One of two Ets-binding sites in the cytokeratin EndoA enhancer is essential for enhancer activity and binds to Ets-2 related proteins

Yasuo Fujimura, Hideyuki Yamamoto+, Fumiaki Hamazato§ and Masami Nozaki*
Department of Microbial Genetics, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan

Received November 3, 1993; Revised and Accepted January 25, 1994

ABSTRACT

Expression of the mouse cytokeratin EndoA gene is restricted in endodermal and epithelial cells, and is regulated by an enhancer that is located 1 kilobase (kb) 3' downstream from the gene. The enhancer consists of six direct repeats, of which each contains two predicted Ets binding sites (EBS1 and EBS2) containing GGAA as a core. Mutation analysis showed that EBS1 is essential for the enhancer activity and additional effects of EBS2, suggesting that some Ets-related proteins bind and activate the enhancer through EBS1. We also showed that Ets-2 mRNA is expressed in PYS-2 cells and that Ets-2 protein produced by E. coli interacts with EBS1 but not with EBS2. Using co-transfection assays, we showed that Ets-2 can trans-activate the enhancer in PYS-2 cells. Mutations that impair Ets-2 binding abolish the activity of the EndoA enhancer. The results obtained from the binding competition assay using an Ets-2 specific antibody, however, suggest that EBS1 binds to an Ets protein which is distinct from Ets-2. These data show that Ets-2 related protein binds and activates the EndoA enhancer in a sequence-specific fashion.

INTRODUCTION

Mouse cytokeratin EndoA is a member of the type-II keratin subfamily, that constitutes keratin intermediate filaments with a type-I keratin partner, EndoB or EndoC, in simple epithelial cells during embryogenesis and in adults (1–4, unpublished data). The EndoA gene is first expressed in the 8-cell stage embryo and in trophoderm, which is one of two distinct cell types emerging during the first differentiation process, but not in the inner cell mass. Subsequently, the EndoA gene is expressed in endodermal cells derived from inner cell mass cells, but not in ectodermal cells (5,6). Thus, the study of EndoA gene expression should provide insight into the mechanisms of early differentiation as well as into epithelial differentiation. Previously, we showed that the tissue specific enhancer is located 1 kb downstream of the gene. The EndoA enhancer activity is prominent in endodermal cells, in which the EndoA gene is also expressed. The nucleotide sequence of the EndoA enhancer consists of six direct repeat units of 22 base pairs (bp), with two Ets binding sites (EBSs) (7,8). We also found that the products of Ets-1 bind each unit in the EndoA enhancer (9), suggesting that Ets is involved in regulation of the EndoA gene.

The ETS gene family comprises sequences related to the v-ets oncogene sequence from the avian erythroblastosis virus E26, a retrovirus that causes erythroblastosis and myeloblastosis in chickens (10,11). The proto-oncogene c-ets encodes a DNA binding protein (12) and is found in a wide variety of animals including Drosophila, sea urchins, Xenopus, chickens, mice, and humans. In each species there are several ets genes (13,14). Among these, Fli-1, PU.1, Elf-1 and ets-1 are preferentially expressed in lymphoid cells (15–19) and GABPa, SAP-1 and ets-2 are expressed in a wide variety of tissues (20–23). In Drosophila (24,25), sea urchins (26), and Xenopus (27,28) mRNAs of some of the ets family including ets-2 are present in eggs and constitutively expressed during early developmental stages at an elevated level. Moreover, introducing antisense oligonucleotides to the ets-2 sequence into Xenopus oocytes blocked germinal vesicle breakdown, suggesting the involvement of ets-2 in oocyte maturation (27). It has therefore been proposed that the ets family genes are intrinsically linked to development. Thus, the Ets product may also play a role in the mouse embryo, although the expression profile of ets mRNA during early mouse development has not been examined.

We report here that EBS1 is essential for EndoA enhancer activity and Ets-2-related protein binds EBS1 in endodermal PYS-2 cells.

MATERIALS AND METHODS

Cloning of the Ets-2 cDNA

The Ets-2 cDNA was amplified by means of reverse transcriptase polymerase chain reaction (RT-PCR) (9,29) using total RNA extracted from the mouse thymus with acid guanidinium
Plasmid construction
The basic CAT plasmid, p150CAT, was prepared as follows: A minimal EndoA promoter sequence (−150 to +79) generated by PCR was fused to the HindIII site in the CAT gene of pSV0CAT (31). Wild-type and mutant oligonucleotides were kinased, mixed with BamHI adapter, ligated and digested with BamHI. Six copies of direct repeated oligonucleotides were selected and inserted into the BamHI site beyond the termination site of the CAT gene in p150CAT, and used as reporter plasmids (6WT, 6M1, 6M2, 6M3 and 6M4). The open reading frame of the mouse Ets-2 described above was cloned into BamHI site of pSG5, which was used as the effector plasmid (pSG5-Ets-2).

Chloramphenicol acetyltransferase (CAT) assay
Cell culture and transfection were performed as described (7,32). Three micrograms of reporter plasmids, 3 μg of internal control pRSV-gal (33) or pact-/β-gal (9) and 9 μg of effector plasmids (pSG5-Ets-2) were transfected into PYS-2 cells.

Electrophoresis mobility shift assays
Mobility shift assays were performed essentially as described (34). Glutathione S-transferase and the DNA binding domain of Ets-2 fusion protein (GST-Ets-2D) was synthesized in E. coli and purified as described (35). Nuclear extract was prepared as described (36) with minor modifications. Briefly, cells were scraped into phosphate-buffered saline, and collected by sedimentation at 500 × g in a refrigerated centrifuge. The cells were resuspended and lysed in buffer 2 (10 mM Hepes, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 5 mM MgCl2). The nuclei then were pelleted at 1500 × g for 5 min in a 50 ml of centrifuge tube (CORNING). The pellet was resuspended in buffer 2 at 108 nuclei per ml. Spermidine was added to a final concentration of 5 mM and NaCl to 0.3 M. The suspension was incubated on ice for 30–60 min, then transferred to an Eppendorf tube and centrifuged for 10 min in a microcentrifuge. The supernatant was dialyzed against buffer 3 (10 mM Hepes, pH 7.9, 50 mM dithiothreitol, 50% glycerol, 50 mM NaCl) overnight, divided into aliquots and stored at −70°C until use. Buffers 2 and 3 contained 0.2 mM (γ-32P)dATP end-labeled probe (3.2 × 108 cpm/μg) was added and incubated for 30 min at 25°C. The reaction mixtures were loaded and electrophoresed on a 5% polyacrylamide gel. For incubation with antibody, ets-2 specific antisera (Cambridge Research Biochemicals) was added to the binding reactions without labeled probes for 30 min at 4°C. The probe was then added and incubated for a further 30 min at 25°C before loading the gel.

Northern hybridization
Total RNA was prepared from PYS-2 cells by acid guanidium thiocyanate-phenol-chloroform extraction (30) and eluted through oligo(dT)-cellulose. Poly(A)+ RNA was fractionated by electrophoresis through a 1% agarose gel containing formaldehyde, transferred onto Nitroplus 2000 (Micron Separations Inc.) and analyzed by hybridization with random primed 32P-labeled cDNAs (4 × 108 cpm/μg).

RESULTS
The Ets binding sequence (EBS) in each unit of the EndoA enhancer is essential for the enhancer activity
The EndoA enhancer consists of six direct repeated units of 22 bp, containing the two Ets binding core sequences, GGAA, as shown in Fig.1. The activity of the enhancer is remarkable in parietal yolk sac endoderm like PYS-2 cells, in which the EndoA gene

---

Figure 1. Sequence of the oligonucleotide and structure of the reporter plasmids used for CAT assay. (A) Sequences of wild-type and mutant oligonucleotides. EBS1 and EBS2 represent two predicted Ets-binding sites. Asterisks indicate base substitutions. WT, oligonucleotide corresponding to the 22 bp unit. M1, oligonucleotide corresponding to the sequence mutated in both of EBS1 and EBS2. M2, M3 and M4 are oligonucleotides corresponding to the sequences mutated in EBS2, EBS1 and the flanking sequence, respectively. (B) Structure of the reporter plasmid. The thin line represents the plasmid backbone. Hatched box represents the Ets binding region inserted at HindIII site and open box shows the CAT coding region (p150-CAT). Angular arrow indicates the transcriptional initiation site. Short arrows indicate six tandem repeats of oligomers described in (A).
is highly expressed. To determine whether the two EBSs were essential for enhancer activity, we analyzed four substitution mutations (Fig. 1) at these sites, by means of transfection assays in PYS-2 cells. The M4 mutation did not significantly affect the activity from WT. On the other hand, the M2 mutation reduced, but did not abolish, enhancer activity. The decrease in activity was about 30% relative to WT. M1 and M3 mutations, however, decreased the activity to background levels (Fig. 2). Thus, EBS1 was essential for enhancer activity, whereas EBS2 contributed quantitatively to raise the amount of transcription, whereas the flanking sequence had no effects. These results showed that EBS1 to which some ETS family members have the potential to bind, is important for enhancer activity.

**Ets-2 mRNA is expressed in PYS-2 cells**

The expression of *ets* family genes including *ets-2* during early development in organisms other than mice has been reported (24–28). Levels of Ets-2 expression are high in mouse organs during development and are detectable in all organs of adult mice. The Ets-2 mRNA is present at low levels in undifferentiated F9 cells and increases during differentiation (38). To determine which of the Ets family is expressed in endodermal cells, we examined PYS-2 cells by Northern blotting using the Ets-1 and Ets-2 cDNAs corresponding to the open reading frame as probes. We detected a single 3.5 kb RNA band using Ets-2 cDNA as a probe in RNA isolated from PYS-2 cells (Fig. 3), which is consistent with the reported size of the mouse Ets-2 mRNA (22, 38). On the other hand, we could not detect a band corresponding to Ets-1 mRNA under the same conditions (exposure time, 14 hr). However, we detected two RNA species of about 5 and 3 kb, from the Ets-1 gene in PYS-2 mRNA after a 7 day exposure (data not shown). The larger Ets-1 product is consistent with the reported size of the murine Ets-1 transcript (22, 38, 39) and the lower band probably corresponds to a minor product found in the thymus and ovary (22, 39). These results showed that the level of Ets-2 mRNA is more than 10-fold higher than that of Ets-1 mRNA in PYS-2 cells.

**The Ets-2 DNA binding domain preferentially binds to EBS1 in the EndoA enhancer**

To investigate whether Ets-2 can bind directly to the EndoA enhancer, a 22 bp wild type enhancer fragment was end-labeled and used in a mobility shift assay with a recombinant GST-Ets-2D fusion protein. As shown in Figure 4 (lane 2), Ets-2 protein bound to a 22 bp unit of the EndoA enhancer with very high affinity. This binding was sequence specific because the mobility shift was efficiently blocked by competition with the same unlabeled sequence (Fig. 4, lanes 3–6). It was unlikely that the GST region in this fusion protein affected the binding, since no bands were detected using purified GST protein (Fig. 4, lane 1). We concluded that Ets-2 binds specifically to the 22bp fragment. To identify further important sequences within the 22 bp which are responsible for Ets-2 binding, we performed competition studies...
Figure 5. Gel shift analysis of Ets-2 using mutated oligonucleotides as probes. Wild-type (lanes 1, 2 and 3) and mutant (M1, lanes 4 and 5; M2, lanes 6 and 7; M3, lanes 8 and 9; M4, lanes 10 and 11) oligomeric probes were 32P-labeled and incubated with increasing amounts of GST-ETS-2D (none, lane 1; 10 ng, even numbered lanes; 100 ng, odd numbered lanes).

Figure 6. Trans-activation of the EndoA enhancer by Ets-2. The reporter described in the legend to Fig. 2 was co-transfected with the effector plasmid pSG5-Ets-2 or with pSG5. CAT activity is represented as described in the legend to Fig. 2. Closed and stippled boxes represent the activities of pSG5 and pSG5-Ets-2, respectively. The standard deviation is represented as vertical bars. All experiments were performed three times with two separately prepared DNAs.

Figure 7. Gel shift analysis of nuclear extract from PYS-2 cells. (A) Competition assays with mutant oligonucleotides. Gel shift analyses were performed using the labeled WT oligonucleotide as a probe and either without (lane 1) or with (lanes 2–17) nuclear extract. Assays contained either no competitor (lane 2), or WT (lanes 3–5), M1 (lanes 6–8), M2 (lanes 9–11), M3 (lanes 12–14) or M4 (lanes 15–17). A molar excess of unlabeled competitor DNA (lanes 3, 6, 9, 12 and 15, 5-fold; lanes 4, 7, 10, 13 and 16, 50-fold; lanes 5, 8, 11, 14 and 17, 500-fold) is shown. The shift bands are indicated by an arrowhead at right. (B) Nuclear extracts were incubated in the anti-Ets-2 antibody. Gel shift analyses were performed using the labeled WT oligonucleotide as a probe, and with no protein (lane 1), GST-Ets-2D (lanes 2 and 3) and nuclear extracts (lanes 4 and 5). Prior to the addition of the labeled probe, anti-Ets-2 antibody (Ab) was incubated (lanes 3 and 5). The shift bands are indicated by arrowheads as in (A).

Ets-2 activates the EndoA enhancer
The binding of the Ets-2 DNA binding domain to the EndoA enhancer suggests that Ets-2 or related proteins, which share same DNA-binding specificity, function in transcriptional control through the EndoA enhancer. We directly tested whether Ets-2 activates the EndoA enhancer. We co-transfected the EndoA promoter-CAT-enhancer (p150CAT-enhancer) containing six repeated wild-type (WT) or mutated-type (M1–M4) oligonucleotides as reporter and an effector plasmids, respectively, containing Ets-2 cDNA in pSG5 (pSG5-Ets-2) into PYS-2 cells, and measured the CAT activity. The activity of WT was increased 2.5-fold when the Ets-2 expression plasmid was included in the assay instead of a control (Fig. 6, WT). Furthermore, trans-activation required EBS1 (Fig. 6, M2 and M4) and no activity was seen in the presence of the six corresponding EBS1 mutant oligonucleotide repeats (Fig. 6, M1 and M3). These results provided evidence that the product of the Ets-2 proto-oncogene binds and activates the EndoA enhancer through the EBS1.

An Ets-related protein distinct from Ets-2 binds to EBS1 of the EndoA enhancer unit in PYS-2 cells
As shown above, Ets-2 products bind and trans-activate the enhancer through the EBS. To determine whether Ets-related proteins actually bind to the EBS1 in PYS-2 cells in which the enhancer is active, a gel shift analysis using a nuclear extract was performed. As shown in Fig. 7A, four retarded bands were identified (Fig. 7A, lane 2) and designated B1 to B4. The specificity of each was examined by competition with various wild (WT) or mutant (M1 to M4) oligonucleotides, of which sequences are shown in Fig. 1. All of these bands are efficiently competed by an excess of an unlabeled WT fragment (Fig. 7A, lanes 3 to 5). Band B1 was competed by either WT or mutants,
indicating that it is nonspecific. Since B2 is competed by WT, not by mutant fragments (M1 to M4), the B2 complex requires extending region of 22 bp sequence. The B3 band is efficiently competed by WT, mutant M2 and M4 fragments, all of which include intact EBS1 (Fig. 7A, lanes 3 to 5, 9 to 11 and 15 to 17). Competition with the M3 fragment also eliminates B3, but less effectively than M2 and M4 (Fig. 7A, lanes 12 to 14). The M1 fragment does not compete with B3 (Fig. 7A, lanes 6 to 8).

Therefore, Ets-related protein binds EBS1, generating the B3 complex, which correlates with the enhancer activity.

To determine whether or not the factor in B3 complex was Ets-2, a gel shift analysis was performed in the presence of Ets-2 specific antibody (Fig. 7B). This antibody shifted the Ets-2/DS/DNA complex (Fig. 7B, lanes 2 and 3), indicating that the antibody actually reacts mouse Ets-2 protein. On the other hand, the antibody neither inhibited the formation of B3 nor shifted the position of the complex to a higher-molecular-weight region (Fig. 7B, lanes 4 and 5), suggesting that the specific Ets-2 epitope recognized by the antibody is not present in the factor.

**DISCUSSION**

We demonstrated that one (EBS1) of two predicted Ets-binding sites (EBSs), in the EndoA enhancer is necessary for the enhancer activity in endodermal PYS-2 cells. Mutation analyses also demonstrated that the other EBS, EBS2, additionally affects the enhancer activity of EBS1. Substitution mutations, such as changing GGAA to AGTA, should abolish the binding of any ETS family members, because GGAA/T is essential for binding all ETS family members examined (40). These data suggested that some Ets family members expressing in PYS-2 cells are involved in the regulation of the EndoA enhancer activity through EBS1. We also found that Ets-2 mRNA is expressed in PYS-2 cells and mutation analyses demonstrated that Ets-2 protein synthesized in E.coli specifically binds EBS1 but not EBS2 in vitro. Moreover, trans-activation the enhancer through EBS1 by Ets-2 was demonstrated, and a mutation in EBS2 quantitatively affects Ets-2 binding and the activation of EBS1. The data imply that Ets-2 is a trans-acting factor for regulating the EndoA enhancer in vivo. Nevertheless, we showed by gel shift analysis with nuclear extract of PYS-2 cells that Ets-related protein, in which specific Ets-2 epitope recognized by the antibody tested is not present, binds to the EBS1 in PYS-2 cells. These data suggested that Ets-related protein, which has the same binding specificity as Ets-2 but is not Ets-2, binds the EndoA enhancer and regulates its activity in vivo.

There are many Ets containing a similar DNA binding region that binds a motif similar to that of Ets-2. Binding sequences recognized by the products of Ets-1, ERG, PEA3 and ELK1 show more than 80% homology with EBS1, 5'-ACAGGAAGTA-3' (40). The endodermal activity of the EndoA enhancer is likely to be regulated by a cell-type-specific factor interacting within the enhancer region, because enhancer activity is remarkable in endodermal cells but weak in other cell types (7, unpublished data). Recently, we identified Elk-1, New Ets and Elf-1 as well as Ets-2 expressed in PYS-2 cells (unpublished data). Therefore, some of these may be intrinsic factors affecting the enhancer activity.

Since both EBSs include GGAA as a core sequence, the cause of the preferential binding of Ets-related factors as well as Ets-2 to EBS1 should be due to variety of adjacent sequences around the GGAA core. Previous reports showed that ETS family members bind similar but not identical sequences except that GGAA/T is the core (40). Because Ets-2 and Ets-1 are nearly identical within their ETS domains (82 of 85 amino acids are identical; 41,42), we predicted that both would bind identical EBS. The sequence of EBS2, 5'-GTAGGAACAG-3' is very similar to the EBS in the HIV-2 LTR, 5'-ACAGGAACAG-3' (8 of 10 nucleotides are identical), which binds to Elf-1 but not to Ets-1 (43). Thus indicates that EBS2 has the potential to bind to other ETS family members including Elf-1, although no trans-acting factors were detected that bound EBS2 in PYS-2 cells.

The Ets-2 mRNA was detected in PYS-2 cells by Northern blotting, but we could not detect a gel shift band recognized by an Ets-2 specific antibody. These results indicated that either Ets-2 protein is synthesized at extremely low level, or Ets-2 products can not bind EBS1 in PYS-2 cells. Since the Ets-2 DNA binding domain synthesized in E.coli binds EBS1 in vitro, Ets-2 products can intrinsically bind EBS1. On the other hand, expression of Ets-2 activated the EndoA enhancer as determined by cotransfection analysis in PYS-2 cells (in this study) and in other some cell lines (unpublished data), although activation is at a relatively low level (2- to 3-fold). These results indicated that, although Ets-2 protein is synthesized at a low level in PYS-2 cells, other Ets-related proteins are synthesized sufficiently high levels and preferentially bind EBS1. Thus, a complete understanding of the regulatory mechanisms of the EndoA enhancer activity awaits identification of the Ets-related proteins that are distinct from Ets-2, which preferentially bind EBS1 in PYS-2 cells.

**REFERENCES**