

# Functional and Cooperative Interactions between the Homeodomain PDX1, Pbx, and Prep1 Factors on the Somatostatin Promoter\*

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**Expression of the somatostatin gene in endocrine pancreatic cells is controlled by several regulatory *cis*-elements located in the promoter region. Among these, the adjacent UE-A and TSE<sub>I</sub> elements, located from -113 to -85 relative to the transcription initiation site, function in combination and act as a pancreas-specific mini-enhancer. The TSE<sub>I</sub> element is recognized by the pancreatic homeodomain factor PDX1. In the present study, we show that the UE-A element binds a heterodimeric complex composed of a Pbx factor and the Prep1 protein, both belonging to the atypical three-amino acid loop extension homeodomain family. Recombinant Pbx1 and Prep1 proteins bind cooperatively to the UE-A site, whereas neither protein can bind this site alone. Transient transfection experiments reveal that both Pbx1 and Prep1 are required to generate a strong transcriptional activation from the UE-A element when this element is inserted close to the TATA box. In contrast, in the context of the intact somatostatin promoter or mini-enhancer, Pbx1 and Prep1 alone have no effect, but they produce a drastic activation when the pancreatic homeodomain factor PDX1 is also coexpressed. Thus, the activity of the somatostatin mini-enhancer is mediated by a cooperative interaction between the Pbx-Prep1 heterodimeric complex and the pancreatic factor PDX1.**

Somatostatins are peptides of 14 and 28 amino acid residues that regulate the secretion of various hormones, including growth hormone, thyrotropin, glucagon, and insulin (1). The gene encoding the preprosomatostatin is strongly expressed in the  $\delta$  endocrine cells of the pancreas and in neurons of the hypothalamus, but its expression is also detected in other cell types, such as the D-cells of the digestive tract, the C-cells of the thyroid gland, and sensory neurons (1).

Previous studies have shown that transcription of the somatostatin gene is controlled by several *cis*-elements present in its 5' flanking sequence (see Fig. 1). A crucial activating sequence is the CRE element located between -35 and -55 relative to the transcription start site, which is recognized by the factor

CREB and other related nuclear proteins (2, 3). In addition, the activity of the somatostatin promoter in pancreatic cell lines is stimulated by two tissue-specific elements, TSE<sub>I</sub> and TSE<sub>II</sub>, located respectively at -85/-99 and at -280/-300 (4–6) (see Fig. 1). These TSEs are both recognized by the pancreatic-specific homeodomain factor PDX1 (also named STF1, IDX1, and IPF1) (6–8), which plays an important role in pancreas organogenesis (9). Whereas PDX1 binds the TSE<sub>I</sub> element as a monomer, it recognizes the TSE<sub>II</sub> element mainly as a heterodimer with the Pbx factors (6, 10). The Pbx proteins, including the proto-oncogene Pbx1 and the closely related factors Pbx2 and Pbx3, contain an atypical three-amino acid loop extension-class homeodomain and share extensive sequence homology with the *Drosophila* protein extradenticle (EXD)<sup>1</sup> (11–13). Genetic and biochemical studies in *Drosophila* have shown that EXD acts as a co-factor for the homeotic selector proteins (HOM-C) to regulate their target genes (14–17). EXD binds cooperatively with HOM-C proteins to DNA target sites, thereby increasing their DNA binding specificity (18, 19). Similarly, in mammals, the Pbx proteins interact with the Hox factors (mammalian homologs of HOM-C factors) and modulate their DNA binding activity (20–24). The conserved pentapeptide motif YPWMK present in many metazoan Hox/HOM-C proteins is necessary for the interaction with the Pbx/EXD factors (21–23). The pancreatic factor PDX1, which is very similar to the *Antennapedia* class of homeodomain proteins, also contains this pentapeptide motif, and this motif is absolutely required for the cooperative binding of PDX1 and Pbx to the somatostatin TSE<sub>II</sub> element (10). All of these results show that the function of the Pbx/EXD proteins is to act as co-factors for the Hox/HOM-C proteins but also for orphan homeodomain proteins, such as PDX1.

Full expression of the somatostatin gene in pancreatic cells results from the synergistic actions of the different *cis*-elements present in the promoter. Indeed, previous reports have shown that the activity of the TSE<sub>I</sub> element is dependent of the immediately adjacent sequence UE-A (located from -100 to -113; see Fig. 1) (4). The bipartite element UE-A/TSE<sub>I</sub> acts as a pancreatic  $\delta$ -cell-specific mini-enhancer. The UE-A site, although devoid of intrinsic activation capacity (4), the bipartite UE-A/TSE<sub>I</sub> element acts also in synergy with the nearby CRE sequence to generate high somatostatin expression levels in pancreatic cells (4, 5). The protein binding the TSE<sub>I</sub> element was identified as a monomer of the pancreatic factor PDX1 (6, 7). However, characterization of the cellular factor(s) recognizing the UE-A element has not been reported to date (25).

In the present study, we have identified the factors binding

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<sup>1</sup> The abbreviations used are: EXD, extradenticle; EMSA, electrophoretic mobility shift assay.

the UE-A element. This sequence recognizes a heterodimeric complex containing a Pbx factor and the Prep1/pKnox protein (36, 40) (referred to here as Prep1). Although these two factors cannot bind separately the UE-A element, they bind strongly when added together. Furthermore, transfection experiments show that full activation of the intact somatostatin promoter is only obtained when the three transcription factors PDX1, Pbx1, and Prep1 are coexpressed. Thus, we have been able to reconstitute the strong pancreatic-specific enhancer complex of the somatostatin gene by using the two ubiquitous factors Pbx1 and Prep1 and the pancreatic homeodomain factor PDX1.

#### EXPERIMENTAL PROCEDURES

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were carried out exactly as described previously (26). Briefly, 2  $\mu$ g of nuclear extract prepared as described by Shreiber *et al.* (27) or 1  $\mu$ l of *in vitro* translated protein was incubated with 0.1 ng of a double-stranded oligonucleotide ( $^{32}$ P-labeled using Klenow polymerase) in presence of 1  $\mu$ g of poly(dI-dC). In supershift experiments, the nuclear cell extracts were preincubated with 1  $\mu$ l of antiserum for 15 min at room temperature before adding the probe. In competition experiments, the cold oligonucleotides were mixed with the probe before addition of the nuclear extract. Competitions with the Hoxa5 peptide (QPQIYPWMRKLH) were carried out by preincubating the peptide with the protein extract for 15 min and subsequent addition to the DNA probe. The sequences of the oligonucleotides (Eurogentec, Liège, Belgium) are GATCTTCCTTGATT-GATTTTGC for the UE-A element, GATCTCTTTCCCTCCTTTGC for UE-Amut, GATCTCAGTAATTAAATCATGCAGATC for TSE<sub>II</sub>.

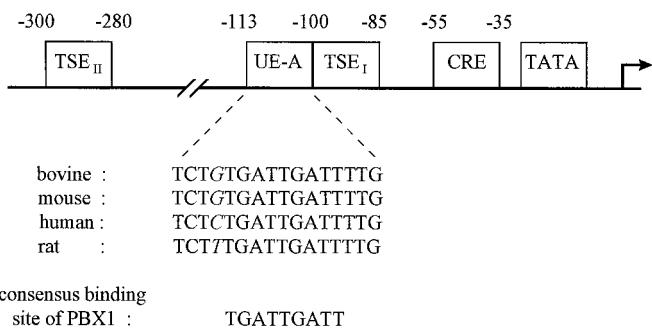
**Plasmid Construction and Expression of Proteins**—The cDNAs coding for Meis1 (28) (generous gift of Dr. Neal Copeland) and Pbx1a tagged with the Flag epitope (29) (generous gift of Dr. C. Murre) were inserted in the pCDNA3 eukaryotic expression vector. The Prep1 cDNA was amplified by PCR from human placental cDNA using the primers GGCTCGAGATGATGGCTACACAGACAT and GCGTCTAGACCTGC-CCCTACTGCAGGG, and cloned in the pCS2 expression vector that contains the bacteriophage SP6 and eukaryotic CMV promoters. The Pbx1a, Flag-Pbx1a, Meis1, and Prep1 proteins were produced *in vitro* using the Promega TNT transcription-translation system, according to the protocol of the manufacturer. PDX1 protein was expressed in *Escherichia coli* using the pGEX3X vector as described previously (26).

The reporter plasmids UE-A-Luc and UE-Amut-Luc each contain one copy of the wild type and mutated UE-A element (see above for the sequences), respectively, inserted in the *Bam*HI site of the GH32Luc plasmid. This GH32Luc vector contains the minimal growth hormone promoter (from -35 to +8 relative to the transcription initiation site) fused to the luciferase gene. The UE-A/TSE<sub>I</sub>-Luc plasmid contains one copy of the somatostatin mini-enhancer (from -120 to -80) in the *Bam*HI site of the GH32Luc vector. The pSRIF-Luc plasmid contains the somatostatin promoter sequence (from -192 to +50) directly linked to the luciferase gene.

**Cell Transfection**—Transient transfection experiments were performed in the colon carcinoma cells HCT116 using the calcium phosphate coprecipitation method. HCT116 were grown in Dulbecco's modified Eagle's medium supplemented with 10% of fetal calf serum in 4 cm dishes. DNA precipitates, containing with 10  $\mu$ g for reporter plasmid, 1  $\mu$ g of expression vector, and 1  $\mu$ g of Rous sarcoma virus- $\beta$ -galactosidase plasmid, used as an internal control, were added to the cells. 24 h after the glycerol shock, the cells were harvested, lysed, and assayed for the luciferase and  $\beta$ -galactosidase activities. Luciferase activities were normalized to  $\beta$ -galactosidase activity in each cell extract.

#### RESULTS

**The UE-A Element Is Recognized by a Cellular Complex Containing Members of the Pbx Factor Family**—Comparison of the nucleotide sequence of all known somatostatin promoters shows that the UE-A element is well conserved (see Fig. 1). The core of the UE-A element, previously identified by linker scanning mutagenesis (4), contains a TGATTGATT motif corresponding exactly to the consensus binding site of the Pbx1 homeodomain (30–32). This prompted us to investigate whether the cellular factor binding the UE-A element is Pbx1 or a member of the Pbx protein family. EMSAs were performed on the UE-A probe using nuclear extract from the somatostatin-producing TU6 cell line. Two closely migrating complexes



**FIG. 1. Sequence of the somatostatin UE-A element.** The position of the UE-A element within the somatostatin gene promoter is shown with the other identified regulatory sequences. *Below* the diagram presents a sequence comparison of the UE-A element from the bovine, mouse, human, and rat somatostatin genes together with the Pbx consensus binding site.

(Fig. 2, *S* and *L*) were observed. Both complexes were easily displaced by adding a 100-fold molar excess of unlabeled UE-A element but were not affected by addition of a UE-A element mutated in the Pbx consensus motif (Fig. 2, *lanes 2–6*). Preincubation of the nuclear extract with a polyclonal anti-Pbx antiserum (recognizing all Pbx members) produced a supershifted band and affected the formation of these two complexes, whereas an anti-PDX1 antiserum or the preimmune serum had no effect (Fig. 2, *lanes 7–9*). To confirm the presence of Pbx factors in these two complexes, we also used another antibody reacting specifically with the long Pbx isoforms (Pbx1a, Pbx2, and Pbx3a) but not with the short isoforms, Pbx1b and Pbx3b, resulting from differential splicing of the 3'-end of the Pbx RNA (11). This antibody blocked the formation of the slower migrating complex *L* only (see Fig. 2, *lane 16*). When the mutated UE-A element was used as probe, no complexes could be observed (Fig. 2, *lanes 10–14*). These results indicate that the cellular complexes binding specifically the UE-A sequence contain Pbx factors. The slower-migrating complex *L* contains the long Pbx isoforms, whereas the *S* complex is probably due to the short Pbx isoforms. Furthermore, as the anti-PDX1 antiserum had no effect, we conclude that these complexes are completely different from the PDX1-Pbx heterodimeric complex observed on the somatostatin TSE<sub>II</sub> element (10).

To investigate the cell type distribution of complexes *S* and *L*, nuclear extracts were prepared from different pancreatic and nonpancreatic cell lines and tested by EMSA (Fig. 3, *lanes 1–11*). Complex *L* was detected with extracts of all tested cell lines, and complex *S* was less abundant in some cell lines, such as COS, Jurkat, or HT29. The presence of Pbx proteins in these complexes was confirmed in all extracts by supershift with the Pbx antibodies (data not shown). These results are consistent with previous reports showing the expression of Pbx factors in all cell lines tested so far (11).

We next investigated whether a recombinant Pbx protein could bind the UE-A element. Pbx1a protein was produced *in vitro* using wheat germ extract and tested in EMSA. No protein-DNA complexes could be reconstituted on the UE-A element with Pbx1a (see Fig. 3, *lane 12*), whereas a heterodimeric complex with PDX1 was formed on the TSE<sub>II</sub> element (Fig. 3, *lane 16*). Identical results were obtained using recombinant Pbx1a produced in *E. coli* (data not shown), suggesting that binding of Pbx factors to UE-A requires a co-factor present in cell extracts. As Hox factors are known to bind cooperatively with Pbx proteins on some target elements, various Hox proteins were produced *in vitro* and tested in the presence of Pbx1 by EMSA; however, no heterodimeric complexes could be generated on UE-A (data not shown). Formation of Hox-Pbx com-

FIG. 2. A member of the Pbx homeodomain family binds to the somatostatin UE-A element. EMSAs were performed on wild type or mutated UE-A element (sequences depicted below the lanes) using nuclear extract of pancreatic TU6 cells. 1 or 10 ng of unlabeled oligonucleotides UE-A or mutated UE-A were added as competitor, as indicated above the lanes. *Pl*, preimmune serum; *Pbx Ab*, antibody raised against PBX1 protein and recognizing all Pbx family members (generous gift of M. Kamps); *PDX1 Ab*, PDX1 antiserum; *PbxL Ab*, antiserum recognizing specifically the long Pbx isoforms (Pbx1a, Pbx2, and Pbx3a) (Santa Cruz Biotechnology)

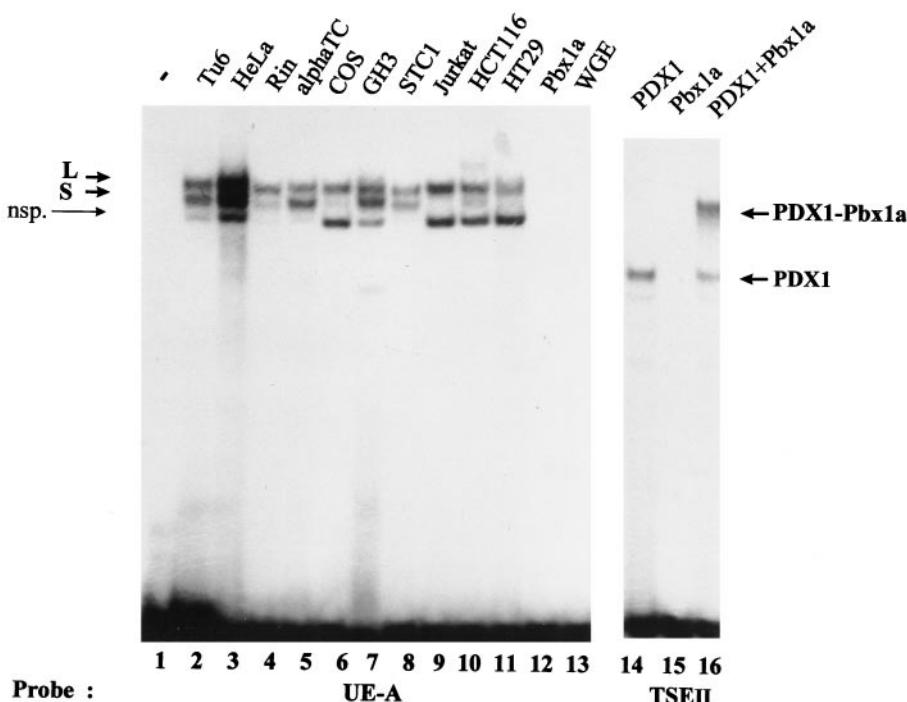
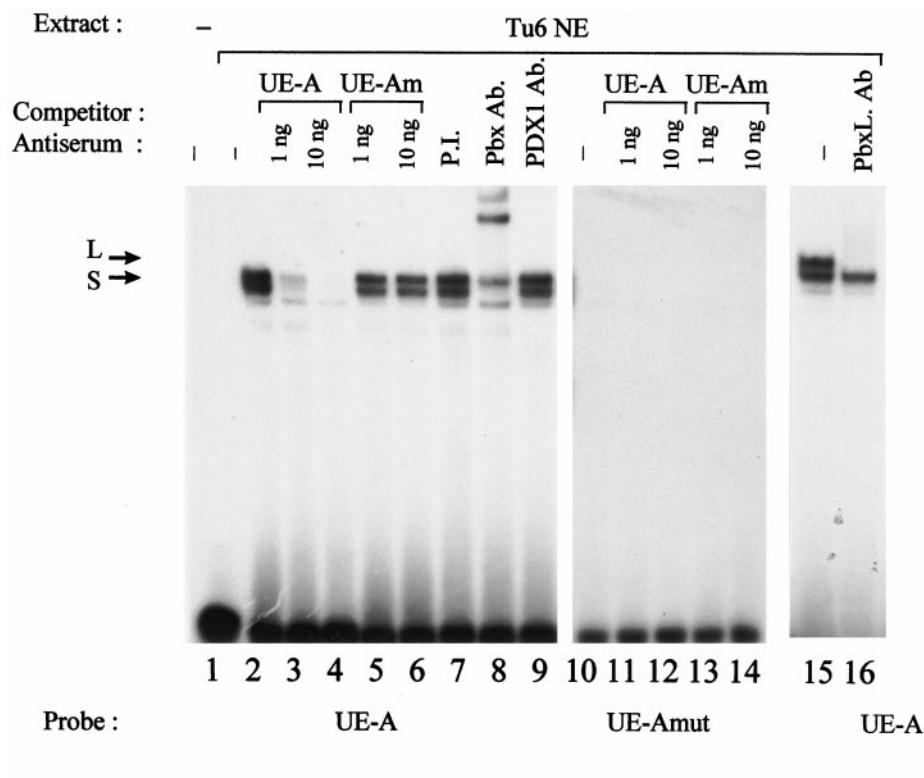


FIG. 3. Formation of the protein-DNA complexes S and L on the UE-A element with nuclear extract of various cell lines. EMSAs were performed on UE-A probe with extracts from TU6, Rin,  $\alpha$ TC cells (pancreatic endocrine type), GH3 cells (pituitary type), STC1 cells (intestinal type), Jurkat cells (T lymphocyte type), HCT116, HT29 cells (colon carcinoma type), Hela, or COS cells. Positions of complexes L and S are indicated by the arrows. *Nsp*, nonspecific complex. *Pbx1a*, recombinant Pbx1a protein produced *in vitro* in a wheat germ extract (WGE) tested on the UE-A probe (lane 12) and on TSE<sub>II</sub> probe in absence (lane 14) and presence (lane 15) of recombinant PDX1 protein.

plexes requires the pentapeptide motif YPWMK present in many homeodomain proteins, and previous experiments have shown that Hox-Pbx complexes can be disrupted by adding high concentrations of synthetic peptide containing this conserved motif (29, 33). Thus, in order to test whether the complexes S and L are Hox-Pbx type complexes, we incubated increasing amount of this synthetic peptide with TU6 cell extract and then performed EMSA. Fig. 4 shows that the two complexes L and S are not substantially affected by the pen-

taapeptide motif in contrast to the complete destabilization of the PDX1-Pbx complex on the TSE<sub>II</sub> element.

As the Pbx1a protein produced *in vitro* or in bacteria is not able to bind UE-A, we decided to test whether it could reconstitute a protein-DNA complex when expressed in eukaryotic cells. Thus, COS cells were transfected by the expression vector pFlag-Pbx1 containing the coding sequence for Pbx1a protein tagged at its N terminus with the FLAG epitope (29). Nuclear extract from transfected cells were tested by EMSA on the

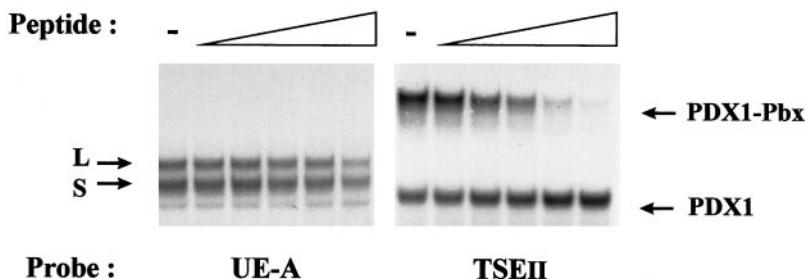


FIG. 4. The protein complexes bound on the UE-A element are not destabilized by the conserved Hox pentapeptide in contrast to the PDX1-Pbx complex bound on the TSE<sub>II</sub> element. EMSAs were performed with increasing concentration of the Hox-B5 peptide QPQIYPWMRKLH (from 1  $\mu$ M to a final concentration of 1 mM), as indicated by the *crescendo* above the lanes. The complexes L and S formed with UE-A probe are indicated, as well as the PDX1 and PDX1-Pbx complexes formed on TSE<sub>II</sub> probe. The free probes are not shown in the figure.

pcDNA3	+	+	-	-
pFlagPBX1	-	-	+	+
Flag Ab.	-	+	-	+



FIG. 5. Pbx1a protein binds the UE-A element when expressed in eukaryotic COS cells. EMSAs were performed on UE-A probe with extracts of COS cells transfected with (+) or without (-) the expression vector containing the coding region of the Flag-tagged Pbx1a (*pFlag-PBX1*) or cDNA insert (*pcDNA3*). 1  $\mu$ l of monoclonal antiflag antibody (*Flag Ab.*) was added as indicated.

UE-A probe (Fig. 5). As expected, the complexes S and L were observed with extract of transfected cells; addition of an anti-FLAG monoclonal antibody produced a supershifted band only in extracts of COS cells transfected with the *pFlag-Pbx1* expression vector. No supershift was observed when the control expression vector *pcDNA3* was used. These results prove that Pbx1a protein can bind the UE-A sequence, but only when it is expressed in eukaryotic cells. This suggests that binding of Pbx1 to UE-A requires either a posttranslational modification of Pbx1 or a cellular co-factor present in cell extracts.

**The Prep-1 Factor Binds Cooperatively with Pbx1a to the UE-A Element**—Recently, Pbx factors have been found to associate with a specific subclass of three-amino acid loop extension homeodomain proteins that lack the YPWMK motif: the Meis/Prep1 protein family. Indeed, Meis1 and the related factor Prep-1 (also named pKnox1) stably interact in solution with the Pbx proteins (34–37). This observation prompted us to investigate whether one of these two factors could bind the UE-A element together with Pbx1. Meis1 and Prep-1 proteins were translated *in vitro* using reticulocyte lysate and tested by EMSA in the presence or absence of Pbx1a. Fig. 6 shows that a very strong cooperative binding occurs using Pbx1a and Prep-1 (*lane 6*), whereas formation of a Pbx1-Meis1 complex was also

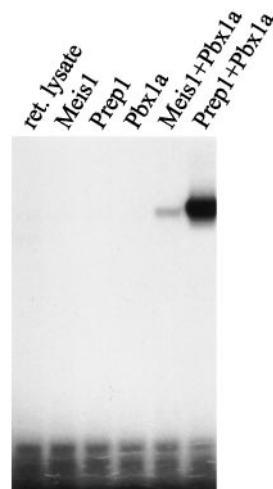


FIG. 6. Prep1 and Pbx1a cooperatively bind the somatostatin UE-A element. Meis1, Prep1, and Pbx1a were translated *in vitro* in reticulocyte lysate and were tested by EMSA as indicated above each lane using the probe UE-A. *Ret. lysate*, control reaction containing the unprogrammed reticulocyte lysate. Roughly equal efficiency of protein translation was obtained for Meis1 and Prep1, as demonstrated by [<sup>35</sup>S]methionine labeling (data not shown).

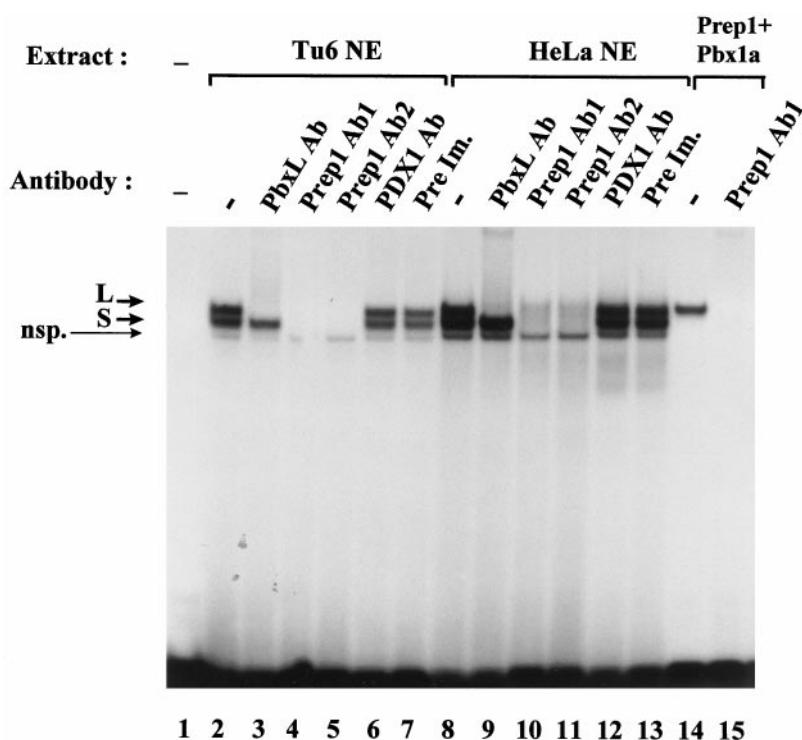
detectable, but much less efficient (*lane 5*). This difference was not due to a lower amount of Meis1 protein as comparable amount of <sup>35</sup>S-labeled Meis1 and Prep-1 proteins were detected on SDS-polyacrylamide gel electrophoresis (data not shown).

To determine whether Prep-1 is the co-factor in the cell extracts binding with Pbx factors to the somatostatin UE-A element, we used two antisera raised specifically against Prep1 (Fig. 7). Addition of these two antisera to Tu6 or HeLa cell extracts completely abolished the formation of the two complexes S and L, whereas the preimmune or the PDX1 anti-serum had no effect (Fig. 7, *lanes 2–13*). Furthermore, the cellular complex L, which was specifically blocked by the long isoform Pbx antibody (Fig. 7, *lanes 3 and 9*), co-migrated with the heteromeric complex obtained with the recombinant Prep-1 and Pbx1a proteins (*lane 14*). In contrast, an anti-Meis1 antibody did not affect the complexes S and L (data not shown). As the Prep1 antibodies do not cross-react with the recombinant Meis1 protein (data not shown), these results clearly indicate that Prep1 is the major cellular factor binding UE-A in combination with Pbx proteins.

**Synergistic Activation of the UE-A Element by Pbx and Prep1 Factors**—By transient transfection experiments, we next investigated whether Pbx and Prep1 factors could stimulate the transcriptional activity of the UE-A element. To that end, we generated reporter plasmid in which the UE-A element was inserted in a single copy directly upstream from a TATA box followed by the luciferase gene. Fig. 8 shows that the activity of

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FIG. 7. Prep1 is the major partner of Pbx factors for the cooperative binding of the somatostatin UE-A element. EMSAs were performed on the UE-A probe using TU6 or HeLa cell extract as indicated. Various antibody were preincubated with the extracts as depicted above each lane. *PbxL Ab*, anti-serum recognizing the long isoforms of Pbx factors. *Prep1 Ab1* and *Prep1 Ab2*, antisera raised specifically against Prep1 protein (generous gift of Prof. F. Blasi). *In vitro* translated Prep1 and *Pbx1a* were tested in lanes 14 and 15. *nsp*, nonspecific complex.



the UE-A element was not affected by co-transfection of *Pbx1a* or Prep1 expression vectors when tested separately. In contrast, when both expression vectors were combined, a strong synergistic stimulation was observed. This activation is mediated through the binding of Pbx-Prep1 heteromeric complex to the UE-A element, as the mutation in the TGATTGATT motif of UE-A preventing the binding of these factors completely blocked this synergistic stimulation. Thus, the Pbx-Prep1 complex displays a strong transcriptional activation potential on an isolated UE-A element.

**PDX1, Pbx, and Prep1 Factors Synergistically Activate the Mini-enhancer and the Intact Promoter of the Somatostatin Gene**—Previous reports have shown that the sequence  $-120$  to  $-80$  of the somatostatin promoter acts as a pancreatic-specific mini-enhancer (25). This mini-enhancer is actually a bipartite element composed of the UE-A domain ( $-113$  to  $-100$ ) and the TSE<sub>I</sub> domain ( $-85$  to  $-99$ ) binding the PDX1 homeodomain factor as a monomer (6, 7) (see Fig. 1). As both sites are required for the enhancer function and act synergistically, we decided to test the effect of the three factors, PDX1, *Pbx1a*, and Prep1, on the somatostatin mini-enhancer. The reporter plasmid UE-A/TSE<sub>I</sub>-Luc, containing one copy of the mini-enhancer (sequence from  $-120$  to  $-80$ ) directly upstream from the TATA box-luciferase fusion construct, was transfected either alone or with various combination of expression vectors for PDX1, *Pbx1a*, or Prep1 in carcinoma HCT116 cells (see Fig. 9). In contrast to the effect on the UE-A element alone, co-transfection of both Prep1 and *Pbx1a* vectors did not produce a strong activation of the mini-enhancer. PDX1 alone caused a stimulation that was slightly increased in the presence of *Pbx1a*. However, expression of all three factors (PDX1, *Pbx1a*, and Prep1) gave a very strong synergistic activation of the mini-enhancer (about 230-fold).

Similarly, when the intact somatostatin promoter was tested as a reporter (Fig. 9, plasmid pSRIF-Luc), co-transfection of both *Pbx1a* and Prep1 again had no significant effect, similar to the mini-enhancer. Also, the modest activation generated by expression of PDX1 was strongly increased by additional expression of both *Pbx1a* and Prep1.

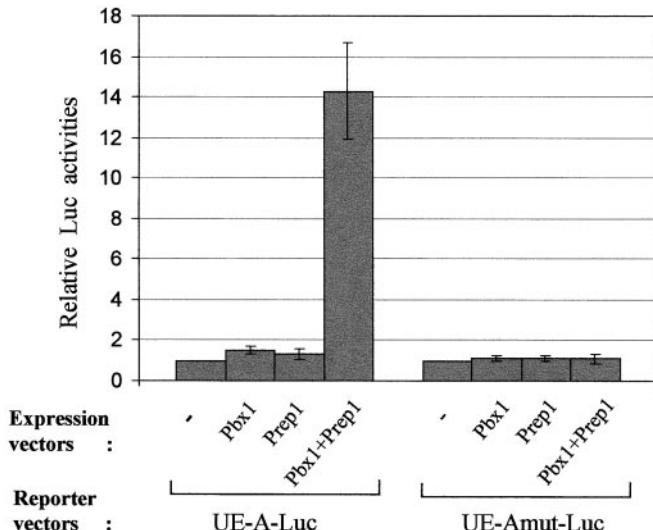
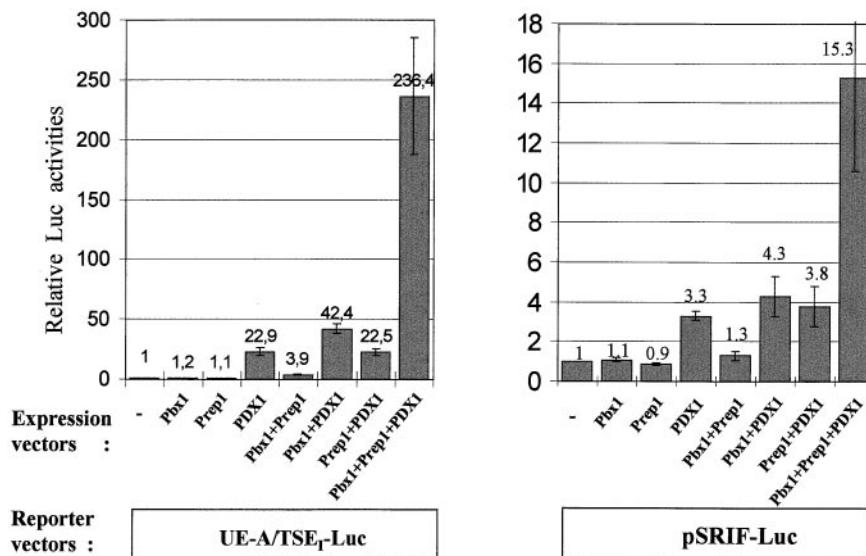


FIG. 8. Synergistic activation of the UE-A element by *Pbx1a* and Prep1 factors. HCT116 cells were transfected by the UE-A-Luc and UE-Amut-Luc reporter plasmids. These vectors contain the luciferase gene under the control of a minimal growth hormone promoter ( $-32$  to  $+8$ ) and one copy of the wild type UE-A sequence (for the UE-A-Luc plasmid) or mutated UE-A sequence (for the UE-Amut-Luc plasmid) (see Fig. 2 for the sequences). These reporter plasmids were cotransfected with an expression vector for *Pbx1a* and/or Prep1 as indicated. Luciferase activities were normalized to  $\beta$ -galactosidase activity generated by the internal control plasmid Rous sarcoma virus- $\beta$ -galactosidase. Normalized Luc activity obtained in the cell transfected without expression vector was arbitrarily set at 1. The data are means  $\pm$  S.D. of four transfection experiments, each performed in duplicate.

## DISCUSSION

The UE-A element is a well conserved regulatory element of the somatostatin gene and is required for the optimal function of the promoter in pancreatic cells. Whereas the UE-A site is part of a pancreas-specific mini-enhancer, it has no intrinsic transactivating capacity (data not shown and Ref. 4). This



**FIG. 9. Synergistic activation of the somatostatin mini-enhancer and the intact somatostatin promoter by Prep1, Pbx1a, and PDX1.** The reporter plasmid UE-A/TSE<sub>1</sub>-Luc contains one copy of the somatostatin mini-enhancer sequence (from -120 to -80) inserted upstream from the minimal growth hormone promoter (-32 to +8) fused to the luciferase gene. The reporter plasmid pSRIF-Luc contains the somatostatin promoter sequence (from -195 to +52) fused to the luciferase gene. These reporter vectors were co-transfected in HCT116 cells together with the internal control plasmid Rous sarcoma virus- $\beta$ -galactosidase and the expression vectors coding for PDX1, Pbx1a, and/or Prep1 factors. Normalized luciferase activity obtained in the cells transfected without expression vector was arbitrarily set at 1. The data presented are the results of nine transfection experiments for the UE-A/TSE<sub>1</sub>-Luc plasmid and four transfection experiments for the pSRIF-Luc plasmid.

element acts by potentiating the transcriptional activation of the nearby TSE<sub>1</sub> element.

In the present study, we demonstrated that the UE-A site is recognized by heterodimers composed of a Pbx family member and the Prep1 protein. The two protein-DNA complexes L and S formed on UE-A with the cell extracts correspond to Pbx-Prep1 heterodimers containing, respectively, the long and short isoforms of Pbx proteins. This is demonstrated by the supershift obtained using the specific Pbx and Prep1 antibodies and the comigration of the L complex with the *in vitro* translated Pbx1a and Prep1 proteins. In transient transfection experiments, we observed that the somatostatin enhancer was strongly stimulated by the co-expression of the three factors, PDX1, Prep1, and Pbx1a. Similar stimulations were obtained by replacing the Pbx1a expression vector by a Pbx1b or Pbx2 expression plasmid (data not shown). Taken together, these results indicate that somatostatin gene expression is under the control of Pbx-Prep1 heterodimers.

In addition to the somatostatin gene, the only known target of the Prep1 factor is the urokinase plasminogen activator gene (36). Similar to the somatostatin UE-A element, Prep1 binds the COM element of the urokinase plasminogen activator enhancer as a heterodimer with Pbx factors, and the COM element is not able to activate transcription on its own. In fact, COM seems to act by increasing the transcriptional activation produced by the transcription factors bound to neighboring sites (*i.e.* Jun, ATF, and Ets factors) (38). In the present study, we also observed that the Pbx-Prep1 heterodimer, bound on the UE-A element, strongly synergizes with the PDX1 factor bound to the nearby TSE<sub>1</sub> site, producing a full activation of the somatostatin mini-enhancer. EMSA failed to show that this synergism is due to a cooperative binding of Pbx-Prep1 heterodimer and PDX1 to the bipartite UE-A/TSE<sub>1</sub> element (data not shown). However, we cannot completely rule out the possibility that DNA binding cooperativity might occur within the cell, perhaps requiring the presence of an unknown cellular factor or eventually requiring the natural context of chromatin.

In this study, we show for the first time that the UE-A element can generate a transcriptional activation, but only

when Pbx and Prep1 are overexpressed and if the UE-A sequence is inserted immediately upstream the TATA box. Increasing the distance between the UE-A site and the TATA box strongly reduces (see Fig. 9, UE-A/TSE<sub>1</sub> reporter plasmid) or completely abolishes the activation (see Fig. 9, pSRIF-Luc reporter). These observations suggest that the Pbx-Prep1 heterodimers possesses an activation capacity that is highly dependent on spatial organization. We can also postulate that in the context of the somatostatin promoter, one function of PDX1 is to promote functional interactions between the basal machinery and the Pbx-Prep1 heterodimer. However, further experiments are required to determine the mechanism of the transactivation mediated by the Pbx-Prep1 heterodimers.

The protein Prep1 shares sequence similarity with the Meis proteins (Meis1, Meis2, Meis3, mrg1, and mrg2) not only in the homeodomain, but also in the two N-terminal domains involved in dimerization with Pbx factors (36, 39–41). Despite this sequence similarity, the Pbx1-Meis1 heterodimer binds much less efficiently to UE-A than the Pbx1-Prep1 heterodimer. Furthermore, we were unable to detect binding of Pbx-Meis heterodimers on UE-A using cell extracts, and all protein complexes observed were Pbx-Prep1 heterodimers. This indicates that Pbx-Prep1 and Pbx-Meis1 heterodimers could have different DNA binding specificity and could regulate distinct target genes. Actually, it has been shown that the Pbx1-Meis complex preferentially recognizes the TGATTGACAG motif (34). We are currently investigating in more detail this DNA binding specificity.

The mechanism of action of the Pbx factors has been remarkably maintained through animal evolution. Indeed, both in mammals and in *Drosophila*, Pbx/EXD factors interact and cooperate with the Hox/HOM-C-like factors (reviewed in Ref. 19), and the interaction between mammalian Pbx and the Meis/Prep1 related factors also occurs in *Drosophila* as EXD interacts with the homothorax protein, which seems to be the ortholog of the murine Meis factors (42–45). Homothorax was shown to be necessary for the nuclear localization of EXD and for its function. Remarkably, the murine Meis1 was able to rescue the homothorax mutant phenotype (42) and to induce

nuclear translocation of EXD. Therefore, it is probable that the nuclear translocation of Pbx factors in mammals is controlled by the Meis factors. Thus, it will be interesting to test whether Prep1 could have such a role. As Rieckhof *et al.* (42) observed nuclear EXD without homothorax expression in some cells of *Drosophila* embryo (42), it is possible that another factor, similar to homothorax, exists in these cells. This factor could eventually correspond to the ortholog of Prep1. A sequence comparison of homothorax, Meis, and Prep1 reveals that Meis1 is much more related to homothorax than to Prep1, suggesting that before the divergence of vertebrates and invertebrates, an ancestral gene duplicated and generated the Prep1 and the Meis/homothorax genes. Thus, this is consistent with the hypothesis that a Prep1-like gene could exist in *Drosophila*.

The somatostatin promoter is a good model to study the regulation of gene expression by the Pbx factors. This promoter contains two distinct Pbx binding sites, the TSE<sub>II</sub> and the UE-A element. Pbx binds TSE<sub>II</sub> cooperatively with the pancreatic factor PDX1, and the formation of this heterodimer requires the FPMWK motif of PDX1 (10). On the UE-A element, Pbx binds as a heterodimer with the Prep1 factor. This Pbx-Prep1 heterodimer functionally cooperates with the PDX1 factor bound to the adjacent TSE<sub>I</sub> site. The somatostatin promoter is the only regulatory sequence shown to bind the three factors Pbx, Prep1, and PDX1 (Hox-like) with a high affinity; this gene is thus a good model in order to investigate, at the molecular level, the mechanisms of action of the Pbx/EXD, Meis/Prep homothorax and Hox-like homeodomain factors.

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## REFERENCES

1. Reichlin, S. (1983) *N. Engl. J. Med.* **309**, 1495–1501
2. Montminy, M. R., Gonzalez, G. A., and Yamamoto, K. K. (1990) *Recent Prog. Horm. Res.* **46**, 219–229
3. Vallejo, M., Ron, D., Miller, C. P., and Habener, J. F. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 4679–4683
4. Vallejo, M., Miller, C. P., and Habener, J. F. (1992) *J. Biol. Chem.* **267**, 12868–12875
5. Leonard, J., Serup, P., Gonzalez, G., Edlund, T., and Montminy, M. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6247–6251
6. Leonard, J., Peers, B., Johnson, T., Ferreri, K., Lee, S., and Montminy, M. R. (1993) *Mol. Endocrinol.* **7**, 1275–1283
7. Miller, C. P., McGehee, R. E., Jr., and Habener, J. F. (1994) *EMBO J.* **13**, 1145–1156
8. Ohlsson, H., Karlsson, K., and Edlund, T. (1993) *EMBO J.* **12**, 4251–4259
9. Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994) *Nature* **371**, 606–609
10. Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. R. (1995) *Mol. Cell. Biol.* **15**, 7091–7097
11. Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M. L. (1991) *Mol. Cell. Biol.* **11**, 6149–6157
12. Rauskolb, C., Peifer, M., and Wieschaus, E. (1993) *Cell* **74**, 1101–1112
13. Burglin, T. R. (1997) *Nucleic Acids Res.* **25**, 4173–4180
14. Chan, S. K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. S. (1994) *Cell* **78**, 603–615
15. Rauskolb, C., Smith, K. M., Peifer, M., and Wieschaus, E. (1995) *Development* **121**, 3663–3673
16. Sun, B., Hursh, D. A., Jackson, D., and Beachy, P. A. (1995) *EMBO J.* **14**, 520–535
17. Gonzalez-Crespo, S., and Morata, G. (1995) *Development* **121**, 2117–2125
18. van Dijk, M. A., and Murre, C. (1994) *Cell* **78**, 617–624
19. Mann, R. S., and Chan, S. K. (1996) *Trends Genet.* **12**, 258–262
20. Popper, H., Biehn, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S., and Krumlauf, R. (1995) *Cell* **81**, 1031–1042
21. Neuteboom, S. T., Peltenburg, L. T., van Dijk, M. A., and Murre, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 9166–9170
22. Phelan, M. L., Rambaldi, I., and Featherstone, M. S. (1995) *Mol. Cell. Biol.* **15**, 3989–3997
23. Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C., and Cleary, M. L. (1995) *Genes Dev.* **9**, 663–674
24. Lu, Q., and Kamps, M. P. (1996) *Mol. Cell. Biol.* **16**, 1632–1640
25. Vallejo, M., Penchuk, L., and Habener, J. F. (1992) *J. Biol. Chem.* **267**, 12876–12884
26. Peers, B., Leonard, J., Sharma, S., Teitelman, G., and Montminy, M. R. (1994) *Mol. Endocrinol.* **8**, 1798–1806
27. Schreiber, E., Matthias, P., Muller, M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419
28. Moskow, J. J., Bullrich, F., Huebner, K., Daar, I. O., and Buchberg, A. M. (1995) *Mol. Cell. Biol.* **15**, 5434–5443
29. Peltenburg, L. T., and Murre, C. (1996) *EMBO J.* **15**, 3385–3393
30. Van Dijk, M., Voorhoeve, M., and Murre, C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6061–6065
31. Lu, Q., Wright, D. D., and Kamps, M. P. (1994) *Mol. Cell. Biol.* **14**, 3938–3948
32. LeBrun, D. P., and Cleary, M. L. (1994) *Oncogene* **9**, 1641–1647
33. Knoepfler, P., and Kamps, M. (1995) *Mol. Cell. Biol.* **15**, 5811–5819
34. Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G., and Cleary, M. L. (1997) *Mol. Cell. Biol.* **17**, 5679–5687
35. Knoepfler, P. S., Calvo, K. R., Chen, H., Antonarakis, S. E., and Kamps, M. P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14553–14558
36. Berthelsen, J., Zappavigna, V., Mavilio, F., and Blasi, F. (1998) *EMBO J.* **17**, 1423–1433
37. Bischof, L. J., Kagawa, N., Moskow, J. J., Takahashi, Y., Iwamatsu, A., Buchberg, A. M., and Waterman, M. R. (1998) *J. Biol. Chem.* **273**, 7941–7948
38. De Cesare, D., Palazzolo, M., and Blasi, F. (1996) *Oncogene* **13**, 2551–2562
39. Nakamura, T., Jenkins, N. A., and Copeland, N. G. (1996) *Oncogene* **13**, 2235–2242
40. Chen, H., Rossier, C., Nakamura, Y., Lynn, A., Chakravarti, A., and Antonarakis, S. E. (1997) *Genomics* **41**, 193–200
41. Steelman, S., Moskow, J., Muzinski, K., North, C., D. T., Montgomery, J., Huebner, K., Daar, I., and Buchberg, A. M. (1997) *Genome Res.* **7**, 142–156
42. Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M., and Mann, R. S. (1997) *Cell* **91**, 171–183
43. Casares, F., and Mann, R. S. (1998) *Nature* **392**, 723–726
44. Kurant, E., Pai, C. Y., Sharf, R., Halachmi, N., Sun, Y. H., and Salzberg, A. (1998) *Development* **125**, 1037–1048
45. Pai, C. Y., Kuo, T. S., Jaw, T. J., Kurant, E., Chen, C. T., Bessarab, D. A., Salzberg, A., and Sun, Y. H. (1998) *Genes Dev.* **12**, 435–446