Interaction of Maf Transcription Factors with Pax-6 Results in Synergistic Activation of the Glucagon Promoter*

Received for publication, May 17, 2001, and in revised form, July 5, 2001
Published, JBC Papers in Press, July 16, 2001, DOI 10.1074/jbc.M104523200

Nathalie Planque, Laurence Leconte, Frédéric M. Coquelle, Sofia Benkhelifa, Patrick Martin, Marie-Paule Felder-Schmittbuhl, and Simon Saule‡

From the CNRS-UMR 146, Institut Curie-Section de Recherche, Bât 110, Centre Universitaire, 91405 Orsay, France

In the endocrine pancreas, α-cell-specific expression of the glucagon gene is mediated by DNA-binding proteins that interact with the G1 proximal promoter element. Among these proteins, the paired domain transcription factor Pax-6 has been shown to bind to G1 and to transactivate glucagon gene expression. Close to the Pax-6-binding site, we observed the presence of a binding site for a basic leucine zipper transcription factor of the Maf family. In the present study, we demonstrate the presence of Maf family members in the endocrine pancreas that bind to G1 and transactivate glucagon promoter expression. In transient transfection experiments, we found that the transactivating effect on the glucagon promoter was greatly enhanced by the simultaneous expression of Maf transcription factors and Pax-6. This enhancement on glucagon transactivation could be correlated with the ability of these proteins to interact together but does not require binding of Maf proteins to the G1 element. Furthermore, we found that Maf enhanced the Pax-6 DNA binding capacity. Our data indicate that Maf transcription factors may contribute to glucagon gene expression in the pancreas.

Pax-6 is a member of the paired domain family of transcription factors and has a specialized homeodomain downstream from the DNA-bindingpaired domain and upstream from the C-terminal activation domain (1, 2). Pax-6 encodes five proteins through alternative splicing and internal initiations (3). Three proteins of 48, 46, and 43 kDa contain the paired domain, whereas two proteins of 33 and 32 kDa are devoid of this DNA binding domain. Pax-6 is known to be critical for eye development (4, 5), where it is required for lens differentiation and crystalline gene expression (6, 7). Pax-6 is also required for the development of all pancreatic endocrine cells and of duodenal GIP-positive cells and gastrin- and somatostatin-producing cells in the stomach (8–10). The α-cell-specific glucagon gene expression in pancreas is conferred by the proximal G1 promoter element (11). The G1 element contains two AT-rich sequences that are recognized by the homeodomain containing Cdx-2/3, Pax-6, and brain-4 (12–14). Pax-6 and Cdx-2/3 have been shown to bind directly to each other and to transactivate synergistically the glucagon gene via their interaction with the G1 element (14). This effect is even enhanced by interaction of these proteins with the p300 coactivator (15). Fine-tuning of cell type-specific gene expression and lineage-specific cell differentiation seems to be achieved by cooperative and inhibitory interactions of transcription factors. To understand the mechanisms of transcriptional regulation in pancreas, it is important to clarify the regulatory cross-talk that occurs with other transcription factors on the G1 element. Examination of the G1 element nucleotide sequence revealed the presence of a potential binding site (16) for a basic leucine zipper (bZip) domain transcription factor of the Maf family, located close to the Pax-6-binding element.

Loss-of-function of the c-Maf bZip1 factor results in defective differentiation of lens fiber cells (17, 18). Ectopic expression of L-Maf/MaFA results in conversion of ectodermal cells into lens fibers cells, and this factor is able to activate the promoters of crystalline genes (19). L-Maf/MaFA, which is expressed in both lens and retina, displays mitogenic capacity when overexpressed in avian neuroretina cells (20). A variety of developmental roles and transcriptional targets has been proposed for Maf transcription factors. The v-maf oncogene is the earliest described member of the family (21). Large Maf subfamily members (c-Maf, L-Maf/MaFA, MaFB, and Nr1) contain an activation domain at the N terminus, whereas small Maf subfamily members (MaFF, MaFK, and MaFG) lack a distinct activation domain (22). Maf transcription factors share structural similarity both within and outside the basic leucine zipper domain and bind common recognition elements, 12-O-tetradecanoylphorbol 13-acetate type Maf response element or cyclic AMP response element type (16, 23). Homo- and heterodimerization through leucine zipper domains is one of the most important mechanisms underlying transcriptional regulation by bZip factors. All the Maf family members can form heterodimers with other bZip factors like Fos and Jun, and these heterodimers are different in their DNA binding specificity from Maf homodimers or AP-1 complexes (16, 24). BZip transcription factors are also able to interact with unrelated transcription factors like glucocorticoid receptors or Ets family members (25, 26). In the case of c-Maf, interaction with the transcription factor c-Myb plays a role during myeloid cell differentiation (27), whereas MaFB interaction with c-Ets-1 represses its transcriptional activity, resulting in the inhibition of erythroid cells differentiation (28). Recently, Maf family members were shown to associate with a set of Hox proteins, resulting in the inhibition of Maf DNA binding, transactivation, and transforming activities (29). Thus, in view of the involvement of both Pax-6 and Maf in common developmental processes (such as lens formation), and the presence of the binding sites for both fac-

* This work was supported by grants from the CNRS, the Institut Curie, the Association Retina France, the Association pour la Recherche Contre le Cancer, the Ligue Nationale Contre le Cancer, and L’Association de Secours Des Amis des Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 33 1 69 86 71 53; Fax: 33 1 69 07 45 25; E-mail: Simon.Saule@curie.u-psud.fr.

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

35751

This paper is available on line at http://www.jbc.org
tors in the G1 element, we tested whether they could interact with and influence each other.

We show that Pax-6 and MafA/L-Maf interact through their transactivation domains. This interaction results in Pax-6-enhanced DNA binding capacity on the G1 element where MafA/L-Maf is indeed able to bind. Cotransfection of a MafA/L-Maf expression plasmid and the G1-containing glucagon promoter results in an increase in CAT activity relatively to the control vector, showing that MafA can activate the glucagon promoter. Cotransfection of MafA/L-Maf expression plasmid with Pax-6 expression vector results in a 5-fold increase in CAT activity over the effect of each factor alone, suggesting a synergistic effect in expression of glucagon promoter through the G1 element by Pax-6 and MafA. This synergistic effect requires a functional paired domain but not the homeodomain. In contrast, as shown with two DNA-binding mutants, the DNA binding capacity of Maf is dispensable. Surprisingly, when the transactivation domain of MafA/L-Maf is removed, this truncated factor still increases the Pax-6 effect on the glucagon promoter. Maf protein strongly enhances the binding of Pax-6 to the G1 element, even when truncated in the transactivation domain or without DNA-binding properties. Finally, we demonstrate the presence of MafA/L-Maf in pancreas and c-Maf in endocrine cells, suggesting that Maf family members may play a role in the pancreas function.

**Experimental Procedures**

Constructs—pcDNA3-MafA expression vector was obtained by inserting an EcoRI fragment corresponding to MafA open reading frame into the EcoRI site of a modified pcDNA3 vector, in frame with HA1 epitope coding sequence. Generation of mutant MafA containing the L2P4AP mutation and of truncated MafA corresponding to the bZip domain was described previously (20). For eukaryotic expression, EcoRI fragments corresponding to these open reading frames were inserted into the EcoRI site of modified pcDNA3 vector as for wild type MafA.

For the synthesis of GST-MafA recombinant proteins, the EcoRI fragment corresponding to MafA open reading frame was subcloned into the EcoRI site of pGex-4T1 vector (Amersham Pharmacia Biotech). For the synthesis of GST-MafA(1–151), proteins an EagI deletion, eliminating the bZip domain, was performed in the previous construct. For the synthesis of GST-MafA(151–286) proteins, an EagI fragment corresponding to MafA bZip domain was subcloned into the Ndel site of pGex-4T2 (Amersham Pharmacia Biotech).

Reporter construct (QR1-AboxX4-TK) carrying a multimerized A box from the neuroretina-specific QR1 gene, upstream of the thymidine kinase promoter, was described previously (20).

The glucagon promoter construct –135M3 was mutated in the MafA-binding site. To generate this mutant we used the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) using the –138 CAT as a template and oligonucleotide G1–51M3 (5‘-CAG ACC CCC ATT ATT TAC AGA TGA GAA ATT ATT TCT GTG GTA ATA ATC TCT-3‘).

**In Vitro Translation and Glutathione S-transferase (GST) Pull-down Assays**—The 35S-radioabeled proteins were translated in vitro using the TNT system (Promega). The GST chimerical proteins were extracted from bacteria following the Amersham Pharmacia Biotech instructions. The labeled proteins were preincubated with empty glutathione-Sepharose beads 30 min at 4°C. The GST proteins on glutathione-Sepharose beads were preincubated with 40 μg of BSA. Bead volumes were kept constant by adding empty beads. The pull-down assays were performed in 25 mm Hepes, pH 7.5, 150 mm KC1, 12.5 mm MgCl2, 0.1% Nonidet P-40, 20% glycerol. Proteins were incubated for 1 h at 4°C and then the beads were washed four times in 20 μl Tris-HCl, pH 8, 100 mm NaCl, 1 mm EDTA, 0.5% Nonidet P-40.

**Cell Cultures, Transfections, Luciferase, and CAT Assays**—Baby hamster kidney (BHK)-21 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal calf serum, 1% vitamins, 100 mg/ml sodium pyruvate, 10 μg/ml ascorbic acid. Cells were seeded 24 h prior to transfection. BHK21 cells were transfected with polyethyleneimine (exgen 5000, Euromedex, Souffelweyersheim) reagent according to the instructions of the manufacturer. 200 ng of expression plasmids were cotransfected except where mentioned in the figure legend. αTC cells transfections were performed by the calcium-phosphate method. The total amount of transfected DNA was kept constant by addition of empty expression vector DNA. A LTRSV-

**Fig. 1. Pax-6 and MafA/L-Maf interact in vitro.** A, the 35S-labeled full-length MafA protein was translated in vitro and incubated with GST, GST fused to the paired domain of p46, GST homeodomain, or GST full-length p46 proteins. The GST protein showed no interaction with the translated MafA protein (lane 1). The paired domain of p46 interacts with MafA (lane 2), such as the p46 homeodomain (lane 3). Stronger interaction was observed with the full-length protein (lane 4). Lane 5, 40% of the reticulocyte lysate was loaded onto the glutathione-Sepharose beads. Numbers refer to amino acids. B, the 35S-labeled full-length Pax-6 protein was translated in vitro and incubated with GST alone (lane 1), GST fused to the N-terminal part of MafA (lane 2), to the C-terminal part of MafA (lane 3), or to the full-length MafA protein (lane 4). Lane 5, 20% of the reticulocyte lysate was loaded onto the glutathione-Sepharose beads. p46 and p30 identify the full size Pax-6 and the paired-less proteins, respectively (3). C, the different GST-Maf fusion proteins used in the pull-down of B are shown after Coomassie Blue staining of the gel. D, the 35S-labeled full-length MafA protein was translated in vitro and incubated with GST-MafA(1–286). A specific interaction with 35S-labeled full-length MafA protein is shown in lane 1, and no binding could be observed with the GST alone (lane 3). 20% of the reticulocyte lysate loaded onto the GST-MafA(1–286) glutathione-Sepharose beads is shown in the lane 2. E, as a control of binding specificity, the GST-MafA(1–286) proteins were incubated with 35S-labeled GFP protein translated in vitro (lane 1). No binding was observed. Lane 2, 20% of the reticulocyte lysate was loaded onto the GST-MafA(1–286) glutathione-Sepharose beads.

LacZ vector or a pcDNA3-LacZ was cotransfected for normalization of CAT assays by controlling the β-galactosidase activity. CAT assays were performed as described previously (30). Levels of CAT activity were quantified after exposure of the thin layer chromatograms to a PhosphorImager screen (Molecular Dynamics).

**Electrophoretic Mobility Shift Assay**—The DNA used as probes were G1–51, G1–51M10, and G1–51M3 and as a control the MITF-binding site in the QNR-71 promoter. G1–51 (5′-CAAGACCCCATTATTAGACCGAGATTTCTCT-3′) is a wild type glucagon promoter element described previously (14) to bind Pax-6. G1–51M10 (5′-CAAGACCCCATTATTACGGAGAGATTTCTCT-3′) is mutated in the Pax-6-binding site (14). G1–51M3 (5′-CAAGACCCCATTATTACGGAGAGATTTCTCT-3′) is mutated in the presumptive Maf-binding site. Underlines represent mutations introduced in the oligonucleotides. QNR-71 oligonucleotide is 5′-GCTTTATACTCCAGATCCTTGAACTACCCATATTATCTGACAGATTTCTCT-3′.
CAT GAT GAG TCC TG-3’. The DNA probes were double-strand oligonucleotides [γ-32P]ATP-labeled with the polynucleotide kinase T4. Gel retardation assays were performed as described previously (30) with 100 ng of bacterially expressed proteins.

**GFP and DsRed Fusion Protein Constructs and Wide Field Optical Scanning Fluorescence Microscopy—**To visualize Pax-6 and MafA proteins, green fluorescent protein (GFP, CLONTECH) and Discosoma strigata red protein (DsRed, CLONTECH) were used as fusion partners of p46 and MafA, respectively, and the following plasmids were constructed. An Nhel-BglII EGFP fragment was ligated to the XhoI and BgIII sites of pVNC3 (modified from pVM116, see Ref. 31) to produce pVNC3 EGFP C1. A BamHI-Apal MafA fragment was inserted into the BgIII and ApoI sites of pVNC3 EGFP to make pVNC EGFP MafA. pVNC3 MiRed (32) was digested with XhoI and Bsp120I to excise the Mifl open reading frame and to replace it by a XhoI-NolI p46 fragment to generate pVNC3 Pax6Red. As controls, we used EGFPMyC and Pax6EGFP (32).

pVNC3 Pax6Red and pVNC EGFP MafA were cotransfected in BHK21 cells. To determine the localization of the chimerical proteins into the nuclei, cells were fixed for 20 min at room temperature in 3% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM magnesium acetate, pH 6.9). Cells were washed three times in PBS and permeabilized for 25 min in 0.1% Triton X-100 in PBS. Chromosomes were stained with 4,6-diamidino-2-phenylindole (Sigma) for 5 min. After a rinse in PBS, coverslips were mounted in 50% PBS/glycerol containing anti-fading reagent 1.4 diazabicyclo[2.2.2]octane (Sigma) at 100 ng/ml.

Pictures of fixed cells were collected using a three-dimensional deconvolution imaging system, the detailed description and validation of which will be published elsewhere. Briefly, it consisted of a Leica DM RXA microscope, equipped with a piezoelectric translator (PIFOC, PI, Germany) placed at the base of a 100× PlanApo N.A. 1.4 objective, and a 5-MHz Micromax 1300Y interline CCD camera (Roper Instruments, France). For the acquisition of Z-series, the camera was operated at full speed and controlled the piezo translator at the start of each CCD chip read out. Stacks containing fluorescence images were collected automatically at 0.2-μm Z-intervals (Metamorph software, Universal Imaging). Wavelength selection was achieved by switching to the corresponding motorized selective Leica filter block before each stack acquisition. Tests using 50 nm tetraspec beads (Molecular Probes) showed that the system did not generate x-y pixel shifts and that Z-plane shifts between colors, due to chromatic aberrations, were reproducible and could be corrected. Exposure times were adjusted to provide ~3000 gray levels at sites of strong labeling. Automated batch deconvolution of each Z-series was computed using a measured point spread function and constrained iterative deconvolution with a custom made software package. The point spread function of the optical system was extracted from three-dimensional images of fluorescent beads of 0.1 μm in diameter (Molecular Probes) collected at each wavelength. The Z-series were pseudo-colored and overlaid, and maximal pixel intensity projections were calculated with the help of Metamorph software (Universal Imaging).

**RT-PCR—**Total RNA from pancreas from 8-day-old quail embryos were digested with RQI DNase. After phenol-chloroform extraction and ethanol precipitation, 2 μg of these RNA were annealed with 0.5 μl of oligonucleotide dT (Perkin-Elmer) (50 μM) for 10 min at 70 °C. The cDNA were obtained by adding 1 μl of 0.1 M dithiothreitol, 2 μl of 5 mM dNTP mix (Eurogentec), 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and 4 μl of 5× buffer in a final volume of 20 μl. The mixture was incubated for 1 h at 42 °C. For the PCR, a 2-μl aliquot was added to 1.6 μl of 25 mM MgCl2, 1 μl of 5 mM dNTP mix (Eurogentec), 0.5 units of Goldstar Taq (Eurogentec), 2 μl of 10× buffer, 1 μl of 20 μM oligonucleotide primers 5′-TTC CAC CCC TCT CAG-3′ and 5′-CTC CCG AAC CGA CAT ACT-3′, in a final volume of 20 μl. Amplifications were carried out in a PTC-200 (MJ Research) as follows: 4 min at 94 °C; 2 min; 30 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 45 s; 72 °C for 10 min. Reaction products were cloned into PCR2.1 (TA cloning Kit, Invitrogen). Individual clones were isolated and sequenced. Similar experiments were performed using dT cells RNA and oligonucleotide primers Maf5′, Maf5′2, and Maf5′3 as described (20).
Fig. 3. Synergistic effect on the glucagon promoter transactivation by different Pax and MafA constructs. A, Pax-2 and MafA did not act synergistically on the glucagon promoter. Two Pax-2 isoforms with distinct transactivation efficiency were used. B, Pax-6 homeodomain is dispensable, but a functional paired domain is required. A schematic representation of the Pax-6 constructs used is shown above the histogram. p46 and p48 Pax-6 isoforms contain two DNA-binding domains, the paired box and the homeobox. p48 contains an addition of 14 amino acids in the paired domain. Numbers refer to the amino acids. Pax-6A170–270 is devoid of the homeodomain but still contains the paired domain. Pax-6A170–270 transactivated the G3–138 glucagon promoter as efficiently as the wild type construct and acted synergistically with MafA (compare with Fig. 1B), suggesting that the homeodomain is not required both for the glucagon promoter transactivation and for the synergy with MafA. The p48 isoform did not transactivate the glucagon promoter and did not modulate the MafA transactivation. C, MafA was still able to act synergistically with Pax-6 as a monomer without DNA binding activity (MafAL2PL4P) or with DNA binding activity but without transactivation domain (MafA(173–286)). A schematic representation of the constructs used is shown above the histogram. Basic domain; LZ, leucine zipper. Both MafAL2PL4P and MafA(173–286), without effect alone, increased the p46 activity.

RESULTS

Pax-6 Interaction with MafA—To test the hypothesis that Pax-6 physically interacts with MafA, we prepared glutathione-Sepharose beads coupled with a GST-paired fusion protein containing the paired domain from the p46 (GST-Prd46(3–131)) and GST-Hom (224–284), containing the p46 homeodomain, and the GST-p46(1–416), containing the full-length Pax-6 product. We also used GST fusion proteins containing either the N-terminal (residues 1–151) or the C-terminal domains (residues 151–286) of MafA or the full-length protein. Glutathione-Sepharose beads coupled to GST served as a negative control. In vitro radiolabeled MafA/L-Maf or Pax-6 proteins were loaded onto these beads and washed, and bound proteins were eluted by boiling and analyzed in SDS-PAGE. The percentage of bound radioactivity was calculated using PhosphorImager. A similar amount of radiolabeled MafA was recovered from GST-Prd46 (0.4%) and from GST-Hom (0.2%). In contrast, a much higher amount of radiolabeled MafA protein (5.6%) was recovered from the GST-p46, suggesting that the transactivation domain of Pax-6 is involved in the protein-protein interaction (Fig. 1A).

As shown in Fig. 1B, both the N terminus of MafA (amino acids 1–151, 1%, lane 2) and the C-terminal (amino acids 151–286, 0.8%, lane 3) domains were independently able to bind the radiolabeled p46. However, it is important to note that a greater amount of GST-N-terminal protein has been used in the experiment (Fig. 1C, compare lanes 1 with 2 and 3) suggesting in fact a lower efficiency of this part of the MafA protein in the interaction with the p46. The full size Maf bound with a greater efficiency (19%) than the two separated parts of the protein. The GST alone did not interact with the radiolabeled MafA (Fig. 1B, lane 1). As a positive control, we incubated the full size GST-MafA(1–286) with the radiolabeled MafA (Fig. 1D). The amount of recovered protein (10%, lane 1) was similar to the amount p46 recovery (Fig. 1B, lane 4), suggesting a strong interaction between the two proteins. As a negative control, we incubated the full size GST-MafA(1–286) with the nonrelevant radiolabeled GFP protein. No interaction could be observed between these two proteins (Fig. 1E). As for Engrailed (33) or Mitf (32), two p46-interacting proteins, the paired-less p30 proteins did not significantly bind to either part of MafA (Fig. 1B).

Effects of Pax-6/Maf Coexpression on Their Transcriptional Activities—Nrl, a large Maf family member, acts synergistically with the paired-like homeodomain transcription factor Crx to activate the rhodopsin gene (34, 35). A potential Maf-binding site (TCAGCG) is present close to the Pax-6-binding site in the G1 element of the glucagon promoter. Therefore, we tested if the observed Pax-6/Maf interaction was able to regulate expression of Pax-6-dependent glucagon promoter. BHK21 cell lysates were collected 2 days after transfection, and the levels of CAT and β-galactosidase activities present in the lysates were determined. Cotransfection of the G1 containing −138 glucagon promoter with the vector expressing the MafA/L-Maf protein resulted in an increase of CAT activity relative...
to that of the control vector (Fig. 2). Similar increase of CAT activity was observed with the −138 glucagon promoter in response to Pax-6 protein expression. Cotransfection of the −138 glucagon promoter with the vectors expressing MafA and Pax-6 proteins resulted in a 5-fold increase of CAT activity relative to the level of expression obtained with each transcription factor alone. The synergistic activation obtained was specific of this promoter because no synergistic effect was observed on the CMV- or RSVLTR-lacZ cotransfected in the experiment. The G3−138, containing an additional Pax-6-binding site (14), also exhibited this synergistic response, except that the CAT activity was increased (Fig. 2B) and the promoter behaved similarly (Fig. 2C). In contrast, the G3 element linked to the Maf sequence, showed a marginal activation by both Pax-6 and MafA/L-Maf, suggesting that the G1 element is indeed essential in the Maf response of the glucagon promoter and synergy with Pax-6.

Another Pax family member (Pax-2) has been described to activate the glucagon promoter through the G1 element (36). We tested the effect of the simultaneous expression of Pax-2 and MafA on the G3−138 glucagon promoter. The D2 form of Pax-2 (a splicing variant in the transactivation domain) was a better transactivator than Pax-6, whereas the C form (36) exhibited only a marginal effect on glucagon expression (Fig. 3A). In contrast to Pax-6, Pax-2 contains only a partial homeodomain (37). Therefore, we asked whether the homeodomain of Pax-6 was required for the synergistic transactivation of the glucagon promoter observed with MafA. We used a Pax-6 mutant, which is devoid of homeodomain, (Pax-6/H9004−270, see Ref. 2). This Pax-6 mutant transactivated the G3−138 glucagon promoter to an extent similar to that of the wild type, suggesting that the
homeodomain is dispensable for G3–138 glucagon promoter activation (Fig. 3B). Cotransfection of the MafA/L-Maf revealed that the Pax-6A170–270 mutant increased glucagon promoter transactivation with MafA. We next asked whether a functional paired domain was required. For that purpose, we used the p48 isoform of Pax-6, which recognizes a distinct DNA-binding sequence due to the insertion of 14 amino acid residues in the paired domain (38). p48 is unable to transactivate the glucagon promoter (Fig. 3B). Coexpression of p48 and MafA proteins did not modify the level of CAT activity when compared with MafA alone (Fig. 3B), indicating that the binding of Pax-6 to DNA through the paired domain is required in order to increase the MafA/L-Maf effect on this promoter.

These results prompted us to study the effect of MafA/L-Maf truncated proteins devoid of transactivation domain in the cooperative transcriptional activation with Pax-6. Fig. 3C shows that this mutant MafA173–286 was no longer able to activate the glucagon promoter but still increased the effect of Pax-6 on the G3–138 promoter, suggesting that binding of MafA to the DNA was sufficient to increase the Pax-6 transactivation properties. However, the use of additional mutants indicated that MafA/L-Maf was also able to increase the Pax-6 activity without binding to DNA. Indeed, mutant MafA2PL4P was unable to activate the glucagon gene promoter (Fig. 3C) but still increased the Pax-6 effect on this promoter (Fig. 3C). MafA2PL4P was unable to form a coiled-coil structure because 2nd and 4th leucine residues were replaced by proline residues in the leucine zipper (20).
We next tested the effect of other Maf family members (Fig. 4A) on Pax-6 activity. Fig. 4B indicates that v-Maf and MafB were indistinguishable from MafA/L-Maf with respect to their effect on glucagon activation and synergy with Pax-6. In addition, a v-Maf mutant in the basic domain (R22E), which is unable to bind DNA (39) and to activate the glucagon promoter, (Fig. 4B), still increased the Pax-6 effect on the G3–138 (Fig. 4B).

Therefore, we conclude that MafA/L-Maf is able to increase the Pax-6 activity in two distinct ways: first in a DNA-binding dependent way (the transactivation domain is dispensable) and second in a DNA-binding independent way. In contrast, Pax-6 must bind to the DNA in order to allow synergistic interaction with Maf. Additional evidence was provided by the use of a thymidine kinase promoter linked with four repeats of a Maf-binding element from the QR1 promoter (20). Fig. 4C shows that coexpression of p46 and MafA/L-Maf did not modify the level of QR1-Abox(x4)-TK promoter expression when compared with MafA/L-Maf expressed alone. Pax-6 alone was unable to activate expression of this promoter (Fig. 4C).

MafA Increases the DNA Binding Activity of Pax-6 in Vitro—Many eukaryotic bZIP proteins are able to bind the DNA, and the resulting DNA curvature may facilitate protein-protein or protein-DNA contacts that are essential in transcriptional regulation (40). Because of Maf and Pax-6 interactions, we tested whether Maf was able to modulate the DNA binding activity of Pax-6. We used glucagon promoter-derived probes to study the binding of GST-Prd46, GST-p46, and GST-MafA, as well as their reciprocal effect on DNA binding. MafA was indeed able to bind the G3–138 DNA sequence, confirming that MafA binds to the glucagon promoter (data not shown).

To demonstrate that the TCAGCG sequence found in the G1 element was the binding site for MafA/L-Maf, we used an oligonucleotide encompassing the G1 nucleotide sequence (Fig. 5A) from −100 to −50 (G1–51) as probe. We also prepared two mutated versions of this oligonucleotide as a probe, one in the Pax-6-binding site (mutated nucleotides from −87 to −78; G1–51M10) and one in the expected MafA-binding site (mutated nucleotides from −60 to −58, G1–51M3). GST-MafA alone was able to bind to G1–51 (Fig. 5B, lane 1), and the resulting complex was competed for by an excess (100×) of unlabeled G1–51 oligonucleotides (Fig. 5B, lane 2) or G1–51M10 (Fig. 5B, lane 4) but not by a similar excess of the G1–51M3 containing the mutation in the expected MafA/L-Maf-binding site (Fig. 5B, lane 3). Gel shift experiments performed with a GST fusion protein containing the p46 showed a specific binding (Fig. 5C, lane 1) competed for by unlabeled G1–51 oligonucleotide (Fig. 5C, lanes 2 and 3) but not by the G1–51M10 oligonucleotide containing the mutated binding site (Fig. 5C, lane 4). We conclude that the TCAGCG is a functional binding site for the MafA protein. Moreover, coinuclease of GST-MafA and GST-Prd46 showed an increase in the binding of GST-Prd46(63–131) to the DNA probe (Fig. 6A, compare lanes 2 and 4).

Since a synergistic transactivation was observed between the p46 Pax-6 and mutants of MafA/L-Maf (mutants in the LZ or in the basic domain that are unable to bind DNA as well as a mutant devoid of transactivation domain but able to bind DNA), we studied whether the GST-Prd46 or GST-p46 binding to G1–51 was increased by GST-MafA/L-Maf as well as by the GST-MafA(151–286). Fig. 6 shows that both GST-MafA mutant proteins increased the Pax-6 binding to the DNA (Fig. 6, B and C) in a very similar way to that of the wild type MafA protein (Fig. 6A). We also investigated whether MafA was able to modulate the binding of p46 to the G1–51M10 oligonucleotide. When this DNA was used as probe, no binding of the p46 was found (Fig. 6D, lane 5), as expected since this oligonucleotide was not able to compete for the binding to the wild type G1–51 glucagon promoter. In contrast to Pax-6, neither GST-MafA nor GST-p46 (Fig. 5C). In contrast, in the presence of GST-MafA protein, p46 was able to bind to the mutated DNA, albeit less efficiently than the wild type sequence (Fig. 6D, lane 6, compare with lane 3). Further confirmation that Maf binding to DNA was not required to increase the efficiency of Pax-6 DNA binding, we observed an increase in p46 binding in the presence of Maf protein on the G1–51M3 oligonucleotide (Fig. 6D, compare lanes 8 and 9).

Since the Pax-6 p48 protein was unable to activate the glucagon promoter and to increase MafA transactivation properties on this promoter (Fig. 3B), we asked whether the paired domain of p48 was able to recognize the G1–51 element and whether GST-MafA protein was able to modulate p48 DNA binding. Fig. 6E shows that, in contrast to GST-Prd46, GST-Prd46 was unable to bind to G1–51 probe without GST-MafA. However, a very faint binding was induced in the presence of GST-MafA (Fig. 6E, compare lanes 4 and 5). To demonstrate further that GST-MafA proteins specifically increased Pax-6 binding to the DNA, we performed a gel shift experiment using a GST-MafA (193–413) fusion protein and the QNR-71 oligonucleotide probe, with and without GST-MafA. As shown in Fig. 6F, the MafA/FN complex was not modified in the presence of MafA.

To investigate the functional role of the MafA-binding site in the glucagon promoter, we performed transfection experiments in BHK21 cells using the −138 glucagon promoter or the promoter bearing the MafA mutations inserted in the G1–51 oligonucleotide (−138M3). Fig. 7 shows the −138M3 glucagon promoter was still activated by Pax-6. In contrast, this mutant promoter exhibited only a marginal activity in response to MafA when compared with the wild type −138 promoter. Therefore, we conclude that the TCAGCG site that we identified in the G1 element constitutes a functional binding site for Maf proteins and that no additional Maf-binding site exists in the −138 glucagon promoter. In contrast to the wild type promoter, concomitant expression of MafA and Pax-6 resulted in a limited effect on the −138M3 promoter, but transactivation was still greater than that obtained with Pax-6 alone, further
indicating that Maf could increase Pax-6 transactivation without binding to the DNA.

**Pax-6 and MafA Colocalize in the Nucleus**—To demonstrate that Pax-6 and Maf are able to interact in vivo, we cotransfected vectors expressing the p46, tagged with the DsRed protein, and the MafA/L-Maf, tagged with the green fluorescent protein (EGFP). Expression of these vectors into BHK21 cells allowed detection of both proteins in cell nuclei (Fig. 8, A and B). Fig. 8C shows the overlap of the two fluorescent proteins by maximal pixel intensity projections of a representative nucleus. If part of the proteins were localized in distinct areas of the nucleus, a significant amount of the yellow labeling could be observed, suggesting that p46 and MafA proteins colocalize in common areas of the nucleus in vivo. As shown in Fig. 8F, the chromophore did not influence by itself the protein localization, since Pax-6 tagged either with EGFP (Fig. 8D) or with DsRed (Fig. 8E) shows a perfect overlapping (Fig. 8F) in COS cells transfected with the two expression vectors. To demonstrate that such a colocalization is not an artifact due to a random effect, we performed a similar cotransfection experiment with a v-Myc protein (which has been shown to not interact with Mitf in vitro, see Ref. 41) tagged with EGFP and Mitf tagged with DsRed. In contrast to Pax-6 and MafA, the overlap of the two fluorescent proteins revealed, as described previously (32), that Mitf and v-Myc do not substantially colocalize (Fig. 8I).

**c-Maf and MafA Are Expressed in the Pancreas**—To identify the maf genes expressed in the pancreas, we first performed RT-PCR amplifications using MafA-specific oligonucleotide primers located upstream from the basic domain and at the end of the zipper domain, respectively. As shown in Fig. 9A, a 396-bp fragment was amplified from the Quail E8 pancreas RNA, which was reverse-transcribed using a dT-priming oligonucleotide. The sequence obtained from this Quail cDNA was indeed identical to that of MafA sequence (20). To demonstrate further that Maf products are present in Pax-6-positive endocrine pancreas cells, we performed a similar experiment using RNA extracted from mouse glucagon producing α-cell line (αTC, see Ref. 42). We performed PCR using Maf-degenerated oligonucleotides, and we identified the c-Maf corresponding fragment after sequencing. This result is in good agreement with the report of Sakai et al. (43) showing that c-maf is activated by Pax-6. To demonstrate further the presence of c-Maf proteins into the nucleus of the αTC cells, we performed an immunodetection experiment using a rabbit anti-v-Maf serum. Fig. 9B shows an example of immunostained αTC nuclei. Since αTC cells expressed c-Maf, we performed transfection experiments in αTC cells using the −138 glucagon promoter or the promoter bearing the MafA mutations inserted in the G1–51 oligonucleotide (−138G1M3). Fig. 9C shows that the mutant promoter exhibits a reduced CAT activity when compared with the wild type −138 promoter. Therefore, we conclude that endogenous c-Maf is important for glucagon promoter expression in the αTC cells.

**DISCUSSION**

This study presents evidence that Pax-6 interacts with Maf transcription factors and that this interaction supports synergistic transactivation on the glucagon promoter. It has been shown that Pax-6 and Maf family members are involved in cellular proliferation and differentiation. These factors are expressed in overlapping regions in the eye or in the central nervous system. For example, MafB has been shown to establish specific rhombomeres in the developing hindbrain and to be responsible for the Kreisler mutation in mouse (44). MafA/L-Maf, Nrl, and Pax-6 are expressed in the neuroretina (20); c-Maf, MafB, Nrl, MafA/L-Maf, and Pax-6 are coexpressed in the lens (17–19, 45, 46). Both Pax-6-binding sites and Maf response elements were identified in transcriptional regulatory regions of cryalline genes (7, 18), as well as in the c-maf promoter (43). All these promoters responded to Pax-6 expression. We therefore examined whether Maf proteins and Pax-6 were able to interact, and we investigated their effects on promoters responsive to these factors. We show here that Pax-6 and Maf proteins interact in pull-down experiments in vitro. Pax-6 activates the glucagon promoter through binding sites located in the G3 and G1 enhancer elements (14), and we demonstrate here that Maf proteins are also effective in activating the glucagon promoter. A Maf-binding site is located in the G1 element, close to the Pax-6-binding site. Mutation of the TCAGCG sequence suppresses both Maf DNA-binding and Maf-
dependent transactivation of the promoter, indicating that the glucagon promoter is a target for Maf transcription factors. Furthermore, we find that both MafA/L-Maf (in quail) and c-Maf (in mouse) are expressed in the pancreas.

Coexpression of Pax-6 and Maf transcription factors resulted in a synergistic activation of the glucagon promoter. This effect was observed not only with all Maf proteins tested (c-Maf, MafB, MafA, and v-Maf) but also with Maf mutants. We tested two Maf mutants, unable to bind DNA and to transactivate the glucagon promoter (a point mutation in the basic domain of v-Maf and a proline mutant in the leucine zipper of MafA), and both of them increased Pax-6-dependent transactivation of the promoter. Likewise, a truncated MafA mutant, lacking the activation domain and unable to activate the glucagon promoter expression, was still able to increase Pax-6 activity. A common effect of these proteins was to increase the DNA-binding efficiency of Pax-6 on its target DNA, as shown by gel shift experiments on the G1 element probe (G1–51). Interestingly the Pax-6 p48 isofrom that contains an alternative exon in the paired domain and recognizes a DNA sequence different from the binding site of p46 (38, 47) did not activate the glucagon promoter. Paired p48 did not bind significantly to the G1 element, even in the presence of MafA proteins. Therefore, there is a positive correlation between the synergistic transactivation and the enhanced binding of Pax-6 to the DNA.

What could be the mechanism leading to the synergistic activation of the glucagon promoter by Pax-6 and Maf transcription factors? Interactions among transcription factors that bind to separate promoter elements may depend on distortion of DNA structure. Fos and Jun, two AP-1 family members related to Maf, have been shown to induce DNA bending, and both the bZip domains and regions overlapping their transcription activation domain induce DNA bending (48). Therefore, it is possible that the common effect of Maf and Maf mutants includes an effect on DNA bending resulting in a more efficient Pax-6 binding and transactivation. Since Maf family members and Pax-6 are coexpressed in the lens and are both important for lens development and crystalline genes expression, we tested the effect of Pax-6 and MafA coexpression on the αA-Crystalline promoter (49). Contrasting with the transactivation synergy observed on the glucagon promoter, no such effect was observed on αA-Crystalline promoter (data not shown), although MafA efficiently activated this promoter. Moreover, gel shift experiments using an αA-Crystalline promoter oligonucleotide containing both Pax-6- and Maf-binding sites showed no increase of Pax-6 binding in the presence of MafA protein, further suggesting that this increased Pax-6 binding was a key event in the synergistic promoter activation. This increase in the DNA binding of Pax-6 in the presence of Maf proteins may depend on the nature of the Pax-6-binding site, since the affinity of Pax-6 differs from one site to another (50).

Close examination of the glucagon promoter G1 element indicated that this sequence is A/T-rich, suggesting that a natural bending may occur (51) that could be increased by Maf proteins. Conversely, this natural bending may also be important for the activity of the Maf proteins, since DNA targets for bZip proteins are distinguished by an intrinsic bend (52). Therefore a functional synergy between Maf family members and Pax-6 may occur depending on the promoter context.

To date several classes of transcription factors are known to interact with Maf family proteins but, except for the related Fos and Jun AP-1 family members (16), most of these interactions act in an inhibitory way. This is the case for USF2, a member of the basic helix-loop-helix zipper transcription factor family, that has been shown to interact with c-Maf and to inhibit its DNA binding activity (53). c-Maf has been also shown to form a transcriptionally inert complex with c-Myb in myeloid lineage cells (27). Similarly, MafB has been reported to block the transcriptional activity of c-Ets-1 through direct interaction and subsequently to repress erythroid differentiation (28). However, it has been reported that Nrl and a paired type homeodomain transcription factor (CRX) synergistically regulate the photoreceptor cell-specific expression of the rhodopsin gene (34, 35, 54). Direct physical interaction of Nrl and Crx has been demonstrated (55). This positive effect on Maf activity is not a general property of home-
odomain-containing transcription factors, since Hoxd12 and Mbox can form complexes with the bZip domain of Maf proteins, resulting in a loss of Maf DNA binding activities (29). This latter result may be of interest for glucagon promoter expression, since several homeodomain-containing proteins are expressed in α-endocrine pancreas cells. For example, Pdx-Prep1 exhibits a repressive activity on Pax-6 transactivation (56), but Cdx-2/3, in contrast, acts synergistically with Pax-6 (14, 15). Therefore, the balance between positive and negative Maf/homeodomain-containing transcription factor interactions may dictate the final Pax-6/Maf synergy status in vivo. The glucagon promoter is also under the control of another paired transcription factor, Pax-2, which in contrast to Pax-6 does not act synergistically with Maf on the glucagon promoter expression. Thus, these different classes of transcription factors may form a regulatory network through protein-protein and protein-DNA interactions in the endocrine pancreas, allowing the fine tuning of the regulation of glucagon gene expression.

Acknowledgments—We thank Georges Calothy for critical reading of the manuscript; Beate Ritz-Laser for the gift of glucagon promoter constructs; Ales Cvekl for α-crystalline promoter; Makoto Nishizawa for v-Maf, L-Maf, and MafB-expressing pEF-Bo vectors; Cherie Stayner for the Pax-2 expression vectors; and Didier Monte for the GST-p46 expression vector.

REFERENCES
1. Martin, P., Carriere, C., Dozier, C., Quatannens, B., Mirabel, M. A., Vandenbunder, B., Stiehelin, D., and Saule, S. (1992) Oncogene 7, 1721–1728.
2. Carriere, C., Plaza, S., Caboche, J., Dozier, C., Bailly, M., Martin, P., and Saule, S. (1995) Cell Growth Differ. 6, 1531–1540.
3. Carriere, C., Plaza, S., Martin, P., Quatannens, B., Bailly, M., Stiehelin, D., and Saule, S. (1993) Mol. Cell. Biol. 13, 7257–7266.
4. Callaerts, F., Helder, G., and Gehring, W. J. (1997) Annu. Rev. Neurosci. 20, 483–532.
5. Chow, R. L., Altmann, C. R., Lang, A. R., and Hemmati-Brivanlou, A. (1999) Development 126, 4213–4222.
6. Dunne, M. K., Kozmik, Z., Cveklova, K., Piatigorsky, J., and Cvekl, A. (2000) J. Cell Sci. 113, 3173–3185.
7. Cvekl, A., and Piatigorsky, J. (1996) BioEssays 18, 621–630.
8. Larsson, L. I., St-Onge, L., Hougaard, D. M., Sosa-Pineda, B., and Gruss, P. (1998) Mech. Dev. 79, 153–1599.
9. St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A., and Gruss, P. (1997) Nucleic Acids Res. 25, 2398–2409.
10. Sander, M., Neubauer, A., Kalamaras, J., Ee, H. C., Martin, G. R., and German, M. S. (1997) Genes Dev. 11, 1662–1673.
11. Philippe, J., Drucker, D. J., Koseki, H., Lepeil, Z., and Habener, J. F. (1988) Mol. Cell. Biol. 11, 4877–4888.
12. Laser, B., Meda, P., Constant, I., and Philippe, J. (1996) J. Biol. Chem. 271, 28984–28994.
13. Sussman, M. A., Lee, J., Miller, C. P., and Habener, J. F. (1997) Mol. Cell. Biol. 17, 7186–7194.
14. Ritz-Laser, B., Estreicher, A., Klages, N., Saule, S., and Philippe, J. (1999) J. Biol. Chem. 274, 4124–4132.
15. Sussman, M. A., and Habener, J. F. (1999) J. Biol. Chem. 274, 28956–28957.
16. Kataoka, K., Noda, M., and Nishizawa, M. (1994) Mol. Cell. Biol. 14, 700–712.
17. Kawachi, S., Takahashi, S., Nakajima, O., Ogino, H., Morita, M., Nishizawa, M., Yasuda, K., and Yamamoto, M. (1999) J. Biol. Chem. 274, 19254–19260.