Glucagon Gene Transcription Activation Mediated by Synergistic Interactions of pax-6 and cdx-2 with the p300 Co-activator

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In the endocrine pancreas, α-cell-specific expression of the glucagon gene is mediated by DNA-binding proteins that interact with the proximal G1 promoter element, which contains several AT-rich domains. The homeodomain transcription factors brain-4, pax-6, and cdx-2 have been shown to bind to these sites and to transactivate glucagon gene expression. In the present study, we investigated the interaction of cdx-2 and pax-6 with p300, a co-activator coupled to the basal transcription machinery. In transient transfection-expression experiments, we found that the transactivating effects of cdx-2 and pax-6 on the glucagon gene were greatly enhanced by the additional expression of p300. This enhancement was due to direct protein-protein interactions of both pax-6 and cdx-2 with the N-terminal CH1 domain of p300. pax-6 and cdx-2 also directly interacted with one another at the protein level. pax-6, bound to its DNA recognition site in the glucagon G1 promoter element, tethered cdx-2 to the molecular complex of pax-6 and p300. Further, we found that the presence of cdx-2 enhanced the interaction of pax-6 with p300, thus establishing a molecular complex of transcription factors implicated in tissue-specific glucagon gene expression with the basal transcriptional machinery.

The glucagon gene is expressed in the pancreatic endocrine α-cells of the pancreatic islets, the L cells of the intestine, and specific areas of the brain (1). The cell-specific expression of the proglucagon gene is best studied in the pancreatic α-cells, in which expression is conferred by the proximal G1 promoter element (1). The G1 element contains two AT-rich motifs that are binding sites for homeodomain transcription factors. Recently, the transcription factors cdx-2, pax-6, and brain-4 have been shown to bind to the AT-rich elements and transactivate glucagon gene expression (2–4). Nuclear proteins of glucagon-producing cell lines form three main protein complexes with the G1 promoter element (5). One of these protein complexes contains the POU domain transcription factor brain-4 (4). Of the two other complexes, the lower molecular weight complex has been reported to contain the paired-domain factor pax-6 as monomer, whereas the higher molecular weight complex contains pax-6 in a heterodimer with the caudal-related factor cdx-2 (6, 7). Of these transcription factors, brain-4 is expressed specifically in pancreatic α-cells (4), and pax-6 is expressed in all pancreatic endocrine cells implicated in islet cell development, predominantly the pancreatic α-cells (8, 9). pax-6 also binds to the G3 element of the proglucagon gene enhancer at a site that confers insulin inhibition of proglucagon gene expression (8). cdx-2 is found in both pancreatic α- and β-cell lines (3), as well as in intestinal epithelial cells (3, 4, 10). Recently, pax-6 and cdx-2 have been shown to directly bind to each other and to synergistically transactivate the proglucagon gene via interaction with the G1 element of the proglucagon gene promoter (6, 7).

cAMP response element-binding protein (CREB)1-binding protein (CBP) and a closely related homologue p300 are known to integrate a number of cell-signaling pathways and to serve as co-activators of various transcription factors, including helix-loop-helix and homeodomain proteins (11–14). Both CBP and p300 serve as adapter proteins linking DNA binding transcription factors with the basal transcriptional machinery. Furthermore, CBP and p300 are proposed to both activate histone acetyltransferase and displace nucleosomes, as well as recruit RNA polymerase II to the transcription complex (15).

In an attempt to understand the mechanisms underlying the tissue-specific transcription of the proglucagon gene, we have examined the mediation of the transactivating properties of pax-6 and cdx-2. We report here that the transactivating effects of both pax-6 and cdx-2 are mediated through their interaction with the transcriptional co-activator p300. Furthermore, our results suggest that the binding of pax-6 to p300 is weak and that the presence of cdx-2 enhances the physical interaction of pax-6 with p300. Whereas the transcriptional activity of pax-6 is dependent on its binding to its DNA recognition site within the G1 promoter element, binding of cdx-2 to the promoter is not absolutely required since pax-6 tethers cdx-2 to form a complex with cdx-2 and p300.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Baby hamster kidney (BHK)-21 cells were obtained from the American Tissue Culture Collection (Manassas, VA). αTC-1 cells were a generous gift from S. Efrat (Albert Einstein College of Medicine, Bronx, NY). All cells were grown at 37 °C in humidified 5% CO2, 95% O2 in Dulbecco’s modified Eagle’s medium (DMEM, 4.5 g of glucose per liter (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 100 units of penicillin and 100 μg/ml streptomycin. Transfections were performed with LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions.

1 The abbreviations used are: CREB, cyclic AMP response element-binding protein; bp, base pair(s); C/EBPβ, CAAT element-binding protein β; CHOP, C/EBP homologous protein; CBP, CREB-binding protein; EMSA, electrophoretic mobility shift assay; ELB, EDTA low salt buffer; GST, glutathione S-transferase; NEN, NaCl-EDTA-Nonidet P-40, p300, protein homologous to CBP; RSV, Rous sarcoma virus; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.

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Glucagon Gene Transcription

**Results**

**Analysis of the Protein-DNA Complexes of the G1 Element of the Glucagon Gene**—Electrophoretic mobility shift assays of aTC-1 nuclear extracts with bp −93 to −60 proximal element G1

| Oligonucleotide | Sequence |
|-----------------|----------|
| −93 to −60 | 5′CCCCATTATTTACAGTGAGAAATTTATATGTACGCGTAA3′ |
| −93 to −60 | 5′GGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
of the glucagon promoter reveal four major protein-DNA complexes. Of these, complex A, which is found at various intensities and is usually weaker than complexes B and C, is most likely formed by a heterodimer of pax-6 and cdx-2 (Fig. 1A). Complex B contains the POU domain transcription factor brain-4 (4), and complex C most likely contains pax-6 homodimer (6, 7). Complex D appears to contain all three homeoproteins, although an unspecific effect of antibodies cannot be definitively ruled out with the present data. Of note, cdx-2 antisera does not clearly disrupt but attenuates the intensity of complex A. This suggests that complex A is composed of a pax-6/cdx-2 heterodimers (Fig. 1A), as has previously been reported by others (6, 7). In EMSA experiments with nuclear extracts from BHK-21 cells overexpressing transfected pax-6 and cdx-2, a heterodimer of pax-6 and cdx-2 can form complex A only when both AT-rich binding sites are intact. An interaction of pax-6 and cdx-2 does not become apparent when the proximal 3' AT-rich site, the preferential binding site for cdx-2 (3, 6, 7), is mutated (Fig. 1B). Complex C is formed by pax-6 alone (Fig. 1B). Fig. 1C depicts the preferential binding sites of pax-6, cdx-2, and brain-4.

Transient Transfection Experiments—To further test the functional interaction of pax-6 and cdx-2, transient co-transfection studies with expression plasmids of pax-6 and cdx-2 with firefly luciferase reporter constructs of the glucagon G1 element were conducted. Both pax-6 and cdx-2 independently transactivate the glucagon gene. When the binding 5' and 3' AT-rich sites of the promoter are mutated, the activity of pax-6 and cdx-2 is markedly attenuated, respectively (Fig. 2A). pax-6 and cdx-2 transfected in submaximal doses together elicited a synergistic effect on the transactivation of the proglucagon gene promoter-reporter construct. However, when the preferential DNA binding site for cdx-2 (the proximal 3' AT-rich site) is mutated, a synergistic transactivating effect of pax-6 and cdx-2 together is (although somewhat reduced) still preserved (Fig. 2B). These findings are in line with observations recently reported (6, 7). In addition, in a situation of mutated pax-6 DNA recognition site (−93 m5' GLU-LUC, distal 5' AT-rich site), overexpression of pax-6 showed a tendency (although not significant) of enhancing the transactivation through cdx-2 (Fig. 2B).

We hypothesized that pax-6 may be tethering cdx-2 to a transcriptional complex, possibly involving a co-activator module. Therefore, we next studied the transactivating properties of pax-6 and cdx-2 in combination with p300. Indeed, addition of p300 expression plasmid markedly increased transactivation through pax-6 and cdx-2 alone and in combination (Fig. 2C; note the interrupted y axis in Fig. 2C). The synergistic effect of pax-6 and cdx-2 with the co-activating effect of p300 was also present even when the 3' AT-rich site (binding site of cdx-2) was mutated (Fig. 2C). This result further suggests that the cdx-2 DNA recognition site is not necessary for cdx-2 to participate in the transcription of the glucagon gene. In contrast, disruption of the pax-6 binding site did not allow overexpressed pax-6 to further stimulate the transcription induced by cdx-2 together with p300. Thus, it appears that the pax-6 DNA recognition site (even in the presence of cdx-2) is required for pax-6 to participate in the transactivation of the glucagon gene. Furthermore, disruption of the cdx-2 site leads to a substantial reduction of the transactivation of pax-6 through interaction with p300 (Fig. 2C). This observation suggests that, for optimal transactivation and interaction with p300, an optimal sequence of the DNA within the promoter region is required. This may indicate the importance of steric conformation of interacting proteins and DNA in transcriptional control.

Next, we attempted to inhibit the co-activation through p300/CBP by overexpression of the adenovirus E1A protein, which specifically binds the cystine/histidine-rich 3 (CH3) domain of p300 and CBP and thereby inhibits transcriptional co-activation (11–13). A mutated E1A protein (E1A RG2), which is a much weaker inhibitor of transcriptional co-activation through p300/CBP, was used as a negative control (13). As shown in Fig. 3A, overexpression of E1A, but not of E1A RG2, reduced the basal expression of a −93 glucagon promoter-luciferase reporter in αTC-1 cells. Furthermore, overexpression of E1A, but not the mutant E1A RG2, in heterologous BHK-21 fibroblasts inhibited the transactivation by cdx-2, pax-6, and cdx-2 and pax-6 together, of the glucagon promoter-reporter construct (Fig. 3B). These results suggest that an endogenous co-activator homologue of CBP/p300 is involved in the transcription of the glucagon gene in and that transactivation by pax-6 and cdx-2 involves the co-activator CBP/p300.
We confirmed that cdx-2 binds to GST-pax-6 (Fig. 4) (6, 7). Next, we tested whether cdx-2 and pax-6 bind to the CBP homologue p300. Indeed, GST-cdx-2 interacted with in vitro translated p300 in a GST pull-down assay. Furthermore, we could demonstrate that GST-cdx-2 pulls down both in vitro translated p300 and pax-6 simultaneously. Vice versa, GST-pax-6 pulls down both in vitro translated p300 and cdx-2 simultaneously (Fig. 4C). Neither GST-pax-6 nor GST-cdx-2 interacted with in vitro translated labeled CHOP, suggesting that nonspecific interactions of pax-6 and cdx-2 with p300 do not take place (Fig. 4B).

**Analysis of Protein-Protein Interaction in Vitro and in Vivo**

We confirmed that cdx-2 binds to GST-pax-6 (Fig. 4) (6, 7). Next, we tested whether cdx-2 and pax-6 bind to the CBP homologue p300. Indeed, GST-cdx-2 interacted with in vitro translated p300 in a GST pull-down assay. Furthermore, GST-pax-6 also interacted with p300. Furthermore, we could demonstrate that GST-cdx-2 pulls down both in vitro translated p300 and pax-6 simultaneously. Vice versa, GST-pax-6 pulls down both in vitro translated p300 and cdx-2 simultaneously (Fig. 4C). Neither GST-pax-6 nor GST-cdx-2 interacted with in vitro translated labeled CHOP, suggesting that nonspecific interactions of pax-6 and cdx-2 with p300 do not take place (Fig. 4B).
On the basis of the transient transfection experiments (Fig. 2), we hypothesized that cdx-2 may be enhancing the interaction of pax-6 and the basal transcription machinery through interaction with a co-activator CBP/p300. To test whether cdx-2 may enhance the physical interaction of pax-6 and p300, we added increasing amounts of unlabeled cdx-2 to the GST-pax-6–p300 pull-down assay. As shown in Fig. 5, addition of increasing amounts of recombinant cdx-2 (in contrast to in vitro translated cdx-3) enhanced the recovery of labeled p300 protein by GST-pax-6. In contrast, addition of unlabeled recombinant pax-6 failed to increase recovery of labeled p300 through GST-cdx-2. These results, although not directly quantitative, support the hypothesis that cdx-2 might facilitate the protein–protein interaction of pax-6 with p300.

In vivo labeling of overexpressed proteins in the mouse glucagon-producing tumor cell line aTC-1 cells were in accordance with the in vitro GST pull-down assays (Fig. 6, A–C). As an internal control for the experiments, we used the known interaction of Ser-133-phosphorylated CREB with p300 (Fig. 6D).

To test whether pax-6 and cdx-2 associate with CBP/p300 co-activator at native levels, we performed co-immunoprecipitation experiments with nuclear extracts of aTC-1 cells. As shown in Fig. 7, immunoprecipitation with p300 antiserum and subsequent immunoblotting allows detection of p300 immunoreactivity, suggesting that both homeodomain transcription factors interact with p300 in aTC-1 cells.

We next sought to determine the site of interaction between pax-6 and cdx-2 with p300. GST pull-down experiments with GST constructs encompassing different portions of p300 protein reveal that both pax-6 and cdx-2 interact with the cystine/histidine-rich domain 1 (C/H1) of the p300 protein (Fig. 8).

**DISCUSSION**

This study provides evidence that both homeodomain proteins pax-6 and cdx-2 interact with co-activator p300 in the transcription of the proglucagon gene. Transcriptional activation of pax-6 and cdx-2 is increased by overexpression of p300 in the heterologous system of BHK-21 fibroblasts. Furthermore, our studies confirm the observation that pax-6 and cdx-2 interact synergistically on the proximal G1 promoter element through GST-pax-6. In contrast, addition of oct3 did not have any effect on the interaction of pax-6 and p300. Input lane of in vitro translated protein corresponds to 50% of protein used for GST pull-down assay.
that different transcription factors compete for the interaction with this co-activator (21). Thus, it is conceivable that overexpression of p300 can lead to enhanced transactivating effects as shown in the present study. However, the synergistic effect of pax-6 and cdx-2 together with p300 on the transcription of the proglucagon reporter gene at submaximal doses of pax-6 and cdx-2 argues against the notion that CBP analogues may be limiting in BHK-21 cells. The results of the present study rather support the concept that pax-6 and cdx-2 facilitate or stabilize each other’s interaction with the co-activator p300 (Fig. 9). To this end, in vitro binding studies show that both GST-pax-6 and GST-cdx-2 can pull down in vitro labeled p300. However, the recovery of p300 through GST-pax-6 was relatively low. By adding unlabeled in vitro translated cdx-2 to the GST-pax-6-p300 pull-down assay, the recovery of p300 increased. Although the data do not absolutely prove this idea, they suggest, together with the results of the transient transfection experiments that the interaction of pax-6 and p300 is relatively weak and may be enhanced by the presence of cdx-2 (Fig. 2).

In addition, the facilitated interaction of pax-6 with p300 by cdx-2 does not absolutely require binding of cdx-2 to the proximal AT-rich site of the proglucagon gene promoter. The transient transfection data support the notion that, even in the absence of a cdx-2 DNA binding site (Fig. 2, B and C), cdx-2 participates in the transcriptional regulation of the proglucagon gene. Taken together with the data that pax-6 binds cdx-2 in vitro and in vivo, it is likely that pax-6 tethers cdx-2 to the complex formed together with p300. On the other hand, in the presence of cdx-2, overexpression of pax-6 shows a tendency (although not significant) to further stimulate transcription even when its recognition site is mutated (Fig. 2B). However, in Fig. 2C, this property of pax-6 is not further recapitulated, suggesting that the DNA binding site for pax-6 is necessary for pax-6 to fully participate in the transcription of the glucagon gene through interaction with the G1 element. Another important observation is that mutation of the 3′ AT-rich site (cdx-2 binding site) significantly reduces the transactivation capacity of pax-6 in the experiment with added p300 (Fig. 2C). It may be speculated that, even in the absence of cdx-2, an optimal interaction of pax-6 with p300 requires a certain optimal DNA

modimer (6, 7). The elucidation of the identity of complexes A and C are largely in agreement with references (6, 7). The protein complex B cannot be accounted for binding by brain-4 alone, since nuclear extracts of BHK-21 cells overexpressing brain-4 form a protein-DNA complex, which is smaller in size than complex B (data not shown). In addition, a combination of brain-4 and pax-6 or cdx-2 incubated with the G1 element fail to form complex B in EMSA experiments (data not shown). Therefore, a protein in addition to brain-4 must be assumed to be involved in the formation of complex B (4). Further studies are required to elucidate the identity of this additional factor.

It must be assumed that CBP/p300 would be present in all cells, and further addition of p300 would not result in any substantial change in transcription. However, CBP/p300 is shown to be a co-activator present in limiting amounts such
sequence, which is no longer present when the 3' AT-rich site is mutated. Alternatively, a cryptic pax-6 binding site within the 3' AT-rich site could be suspected, of which mutation leads to a reduction of pax-6 transactivation. However, the EMSA experiments (Fig. 1B) do not suggest that pax-6 binds to the 3' AT-rich site.

The information derived from Fig. 8 suggests that both pax-6 and cdx-2 interact with the same region (C/H1 domain) of p300. However, from the result of transient transfection data, it cannot be assumed that the interaction site with p300 would be absolutely identical, since it pax-6 and cdx-2 do not appear to compete with the binding to p300, but act synergistically at the functional level. Furthermore, the in vitro observations indicate that cdx-2 may enhance binding of pax-6 to p300, possibly by further stabilizing the interaction of these proteins (Fig. 5). One possible explanation for our findings would be that cdx-2 forms a bridge between pax-6 and p300, no longer requiring a direct p300-pax-6 interaction, whereas in the absence of cdx-2, pax-6 has the capacity of interacting directly with p300. Alternatively, the stronger functional and physical interaction of pax-6 with p300 in the presence of cdx-2 may be due to a change in conformation of the participating proteins when interacting with each other and thus altering their capacity to associate with other proteins. Indeed, altered conformation of interacting homeodomain transcription factors has been described previously (22).

Taking all the above considerations into account, it appears that although pax-6, cdx-2, and p300 can interact separately with each other even in the absence of DNA, the optimal functional interaction of these proteins requires an intact proximal G1 element of the proglucagon gene (Fig. 2C). This notion is further supported by the EMSA results, which demonstrate that both AT-rich sites of the G1 element need be intact for the formation of a protein-DNA containing both pax-6 and cdx-2 (Fig. 1B). It is important to note at this point that the mutations in the promoter region may also alter the geometric association of the transcription factors, co-activator, and the basal transcription machinery to such an extent that some of the results generated by these methods may not only be accounted for just by the presence or lack of DNA binding sites for transcription factors but also be altered geometry in the region immediately upstream of the transcriptional start site.

pax-6 has been shown to bind not only to the G1 element but also to the upstream G3 enhancer element (8). Whether pax-6 bound to the G3 element also interacts with cdx-2 and/or p300 cannot be addressed with the present data.

cdx-2 has previously been reported to reduce the proliferation rate of intestinal epithelial cells (10). In other systems, the inhibition of progression through the cell cycle is reported to be mediated by CBP/p300. Thus, the function of cdx-2 in pancreatic α-cells may not only be the expression of the glucagon gene but also inhibition of α-cell proliferation. Little information is available on the pancreatic phenotype in cdx-2 knock-out mice. pax-6, on the other hand, has been implicated in pancreatic islet cell development and phenotype determination (8, 9). pax-6 mutant and knock-out mice have reduced pancreatic endocrine cell mass, with markedly reduced glucagon-producing cells (8, 9). The present results, together with previous reports, further support the notion that the transcription factors implicated in islet cell development are also involved in gene expression of terminally differentiated cells (8, 9, 23).

The in vitro relevance of our findings with regard to glucagon gene expression needs to be addressed by further studies. While the present studies show the importance of the interaction of the transcription factors pax-6 and cdx-2 with the α-cell-specific G1 element of the glucagon gene promoter and with the co-activator p300, it should be stated that expression of neither pax-6 nor cdx-2 is restricted to pancreatic α-cells. Of the transcription factors known to be expressed in pancreatic α-cells, brain-4 appears to be restricted to the glucagon-producing cells.

Fig. 8. Mapping of the interaction site of p300 with cdx2 or pax-6. Both homeoproteins interact with C/H1 domain of p300. GST fusion proteins with different fragments of the p300 protein (generously provided by H. Lu) attached to Sepharose beads were incubated with in vitro translated [35S]methionine-labeled cdx2 or pax-6. GST-protein and protein interacting with the GST-tagged protein were recovered by centrifugation of the Sepharose beads. After repeated washing procedures, the radioactively labeled protein trapped by the GST fusion protein was subjected to SDS-PAGE and detected by autoradiography. Input lane of in vitro translated protein corresponds to 50% of protein present in vitro.

Fig. 9. Proposed model of the interaction of pax-6 and cdx-2 with the co-activator p300. Binding of pax-6 to 5' AT-rich site of the G1 promoter element of the proglucagon gene is strong and required for the interaction to take place. Interaction of cdx-2 to the 3' AT-rich site in the promoter is weaker. pax-6 and cdx-2 interact with each other. Both pax-6 and cdx-2 interact with p300. Interaction of pax-6 with p300 is enhanced by the presence of cdx-2.
It is conceivable that the interaction of cell-specific with non-cell-specific transcription factors leads to an optimal expression of a certain cell-type restricted gene. To this end, in preliminary studies, we have found that overexpression in transgenic mice of brain-4 in the context of other transcription factors required of pancreatic endocrine cell phenotype can lead to ectopic expression of the glucagon gene.2

In conclusion, the present studies demonstrate that pax-6 and cdx-2 both interact with p300 at the protein-protein level. Further, the interaction of pax-6 with p300 is enhanced by the presence of cdx-2. Since cdx-2 participates in the transactivation of the proglucagon gene even in absence of its DNA binding site in the G1 promoter element, it is likely that pax-6 tethers cdx-2 to the complex formed by pax-6, cdx-2, and the transcriptional co-activator p300. Both pax-6 and cdx-2 stimulate proglucagon gene expression in pancreatic α-cells through interaction with the cell-specific G1 element. Thus, pax-6, cdx-2, and p300 form a functional complex in the α-cell-specific expression of the proglucagon gene. The elucidation of these additional transcription factors will lead to a better understanding of the complex nature of the tissue-specific expression of the glucagon gene.

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REFERENCES

1. Habener, J. F. (1996) in The Insulinotropic Gut Hormone Glucagon-like Peptide-I (Fehmann, H. C., and Gecke, B., eds) Vol. 13, Karger, Basel, Switzerland
2. Laser, B., Meda, P., Constant, I., and Philippe, J. (1996) J. Biol. Chem. 271, 28984--28994
3. Jin, T., Trinh, D. K. Y., Wang, F., and Drucker, D. J. (1997) Mol. Endocrinol. 11, 203--209
4. Hussain, M. A., Lee, J., Miller, C. P., and Habener, J. F. (1997) Mol. Cell. Biol. 17, 7186--7194
5. Philippe, J., Drucker, D. J., Kneepk, W., Jepeal, L., Misulovin, Z., and Habener, J. F. (1998) Mol. Cell. Biol. 8, 4877--4888
6. Andersen, F., Keller, R., Petersen, H., Jensen, J., Madsen, O., and Serup, P. (1999) FEBS Lett. 445, 306--310
7. Ritz-Laser, B., Estreicher, A., Klages, N., Saule, S., and Philippe, J. (1999) J. Biol. Chem. 274, 4324--4332
8. Sander, M., Neubuser, A., Kalamaras, J., Ee, H. C., Martin, G. R., and German, M. S. (1997) Genes Dev. 11, 1662--1673
9. St-Onge, L., and Sosa-Pineda, B. (1997) Nature 387, 406--409
10. Suh, E., Chen, L., Taylor, J., and Traber, P. G. (1994) Mol. Cell. Biol. 14, 7340--7351
11. Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. E., and Livingston, D. M. (1994) Genes Dev. 8, 869--884
12. Arany, Z., Sellers, W., Livingston, D., and Eckner, R. (1994) Cell 77, 799--800
13. Lundblad, J. R., Kwek, R. P., Laurence, M. E., Harter, M. L., and Goodman, R. H. (1995) Nature 374, 85--88
14. Xu, L., Lavinsky, R. M., Dassen, J. S., Flynn, S. E., Mcinerney, E. M., Mullen, T. M., Heinzl, T., Szeles, D., Korzus, E., Kurkowial, R., Aggarwal, A. K., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1998) Nature 395, 301--306
15. Ogryzko, V. V., Schiltz, L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 953--959
16. Jin, T., and Drucker, D. J. (1995) Mol. Endocrinol. 9, 1306--1320
17. Walker, W. H., Sanborn, B. M., and Habener, J. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12423--12427
18. Schreiber, E., Matthies, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
19. Lassar, A. B., Davis, R. L., Wright, W. E., Kadesh, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991) Cell 66, 305--315
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. S., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) Short Protocols in Molecular Biology, John Wiley & Sons, Inc., Boston
21. Ron, D., and Habener, J. F. (1992) Genes Dev. 6, 439--453
22. Passner, J. M., Ryo, H. D., Shen, L., Mann, R. S., and Aggarwal, A. K. (1999) Nature 397, 714--719
23. Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G., and Gruss, P. (1997) Nature 386, 399--402

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