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Mechanism of *insulin* Gene Regulation by the Pancreatic Transcription Factor Pdx-1

APPLICATION OF PRE-mRNA ANALYSIS AND CHROMATIN IMMUNOPRECIPITATION TO ASSESS FORMATION OF FUNCTIONAL TRANSCRIPTIONAL COMPLEXES*

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The homeodomain factor Pdx-1 regulates an array of genes in the developing and mature pancreas, but whether regulation of each specific gene occurs by a direct mechanism (binding to promoter elements and activating basal transcriptional machinery) or an indirect mechanism (via regulation of other genes) is unknown. To determine the mechanism underlying regulation of the *insulin* gene by Pdx-1, we performed a kinetic analysis of *insulin* transcription following adenovirus-mediated delivery of a small interfering RNA specific for *pdx-1* into insulinoma cells and pancreatic islets to diminish endogenous Pdx-1 protein. *insulin* transcription was assessed by measuring both a long half-life *insulin* mRNA (mature mRNA) and a short half-life *insulin* pre-mRNA species by real-time reverse transcriptase-PCR. Following progressive knock-down of Pdx-1 levels, we observed coordinate decreases in pre-mRNA levels (to about 40% of normal levels at 72 h). In contrast, mature mRNA levels showed strikingly smaller and delayed declines, suggesting that the longer half-life of this species underestimates the contribution of Pdx-1 to *insulin* transcription. Chromatin immunoprecipitation assays revealed that the decrease in *insulin* transcription was associated with decreases in the occupancies of Pdx-1 and p300 at the proximal *insulin* promoter. Although there was no corresponding change in the recruitment of RNA polymerase II to the proximal promoter, its recruitment to the *insulin* coding region was significantly reduced. Our results suggest that Pdx-1 directly regulates *insulin* transcription through formation of a complex with transcriptional coactivators on the proximal *insulin* promoter. This complex leads to enhancement of elongation by the basal transcriptional machinery.

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Insulin is produced almost exclusively by the β cells of the pancreatic islets of Langerhans. This restriction of insulin production derives primarily from constraints imposed at the level of transcription of the gene encoding preproinsulin (the *insulin* gene), rather than at the level of translation of the nascent mRNA (1, 2). Studies of the rodent *insulin* genes indicate that ~400 base pairs (bp) of DNA 5′ of the transcriptional start site (the *insulin* promoter) are sufficient to confer cell type-specific expression of *insulin* (3–7). Multiple discrete sequence elements within the proximal promoter region contribute to both the specificity and magnitude of *insulin* expression, and these elements are believed to serve as binding sites for several islet transcription factors, including Pdx-1, MafA, and BETA2/NeuroD (see Ref. 8 for a review). In the prevailing hypothesis of *insulin* transcription, the association of these transcription factors with the promoter and their subsequent interaction with ubiquitously expressed factors (*e.g.* E47 and p300) (9, 10) leads to the recruitment of the basal transcriptional machinery to the *insulin* gene. This hypothesis, however, has never been rigorously tested for the endogenous *insulin* gene in islet β cells.

The Hox-like homeodomain protein Pdx-1 is perhaps the most extensively studied β cell transcription factor. Much of the evidence implicating Pdx-1 in the regulation of *insulin* transcription is circumstantial, and is derived from studies of interaction of Pdx-1 with *insulin* promoter elements *in vitro*, reporter gene expression studies in non-β cell lines, and loss-of-function studies in animals (11–17). It is unknown from these experiments whether Pdx-1 might control *insulin* transcription by a direct mechanism involving interaction with upstream promoter elements or an indirect one via regulation of other genes. In this regard, Pdx-1 is known to regulate a complex genetic hierarchy, because its disruption in both mice and humans results not only in the absence of insulin production, but also in arrested development of the early pancreatic anlage (12, 14, 18). More recent studies involving functional or physical attenuation of Pdx-1 protein in late mouse development or in cell lines (through targeted gene knock-out, antisense, or dominant negative strategies) have suggested a potentiating effect of Pdx-1 on the endogenous *insulin* gene (16, 19–23). On the one hand, these latter studies provide evidence for the overall positive regulation of *insulin* transcription by Pdx-1; on the other, many of these studies employed use of

dominant negative proteins (which may have squelching effects on gene expression) and/or involved long-term functional knock-down of Pdx-1 (days to weeks), where its impact on a wide range of islet genes (*glut2*, *glucokinase*, *pdx-1*, *nkx6.1*, *glucagon*, and others) may have indirectly influenced *insulin* expression. Few of these studies have quantitatively examined the effect of physical knock-down of endogenous Pdx-1 in the short term (2–4 days); those that have reveal widely conflicting results on *insulin* transcription, from virtually no effect (24) to near-complete ablation (23).

To address definitively the role of Pdx-1 in the regulation of *insulin* transcription, we delivered small interfering RNA (siRNA)[1] into mouse β cell lines and islets to diminish endogenous Pdx-1 protein; we subsequently measured transcription by reverse transcriptase (RT)-PCR and quantitated transcription factor occupancy at the *insulin* promoter by chromatin immunoprecipitation (ChIP). To assess precisely the transcription of the *insulin* gene by RT-PCR, we employed a unique strategy whereby we measured total mouse *insulin I* and *II* mRNA (which reflects primarily fully processed or mature mRNA) and a mouse *insulin II* pre-mRNA species containing intron 2 by quantitative real-time RT-PCR. We demonstrate here that intron 2-containing transcripts possess short half-lives (9–30 min) in both mouse β cell lines and islets, whereas mature mRNA transcript displays substantially extended half-life (perhaps in excess of 24 h). We show that Pdx-1 knock-down in β cell lines and islets results in a coordinate decline in *insulin* transcription as measured by *insulin* pre-mRNA, but not as measured by mature mRNA. This decline in insulin transcription is accompanied by a fall in the association of Pdx-1 and its interacting coactivator p300 with the *insulin* gene, and a fall in the occupancy of RNA polymerase II (Pol II) at the *insulin* coding region. Our results suggest a model whereby Pdx-1 directly regulates *insulin* transcription through formation of a complex with transcriptional coactivators on the proximal *insulin* promoter. This complex appears to lead to enhancement of transcriptional elongation by components of the basal transcriptional machinery.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antiserum against p300 (N-15), RNA polymerase II (N-20), and actin were from Santa Cruz Biotechnology. Rabbit polyclonal antiserum against acetylated histone H3 and acetylated histone H4 were from Upstate Biotechnology. Rabbit polyclonal antiserum against Pdx-1 was a gift from Dr. M. German (University of California, San Francisco).

Cell Culture—The mouse insulinoma cell lines βTC3 (25) and MIN6 (26) were maintained in Dulbecco's modified Eagle's medium as previously described (26, 27) in 6-well tissue culture plates at a density of 5×10^5 cells/well. Mouse pancreatic islets were provided by the University of Virginia Diabetes Center Islet Isolation Core Facility. Islets were picked by hand from collagenase-digested 6–8-week-old CD-1 mouse pancreas (28) using a protocol approved by the Institutional Animal Care and Use Committee. Islets were cultured in RPMI medium containing 25 mM glucose overnight, then plated in 6-well tissue plates at a density of 150 islets/well.

For RNA decay experiments, cells were incubated in medium containing 0.1 mM glucose for 4 h, then switched to fresh medium containing either 0.1 or 25 mM glucose for 30 min and subsequently treated with 50 μg/ml of actinomycin D. At the times indicated, cells were washed once with PBS and processed for RNA isolation using the RNeasy® kit (Qiagen) according to the manufacturer's instructions. For isolation of whole cell extract, cells were washed twice in PBS and subsequently lysed directly in the plates by adding 50 μl of Laemmli buffer containing 4% SDS.

Adenoviral Vector Construction and Infection—A recombinant adenovirus (Ad-siPdx) containing an siRNA sequence (5′-GAAAGAGGAAGATAAGAAA-3′) corresponding to nucleotides 706–724 of *pdx-1* (GenBank™ accession number NM_022852) was recently prepared[2] using vector EH006 as described (29). An siRNA sequence targeting the *Photinus pyralis* luciferase gene, GL2 (30), was cloned into adenovirus (Ad-siLuc) for use as controls. Resulting adenoviruses were purified using the AdenoX® purification kit (BD Biosciences). Infectious titers of virus, as determined by end point dilution assay in HEK293 cells, were on the order of 10^{10}–10^{11} pfu/ml.

For adenoviral transduction of insulinoma cell lines, βTC3 and MIN6 cells were cultured overnight at a density of 5×10^5 cells/well in 6-well tissue culture plates as described above, followed by addition of recombinant adenoviruses at a multiplicity of infection of ~2500 pfu/cell. After 5 h, virus-containing medium was removed and cells were washed once with PBS and cultured in fresh medium. At the times indicated, cells were processed for isolation of total RNA or whole cell extracts, as described above. Islets were transduced with adenoviruses as described previously (29). Briefly, islets were maintained in 6-well plates at a density of 150 islets/well; adenoviruses were added at a multiplicity of infection of 1.3×10^6 pfu/islet in a total of 0.8 ml of serum-free medium. After 24 h, 2 ml of fresh medium containing serum was added and the islets were incubated for an additional 48 h, after which they were processed for total RNA isolation and whole cell extracts.

Real-time RT-PCR—Five micrograms of total RNA from β cell lines and islets were reverse transcribed at 37 °C for 1 h using 15 μg of random hexamers, 0.5 mM dNTPs, 5× first strand buffer, 0.01 M dithiothreitol, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a final reaction volume of 20 μl. Real-time RT-PCR was performed as described previously (27, 31, 32), but using forward and reverse primers to amplify mature *insulin* mRNA (5′-TGGCTTCTTCTACACACCCAAG-3′ and 5′-ACAATGCCACGCTTCTGCC-3′), *insulin* pre-mRNA (5′-GGGGAGCGTGGCTTCTTCTA-3′ and 5′-GGGGACAGAATTCAGTGGCA-3′), or *β-actin* (5′-AGGTCATCACTATTGGCAACGA-3′ and 5′-CACTTCATGATGGAATTGAATGTAGTT-3′). PCR were cycled 50 times using the following conditions: 95 °C for 15 s, 64 °C for 1 min. Homogeneity of products from each reaction was confirmed by melt curve analysis (as shown in Fig. 1*C*). *Pdx-1* mRNA levels in reverse-transcribed RNA were determined using the Assay on Demand® RT-PCR kit (product number Mm00435565_m1, Applied Biosystems) according to the manufacturer's instructions.

Amplified products from initial PCR were subcloned into the T/A cloning vector pCR2.1 (Invitrogen), and 3–4 clones were sequenced to confirm the identity of the amplified product. The threshold cycle (C_T) methodology (27, 31) was used to calculate relative quantities of mRNA products from each sample; all samples were corrected for total input RNA by normalizing C_T values to the C_T value of *β-actin* message. These corrections were verified by quantitation of total RNA in each sample by fluorescence using RiboGreen® dye (Molecular Probes).

Primers for mature mRNA and pre-mRNA displayed equal efficiency for amplification of target DNA as determined in the following manner: total genomic DNA from βTC3 cells was used as a template for PCR using each primer set. Because the mature mRNA primers recognize both mouse *insulin I* and *insulin II* (total = 4 copies in genomic DNA), whereas pre-mRNA primers recognize only mouse *insulin II* (total = 2 copies in genomic DNA), it would be expected that C_T values would occur 1 cycle sooner for mature mRNA primers than for pre-mRNA primers (assuming both primer sets amplified with equal efficiency). We observed amplification of mature mRNA with a C_T of ~17, and amplification of pre-mRNA with a C_T of ~18. This difference of 1 cycle was reproducible across a range of dilutions of genomic DNA.

Mathematical Modeling of RNA Decay—RNA decay curves shown in Fig. 2 were optimized (by a modified Gauss-Newton nonlinear least squares algorithm) (33) to an exponential decay function of the form,

$$y(t) = \alpha \exp\left(\frac{-t}{\tau}\right) + c \qquad \text{(Eq. 1)}$$

where $y(t)$ is the observed time series data; t is time; α is the magnitude of exponential decay; τ is the lifetime of the exponential decay process; and c is an additive, offset constant.

Quantitative ChIP—ChIP assays, including quantitation of co-immunoprecipitated DNA fragments by real-time PCR, were performed as

[1] The abbreviations used are: siRNA, small interfering RNA; Ad-siLuc, adenovirus encoding siRNA against *luciferase* mRNA; Ad-siPdx, adenovirus encoding siRNA against *pdx-1* mRNA; ChIP, chromatin immunoprecipitation; Pol II, RNA polymerase II; RT, reverse transcriptase; PBS, phosphate-buffered saline; pfu, plaque-forming unit.

[2] J. Schisler, P. Jensen, D. Taylor, T. Becker, F. Knop, M. German, G. Weir, D. Lu, R. Mirmira, and C. Newgard, submitted for publication.

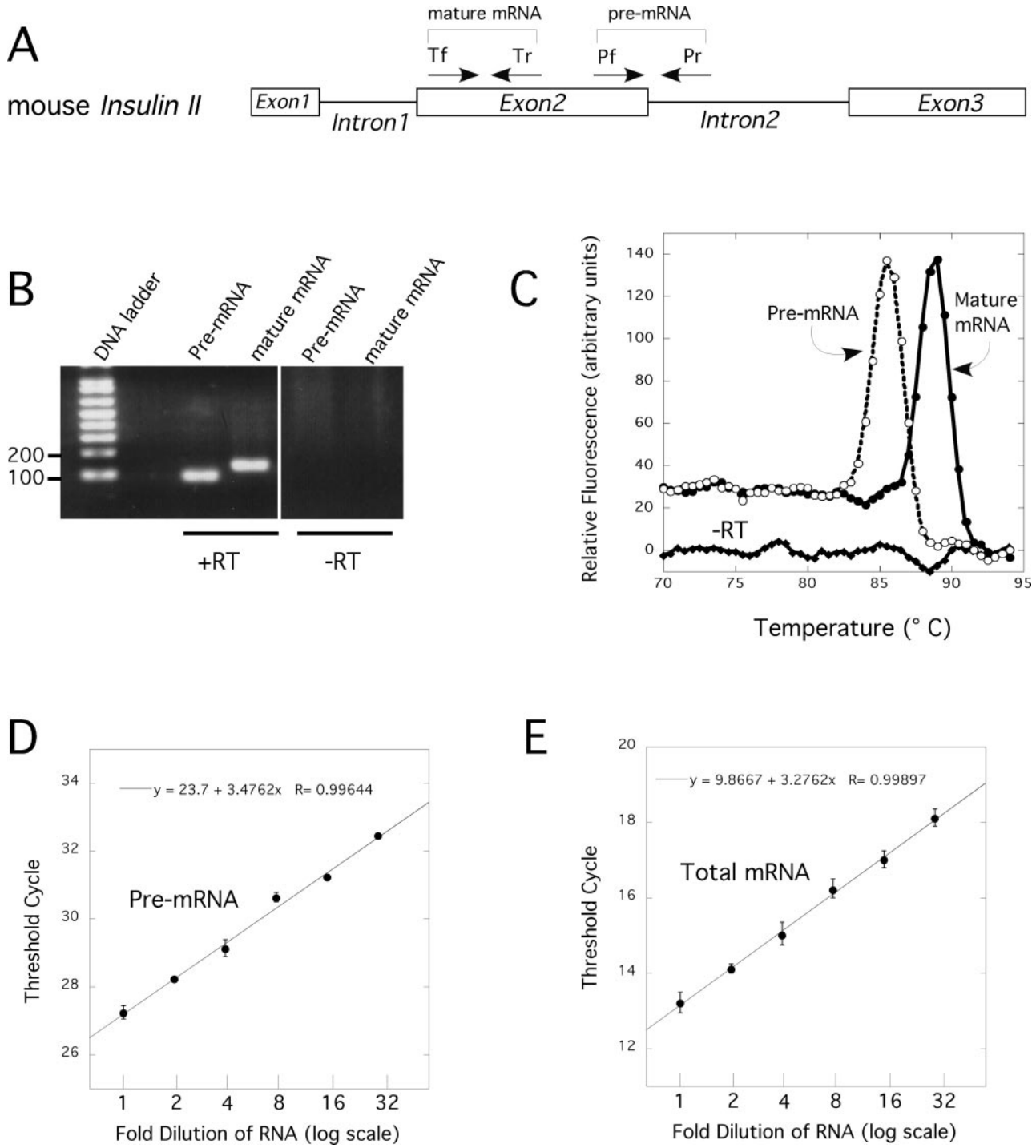

FIG. 1. **Real-time RT-PCR amplification of *insulin* pre-mRNA and mature mRNA.** *A*, schematic representation of the mouse *insulin II* gene, indicating positions of PCR primers (*Tf*, *Tr*, *Pf*, and *Pr*) relative to the introns and exons of the gene. *B*, a 2% agarose gel showing amplification of products from reverse-transcribed islet RNA corresponding to *insulin* pre-mRNA and mature mRNA. *C*, SYBR Green I melt curve analysis of products following real-time PCR amplification from reverse-transcribed islet RNA. *D* and *E*, real-time PCR threshold cycles demonstrating linearity of amplification of pre-mRNA (*D*) and mature mRNA (*E*) from serial 2-fold dilutions of islet RNA.

described previously (27, 31, 32) with the following modifications. βTC3 cells and islets were plated in 6-well dishes at 5×10^5 cells/well or 150 islets/well, respectively, for infection with adenovirus (as described above). Following incubation with adenovirus for 72 h, each well was washed with PBS, cross-linked for 15 min in 1% formaldehyde (in PBS), then all wells were pooled. Pooled samples were sonicated as described (27) in 600 μl of sonication buffer to yield chromatin fragments of ~500–800 bp in length. Approximately 150 μl of sonicated chromatin was diluted to 1 ml in ChIP buffer (27) and subject to immunoprecipitation with the antibodies indicated in Fig. 6. 15 μl of sonicated sample was saved for quantitation of input chromatin. ChIP assays were performed on at least 3 independent occasions; for each ChIP assay, promoter samples were quantitated in triplicate as previously described (27, 31) using SYBR Green I-based real-time PCR. Data in Fig. 6 are presented as percent of input DNA recovered. Forward and reverse primer sequences, respectively, used for PCR were: proximal *insulin* promoter: 5′-TACCTTGCTGCCTGAGTTCTGC-3′ and 5′-GCATTTTC-CACATCATTCCCC-3′; *insulin* coding sequence: 5′-TGGCTTCTTCTA-CACACCCAAG-3′ and 5′-ACAATGCCACGCTTCTGCC-3′.

Immunoblot Analysis—Whole cell extracts from cell lines and islets were prepared as described above. Aliquots of 5 μg of protein were resolved by electrophoresis on a 15% SDS-polyacrylamide gel followed by immunoblot analysis using anti-Pdx-1 or anti-actin antibodies (both at 1:5000 dilution). Western blots were visualized using the ECL-Plus®

TABLE I
Real-time RT-PCR threshold cycles (C_T) for mouse mature insulin mRNA and insulin II pre-mRNA in β cell lines and islets

	Insulin II pre-mRNA[a]		Mature *insulin* mRNA[a]		Relative abundance[b] (pre-mRNA:mature mRNA)
	+RT	−RT	+RT	−RT	
βTC3 cells	17.5 ± 0.1	>35	12.3 ± 0.6	>40	0.027
MIN6 cells	20.3 ± 0.8	>35	13.9 ± 1.4	>40	0.012
Islets	28.4 ± 2.6	>35	14.2 ± 2.7	>40	5.3×10^{-5}

[a] To correct for variations in input RNA, threshold cycles were normalized to the *actin* mRNA threshold cycle in each sample, which was approximately 17 for all cell types.
[b] Relative abundance was calculated using the following formula: $2^{[CT(\text{mRNA})-CT(\text{pre-mRNA})]}$. As noted under "Experimental Procedures," RT-PCR primers for *insulin* mRNA and pre-mRNA were equally efficient.

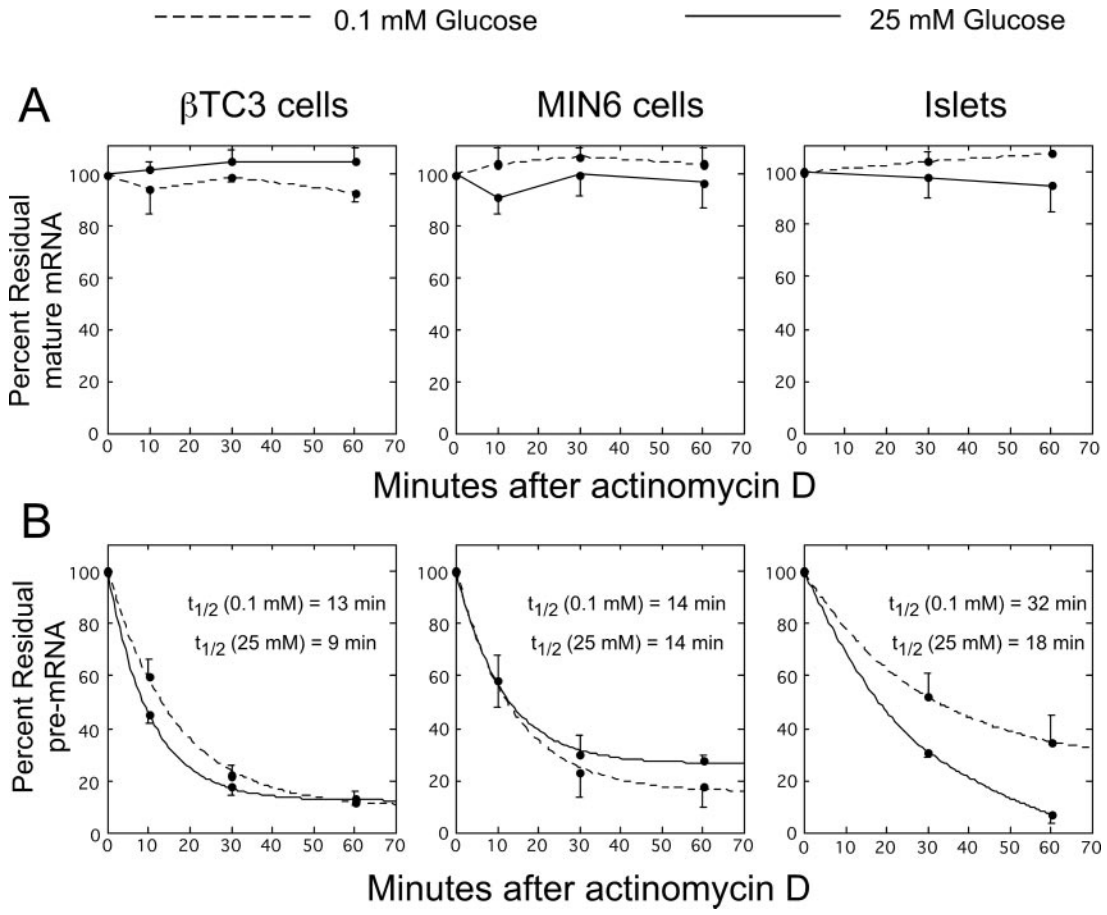

FIG. 2. ***insulin* transcript half-lives.** βTC3 cells, MIN6 cells, and islets in either 0.1 or 25 mM glucose were incubated with actinomycin D for the times indicated, then total RNA from the cells was isolated, reverse-transcribed, and subject to quantitation for either mature *insulin* mRNA (*panel A*) or *insulin* pre-mRNA (*panel B*) by real-time PCR. To correct for variations in input RNA, all data were normalized to the quantity of *β-actin* message (also determined by real-time PCR). The half-lives ($t_{½}$) indicated in *panel B* were determined by fitting the data to an exponential decay model, as detailed under "Experimental Procedures." Experiments were performed on a minimum of three independent occasions. The curves in *panel B* represent the best-fit model.

system (Amersham Biosciences), and quantitated by densitometry using 1D® software (Kodak).

RESULTS

Quantitation of Mature insulin mRNA and insulin Pre-mRNA and Determination of Their Biologic Half-lives in β Cells—In most mammals (including the human), the gene encoding preproinsulin (*insulin*) exists as a single gene containing two introns. However, in rats and mice the gene has undergone a duplication event during evolution, resulting in two homologous non-allelic genes (*insulin I* and *insulin II*) (1, 2). Similar to other mammals, the rodent *insulin II* gene contains two introns (see Fig. 1*A*), whereas the *insulin I* gene lacks the second intron. Prior studies have suggested very long half-lives (up to perhaps 80 h) of many spliced and unspliced forms of *insulin* mRNAs in mouse islets and β cell lines (34–38); a notable exception, however, is the *insulin II* intron 2-containing transcript, which appears to have a half-life on the order of minutes (37). Because both *insulin* genes appear to be coordinately regulated in the β cell (39, 40), we hypothesized that measurement of a short half-life *insulin II* transcript species would more accurately reflect immediate changes (particularly decreases) in overall transcriptional rates that might occur in our studies of *insulin* gene regulation.

To measure *insulin* mRNA species, we designed two primer sets (shown in Fig. 1*A*) for quantitative real-time RT-PCR. The primer set Tf/Tr was designed to amplify a 130-bp sequence in exon 2 of the mouse *insulin I* and *II* genes, and therefore targets both spliced and unspliced mRNA (total mRNA). Because unspliced *insulin* mRNA represents a very small fraction of total *insulin* mRNA (37), this primer set can be viewed as

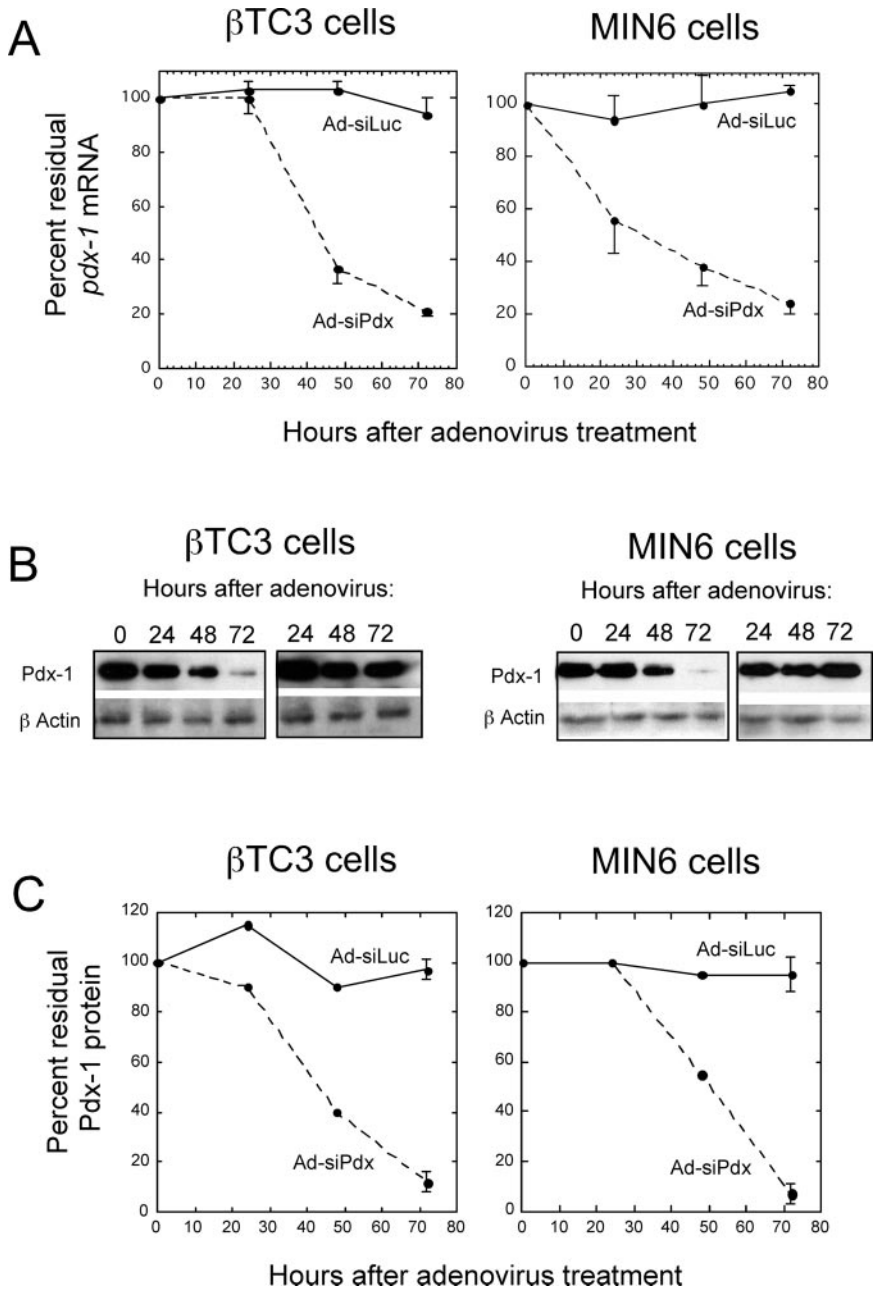

FIG. 3. **siRNA-mediated knockdown of Pdx-1 in insulinoma cell lines.** βTC3 and MIN6 cells were left uninfected or infected with adenovirus encoding siRNA against either *luciferase* (Ad-siLuc) or *pdx-1* (Ad-siPdx) at a multiplicity of infection of ~2500 pfu/cell. Cells were harvested at the time points indicated for isolation of either total RNA or whole cell extract. *A*, quantitation of *pdx-1* mRNA by real-time PCR in infected cells. To correct for variations in input RNA, data were normalized to the quantity of *β-actin* message (also determined by real-time PCR). Data at each time point represent percent of *pdx-1* mRNA relative to uninfected cells (for which levels of *pdx-1* mRNA did not change significantly over the time course). *B*, representative immunoblot for Pdx-1 and β-actin proteins from whole cell extracts of infected βTC3 and MIN6 cells. *C*, phosphorimager quantitation of Pdx-1 protein levels from Western blots shown in *panel B*. Data represent the average of at least three independent experiments.

representing primarily mature (or fully spliced) mRNA. The primer set Pf/Pr was designed to amplify an 82-bp fragment spanning the exon 2/intron 2 boundary, thereby targeting only a specific unspliced mRNA (pre-mRNA) of *insulin II* containing intron 2. Fig. 1*B* shows that PCR amplification of both species of mRNA from reverse-transcribed total islet RNA results in homogenous products of expected molecular weight. Primers were subsequently used for SYBR Green I-based real-time PCR (27, 31). Fig. 1*C* confirms, by melt curve analysis, that homogenous products were obtained following 50 cycles of PCR; Fig. 1, *D* and *E*, shows that the real-time PCR quantitation of both *insulin* pre-mRNA and mature mRNA was linear over the relatively narrow range of total RNA concentrations observed in our studies, thereby demonstrating the reproducibility and linearity of our real-time assay in distinguishing small differences in target mRNA quantities. Similar linearity data were obtained for the real-time amplification of *β-actin* message, and for all messages amplified from reverse-transcribed RNA isolated from two mouse β cell-derived insulinoma cell lines, βTC3 and MIN6 (data not shown).

Table I shows the real-time PCR amplification threshold values for *insulin* mature mRNA and pre-mRNA in mouse insulinoma cell lines and islets maintained in 25 mM glucose. Interestingly, these data reveal no substantial differences in the relative amounts of mature *insulin* mRNA in these cells, but do show striking differences in pre-mRNA levels. Based on the calculated ratio of pre-mRNA to mature mRNA, the quantities of pre-mRNA are substantially lower than mature mRNA in all cell types. (Importantly, as noted under "Experimental Procedures," both primer sets appeared to have identical amplification efficiencies based on a comparison of threshold values from genomic DNA.) However, although this ratio was similar for the two insulinoma cell lines, by comparison it was lower by 200–500-fold in islets. These data suggest that the cell lines produce initial transcripts in far greater quantity than islets, a finding consistent with their tumorigenic origin.

To determine the biologic half-lives of *insulin* mRNA species, βTC3 cells, MIN6 cells, and mouse islets were treated with actinomycin D to block transcriptional elongation, and cells were harvested at various time points for isolation and quan-

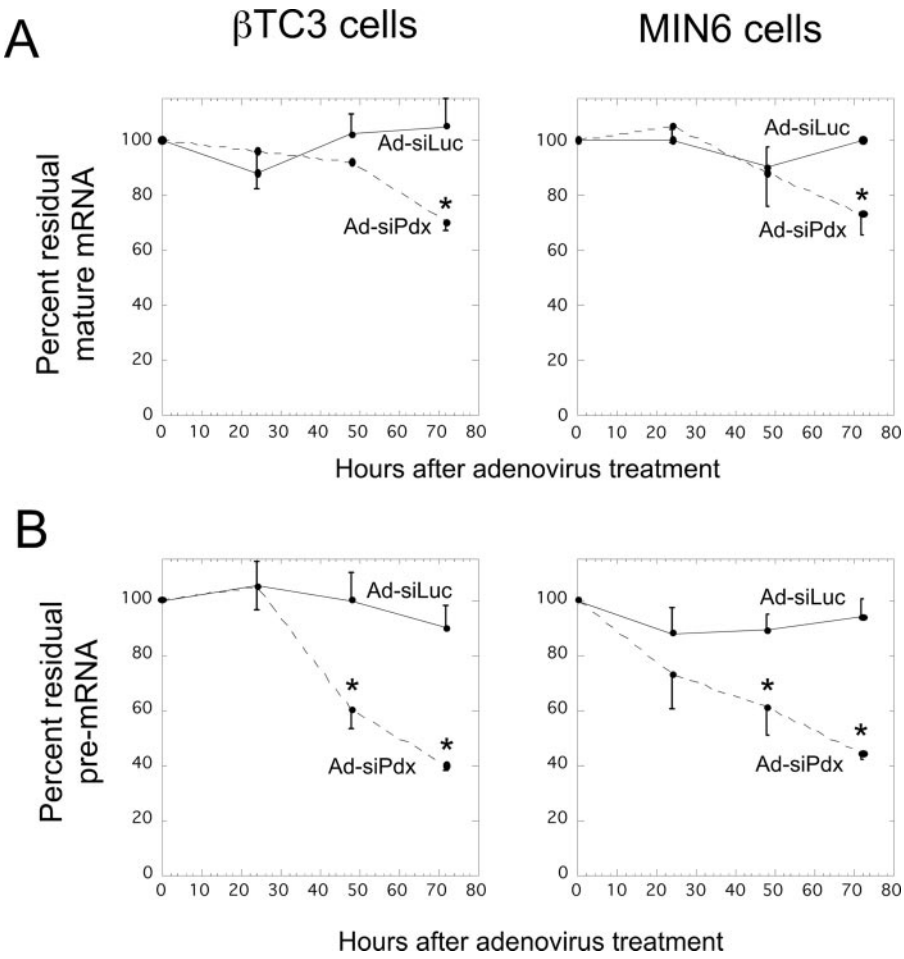

FIG. 4. **Quantitation of mature *insulin* mRNA and *insulin* pre-mRNA in siRNA-treated β cell lines.** βTC3 and MIN6 cells were left uninfected or infected with adenovirus encoding siRNA against *luciferase* (Ad-siLuc) or *pdx-1* (Ad-siPdx) as in Fig. 3, and total RNA was isolated from cells at the indicated time points. Levels of mature *insulin* mRNA (*A*) or *insulin* pre-mRNA (*B*) were quantitated by real-time PCR. To correct for variations in input RNA, data were normalized to the quantity of *β-actin* message (also determined by real-time PCR). Data at each time point represent percent of mRNA or pre-mRNA relative to uninfected cells (for which levels of these species did not change significantly over the time course). *Asterisk* (*) signifies that the value for Ad-siPdx-treated cells is statistically different ($p < 0.05$) from the corresponding value for untreated or Ad-siLuc-treated cells. Data represent the average of at least three independent experiments.

titation of mRNAs. As shown in Fig. 2, mature *insulin* mRNA was observed to have an extended half-life (no discernable decay after 60 min) in all cell types grown under high (25 mM) or low (0.1 mM) glucose conditions. By contrast, intron 2-containing pre-mRNA showed short half-lives of 9, 14, and 18 min in βTC3, MIN6, and islet cells, respectively, under high glucose conditions (see Fig. 2*B*). In βTC3 cells and islets, this half-life was extended about 1.5-fold (to 13 and 32 min, respectively) under low glucose conditions, in confirmation of prior studies showing that glucose concentration can affect *insulin* mRNA processing rates (37). The data in Fig. 2 were normalized to levels of *β-actin* mRNA, which showed no discernable decay during the time course (data not shown).

siRNA-induced Knock-down of Pdx-1 Levels in β Cell Lines Coordinately Diminishes insulin Pre-mRNA Levels—To determine the role of Pdx-1 in *insulin* transcription, we knocked down endogenous Pdx-1 protein in β cell lines and subsequently analyzed cells for *insulin* transcription by real-time RT-PCR. To knock-down Pdx-1 levels, we treated cells with an adenovirus encoding siRNA specific for *pdx-1* (Ad-siPdx). Fig. 3*A* shows that Ad-siPdx treatment caused a time-dependent decrease in *pdx-1* mRNA levels to a nadir of ~20% of starting concentrations in both βTC3 and MIN6 cells. Conversely, untreated cells or cells treated with an adenovirus encoding siRNA specific for *luciferase* (Ad-siLuc) showed no change in *pdx-1* mRNA levels (Fig. 3*A*). The fall in *pdx-1* mRNA levels in Ad-siPdx-treated cells corresponded to a decrease in Pdx-1 protein in both cell types as assessed by immunoblot analysis, to levels ~5–15% of those observed in control cells (Fig. 3, *B* and *C*).

To assess the effect of Pdx-1 knock-down on *insulin* gene transcription in βTC3 and MIN6 β cells, we measured both *insulin* mature mRNA and pre-mRNA during a time course following Ad-siPdx or Ad-siLuc treatment (or no treatment). As shown in Fig. 4*A*, Ad-siPdx treatment resulted in no significant decline in mature *insulin* mRNA levels over the first 48 h of the experiment, and a decline of only 30% at 72 h relative to control cells. These results are comparable with a previous report demonstrating virtually no decline in insulin message in both cell types following antisense RNA-induced Pdx-1 knock-down (24). In striking contrast, however, pre-mRNA levels in both cell types were decreased by 40% 48 h after Ad-siPdx treatment, and by 60% 72 h after treatment. The decreases in pre-mRNA levels precisely paralleled the fall in Pdx-1 protein levels observed by immunoblot analysis (*cf.* Fig. 3*C*), highlighting the rapid effect of Pdx-1 knock-down on *insulin* transcription in β cell lines. Importantly, half-lives of *insulin* pre-mRNA and mature mRNA appear unchanged in cells treated with Ad-siPdx and Ad-siLuc, based on actinomycin D experiments performed at 72 h after viral treatment (data not shown).

siRNA-induced Knock-down of Pdx-1 in Primary Mouse Islets Diminishes insulin Pre-mRNA Levels—To determine the contribution of Pdx-1 to *insulin* transcription in primary cells, we treated intact mouse islets with Ad-siPdx and compared these to Ad-siLuc-treated and untreated control islets. As shown in Fig. 5*A*, treatment of islets with Ad-siPdx for 72 h resulted in a 70% decrease in *pdx-1* mRNA compared with control islets. This reduction in *pdx-1* mRNA corresponded to ~70% reduction in Pdx-1 protein levels compared with control islets (Fig. 5*B*). We next measured both mature *insulin* mRNA and *insulin* pre-mRNA by real-time RT-PCR from total RNA isolated from Ad-siPdx-treated and control islets at 72 h. As shown in Fig. 5*C*, mature mRNA levels decreased by ~25% compared with controls, whereas pre-mRNA levels declined by

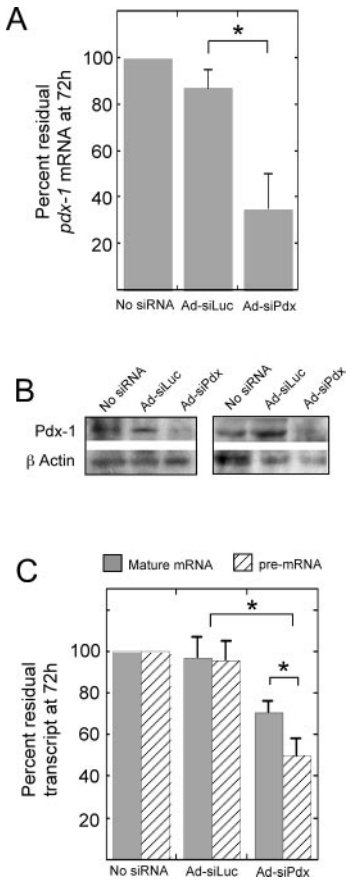

FIG. 5. **siRNA-mediated knock-down of Pdx-1 in isolated mouse islets.** Approximately 150 mouse islets were untreated or treated with adenovirus encoding siRNA against *luciferase* (Ad-siLuc) or *pdx-1* (Ad-Pdx) at a multiplicity of infection of 1.3×10^6 pfu/islet. Islets were harvested at 72 h for isolation of total RNA or whole cell extract. *A*, quantitation of *pdx-1* mRNA by real-time PCR in infected islets. To correct for variations in input RNA, data were normalized to the quantity of *β-actin* message (also determined by real-time PCR). Data represent percent of *pdx-1* mRNA relative to untreated cells (set at 100%). *B*, immunoblot for Pdx-1 and β-actin proteins from whole cell extracts of infected islets. Data from two independent infections are shown. *C*, levels of mature *insulin* mRNA or *insulin* pre-mRNA were quantitated by real-time PCR, and normalized to the quantity of *β-actin* message. Data represent percent of mRNA or pre-mRNA relative to uninfected cells (set at 100%). *Asterisk* (*) signifies that the values are statistically different for the comparisons indicated ($p < 0.05$). Data represent the average of at least three independent experiments.

50%. These results are comparable with those obtained in the β cell lines, and suggest that *insulin* transcription in primary isolated islets is similarly regulated by Pdx-1. Taken together, the results from β cell lines and islets strongly suggest that Pdx-1 directly regulates *insulin* transcription and that *insulin* pre-mRNA levels reflect this regulation more accurately.

Recruitment of Pdx-1, p300, and RNA Polymerase II to the insulin Promoter—To determine the mechanism underlying *insulin* gene activation by Pdx-1, we examined the association of Pdx-1, p300, and RNA polymerase II to the *insulin* promoter in untreated, Ad-siLuc-, and Ad-siPdx-treated βTC3 cells and mouse islets by the ChIP assay. We and others have previously demonstrated by ChIP that Pdx-1 and p300 specifically occupy the proximal *insulin* promoter of βTC3 cells (27, 31, 41). 72 h following Ad-siPdx treatment, we observed an 80% reduction in Pdx-1 occupancy at the proximal promoter in βTC3 cells and a reduction of Pdx-1 occupancy to background levels in mouse islets (Fig. 6, *A* and *B*, respectively); these effects were not observed in Ad-siLuc-treated or untreated control islets. The decrease in Pdx-1 at the proximal promoter in βTC3 cells was accompanied by a 40% reduction in the occupancy of p300 at the proximal *insulin* promoter (Fig. 6*C*). Because we did not observe any decrease in the levels of p300 in Ad-siPdx-treated cells by immunoblot (Fig. 6*C*, *inset*), these results suggest that the knock-down of Pdx-1 impairs the recruitment of p300 to the proximal promoter. (Because of low signal-to-noise ratios, we were unable to obtain meaningful data on p300 recruitment in islets.) Although p300 has histone acetyltransferase activity (42), we did not observe significant falls in histone H3 or H4 acetylation at the proximal *insulin* promoter during the time course of these studies in βTC3 cells (data not shown).

At least one of the mechanisms underlying the action of transcription factors and coactivators is their effect on the initiation and/or elongation of transcription by RNA polymerase II (Pol II) (43). We therefore examined the effect of Pdx-1 knock-down on Pol II occupancy at the *insulin* promoter by ChIP. Interestingly, at the proximal insulin promoter (where transcriptional initiation complex formation is expected to occur), we observed no significant change in Pol II occupancy following Ad-siPdx treatment of βTC3 cells (Fig. 6*D*). However, Pol II occupancy within the downstream coding region of the *insulin* gene was diminished by almost 4-fold following Ad-siPdx treatment (Fig. 6*E*), suggesting that loss of Pdx-1 impairs transcriptional elongation by Pol II.

DISCUSSION

In this report, we provide evidence that the *insulin* gene is a direct downstream target of the pancreatic homeodomain transcription factor Pdx-1. Prior to our work, evidence supporting a direct role of Pdx-1 on insulin transcription derived primarily from electrophoretic mobility shift analysis *in vitro* and studies of reporter gene expression in mammalian cells (11–13, 15, 16). However, those and other studies did not examine the role of endogenous Pdx-1 protein upon transcription of the native, chromatin-embedded *insulin* gene. In this study, we employed a unique RT-PCR strategy following siRNA delivery to study transcription of the native *insulin* gene in the absence of Pdx-1 protein.

One complication to studying the regulation of *insulin* transcription by RT-PCR or Northern blot analysis is that such assays are strongly influenced by the high levels and extended half-life of the mature *insulin* mRNA in insulinoma cell lines and islets. It has been estimated that the β cell contains up to 40,000 *insulin* transcripts, exceeding even the number of *β-actin* transcripts (36, 37). Our results here appear to support this observation (Table I), and indicate further that the half-life of insulin mRNA is considerably in excess of 60 min, but in all likelihood is closer to 30–80 h, as reported by many other investigators (34–38). Thus, an important implication of our studies is that if *insulin* transcription is studied solely by methods that quantitate total mRNA, it will be difficult to interpret the effect of putative transcriptional modulators (transcription factors, glucose, cAMP, etc.), because several days may be necessary to observe changes in the rather large *insulin* mRNA content in β cells. In this context, an earlier report (24) that showed no effect of Pdx-1 knock-down on *insulin* transcription (as assessed by Northern blot analysis) in MIN6 and βTC1 cells is clarified. Indeed, our studies confirm that knock-down of Pdx-1 levels by 80–90% causes only a 30% reduction in mature *insulin* mRNA over 72 h, and well after clear suppression of Pdx-1 has occurred. By themselves, these data may raise doubt as to the immediate role of Pdx-1 in *insulin* transcription.

To overcome the potential barrier imposed by the long half-life of *insulin* mRNA, we examined whether a pre-mRNA species with a shorter half-life could be used to study *insulin* transcription. In this respect, formation of pre-mRNAs are

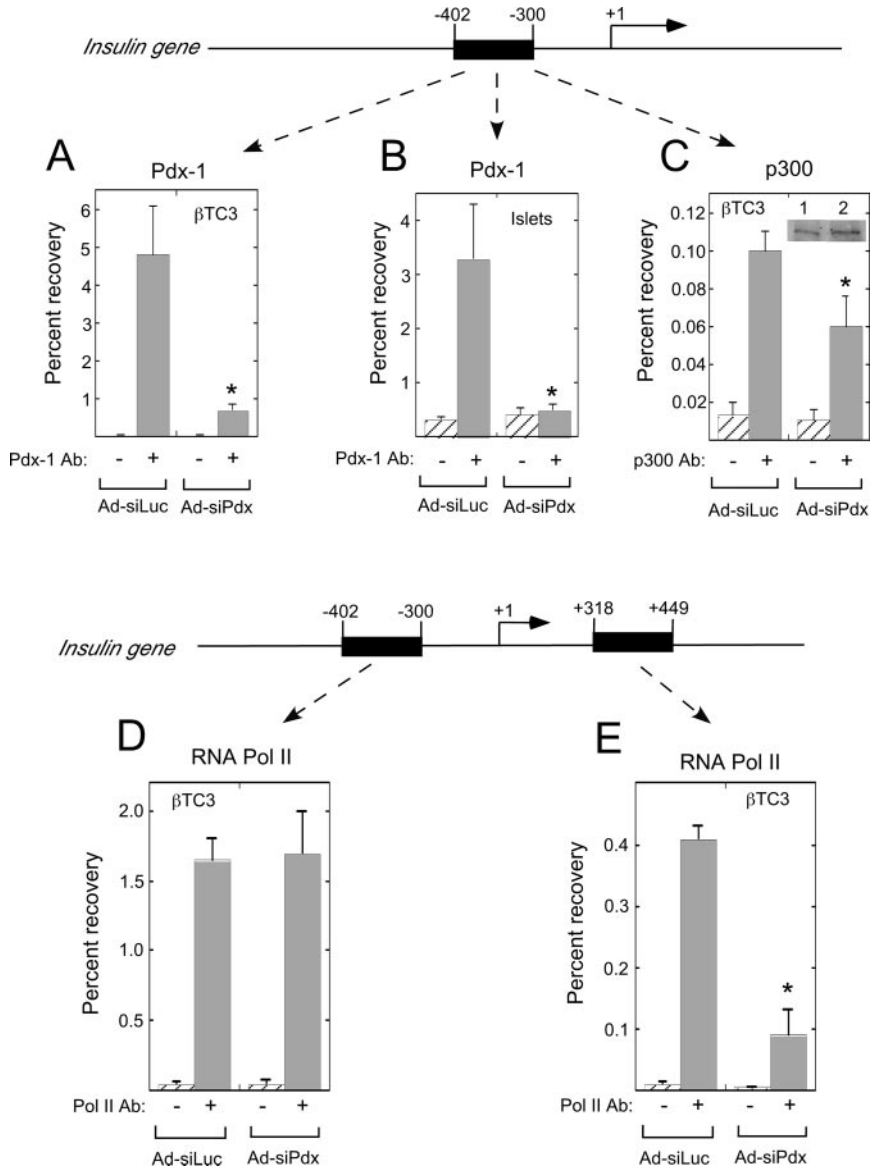

FIG. 6. **Association of Pdx-1, p300, and RNA polymerase II with the *insulin* gene.** βTC3 cells or islets were infected with adenovirus encoding siRNA against either *luciferase* (Ad-siLuc) or *pdx-1* (Ad-siPdx) for 72 h, and cells were subsequently harvested for ChIP as detailed under "Experimental Procedures." Schematic diagrams of the *insulin* gene *above* the panels indicates the DNA fragments (and their positions in bp relative to the transcriptional start site at +1) that were amplified by real-time PCR following ChIP. *A* and *B*, recovery of the proximal *insulin* promoter fragment (−300 to −462 bp) following ChIP using antibody (*Ab*) against Pdx-1 and extracts from infected βTC3 cells (*A*) or islets (*B*). *C*, recovery of the proximal promoter fragment following ChIP using p300 Ab and extracts from infected βTC3 cells. *Inset* shows an immunoblot for p300 from βTC3 cells treated with no virus (*lane 1*) or Ad-siPdx (*lane 2*). *D* and *E*, recovery of the proximal promoter fragment (*D*) or a distal fragment from the *insulin* coding region (*E*) following ChIP using RNA polymerase II (Pol II) Ab and extracts from infected βTC3 cells. All data represent recovery, in percent, of each DNA fragment relative to total input DNA. *Asterisk* (*) signifies that the value for Ad-siPdx-treated cells is statistically different from the corresponding value for Ad-siLuc-treated cells ($p < 0.05$). Data represent the average of at least three independent experiments.

more closely linked to transcription, because no intervening processes such as splicing or cytoplasmic export necessarily occur. Quantitation of such short half-life pre-mRNAs have been used as a reflection of acute transcriptional regulation in other systems such as the *LHβ*, *FSHβ*, and *follistatin* genes, and can closely mimic results obtained from nuclear run-off assays (44, 45). We demonstrate here by real-time RT-PCR that the intron 2-containing transcripts of mouse *insulin II* possess remarkably short half-lives (on the order of minutes) in both β cell lines and islets, a finding consistent with a prior study using the branched DNA assay methodology (37). Interestingly, pre-mRNA levels (relative to mature mRNA levels) appear to be much lower in islets than insulinoma cell lines (Table I). Although this finding suggests that *insulin* transcription rates are lower in islets compared with insulinoma cells, it should be noted that our studies examined only relative message levels in intact islets, which additionally contain endocrine cell types other than β cells. In this regard, other studies using different methodologies have found higher pre-mRNA and mature mRNA levels in islets on a per-cell basis (37).

In contrast to the lesser and delayed decrease in mature *insulin* mRNA following knock-down of Pdx-1, the decline in intron 2-containing *insulin* pre-mRNA levels are observed in close association with the fall in Pdx-1 levels. This finding has four important implications: 1) pre-mRNA levels reflect a downward trend in transcription much sooner (by about 24 h) than mature mRNA levels, a finding that is explained by the shorter half-life of the pre-mRNA species; 2) the close association of *insulin* pre-mRNA and Pdx-1 levels, in conjunction with the decrease in occupancy of Pdx-1 at the proximal *insulin* promoter, points to the *insulin* gene as an immediate downstream target of Pdx-1 (as opposed to a target downstream of a secondary or tertiary gene expression event); 3) our data suggest that a striking fraction (at least 60%) of *insulin* transcriptional activity is dependent upon Pdx-1 action, notwithstanding the fact that a host of β cell-specific transcription factors are believed to participate at the promoter (8). We believe these findings must be considered in the context of transcription factor cooperativity at the *insulin* promoter. Thus, the loss of cooperative interactions between Pdx-1 and factors such as BETA2/NeuroD and E47 (15) may underlie the significant fall in transcriptional rate; and 4) our findings in the β cell lines appear to be similar to those in primary mouse islets, suggesting that the mechanism by which Pdx-1 regulates *insulin* transcription in insulinoma cells is likely to be very similar to that in the primary cell type.

The mechanism governing transcriptional activation by Pdx-1 has been the subject of intense investigation over the

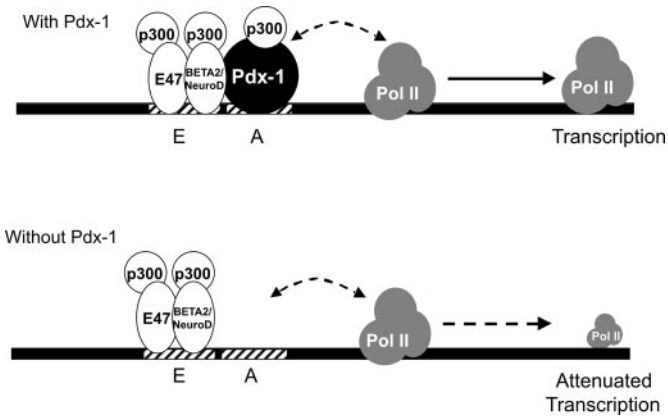

FIG. 7. **Model of *insulin* gene regulation by Pdx-1.** A schematic representation of the *insulin* gene is shown, identifying key "E" and "A" elements within the promoter region (*hatched rectangles*) that serve as binding sites for transcription factors E47, BETA2/NeuroD, and Pdx-1 (*ovals*). *Upper panel*, in the presence of Pdx-1, a complex of transcription factors and cofactors (p300) is formed that physically and/or functionally interact with the RNA polymerase II (*Pol II*) transcriptional machinery. This interaction leads to enhancement of transcriptional elongation by Pol II. *Lower panel*, in the absence of Pdx-1, complex formation is disrupted, p300 concentration at the proximal promoter is diminished, and the physical/functional interactions with Pol II are diminished. In this setting, although recruitment of Pol II to the proximal promoter is unaffected, transcriptional elongation by Pol II is attenuated.

years. Pdx-1 is known to have a uniquely functioning transactivation domain in its amino terminus (46), and to interact with a host of other transcription factors and cofactors (9, 15, 47, 48). Importantly, the net effect of Pdx-1 action at any putative target promoter (degree of transcriptional activation or even repression) likely depends upon the overall balance of these interactions. The coactivator p300 interacts with Pdx-1 and is thought to enhance *insulin* transcriptional activity through multiple mechanisms, including the recruitment and activation of components of the basal transcriptional machinery and histone/protein acetylation (9, 31, 49–51). Our studies show that the association of p300 with the insulin promoter is diminished upon knock-down of Pdx-1, consistent with the notion that its interaction with Pdx-1 may be important in its recruitment to the promoter. Although this decrease in p300 occupancy was not associated with significant changes in histone acetylation at the *insulin* gene, we cannot rule out the possibility that acetylation of other proteins (*e.g.* basal transcriptional components) by p300 is crucial for *insulin* transcription. Importantly, the association of p300 with the *insulin* promoter is only partially eliminated following Pdx-1 knock-down, thereby emphasizing the role of other transcription factors at the promoter that are likely to recruit p300, including BETA2/NeuroD and E47 (10).

Pol II is a crucial component of the basal transcriptional machinery in mammalian cells and is believed to be physically or functionally "bridged" to sequence-specific transcription factors by cofactors such as p300 (43). Despite the decreases in p300 recruitment and *insulin* transcription following Pdx-1 knock-down, we did not observe significant changes in the occupancy of Pol II to the proximal *insulin* promoter; however, the occupancy of Pol II in the downstream coding region was reduced significantly by almost 4-fold. Because it is known that sequence-specific transcription factors can differentially affect either initiation or elongation by Pol II in other systems (52–54), our results suggest a model (see Fig. 7) whereby Pdx-1 directly interacts with sequence elements of the *insulin* promoter and (perhaps in conjunction with its interacting proteins p300, BETA2/NeuroD, and E47) may ultimately be crucial to promoting transcriptional elongation at the *insulin* gene, at least in insulinoma cells.

Taken together, our studies validate the hypothesis that islet transcription factors interact with upstream regulatory sequences in the *insulin* promoter, form complexes with ubiquitously expressed factors, and subsequently activate components of the basal transcriptional machinery (Fig. 7). In the context of disease, our data point to deficiency at the transcriptional level as one factor underlying the insulin secretory defect observed in conditions of functional Pdx-1 deficiency, such as human maturity onset diabetes of the young type 4 syndrome (MODY4) (55). Central to our studies is the measurement of a less abundant *insulin II* pre-mRNA species, in addition to mature mRNA, as a means of assessing changes to *insulin* transcriptional rates. We anticipate that measurement of this pre-mRNA species will likely clarify findings in future studies examining transcriptional regulation of the endogenous *insulin* gene.

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