The Homeoprotein Alx3 Expressed in Pancreatic β-Cells Regulates Insulin Gene Transcription by Interacting with the Basic Helix-Loop-Helix Protein E47

Mercedes Mirasierra and Mario Vallejo

Instituto de Investigaciones Biomédicas “Alberto Sols,” Consejo Superior de Investigaciones Científicas/Universidad Autónoma de Madrid, 28029 Madrid, Spain

The regulation of insulin gene expression in pancreatic β-cells is the result of the coordinate activity of specific combinations of transcription factors assembled on different promoter elements. We investigated the involvement of the aristless-related homeoprotein Alx3 in this process. We found that Alx3 is coexpressed with insulin in pancreatic islets, as well as in the β-cell line MIN6, and it is also present in glucagon- and somatostatin-expressing cells. Chromatin immunoprecipitation assays indicated that Alx3 present in MIN6 cells and in mouse pancreatic islets occupies the promoter of the mouse insulin genes. EMSAs indicated that Alx3 present in MIN6 cells binds to the A3/4 regulatory element of the insulin I promoter. We found that Alx3 transactivates the insulin promoter by acting on the E2A3/4 enhancer in conjunction with the basic helix-loop-helix transcription factors E47/Pan1 and Beta2/NeuroD, and that Alx3 physically interacts via the homeodomain with E47/Pan1 but not with Beta2/NeuroD. Alx3 binds to the A3/4 element as a dimer, and the homeodomain is sufficient to recruit E47/Pan1 to the insulin promoter. Deletion studies in transfected HeLa cells indicated that proline-rich regions located at either side of the Alx3 homeodomain work together with E47/Pan1, and that this requires the integrity of the amino-terminal activation domain to transactivate. Thus, these studies support the notion that Alx3 participates in the regulation of insulin gene expression in pancreatic β-cells. (Molecular Endocrinology 20: 2876–2889, 2006)

Insulin is an essential hormone that regulates glucose homeostasis by acting on target organs such as liver, muscle, and adipose tissue. Circulating insulin is exclusively produced by the β-cells of the islets of Langerhans that constitute the endocrine compartment of the pancreas. In addition to β-cells, which occupy most of the islet mass, the pancreatic islets contain α-cells that produce glucagon, δ-cells that produce somatostatin, and cells that produce pancreatic polypeptide, all of these located in the islet periphery. The elucidation of the molecular mechanisms by which these cell types are generated constitutes a topic of intense study and has important implications for our knowledge of the etiopathological processes that lead to the occurrence of diabetes mellitus.

During development, the pancreatic bud is formed from the primitive gut epithelium (1). The endocrine cells that compose the islets derive from a subpopu-

First Published Online July 6, 2006

Abbreviations: bHLH, Basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; CBP, cAMP response element binding protein-binding protein; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; PCK, phosphoenolpyruvate carboxykinase.

Molecular Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
interactions (7–12). Also important for the regulation of insulin gene expression in β-cells are the paired-type homeodomain transcription factor Pax6 (13) and the basic leucine zipper protein MafA, which bind cis-regulatory elements known as C-boxes (14–16).

Notably, studies carried out mostly in rodents have demonstrated that many of the transcription factors that regulate insulin gene expression are also required for the proliferation of islet cell precursors and for the differentiation and survival of β-cells (17–19). In addition, the discovery of β-cell transcription factors in rodents has been highly predictive for the identification of some causative aspects of human diabetes (18, 20–22), because mutations in the genes that encode some of those transcription factors in humans are associated with different types of diabetes (18, 20, 23–25). Therefore, knowing the complete spectrum of transcription factors that operate in β-cells has important implications for the design of protocols to program this type of cells as a putative tool to treat diabetes.

Alx3 is a paired-class aristaless-like homeoprotein expressed in different tissues during embryonic development (26). Although a partial cDNA clone encoding the Alx3 homeodomain was originally isolated from the hamster insulinoma cell line HITT-15 (27), the expression of Alx3 in pancreatic islets or the target genes that it regulates have not been investigated. In the present study, we present evidence in support of a role for Alx3 on the regulation of insulin gene expression in pancreatic β-cells.

RESULTS

Alx3 Is Expressed in Pancreatic Islet Cells

The initial identification of Alx3 in hamster insulinoma HITT-15 cells (27) prompted us to investigate expression in pancreatic islet cells. Western immunoblot showed that Alx3 is expressed in nuclear extracts of the somatostatin-producing islet-derived RIN-1027-B2 cell line, confirming our previous finding (28), and of the insulin-producing β-cell line MIN6 (Fig. 1A).

In contrast, we did not detect Alx3 expression in BHK-21, COS-7, or HeLa cells (Figs. 1A and 4C). As a positive control, we confirmed the presence of Alx3 in RC2.E10 cells (Fig. 1A), a cell line derived from embryonic rat neuroepithelium, that express Alx3 constitutively (28). Nuclear localization of Alx3 was confirmed by immunocytochemistry in MIN6 and RIN-1027-B2 cells (Fig. 1, B–E).

To test for expression of Alx3 in intact pancreatic cells, we carried out immunohistochemistry on rat pancreata. We found that Alx3 immunostaining is restricted to islets, where Alx3-positive cells were found distributed throughout the entire mass of the islets (Fig. 2). Double-label immunohistochemistry demonstrated that all β-cells that synthesize insulin express Alx3, and that Alx3-positive cells located in the periphery of the islets correspond to glucagon- and somatostatin-expressing cells (Fig. 2). Thus, Alx3 appears to be present in all α-, β-, and δ-cells. In contrast, Alx3 immunoreactivity was not detected in the exocrine pancreas (Fig. 2).

Alx3 Present in β-Cells Recognizes the A3/4 Element of the Insulin Promoter

The expression of Alx3 in islet β-cells suggested that Alx3 may regulate insulin gene expression. To investigate whether Alx3 binds to the endogenous insulin genes in intact cells, we carried out chromatin immunoprecipitation (ChIP) assays on the insulin-expressing islet β-cell line MIN6, using a specific anti-Alx3 antiserum (28). We found that promoter sequences from both insulin I and II genes were selectively amplified by PCR from chromatin immunoprecipitated with the anti-Alx3 antiserum, but not with control pre-immune serum (Fig. 3A). Promoter sequences from the phosphoenolpyruvate carboxykinase (PCK) gene,
used as a control, were not amplified from the anti-AIx3 immunoprecipitated chromatin (Fig. 3A), indicating that Alx3 expressed in MIN6/H9252-cells occupy the endogenous insulin gene, but not the PCK gene. In addition, we carried out ChIP assays on chromatin prepared from mouse pancreatic islets, and we found that a fragment of the insulin I gene was selectively amplified by PCR with the anti-Alx3 antiserum, but not with control preimmune serum (Fig. 3A), thus confirming that Alx3 is bound to the insulin gene promoter in intact islets in vivo.

Next, we sought to identify regulatory elements recognized by Alx3. In the insulin I gene, at least two important regulatory sites that incorporate TAAT sequence motifs recognized by homeodomain proteins, that could serve as potential targets for binding by Alx3, are present in the insulin promoter. Those regulatory sequences correspond to the A2 element, which contains a single TAAT site, and the A3/4 elements, which contain several TAAT sites (Fig. 3B). The A3/4 element forms a functional enhancer together with the adjacently located E2 element, which binds bHLH proteins (5). To determine whether Alx3 binds to any of those sites, we carried out EMSA with synthetic oligonucleotide probes using nuclear extracts from MIN6 cells.

When a probe corresponding to the A3/4 element was used, several DNA-protein complexes were found, whose sequence specificity was determined by competition with unlabeled A3/4 oligonucleotide added in excess to the binding reaction. Addition of an oligonucleotide of unrelated sequence failed to compete (Fig. 3B). To investigate whether any of the complexes observed contain Alx3, we carried out the protein extract-DNA binding reaction in the presence of the anti-Alx3 antiserum. The addition of this antiserum resulted in the disappearance of one of the upper complexes bound to the A3/4 probe, and in the appearance of a supershifted band, whereas the addition of preimmune serum did not disturb the band pattern (Fig. 3C). The specificity of the electrophoretic mobility supershift observed with the anti-Alx3 antiserum was further confirmed by using the antiserum α253 (29, 30), which specifically recognizes the homeodomain transcription factor Pdx1 (also known as IDX1, IPF1, and STF1), because the A3/4 enhancer is a known target for regulation by this homeoprotein (10, 31). Thus, when EMSAs were carried out in the presence of the anti-Pdx1 antiserum, we observed the disappearance of one of the lower complexes bound to the A3/4 probe and the appearance of a supershifted band. The upper complex affected by the anti-Alx3 antiserum was unaffected by the anti-Pdx1 antiserum (Fig. 3B).

When we used the A2 probe, only a single protein-DNA complex was identified. However, we found that this complex contains Pdx1, but not Alx3, as a major protein component, because it specifically disappeared in the presence of the anti-Pdx1 antiserum but was unaffected by the anti-Alx3 antiserum (Fig. 3D). Thus, these data demonstrate that Alx3 present in MIN6 β-cells is able to recognize the insulin promoter by binding specifically to the A3/4 element.

**Alx3 Transactivates the Insulin Promoter**

To investigate whether Alx3 exhibits insulin promoter transactivation activity, we cotransfected the β-cell
observed by the control plasmid RSVCA. In addition, we found that cotransfection of INSICAT with pcDNA-Ax3 did not increase INSICAT activity in these cells (Fig. 4A). Similarly, we observed no increase in chloramphenicol acetyltransferase (CAT) activity when INSICAT was cotransfected in MIN6 cells with expression plasmids encoding known transactivators of the insulin gene such as the homeoprotein Pdx1, or the bHLH proteins E47/Pan1 (32) or Beta2/NeuroD (7) (Fig. 4A).

Similar results were obtained when we used the β-cell line INS-1 (data not shown). Because other homeomain transcription factors expressed in β-cells regulate insulin gene expression in combination with bHLH proteins synergistically (10, 11, 33), we investigated whether Ax3 could transactivate the insulin promoter in the presence of E47/Pan1 and Beta2/NeuroD. We found that cotransfection of INSICAT with expression vectors for E47/Pan1 and/or Beta2/NeuroD did not result in enhanced CAT activity in MIN6 cells. However, INSICAT activity increased 2- to 3-fold when an expression vector for Ax3 was combined with both E47/Pan1 and Beta2/NeuroD expression plasmids (Fig. 4A).

Because MIN6 cells exhibit features of a well-differentiated β-cell phenotype (34), the relatively poor magnitude of this response was interpreted as reflecting the existence of near saturating levels of endogenous Ax3 and of other β-cell-enriched transcription factors that regulate insulin gene expression (such as Pdx1 and Beta2/NeuroD), and to the possible existence of limiting amounts of transcriptional coactivators such as cAMP response element binding protein-binding protein (CBP)/p300, which is known to interact with them (35). In support of this notion, we found that INSICAT activity was increased 3-fold by cotransfection with an expression vector encoding the coactivator CBP (36) (Fig. 4B). We also observed that, although Ax3 had no effect on its own, it was able to stimulate INSICAT activity in the presence of CBP by approximately 2-fold relative to the activity elicited by CBP alone, and 5- to 6-fold relative to basal INSICAT activity (Fig. 4B).

Thus, to minimize interferences with endogenous Ax3 that may regulate insulin gene expression in β-cells, we used HeLa cells in subsequent experiments, because they do not express β-cell-specific transcription factors and we determined that they do not contain detectable levels of Ax3 (Fig. 4C). In HeLa cells, cotransfection of INSICAT with either Ax3 or Pdx1 expression vectors did not result in elevated CAT activity unless an E47/Pan1 expression vector was added, in which case CAT activity was very significantly elevated (Fig. 4D). These results are in agreement with previous studies indicating that homeodomain and bHLH proteins cooperate to regulate insulin gene expression (10, 11, 33), and suggest the existence of functional interactions between Ax3 and E47/Pan1.

Because the E2A3/4 enhancer provides a major site for synergistic interactions between bHLH and home-
odomain proteins (5), we tested the relative transactivational activity of Alx3, alone or in combination with E47/Pan1 or Beta2/NeuroD, in directing transcription from this enhancer. For this purpose, we carried out transfections in HeLa cells using the reporter plasmid 5FF-CAT, which bears five multimerized copies of the rat insulin I E2A3/4 enhancer (37). We found that 5FF-CAT activity in transfected HeLa cells was not increased by Alx3 or E47/Pan1, but Beta2/NeuroD elicited a small increase that was slightly potentiated in the presence of E47/Pan1 (Fig. 4E). However, we observed that, although Alx3 lacks transcriptional transactivation activity on its own, it was able to induce transcription from 5FF-CAT in transfected HeLa cells not increased by Alx3 or E47/Pan1, but Beta2/NeuroD elicited a small increase that was slightly potentiated in the presence of E47/Pan1 (Fig. 4E). However, we observed that, although Alx3 lacks transcriptional transactivation activity on its own, it was able to induce transcription from 5FF-CAT in the presence of E47/Pan1 or NeuroD/Beta2, an effect that further increased significantly when both E47/Pan1 and NeuroD/Beta2 were present at the same time together with Alx3 (Fig. 4E). Thus, these experiments show that Alx3 exerts a synergistic activation of the insulin gene promoter in the presence of bHLH proteins expressed in β-cells. Together with the results from the EMSA experiments described above, these experiments indicate that Alx3 directs transcription of the insulin gene by binding to the E2A3/4 enhancer.

**Alx3 Interacts with E47 But Not with Beta2/NeuroD**

The synergistic effects observed in the transfection assays prompted us to investigate the possible existence of direct protein-protein interactions between Alx3 and E47/Pan1 and/or Beta2/NeuroD, using glutathione S-transferase (GST) pull-down assays. We found that a GST-Alx3 fusion protein is able to interact with [35S]Met-labeled E47/Pan1, but not with [35S]Met-labeled Beta2/NeuroD (Fig. 5A). Because E47/Pan1 and Beta2/NeuroD are known to act as partners to form heterodimers, we used GST-E47 as a positive control to confirm the ability of Beta2/NeuroD to interact (Fig. 5A).

These data indicate that Beta2/NeuroD is unable to interact with Alx3. However, our transfection experiments in HeLa cells indicate the existence of a syner-
gistic increase in CAT activity when Beta2/NeuroD, E47/Pan1, and Alx3 expression plasmids are cotransfected together with the reporter plasmid 5FF-CAT (see Fig. 4C). Thus, it is possible that the functional interaction between Beta2/NeuroD and Alx3 is indirect using E47/Pan1 as a bridging protein interacting both with Beta2/NeuroD and with Alx3. To test this notion, we carried out additional GST pull-down experiments reported in D and E. Proline-rich domains (Pro1, Pro2, and Pro3) are indicated as striped boxes, and the homeodomain (HD) as a black box. E, Purified GST-E47 was incubated with truncated versions of 35S-labeled, in vitro-translated Alx3. Full-length Alx3 (Alx3FL), N-terminal deletions to amino acids 57 (Alx357), 91 (Alx391), or 143 (Alx3143), or C-terminal deletions from amino acids 228 (Alx31–228) or 279 (Alx31–279) were used. Labeled products did not bind to control GST (data not shown). F, Binding of 35S-labeled, in vitro-translated Alx3143–228 to GST-E47 but not to control GST. In this case, the labeled Alx3143–228 polypeptide was resolved in a 20% polyacrylamide gel.

Fig. 5. Alx3 Physically Interacts with E47/Pan1 but Not with Beta2/NeuroD

A and B, GST pull-down assays showing selective interactions of Alx3 with E47/Pan1 but not with Beta2/NeuroD. 35S-labeled in vitro-translated full-length E47/Pan1 or Beta2/NeuroD were incubated with the indicated purified GST fusion proteins expressed in bacteria and bound to glutathione-Sepharose beads. A, E47/Pan1 interacts with GST-Alx3, but not with control GST, whereas Beta2/NeuroD interacts with GST-E47 but not with GST-Alx3. B, Beta2/NeuroD indirectly interacts with GST-Alx3 only when unlabeled E47/Pan1 is present, indicating the formation of a ternary complex composed of GST-Alx3, unlabeled E47/Pan1, and labeled Beta2/NeuroD. C, Interaction between Alx3 and E47/Pan1 in the nuclei of intact cells. Shown is a Western immunoblot carried out with the anti-E47 antibody on samples that had been prepared by immunoprecipitation from MIN6 cell nuclear lysates with either anti-Alx3 antiserum or control nonimmune rabbit serum (NRS). The asterisk denotes the presence of bands corresponding to the immunoglobulins from the immunoprecipitation step. Input indicates a Western immunoblot carried out on MIN6 nuclear proteins not subjected to immunoprecipitation. D, Schematic depiction of the truncated versions of Alx3 used in the GST pull-down experiments reported in D and E. Proline-rich domains (Pro1, Pro2, and Pro3) are indicated as striped boxes, and the homeodomain (HD) as a black box. E, Purified GST-E47 was incubated with truncated versions of 35S-labeled, in vitro-translated Alx3. Full-length Alx3 (Alx3FL), N-terminal deletions to amino acids 57 (Alx357), 91 (Alx391), or 143 (Alx3143), or C-terminal deletions from amino acids 228 (Alx31–228) or 279 (Alx31–279) were used. Labeled products did not bind to control GST (data not shown). F, Binding of 35S-labeled, in vitro-translated Alx3143–228 to GST-E47 but not to control GST. In this case, the labeled Alx3143–228 polypeptide was resolved in a 20% polyacrylamide gel.

antiserum, followed by Western immunoblot with a specific anti-E47/Pan1 monoclonal antibody. The detection of a band corresponding to E47/Pan1 in samples immunoprecipitated with the anti-Alx3 antiserum, but not in those immunoprecipitated with nonimmune rabbit serum (Fig. 5C), confirmed the Alx3-E47/Pan1 interaction in the nuclei of cells.

Besides the homeodomain, Alx3 contains three proline-rich domains (28), two of them (Pro1 and Pro2) located in the amino-terminal region and one (Pro3) located in the carboxyl-terminal region (Fig. 5D). To determine whether these domains are important for the interactions with E47/Pan1, we carried out GST pull-down assays using a GST-E47/Pan1 fusion protein and either full-length or truncated versions of [35S]Met-labeled Alx3 generated by deletion of residues spanning either the carboxyl or the amino terminus of Alx3. As shown in Fig. 5E, full-length Alx3 is able to interact with GST-E47/Pan1. Increasing deletions of either the amino (Alx357, Alx391, and Alx3143) or the
carboxyl terminus of Alx3 (Alx3_{1–228} and Alx3_{1–279}), but leaving intact the homeodomain, did not affect dimerization with the GST-E47/Pan1 fusion protein (Fig. 5E). Dimerization was also observed using labeled Alx3_{143–228}, which corresponds to the Alx3 homeodomain (Fig. 5F). In addition, none of the labeled versions of Alx3 was observed to bind to control GST. Thus, these experiments indicate that the segment of Alx3 corresponding to the homeodomain is sufficient for heterodimerization with E47/Pan1.

The Homeodomain of Alx3 Promotes Binding of E47/Pan1 to the E2A3/4 Enhancer

To evaluate directly the binding of Alx3 and E47/Pan1 to the insulin promoter, we carried out EMSA. Because we have previously determined that deletions of segments of the amino or carboxyl terminus of Alx3 do not affect binding to DNA as long as the homeodomain remains intact (28), we used Alx3_{143} or Alx3_{143–228} synthesized in reticulocyte lysates to better resolve the protein-DNA complexes.

It is known that aristaless-like homeoproteins form dimers cooperatively upon binding to DNA consensus sequences known as P3 sites, which contain two inverted TAAT motifs separated by three nucleotides (28, 38, 39). In addition, Alx3 binds selectively as a monomer to specific TAAT-containing DNA sequences such as the GFAPT3 site found in the promoter of the glial fibrillary acidic protein gene (28).

We found that Alx3_{143} binds to the E2A3/4 probe forming two complexes (Fig. 6B), thus resembling the binding pattern generated by the cooperative dimerization observed upon binding to P3 sites (28). Indeed, a comparison with the binding obtained with a GFAPT3 probe demonstrated that the lower complex corresponds to the monomeric form of Alx3 bound to DNA (Fig. 6B).

On the other hand, consistent with previous reports (7, 10, 11), we found that E47/Pan1, but not Beta2/NeuroD, can bind to the E2A3/4 probe (data not shown). We then carried out titration experiments to calculate the minimum amount of E47/Pan1 to detect binding to the E2A3/4 probe, and investigated whether E47/Pan1 can bind cooperatively with Alx3 to this site. E47/Pan1 was used at a concentration that did not yield appreciable binding to the E2A3/4 probe when used alone.

When E47/Pan1 was incubated simultaneously with Alx3_{143}, the two complexes corresponding to the monomeric and dimeric forms of Alx3 were observed unaltered (Fig. 6C). In addition, a band with lower electrophoretic mobility appeared, an effect that was not observed when Beta2/NeuroD was used instead of E47/Pan1 (Fig. 6C). A similar effect was found when we used Alx3_{143–228}, which only spans the Alx3 homeodomain (Fig. 6D). These results indicate that Alx3 and E47/Pan1 can interact cooperatively forming a ternary complex on the DNA, and that the homeodo-

main of Alx3 is sufficient for the occurrence of this interaction.

The Homeodomain of Alx3 Is Sufficient to Promote E47/Pan1-Dependent Transactivation

In earlier studies, using promoter elements on which Alx3 transactivation activity does not depend on direct interactions with bHLH proteins, we found that Pro1 and Pro2 domains are necessary for transactivation, but Pro3 is dispensable, and that the region spanning the homeodomain is unable to transactivate on its own (28). Thus, we tested for the relative contributions of these domains to the transcriptional activity of Alx3
from the insulin promoter, which depends on interactions with E47/Pan1. For this purpose, we cotransfected HeLa cells with the 5FF-CAT reporter plasmid and expression plasmids encoding E47/Pan1 or truncated versions of Alx3 lacking the amino-terminal or the carboxyl-terminal domains.

As mentioned earlier, Alx3 is unable to transactivate the 5FF-CAT reporter unless E47/Pan1 is present (see Fig. 4E). In these experiments, we found that when residues 1–143 of Alx3 were deleted (Alx3143), eliminating the entire amino-terminal region to a position next to the homeodomain, the CAT activity elicited by both proteins was reduced to approximately 80% of that obtained with the full-length proteins, indicating that the Pro1 and Pro2 domains of Alx3 are not essential for transactivation in the presence of E47/Pan1 (Fig. 7A). To test whether the Pro3 domain located in the C-terminal region of Alx3 is essential for transactivation, we cotransfected an expression vector encoding a truncated version of Alx3 spanning residues 1–228 (Alx31–228), in which the Pro1 and Pro2 domains, as well as the homeodomain, remain intact. We found that Alx31–228 yields levels of CAT activity that are also about 80% of that obtained with full-length Alx3 in the presence of E47/Pan1, indicating that the Pro3 domain is not essential for activity when the other two domains are intact (Fig. 7A). Finally, we cotransfected an expression vector encoding Alx3143–228, a truncated Alx3 protein in which both the amino- and the carboxyl-terminal regions were deleted leaving intact the homeodomain. We found that the activity elicited by Alx3143–228 is about 20% of that elicited by full-length Alx3 (Fig. 7A).

Interestingly, although greatly reduced, this is still a significant degree of activity compared with background levels of 5FF-CAT activity, which are not increased when either E47/Pan1 or Alx3 are transfected alone (Figs. 4E and 7B). Because our previous work demonstrated that the isolated Alx3 homeodomain lacks transcriptional transactivation activity (28), these experiments suggest that the residual activity observed when Alx3143–228 and E47/Pan1 are present together is due to the activity of the E47/Pan1 interacting with the Alx3 homeodomain at the insulin promoter.

To test this notion, we cotransfected the 5FF-CAT reporter plasmid with expression vectors encoding the Alx3 homeodomain (Alx3143–228) and with expression vectors encoding truncated versions of E47/Pan1 corresponding to amino-terminal deletions to residues 91, 334, or 549, that lack one or two of the activation domains, but leave intact the bHLH domain (Fig. 7B). Cotransfection of these plasmids with the plasmid encoding Alx3143–228 resulted in the generation of background levels of CAT activity indistinguishable from those observed when Alx3143–228 was transfected alone. Thus, as predicted, stimulation of 5FF-CAT activity in the presence of the Alx3 homeodomain was dependent on the integrity of the transactivation domains of E47/Pan1.

In summary, all of these experiments taken together support the notion that the Alx3 homeodomain interacts with E47/Pan1 on the insulin promoter, and that full transcriptional transactivation is contributed by Alx3 proline-rich domains and E47/Pan1 transactivation domains, as well as by Beta2/NeuroD complexed with E47/Pan1.

Fig. 7. Functional Interactions between the Homeodomain of Alx3 and E47/Pan1 in Transfected Cells

A, Relative CAT activities elicited in HeLa cells cotransfected with the 5FF-CAT reporter plasmid, an expression vector encoding E47/Pan1, and expression vectors encoding either full-length (FL) or truncated versions of Alx3, as indicated schematically on top. B, Relative CAT activities elicited in HeLa cells cotransfected with the 5FF-CAT reporter plasmid, an expression vector encoding the Alx3 homeodomain (Alx3143–228), and expression vectors encoding either full-length (FL) or truncated versions of E47/Pan1, as indicated schematically on top. In all cases, the total amount of transfected DNA was kept constant by adding the corresponding empty vector. Values represent the mean ± SEM of at least three experiments carried out in duplicate. HD, Homeodomain; AD1 and AD2, activation domains 1 and 2; HLH, helix-loop-helix domain.
DISCUSSION

Several studies using mice carrying mutant alleles have advanced significantly our knowledge on the functions of different aristaless-like genes, including *Cart1*, *Alx3*, and *Alx4*, during embryonic development (38, 40–44). However, despite these advances on the developmental functions of aristaless-like transcription factors, target genes regulated by them have not been identified, and their functions as transcriptional regulators of gene expression in differentiated cells remain unknown.

In the present study, we have identified the insulin gene as the first known target for regulation by Alx3. This notion is based on the expression of Alx3 in pancreatic islet cells, on DNA binding and ChIP assays showing specific interactions of Alx3 with the insulin promoter in β-cells, and on functional transient transfection assays showing that Alx3 transactivates the insulin gene by interacting with bHLH proteins.

Alx3 Interacts with E47/Pan1 to Yield Synergistic Transactivation of Insulin Gene Transcription

In addition to Alx3, the homeodomain proteins Pdx1 and Lmx1.1 have been shown to interact with the class A bHLH protein E47/Pan1 on the insulin E2A3/4 enhancer. In contrast, interactions with the class B bHLH protein Beta2/NeuroD are selective, because only Pdx1 appears to interact directly with Beta2/Neuro (10). Our data indicate that the synergistic transactivation observed between Alx3 and E47/Pan1 could be due to interactions at two different levels. On the one hand, direct protein–protein interactions with the Alx3 homeodomain bound to the E2A3/4 enhancer, bringing in addition Beta2/NeuroD to the transcriptionally active complex, and on the other hand functional interactions of the transactivation domains, perhaps by recruiting coactivator proteins that could not interact stably with each factor in isolation.

Alx3 in pancreatic β-cells recognizes the A3/4 site in the promoter of the insulin gene. Although Alx3 binds the A3/4 site preferentially as a dimer, a less intense complex corresponding to the monomeric form was also observed in EMSA carried out with the synthetic version of the protein (see Fig. 6). The A3/4 element contains the sequence TAATCTAATTA, which resembles a P3 site to which paired class homeoproteins bind preferentially as dimers in a cooperative manner (28, 38, 39, 45). In addition, the palindromic motif TAATTTAA, which resembles a P3 site to which paired class homeoproteins bind preferentially as dimers in a cooperative manner (28, 38, 39, 45). Thus, the palindromic motif TAATTTAA within this sequence serves as a preferred site for binding by Alx3 (28). Thus, these data suggest that Alx3 dimerizes cooperatively upon binding to the A3/4 site. In addition, the ability of Alx3 to interact directly with E47/Pan1 appears to favor the cooperative binding of this bHLH, which in turn would bring Beta2/NeuroD to form a functionally active multiprotein complex assembled on the E2A3/4 enhancer. Thus, these data reinforce the notion that synergistic activation of the insulin promoter occurs as a consequence of cooperative DNA binding that promotes recruitment of multiple activators (10).

Functional interactions at the level of the transactivation domains also appear to be important for synergistic enhancement of insulin transcription. We have previously determined that the integrity of the Pro1 and Pro2 domains of Alx3 is required for transcriptional transactivation from a generic P3 site, thus indicating that the Pro3 domain is unable to function on its own (28). However, in the present study, we have observed that when Alx3 is acting from the E2A3/4 element in the presence of E47/Pan1, the Pro3 domain is still able to synergize with this bHLH protein, whose activity is in turn dependent on the integrity of the transactivation AD1 domain. Interestingly, E47/Pan1 is able to stimulate transcription, although relatively weakly, when all the proline-rich domains of Alx3 have been deleted, further supporting the notion that the homeodomain of Alx3 is sufficient to recruit E47/Pan1 to a transcriptionally active complex assembled on the insulin promoter. The mechanism by which the transactivation domains of Alx3 and bHLH proteins synergize have not been explored in the present study, but previous work (35) and our own data (see Fig. 4B) suggest that interactions with coactivators such as CBP/p300 may be involved.

Finally, it is possible that additional mechanisms involving different types of proteins contribute to the synergistic transactivation of Alx3 and E47/Pan1. For example, recruitment of coactivators (37) or interactions with HMG-type proteins have been shown to be important in the case of the synergism between Pdx1 and E47/Pan1 (10). Also, it is possible that Alx3 can alter chromatin conformation, as has been shown to be the case for HNF1α (46), to favor the binding of other transcription factors or coactivators. The study of these possibilities remains the subject for future research.

Regulation of Insulin Gene Transcription by Alx3 in Pancreatic β-Cells

One important concept emerging from genetic studies on aristaless-like genes is the existence of a relatively high degree of functional redundancy among them due to overlapping functions that only become evident when double or triple mutant mice are generated (38, 43, 44). It is possible that redundancy also exists between Alx3 and other proteins that regulate the expression of the insulin gene in β-cells, because Alx3 appears to function like other homeodomain transcription factors that cooperate with bHLH proteins on the E2A3/4 elements of the insulin promoter, such as Pdx1 or the LIM-domain proteins Lmx1.1 and Lmx1.2 (10, 11, 31, 47). However, ongoing studies in our laboratory using Alx3 mutant mice (44) point to the existence of a mild glucose homeostasis phenotype, lending support to the notion that Alx3 participates in the control of β-cell function.
Pdx1 has emerged as a major regulator of insulin gene transcription in both humans and rodents (11, 48–50). However, although clearly important for β-cell function and survival, it appears that Pdx1 is not specifically required for insulin gene transcription, because insulin gene expression can occur in the absence of Pdx1 (17, 51, 52). These findings have been interpreted on the basis that other homeodomain transcription factors present in β cells can compensate for the loss of Pdx1 by acting via a similar mechanism, i.e. interaction with bHLL proteins at the level of specific insulin promoter elements such as E2A3/4 (5). It is possible that Alx3 could substitute for Pdx1, as the LIM-homeodomain factors Lmx1.1 and Lmx1.2 may also do (5). Thus, loss of a single homeodomain transcription factor appears not sufficient to compromise insulin gene transcription, because this is probably the result of the coordinate activity of several of these factors that can compensate for one another. In this regard, studies aimed at determining functional interactions between Alx3 and Pdx1 and their relative contributions to regulation of insulin gene transcription are currently being carried out in our laboratory.

As mentioned earlier, some of the functions of Alx3 and other aristaless-related genes have been identified after generating mice with combined mutant alleles. On the other hand, it has been shown that combined heterozygous mutations in certain genes expressed in pancreatic β-cells have no effect on pancreatic function as single mutants, such as Hnf1α or Hnf3β, can lead to altered β-cell function resulting in decreased insulin gene transcription or aggravation of diabetes caused by haploinsufficiency of Pdx1 (53). Thus, although not known at the present time, it is possible that mutations in Alx3 in combination with mutations in other diabetes-related genes may alter pancreatic β-cell function as a consequence of decreased insulin synthesis or secretion. In this regard, it is important to note that Alx3 is functionally related to Beta2/NeuroD, Pdx1, and Hnf1α, because it acts in combination with Beta2/NeuroD (this study) and regulates insulin gene transcription upon binding to the same promoter element used by Pdx1 and Hnf1α. These three genes are causatively related to maturity onset diabetes of the young (3). Further studies will determine whether mutations in Alx3 are directly or indirectly related to the development of diabetes.

**MATERIALS AND METHODS**

**Cell Lines and Transfections**

MIN6 cells (54) were cultured in the presence of 15% fetal bovine serum and β-mercaptoethanol (70 μM), and were not used after reaching 35 passages. INS-1E cells, provided by Dr. Claes Wollheim (University of Geneva, Geneva, Switzerland) (55) were cultured in RPMI 1640 in the presence of 10% fetal bovine serum, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Neural RC2.E10 cells derive from cerebral cortex of rat fetuses of 16 d of gestational age, and were cultured at a temperature of 33 C as described (56). COS-7 (ATCC CRL-1651; American Type Culture Collection, Manassas, VA), HeLa cells, BHK-21 cells (baby hamster kidney fibroblasts) (ATCC CCL10), and rat pancreatic islet somatostatin-producing RIN-1027-B2 cells (57) were cultured at a temperature of 37 C. Unless otherwise stated, all cells were cultured in DMEM containing 10% fetal bovine serum in the presence of penicillin (100 U/ml) and streptomycin (10 μg/ml). MIN6 cells were transfected with 5 μg of reporter plasmid and 2 μg of expression plasmids using Lipofectin (Invitrogen Life Technologies, San Diego, CA) as described (56). HeLa cells were transfected with 10 μg of reporter plasmid and 2 μg of expression plasmids using the calcium phosphate precipitation method. When necessary, total amount of plasmid DNA was maintained constant by adding empty expression vector.

CAT activity was measured by a solution assay (58) 48 h after transfection exactly as described (56). Values were normalized to those yielded by a Rous sarcoma virus enhancer reporter plasmid (RSVCAT) transfected in parallel in MIN6 cells, or to CMVCAT in HeLa cells. All of the values are expressed as mean ± SEM of at least three independent experiments carried out in duplicate.

**Plasmids**

INSCAT encodes a CAT reporter gene driven by a fragment of the rat insulin I gene promoter spanning nucleotides –410 to +34 (59). 5FF-CAT is a reporter plasmid bearing five multimerized copies of the rat insulin I E2A3/4 enhancer driving CAT expression (provided by Dr. Melissa Thomas, Massachusetts General Hospital, Boston, MA) (60). Construction of the expression plasmids containing full-length and deleted versions of Alx3 cloned into pcDNA3 has been described (28). The plasmid pcDNA-Pdx1 contains a full-length cDNA encoding rat IDX1 (61) cloned into the HindIII and NotI sites of pcDNA3. The plasmid pCMV-Beta2 (provided by Dr. J. Feller, Hospital Clinic, Barcelona, Spain) contains a full-length cDNA encoding Beta2/NeuroD (7). The E47/Pan1 expression vector used in CAT reporter assays corresponds to pCMV-Pan1 described by Nelson et al. (32).

For GST pull-down experiments and protein synthesis in reticulocyte lysates, we used GST-E47, pcDNA3-E47, and pcDNA3-Beta2 provided by Dr. Amparo Cano (Instituto de Investigaciones Biomédicas, Madrid, Spain) (62), as well as GST-Alx3 and pcDNA3-Alx3 (full-length and deleted versions) previously described (28).

For experiments designed to test deleted versions of E47/Pan1, we used the plasmid pZeoSV2 (Invitrogen Life Technologies, Carlsbad, CA) containing cDNAs encoding amino-terminal deletions to residues 91, 334, or 549. These were kindly provided by Dr. Amparo Cano (Instituto de Investigaciones Biomédicas, Madrid, Spain).

**Western Immunoblot**

Nuclear extracts from cells growing in 60-mm dishes were prepared as described (63), and proteins were resolved by SDS-PAGE and blotted onto a nitrocellulose membrane. Alx3 immunoreactivity was detected with a rabbit polyclonal primary antisemur (28) (1:5000 dilution), followed by incubation with a goat antirabbit peroxidase-conjugated secondary antibody (1:10,000 dilution) (Bio-Rad, Hercules, CA). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Piscataway, NJ).

**Immunocytochemistry**

Immunocytochemistry on MIN-6 and RIN-1027-B2 cells was carried out basically as described (56). Cells plated into
35-mm tissue culture dishes were fixed in 4% paraformaldehyde in PBS for 5 min, washed in PBS, and permeabilized with methanol for 2 min at −20 C. After blocking with normal goat serum for 1 h, cells were incubated overnight with the anti-Alx3 antisera (1:4000 dilution) at 4 C. Immunodetection was carried out with a secondary biotinylated goat anti-rabbit antisera (Bio-Rad) using nickel-intensified immunoperoxidase staining with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

**Immunohistochemistry**

Adult pancreata from Wistar rats (200–250 g body weight) were fixed by transcardial perfusion with 4% paraformaldehyde, removed, postfixed overnight, and cryoprotected in PBS containing 20% sucrose. Cryostat sections (20 μm) were cut and kept at −80 C until used. Sections were brought to room temperature, permeabilized with methanol for 2 min at −20 C, treated with 5% normal goat serum, and then incubated overnight with the anti-Alx3 (1:4000 dilution) antisera at room temperature. Immunodetection was carried out with a secondary biotinylated goat antirabbit antisera using nickel-intensified immunoperoxidase staining. For insulin staining, sections were incubated with guinea pig anti-human insulin (Linco Research, St. Charles, MO; 1:100 dilution), and immunodetection was carried out with a secondary biotinylated goat anti-guinea pig antisera using immunoperoxidase staining. For glucagon staining, we used a guinea pig antibody (Linco Research; 1:100 dilution), detected with a secondary biotinylated goat anti-guinea pig antiserum and immunoperoxidase staining. For somatostatin staining, we used a mouse monoclonal antibody (Acris Antibodies, Hidenhausen, Germany; 1:100 dilution), detected with a secondary biotinylated horse antiamoine antiseraum and immunoperoxidase staining.

For two-color dual antigen immunostaining, Alx3 was first detected using nickel-enhanced immunostaining, which yields a dark blue color. Sections were then washed, and insulin, glucagon, or somatostatin immunohistochemistry was carried as described above, using immunoperoxidase staining without nickel, which yields a brown color.

Experimental protocols involving Wistar rats for the preparation of sections for immunohistochemistry were approved by the institutional committee on research animal care, and meet the requirements of current Spanish and European Community legislation.

**EMSAs**

EMSAs were carried out with either nuclear extracts (63) of MIN-6 cells or with transcription factors synthesized in vitro using a rabbit reticulocyte lysate system (Promega, Madison, WI). Protease inhibitors (Complete protease inhibitor cocktail; Roche, Basel, Switzerland) were added to nuclear extracts, and protein concentrations were determined by the Bio-Rad protein assay. Synthetic complementary oligonucleotides with 5′-GATC overhangs were annealed and labeled by a fill-in reaction using [α-32P]dATP and Klenow enzyme. Binding reactions were carried out in the presence of 20,000 cpm of radiolabeled probe (6–10 fmol) in a total volume of 20 μl containing 2 μg poly(dIdC), 20 mM HEPES (pH 7.9), 70 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol, 0.3 mM EDTA, and 10% glycerol. The sequences of the oligonucleotides used are as follows (coding strand): A2, 5′-GATCCGGACCCTTATGGGCCCAA-3′; A3/4, 5′-GATCCGGCTTATAAATCTATGCA-3′; E2A3/4, 5′-GATCCCTGACGCGCCTGAGCCGCTGTATCTTCCCTAGA-3′; GFAP3, 5′-GATCCTTGCAATTAGTGTGACA-3′.

**ChIP**

ChIP assays were carried out basically as described (64). Cross-linked chromatin from mouse islets was generously provided by Jorge Ferrer (Hospital Clinic, University of Barcelona, Barcelona, Spain). MIN6 cells were treated with 1% formaldehyde for 15 min at room temperature and the cross-linked protein-DNA complexes were isolated. After sonication, chromatin was incubated with anti-Alx3 antisera or control normal rabbit serum. Antibody-protein-DNA complexes were isolated by incubation with protein A-Sepharose. To detect bound DNA, PCR was carried out using oligonucleotide primers that amplify a fragment of the mouse insulin gene I spanning nucleotides −360 to −12. PCR conditions were as follows: 95 C for 5 min, followed by 30 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec, after which a 5-min incubation at 72 C followed. The sequence of the oligonucleotide primers are as follows: forward, 5′-ATATTAGGTTCCAACGTAG-3′ and reverse, 5′-TACTGGATGTCACACCTATATTAAC-3′. To ensure specificity, the 3′-end of the forward oligonucleotide corresponds to a 5-nucleotide insertion absent in the insulin II gene. For amplification of the mouse insulin I gene, we used the following oligonucleotides: forward, 5′-CAGATGACTAGTTGCCCCACTG-3′; reverse, 5′-CCACTACCTTTATAGCAACAGC-3′. PCR conditions were identical with those used for the insulin I sequences. In this case, the 3′-end of the forward oligonucleotide corresponds to a 3-nucleotide insertion absent in the insulin I gene. In addition, specificity of the amplified fragments was checked by digestion with either AatI or PstI, both of which only cut the fragment amplified from the insulin II gene. As a control, we used promoter sequences from the PCK gene (nucleotides −434 to −96) as indicated by Cissell et al. (65). The sequence of the PCK oligonucleotide primers are as follows: forward, 5′-GAGTGGACCTCTGACAGTGG-3′; and reverse, 5′-GGCACGCGCTTTGGAATCATCAGC-3′. PCR conditions were as follows: 95 C for 2 min, followed by 28 cycles of 95 C for 30 sec, 61 C for 30 sec, and 72 C for 30 sec, after which a 5-min incubation at 72 C followed.

In all cases, PCR products were run on a 1% agarose gel, stained with ethidium bromide, and photographed.

**GST Pull-Down Assays**

Full-length or truncated versions of [35S]Met-labeled Alx3 were generated by in vitro translation using a rabbit reticulocyte lysate system (Promega) and cDNAs cloned into pcDNA3 (28). [35S]Met-labeled E47/Pan1 and Beta2/NeuroD were similarly generated using plasmids pcDNA3-E47 and pcDNA3-Beta2 (62). These labeled proteins were incubated with recombinant GST-Alx3, GST-Alx3-143–228 (28), or GST-E47 (62) expressed in bacteria and bound to glutathione-Sepharose beads (Amersham Biosciences, Little Chalfont, UK). Incubations were carried out at 4 C for 1 h in buffer containing 20 mM sodium phosphate (pH 7.9), 150 mM KCl, 0.5 mM EDTA, 0.02% Triton, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin. After extensive washing, the bead-bound proteins were denatured, resolved by SDS-PAGE, and detected by autoradiography.

**Immunoprecipitation**

Nuclei from MIN6 cells (2 × 106) were prepared as described by Schreiber et al. (63) by incubation of cells in hypotonic buffer followed by vigorous agitation in the presence of 0.06% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO). Nuclei were then lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Igepal CA-630, and protease inhibitor cocktail (Roche). Proteins were
incubated overnight with either nonimmune rabbit serum or with anti-Ax3 antiserum at 4 C, and then protein A-Sepharose was added for another 2 h. After this, the samples were centrifuged, washed, and resolved by SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane, and E47/Pan1 immunoreactivity was detected by Western immunoblot using a specific monoclonal antibody (BD Pharmingen, San Diego, CA; 1:500 dilution).

Acknowledgments

We thank Jorge Ferrer and Joan Marc Servitja (Hospital Clinic, Barcelona, Spain) for providing mouse pancreatic islet chromatin for ChiP assays; Claes Wollheim (University of Geneva, Geneva, Switzerland) for INS-1E cells; Joel Habener (Massachusetts General Hospital, Boston, MA) for anti-Pdx1 antiserum; and Amparo Cano (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid, Spain), Melissa Thomas (Massachusetts General Hospital), and Jorge Ferrer for plasmids.

Received November 24, 2005. Accepted June 26, 2006.

Address all correspondence and requests for reprints to:
Mario Vallejo, M.D., Ph.D., Instituto de Investigaciones Biomédicas “Alberto Sols,” Calle Arturo Duperier 4, 28029 Madrid, Spain. E-mail: mvallejo@ibb.uam.es.

This work was funded by grants (to M.V.) from the Spanish Ministry of Science and Technology (PB98-1629-CO2-02) and the Instituto de Salud Carlos III (RGDM G03/212 and PI042374). M.M. was supported by a postgraduate fellowship and the Instituto de Salud Carlos III (Spanish Ministry of Health).

Acknowledgments

We thank Jorge Ferrer and Joan Marc Servitja (Hospital Clinic, Barcelona, Spain) for providing mouse pancreatic islet chromatin for ChiP assays; Claes Wollheim (University of Geneva, Geneva, Switzerland) for INS-1E cells; Joel Habener (Massachusetts General Hospital, Boston, MA) for anti-Pdx1 antiserum; and Amparo Cano (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid, Spain), Melissa Thomas (Massachusetts General Hospital), and Jorge Ferrer for plasmids.

Received November 24, 2005. Accepted June 26, 2006.

Address all correspondence and requests for reprints to:
Mario Vallejo, M.D., Ph.D., Instituto de Investigaciones Biomédicas “Alberto Sols,” Calle Arturo Duperier 4, 28029 Madrid, Spain. E-mail: mvallejo@ibb.uam.es.

This work was funded by grants (to M.V.) from the Spanish Ministry of Science and Technology (PB98-1629-CO2-02) and the Instituto de Salud Carlos III (RGDM G03/212 and PI042374). M.M. was supported by a postgraduate fellowship from the Consejo Superior de Investigaciones Científicas and by a fellowship from the Instituto de Salud Carlos III (Spanish Ministry of Health).

M.M. and M.V. have nothing to declare.

REFERENCES

8. Emens LA, Landers DW, Moss LG 1992 Hepatocyte nuclear factor 1α is expressed in a hamster insulinoma line and transactivates the rat insulin I gene. Proc Natl Acad Sci USA 89:7300–7304
41. Qu S, Niswender KD, Ji Q, van der Meer R, Keeney D, Zhao Q, Behringer RR, de Crombrugghe B 1996 Prenatal
37. Thomas MK, Yao KM, Tenser M, Wong G, Habener JF
35. Qiu Y, Guo M, Huang S, Stein R 2002 Insulin gene
27. Rudnick A, Ling TY, Odagiri H, Rutter W, German MS 1994
24. Perez-Villamil B, Mirasiria M, Vallejo M 2004 The
64. Gerrish K, Cissell MA, Stein R 2001 The role of hepatic nuclear factor 1α and PDX-1 in transcriptional regulation of the pdx-1 gene. J Biol Chem 276:47775–47784

Molecular Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.