cells, and CAT assays were carried out. (A) Schematic drawing of the pUC19 clones of the F9 DNA fragments containing BOX DNA (left) and of the CAT constructs (right). B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. (B) Autoradiographs of the CAT assays. Cm, chloramphenicol; Ac-Cm, acetylated chloramphenicol.

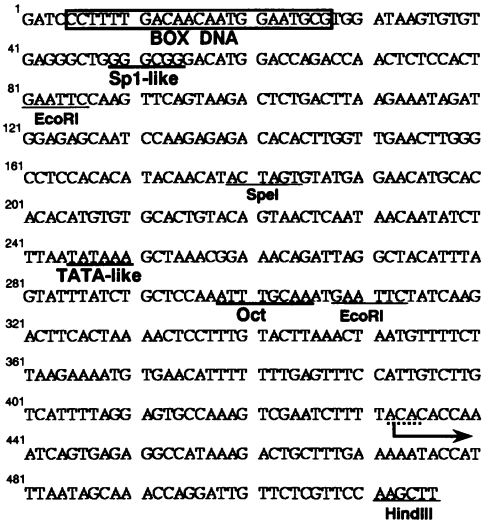


FIG. 11. Nucleotide sequence of BOXF1. BOXF1 consists of 511 nucleotides. The BOX DNA sequence is boxed. Bold or plain underlines indicate the transcriptional regulatory motifs (Sp1-like, TATA-like, and Oct motifs) or the restriction sites, respectively. A dotted underline and an arrow show the initiation site and the direction of transcription deduced from S1 mapping (Fig. 12).

genes of a BOX DNA-binding protein(s) and may be involved in EC cell differentiation.

DISCUSSION

EC cell differentiation is correctly controlled by a complicated network of various genes in definite stages of the process. Expression of these genes is 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 EC cell differentiation. Although changes in their expression levels have been well studied, their roles in EC cell differentiation are little understood.

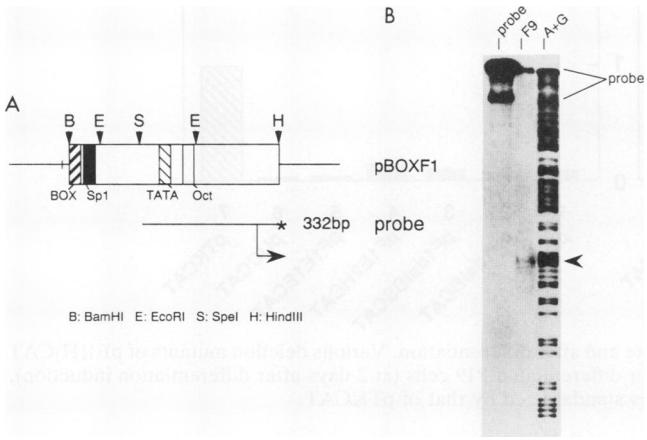


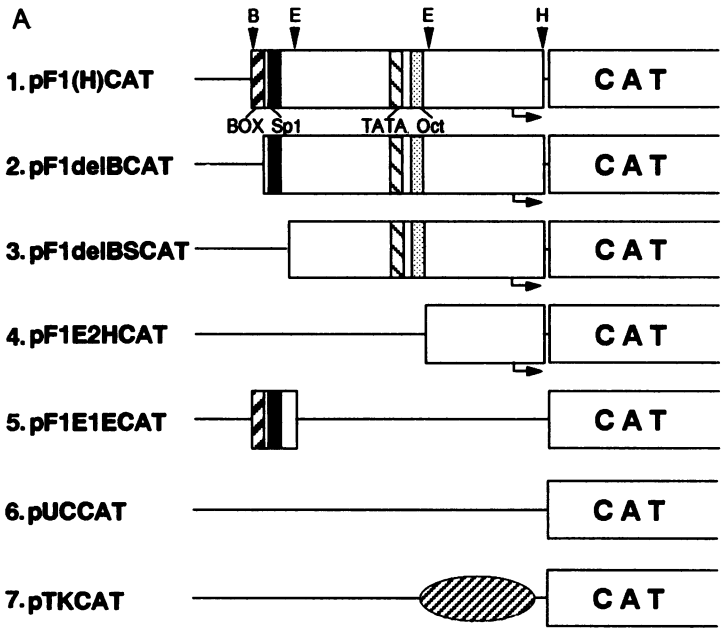
FIG. 12. Determination of the transcription initiation site in BOXF1 by S1 mapping analysis. (A) Structure of BOXF1. Putative sequences for transcriptional regulation, including BOX DNA, are shown. Triangles above indicate recognition sites for restriction enzymes. The *Spe*I-*Hin*dIII fragment of 332 bp (underlined) was labelled at the *Hin*dIII site (asterisk) and used as a probe. (B) Result of S1 mapping. Cytoplasmic RNAs from F9 cells were subjected to 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. Positions of the input probe and the S1-protected band (arrowhead) are indicated at the right.

Here we report on BOX DNA, whose binding proteins would be novel transcription factors involved in EC cell differentiation. BOX DNA functioned as a specific enhancer of 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 of transcription as well as of viral DNA replication. The silencer activity was observed upon transcription from the SV40 or polyomavirus promoter in F9-28, and F9 clone expressing polyomavirus large T antigen (2, 3). 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 cells. Viral T antigen expressed in this cell line might be responsible for the differences.

It was recently reported that several transcription factors function positively or negatively in cell type- or promoter-dependent manners (24). For instance, positive and negative effects of the Oct motif on different promoters are observed in F9 cells. It functions as an enhancer of the HSV TK promoter but as a silencer of the c-*fos* promoter in the cells (23). 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 cells, when E6 was linked to the SV40 enhancer (41). Similarly, BOX DNA may or may not be bifunctional in different situations. Except in the case of F9-28 cells, BOX DNA is considered to be an undifferentiated EC cell-specific enhancer.

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 cells (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 maintenance of the undifferentiated state. The loss or decrease of their activity may therefore result upon promotion of EC cell differentiation. These results combined with those for non-EC cells (discussed below) suggest that the DNA-protein complexes observed in undifferentiated EC cells are required, but not sufficient (as in the case of F9-28 cells), for the enhancer activity of BOX DNA.

BOX DNA-binding proteins were also present in L and BALB/3T3 cells, in which BOX DNA did not activate transcription. In band shift assays, the mobilities of the BOX DNA-protein complexes in these non-EC cells were different from those in EC cells. It is reported that not only the core binding but also coactivators play important roles in potentiation of transcriptional activation in the cases of Oct-2 (26)



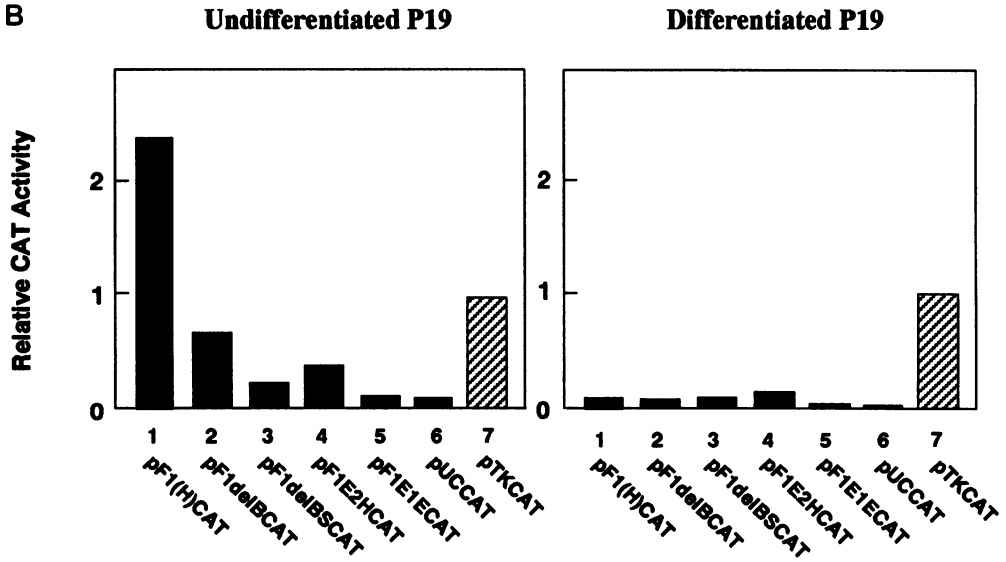


FIG. 13. Effect of BOX DNA on the BOXF1 promoter in EC cells before and after differentiation. Various deletion mutants of pF1(H)CAT (schematically shown in panel A) were transfected into undifferentiated or differentiated P19 cells (at 2 days after differentiation induction), and CAT assays were performed as in Fig. 1. (B) Relative CAT activities standardized by that of pTKCAT.

and Oct-4 and Oct-5 (36). Several possibilities are considered for the inactivity of BOX DNA as an enhancer in non-EC cells. First, the proteins that bind to BOX DNA in EC and non-EC cells are distinct, being functional in EC cells an nonfunctional in non-EC cells. Alternatively, cooperative factors required for the activity may be absent or inactive in non-EC cells, although the same BOX DNA-binding proteins are present. Another possibility is that in non-EC cells, the BOX DNA-binding protein(s) themselves are constitutively inactive, or the modifying enzymes to activate them may be lacking.

To identify the BOX DNA-binding proteins, we carried out Southwestern (DNA-protein) analyses. However, we could not recognize the bands because of the specific protein(s) bound to BOX DNA. Possibly, the BOX DNA-binding proteins cannot be renatured during transfer from the SDS-polyacrylamide gel to the nitrocellulose filter. To analyze the properties and functions of BOX DNA, purifi-

cation of the BOX DNA-binding proteins followed by cloning of the cDNA encoding them should be performed.

What genes are the cellular targets of the BOX DNA-binding protein(s)? 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 *Hin*dIII linker as primers, we searched for the genes containing BOX DNA sequences in 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 one copy per 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. These results indicate that BOXF1 belongs to the enhancer/promoter region of a target gene for BOX DNA-binding proteins and that the gene may be involved in EC cell differentiation. Cloning and characterization of the gene, as well as of cDNAs encoding BOX DNA-binding proteins, would provide a new approach for analyzing the mechanisms of cell differentiation.

ACKNOWLEDGMENTS

We thank T. Tsukiyama and T. Atsumi for providing ECA2 cells and P19 cells, respectively, and S. Sakai and S. Imakado for plasmid pBLCAT2. We are grateful to S. M. M. Iguchi-Ariga and I. Galli for critical reading of the manuscript, K. Takaya for technical assistance, and H. Kawasaki for continuous encouragement.

This work was supported by grants from the Ministry of Science, Culture and Education, Japan, and from the Kowa Life Science Foundation.

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