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Cooperativity between the Preproinsulin mRNA Untranslated Regions Is Necessary for Glucose-stimulated Translation*

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Barton Wicksteed‡, Terence P. Herbert‡§, Cristina Alarcon‡, Melissa K. Lingohr‡, Larry G. Moss¶, and Christopher J. Rhodes‡‖

From the ‡Pacific Northwest Research Institute, Seattle, Washington 98122 and the ¶Departments of Medicine and Physiology, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts 02111

Glucose regulates proinsulin biosynthesis via stimulation of the translation of the preproinsulin mRNA in pancreatic β-cells. However, the mechanism by which this occurs has remained unclear. Using recombinant adenoviruses that express the preproinsulin mRNA with defined alterations, the untranslated regions (UTRs) of the preproinsulin mRNA were examined for elements that specifically control translation of the mRNA in rat pancreatic islets. These studies revealed that the preproinsulin 5′-UTR was necessary for glucose stimulation of preproinsulin mRNA translation, whereas the 3′-UTR appeared to suppress translation. However, together the 5′- and 3′-UTRs acted cooperatively to markedly increase glucose-induced proinsulin biosynthesis. In primary hepatocytes the presence of the preproinsulin 3′-UTR led to reduced mRNA levels compared with the presence of the SV40 3′-UTR, consistent with the presence of mRNA stability determinants in the 3′-UTR that stabilize the preproinsulin mRNA in a pancreatic β-cell-specific manner. Translation of these mRNAs in primary hepatocytes was not stimulated by glucose, indicating that regulated translation of the preproinsulin mRNA occurs in a pancreatic β-cell-specific manner. Thus, the untranslated regions of the preproinsulin mRNA play crucial roles in regulating insulin production and therefore glucose homeostasis by regulating the translation and the stability of the preproinsulin mRNA.

Pancreatic islet β-cells secrete insulin in response to increases in circulating nutrients, the most physiologically relevant of which is glucose (1). To replenish secreted insulin and maintain optimal intracellular insulin stores there is a corresponding rapid and specific stimulation of proinsulin biosynthesis (1–3). Under normal circumstances this occurs by increasing translation of the existing preproinsulin mRNA and is mostly controlled at the initiation phase of the translational mechanism. Preproinsulin mRNA is mobilized from an inert intracellular storage pool (in which most of the mRNA is free) to membrane-bound polyribosomes, marking the entry of newly synthesized proinsulin into the β-cell secretory pathway (4, 5). Translation of the preproinsulin mRNA is targeted to the endoplasmic reticulum through an interaction between its signal peptide and the signal recognition particle (SRP).[1] It has been reported that SRP-mediated translocation of secretory pathway mRNAs to the endoplasmic reticulum is glucose-regulated in pancreatic β-cells (6, 7).

Glucose stimulates general protein synthesis in the β-cell approximately 2-fold (7–9). This occurs through an increase in the activity of the general translation machinery, largely through protein phosphorylation regulation of eukaryotic initiation factor (eIF) activity (10). In β-cells, glucose stimulation of general translation has been shown to occur through the regulation of the activity of two basal translation factors, eIF-2B and PHAS-1/eIF-4E-BP (11, 12). However, glucose-stimulated proinsulin biosynthesis can increase as much as 30-fold in 1 h, implying a specific control mechanism above the general effect of glucose on total protein synthesis. We reveal in this study that such specific translational regulation of preproinsulin mRNA lies within the molecule itself.

Specific translational regulation of individual mRNAs can involve elements within their untranslated regions (UTRs). For example, the translation of the ferritin mRNA is regulated by intracellular iron levels acting through an element in the 5′-UTR (13), known as an iron response element, that includes a *cis*-acting stem-loop secondary structure (dG = −15.4 kcal/mole). This element up-regulates ferritin mRNA translation in response to increased cellular iron through an interaction with a *trans*-acting iron-binding protein. Iron response elements are evolutionarily conserved in ferritin H chain mRNAs from several mammalian species. Intriguingly, the preproinsulin mRNA also has evolutionarily conserved features. RNA structural analysis of the rat preproinsulin II mRNA predicts the presence of a stem-loop structure in the 5′-UTR (dG = −10.8 kcal/mole) that closely resembles structures predicted to form in the 5′-UTR of other mammalian preproinsulin mRNAs (14, 15). By comparative analysis of mammalian preproinsulin mRNA sequences we have also found the presence of a highly conserved 12-bp element within the 3′-UTR (Fig. 1) that lies between the polyadenylation signal (AAUAAA) and the polyadenylation site. Elements within the 3′-UTR of other mRNAs have been shown to be necessary for the regulation of mRNA localization, translation, polyadenylation, and stability (16). These observations are consistent with a role for the untranslated regions of the preproinsulin mRNA in regulating its spe-

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§ Present address: Dept. of Anatomy and Physiology, MSI/Wellcome Trust Bldg., University of Dundee, Dow St., Dundee DD1 5EH, Scotland.

‖ To whom correspondence should be addressed: Pacific Northwest Research Inst., 720 Broadway, Seattle, WA 98122. Tel.: 206-860-6777; Fax: 206-726-1202; E-mail: cjr@pnri.org.

[1] The abbreviations used are: SRP, signal recognition particle; eIF, eukaryotic (translation) initiation factor; UTR(s), untranslated regions; HisPI, 6 Histidine-tagged proinsulin; CMV, cytomegalovirus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PC, prohormone convertase; bp, base pair(s); PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

SPECIES	3′UTR SEQUENCE
rat II	aauaaaaccu**uugaa**agagc
rat I	aauaaagccu**uugaa**ugagc
mouse II	aauaaaaccu**uugaa**ugagc
mouse I	aauaaaacuu**uugaa**uaagc
human	aauaaagccc**uugaa**ccagc
chimp	aauaaagccc**uugaa**ccagc

FIG. 1. **Conserved features exist within the untranslated regions of the preproinsulin mRNA.** Analysis of the sequence of the untranslated regions of mammalian preproinsulin mRNAs indicates that certain features are conserved, including a primary sequence that was identified in the sequences of published mammalian preproinsulin mRNAs that lies between the polyadenylation signal and the site of cleavage and polyadenylation.

cific translation in response to glucose, which has been directly tested in this study.

MATERIALS AND METHODS

Virus Construction—Rat preproinsulin cDNA was cloned from isolated rat islets by RT-PCR, and the sequence was verified by DNA sequencing. A 6-histidine tag was inserted at the *Sma*I site that lies within the coding region, 213 bp downstream from the preproinsulin initiation codon, by the insertion of a hybridized oligonucleotide pair that give the sense sequence GCACCACCACCACCACCACGC. This histidine-tagged preproinsulin (named HisPI) coding region was used to make four gene constructs (Fig. 2). In the first of these (5HisPI3) the His-tagged preproinsulin coding region was flanked by the UTRs of the rat II preproinsulin mRNA. In the second construct (5HisPI) the 3′-UTR of 5HisPI3 was replaced with that of SV40, while retaining the preproinsulin mRNA 5′-UTR. In contrast, in the third construct (HisPI3) the preproinsulin 3′-UTR was retained, but the 5′-UTR was replaced with an artificial sequence (UGAAUACAAGCUCACGACCCACUACACAAGCTACCAGATACAACAACAAGCATCCACC) that was based upon a sequence that is predicted to have little secondary structure and that should act as a strong, unregulated 5′-UTR (17). Finally, both the 5′- and 3′-UTRs of the 5HisPI3 were replaced with the artificial 5′-UTR and the SV40 3′-UTR to create HisPI. These gene constructs were placed under the control of the cytomegalovirus (CMV) promoter and used to construct attenuated, recombinant adenoviruses. A luciferase-expressing adenovirus was generated using the firefly luciferase coding region from the plasmid pSP-luc+NF (Promega). These constructs were subcloned into pAC-CMV (27), between the *Sac*I site that lies in the proximal part of the CMV promoter, 12 bp from the transcription initiation site, and restriction endonuclease sites within the pAC-CMV multiple cloning site. Use of this *Sac*I site within the CMV promoter sequence allowed detailed positioning of the transcription initiation site to match that used in the endogenous preproinsulin mRNA.

Mapping the 5′ and 3′ Termini of mRNAs—The 5′-ends of the virally expressed RNAs were mapped by primer extension using poly(A)+ RNA purified from primary hepatocytes or the β-cell line, INS-1, that had been infected overnight with the virus of interest. Primer extension was carried out as previously described (28) using an oligonucleotide (AGGAAGCGGATCCACAGG) that hybridizes between nucleotides 6 and 23 of the preproinsulin coding region. The 3′-ends of the mRNAs were mapped by performing RT-PCR (Ready To Go kit; Amersham Pharmacia Biotech) upon total RNA isolated from islets infected with the HisPI3 adenovirus using the RNeasy kit (Qiagen). Fragments were digested by *Bam*HI and *Not*I, subcloned into pBluescript (Stratagene), and sequenced with the Sequenase kit (Amersham Pharmacia Biotech) using the universal primer.

Islet Isolation and Culturing—Pancreatic islets were isolated from 200–250-g male Sprague-Dawley rats as described previously (9). Islets were cultured overnight in RPMI 1640 with 5.6 mM glucose, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, either in the absence or the presence of adenovirus at ~10^{10} pfu/ml. Islets were then cultured in Krebs Ringer HEPES-buffered saline with 2.8 mM glucose for 60 min to bring biosynthesis to a basal level, and then at the desired glucose concentration for 40 min, before being labeled with 250 μCi/ml [^{35}S]methionine for 20 min in the same medium. Islets were collected by centrifugation at 500 × *g* for 2 min and lysed.

Hepatocyte Isolation and Culturing—Primary hepatocytes were isolated from 200–250-g male Sprague-Dawley rats according to previously published methods (29). Briefly, the liver was digested with 101 units/ml colagenase type 2 (Worthington Biochemical Corporation) for ~10 min. Digestion was halted with Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The hepatocytes were plated for 4 h in this medium after which adenovirus infection was carried out by including ~10^{10} pfu/ml adenovirus in the overnight culture medium (Dulbecco's modified Eagle's medium supplemented with 100 nM dexamethasome, 100 units/ml penicillin, and 100 μg/ml streptomycin). Subsequent experiments were conducted as described for islet isolation with insulin added where indicated at 100 nM. Lactate levels were assayed using a lactate kit (Sigma) used according to the manufacturers instructions.

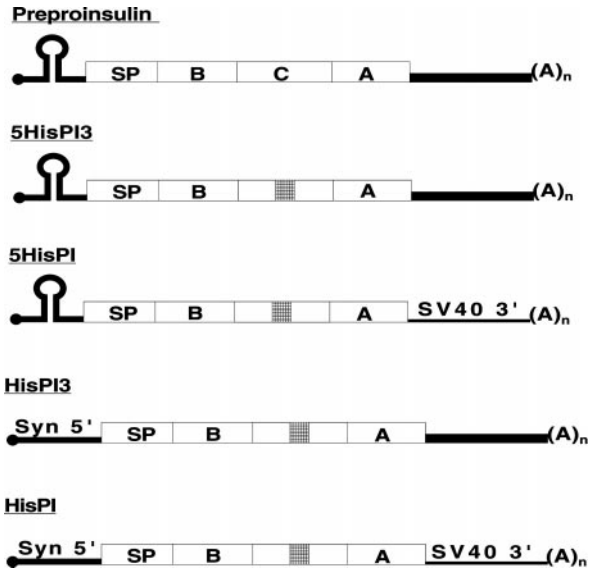

FIG. 2. **Gene constructs were designed to express a His-tagged proinsulin.** Four gene constructs were made in which the preproinsulin coding region (*boxes* are representative as follows: *SP*, signal peptide; *B*, B peptide of insulin; *C*, C peptide; *A*, A peptide) was tagged with 6 histidine residues (*hatched*), leading to production of HisPI. The untranslated regions of 5HisPI3 were replaced with a synthetic (*Syn*) 5′-UTR (HisPI3 and HisPI) and/or the SV40 3′-UTR (5HisPI and HisPI).

Immunoprecipitation, Nickel Affinity Purification, and Protein Gel Electrophoresis—Cultured islets and hepatocytes were either lysed in immunoprecipitation lysis buffer (50 mM HEPES, 0.1% Triton X-100, 1 μM phenylmethylsulfonyl fluoride, 1 μM E64, 1 μM pepstatin A, 1 μM 1-chloro-3-tosylamido-7-amino-2-heptanone, 1.0% sodium azide, pH 8.0) for immunoprecipitation by guinea pig anti-bovine insulin (Sigma) and rabbit anti-firefly luciferase (Cortex) antibodies as described previously (9) or were lysed in a denaturing nickel affinity lysis buffer containing 6 M guanidine hydrochloride, as recommended by Qiagen. Samples were briefly sonicated, and aliquots were taken for trichloroacetic acid precipitation. The 6-histidine-tagged proinsulin was purified from the lysate by addition of nickel-nitrilotriacetic acid-agarose (Qiagen) to the sample and incubation at room temperature for 2 h. The nickel-nitrilotriacetic acid-agarose was washed twice in native wash buffer and eluted in gel loading buffer (24% glycerol, 8% SDS, 10% β-mercaptoethanol, 0.4 M Tris, pH 6.8) containing 400 mM imidazole. Nickel affinity-purified histidine-tagged proinsulin and immunoprecipitated endogenous proinsulin were resolved by Tricine-SDS-polyacrylamide gel electrophoresis. Immunoprecipitated luciferase was resolved by glycine-SDS-polyacrylamide gel electrophoresis. Gels were fixed in 50% methanol/10% acetic acid, dried, and analyzed by phosphorimaging.

RNA Analysis—The RNA levels in islets and hepatocytes cultured as for protein analysis were analyzed by the RNase protection assay, using the Direct lysis kit (Ambion). RNA was protected using [^{32}P]uridine-labeled antisense RNA fragments corresponding to the coding region of the histidine-tagged preproinsulin or to part of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) coding region (Ambion). This yielded protected RNA fragments of 336 bp of the His-tagged preproinsulin-expressing mRNA, two fragments of 200 bp and 110 bp for the endogenous preproinsulin mRNA, and 318 bp of the GAPDH mRNA. Samples were resolved on denaturing 5% acrylamide/Tris-borate EDTA-buffered gels and analyzed by autoradiography and phosphorimaging.

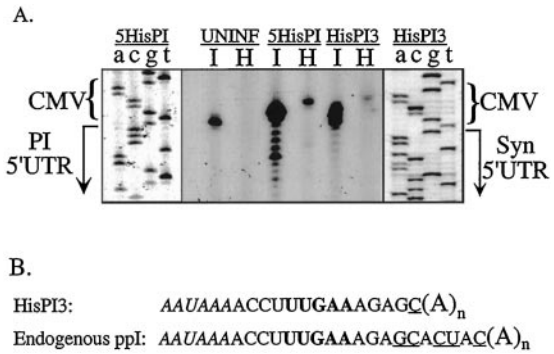

FIG. 3. **Mapping of the termini of the His-tagged proinsulin-expressing mRNAs revealed that they mimic those of the endogenous preproinsulin mRNA.** To determine that the translation of the His-tagged proinsulin-expressing mRNAs mirrored the endogenous preproinsulin mRNA as closely as possible, the termini were mapped. *A*, primer extension performed upon poly(A)$^+$ from the β-cell line, INS-1 (*I*), or primary hepatocytes (*H*), either uninfected (*UNINF*) or infected with adenoviruses expressing the 5HisPI or HisPI3 mRNAs. Polyacrylamide gels showing the primer extension productions are presented alongside sequencing reactions of 5HisPI and HisPI3 that used the same oligonucleotide primer. *CMV* indicates CMV promoter sequences, *PI 5′-UTR* indicates the preproinsulin mRNA 5′-UTR, and *Syn 5′-UTR* indicates the synthetic 5′-UTR. *B*, the 3′ termini of the endogenous preproinsulin (*ppI*) mRNA and the HisPI3 mRNA were mapped by RT-PCR, subcloning, and DNA sequencing. *Italics* indicate the polyadenylation signal (AAUAAA). *Bold* indicates the evolutionarily conserved sequence element. Bases found to immediately precede the poly(A)$^+$ tail are *underlined*. Five sites of polyadenylation were found in the endogenous message, one of which was used in the preproinsulin 3′-UTR of HisPI3.

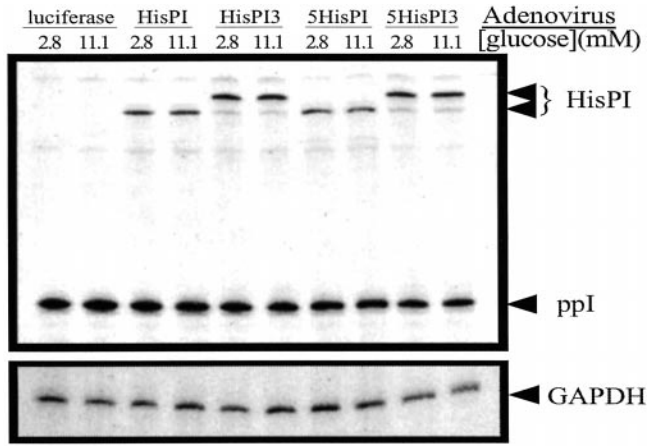

FIG. 4. **Measurement of RNA levels by RNase protection assay.** *Arrows* indicate the positions of HisPI-, endogenous proinsulin (*ppI*)-, and GAPDH-encoding mRNAs in pancreatic islets infected with the indicated adenovirus and cultured for 1 h at 2.8 or 11.1 mM glucose. The His-tagged proinsulin probe fragment protected the preproinsulin coding region and 3′-UTR, thereby protecting a larger fragment in mRNAs carrying the preproinsulin 3′-UTR (HisPI3 and 5HisPI3) compared with those carrying the SV40 3′-UTR (HisPI and 5HisPI).

RESULTS

Virus Construction and Evaluation—To examine the role of the untranslated regions of the rat preproinsulin II mRNA, a series of gene constructs were designed that would express mRNA molecules that mirror the endogenous rat preproinsulin II mRNA as closely as possible, except for defined alterations (Fig. 2). These mRNAs encode a His-tagged proinsulin flanked by the preproinsulin 5′- and 3′-UTRs (5HisPI3), the preproinsulin 5′-UTR and SV40 3′-UTR (5HisPI), the preproinsulin 3′-UTR and a synthetic 5′-UTR that is based upon a previously characterized sequence that is A/T-rich and predicted to have little secondary structure (HisPI3; see Ref. 17), or the synthetic 5′-UTR and the SV40 3′-UTR (HisPI). These gene constructs, under the control of the CMV promoter, were used to construct attenuated recombinant adenoviruses. In addition, a recombinant adenovirus expressing firefly luciferase was used as a control.

The termini of the RNAs expressed by these adenoviruses were mapped and compared with those of the endogenous preproinsulin mRNA. The 5′-end was mapped by primer extension using poly(A) RNA isolated from hepatocytes and the β-cell line, INS-1, infected with the adenoviruses expressing the His-tagged proinsulin (Fig. 3*a*). These results showed that the initiation of transcription of the endogenous preproinsulin mRNA in INS-1 cells occurred 55 bp from the initiation codon. Transcription of the preproinsulin 5′-UTR from the CMV promoter of the 5HisPI adenovirus was shown to occur 1 bp before this (56 bp from the initiation codon) in INS-1 cells and within 2 bp of this in primary hepatocytes. The synthetic 5′-UTR of HisPI3 was shown to be ~58 bp in length. The preproinsulin 5′-UTR of 5HisPI3 and the synthetic 5′-UTR of HisPI matched those of 5HisPI and HisPI3, respectively (data not shown). The 3′-end of the endogenous and virally expressed mRNAs were mapped by RT-PCR, subcloning, and sequencing of 3′-ends generated from RNA prepared from rat islets infected with the HisPI3 adenovirus (Fig. 3*b*). In the endogenous preproinsulin 3′-UTR five sites of polyadenylation were identified over a window of 7 bp, downstream of the conserved UUGAA sequence. Polyadenylation of the HisPI3 mRNA occurred at one of these sites and leaves intact the conserved primary sequence element (Fig. 1) that lies between the polyadenylation signal (AAUAAA) and the polyadenylation site. Thus, the preproinsulin 5′ and 3′ termini in the HisPI constructs mimic those found in the endogenous preproinsulin mRNA.

The Levels of the Endogenous and HisPI-expressing RNAs Do Not Respond to Glucose—Total RNA levels were analyzed by RNase protection assay, allowing assessment of both the endogenous and His-tagged preproinsulin mRNAs. RNA levels were measured relative to the endogenous GAPDH level, in uninfected islets or in islets infected with each of the four His-tagged proinsulin expressing viruses or a luciferase-expressing control virus (Fig. 4). The His-tagged preproinsulin-expressing mRNAs were present at levels below that of the endogenous preproinsulin mRNA but, nonetheless, were at equivalent levels relative to each other. Incubation of isolated islets at basal (2.8 mM) or stimulatory (11.1 mM) glucose for 1 h showed that neither endogenous nor the His-tagged preproinsulin mRNA levels responded to glucose, relative to the GAPDH mRNA, in accordance with previous observations (4, 18).

The 5′-UTR Is Necessary and Acts Cooperatively with the 3′-UTR to Specifically Regulate Preproinsulin mRNA Translation—The level of translation from the endogenous and His-tagged proinsulin-expressing mRNAs was examined in uninfected islets or islets infected with the luciferase or HisPI adenoviruses. Islets were incubated for 1 h over a range of glucose concentrations (2.8–16.7 mM) to determine the biosynthetic response of the endogenous and His-tagged proinsulins to a glucose stimulus (Fig. 5). At a stimulatory (11.1 mM) glucose concentration endogenous proinsulin biosynthesis was strongly stimulated relative to that at a basal (2.8 mM) glucose concentration (Fig. 5*a*), specifically increasing above total islet protein synthesis, which was increased ~2-fold. Biosynthesis of luciferase in islets expressing the control luciferase adenovirus was not regulated by glucose, despite a marked glucose-induced stimulation of endogenous proinsulin synthesis in the same islet β-cells (Fig. 5*a*). As such, regulation of proinsulin biosynthesis was unaffected by the recombinant adenovirus infection. Glucose dose-response analysis of the biosynthesis of

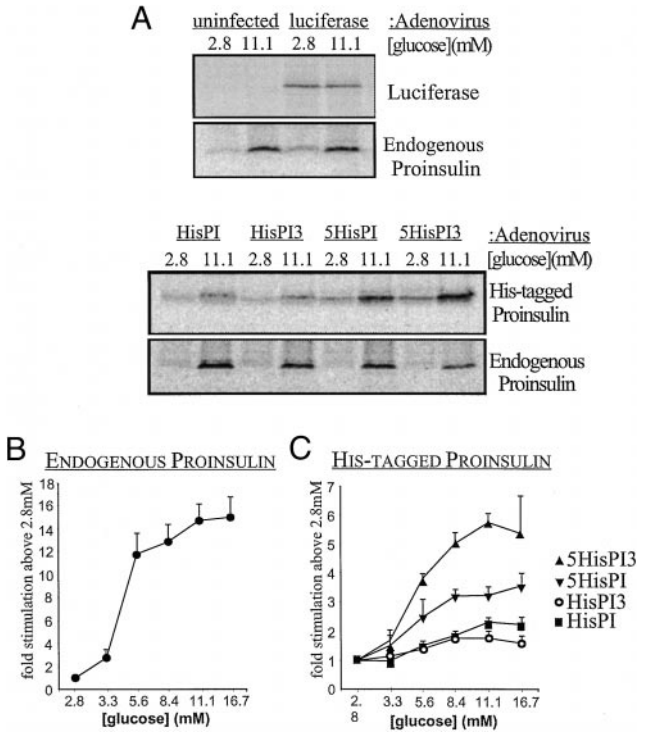

FIG. 5. **Translation of the endogenous and His-tagged preproinsulin mRNAs.** The translation of the endogenous preproinsulin mRNA and the virally encoded HisPI, HisPI3, 5HisPI, 5HisPI3, and luciferase mRNAs were measured. *A*, representative gels of [^{35}S]methionine-labeled endogenous and His-tagged proinsulin and luciferase from isolated rat islets cultured at 2.8 or 11.1 mM glucose. *Graphs* show the dose response to glucose of the translation of endogenous proinsulin (*B*) and His-tagged proinsulin (*C*) measured from islets cultured at a range of glucose concentrations (2.8–16.7 mM). Values are expressed as mean ± S.E. Endogenous proinsulin, $n = 5$; HisPI, $n = 4$; HisPI3, 5HisPI, and 5HisPI3, $n = 3$.

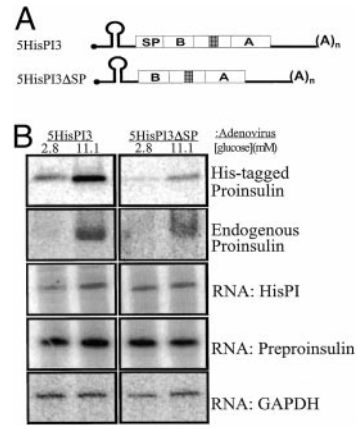

FIG. 6. **The role of the signal peptide in the translational regulation of preproinsulin.** *A*, an adenovirus with a new gene construct, 5HisPI3ΔSP, removed the signal peptide from the coding region of 5HisPI3. *SP*, signal peptide; *B*, B peptide of insulin; *A*, A peptide. *B*, protein synthesis (endogenous and His-tagged proinsulin) and RNA levels (endogenous and His-tagged proinsulin and GAPDH) examined in isolated pancreatic islets infected with the 5HisPI3 and 5HisPI3ΔSP adenoviruses were cultured at the indicated glucose concentrations.

the endogenous proinsulin outlined a threshold response to glucose at about 3 mM glucose, a marked increase between 3 and 8 mM glucose, and maximum stimulation was reached at 11 mM glucose, with proinsulin levels 14.7-fold (S.E. ± 1.4; $n = 5$) above those at 2.8 mM glucose, when corrected for effects of glucose upon general protein synthesis. These results are in accordance with previous reports (9, 19). The biosynthetic response to glucose for the His-tagged proinsulin was dependent upon the UTRs present on the adenovirally expressed mRNA (Fig. 5*a*). Translation of the HisPI mRNA, which lacks preproinsulin mRNA UTR sequences, was stimulated in response to 11.1 mM glucose 2.30-fold (S.E. ± 0.14; $n = 4$) above the level observed at 2.8 mM glucose (Fig. 5, *a* and *c*). In the presence of the 3′-UTR of the preproinsulin mRNA, a 1.76-fold (S.E. ± 0.23; $n = 3$) stimulation of the translation of the HisPI3 mRNA at 11.1 mM glucose (above that at basal, 2.8 mM, glucose) was modestly, yet significantly ($p < 0.05$), below the response observed with the HisPI mRNA. In contrast, the 5′-UTR of the preproinsulin mRNA was stimulatory, in that the translation of 5HisPI at 11.1 mM glucose was 3.22-fold (S.E. ± 0.28; $n = 3$) above the levels at 2.8 mM glucose and significantly above the response observed with HisPI and HisPI3 ($p < 0.05$; see Fig. 5*c*). However, the strongest translational response to glucose was seen with the 5HisPI3 mRNA, which was 5.73-fold (S.E. ± 0.32; $n = 3$) stimulated at 11.1 mM glucose above the level at 2.8 mM glucose and above the responses observed for the HisPI, HisPI3, and 5HisPI translation at 11.1 mM glucose ($p < 0.05$; Fig. 5*c*). This indicated that a cooperativity existed between sequences in the 5′- and 3′-UTRs for glucose-regulated preproinsulin mRNA translation. The glucose dose-response for 5HisPI3 mRNA translation (Fig. 5*c*) followed the same qualitative pattern as that for endogenous proinsulin biosynthesis (Fig. 5*b*), although the magnitude of the response was lower. However, it should be considered that adenovirus-mediated HisPI expression, as driven by the CMV-promoter, would result in constitutive His-tagged proinsulin expression in the non-β-cells of the islet. This, in turn, would obscure the glucose stimulation of the specific control of 5HisPI3 mRNA translation in glucose-sensitive islet β-cells. Indeed there is precedence for this, in that high constitutive expression of prohormone convertase (PC) 2 in the non-β-cells of the pancreatic islets masked the specific glucose-induced translational control of PC2 biosynthesis in the β-cells (20, 21).

The Signal Peptide Is Not Involved in the Specific Regulation of Preproinsulin Translation—A modest response to glucose of the translation of the HisPI mRNA suggested that sequences within the His-tagged preproinsulin coding region might influence translation. Therefore the potential role of the signal peptide-encoding region of the preproinsulin mRNA in glucose-induced translational regulation was examined. An additional recombinant adenovirus was generated in which the signal peptide of 5HisPI3 was removed (5HisPI3ΔSP; see Fig. 6*a*). In isolated pancreatic islets infected with recombinant adenovirus to express the 5HisPI3 or 5HisPI3ΔSP mRNAs, incubation for 1 h at basal (2.8 mM) or stimulatory (11.1 mM) glucose resulted in no change in 5HisPI3 or 5HisPI3ΔSP mRNA levels, in parallel with endogenous preproinsulin and GAPDH mRNAs (Fig. 6*b*). This was despite a marked glucose-induced increase in endogenous proinsulin biosynthesis in the same islets (Fig. 6*b*). Although removal of the signal peptide reduced the response to stimulatory (11.1 mM) glucose 1.44-fold (S.E. ± 0.19; $n = 4$), the translational response to glucose was nonetheless still evident (Fig. 6*b*). Thus, it appeared that specific translational regulation of proinsulin biosynthesis by glucose does not require the preproinsulin signal peptide region. However, 5HisPI3ΔSP mRNA translation was reduced at 11.1 mM glucose compared with that of 5HisPI3 mRNA, implicating a general effect imparted by the signal peptide on the synthesis of proteins destined to the β-cell secretory pathway. The presence of the signal peptide probably also accounted for a modest glucose effect on HisPI mRNA translation (Fig. 5, *a* and *c*).

Translational Regulation of the Proinsulin Biosynthesis Is Specific to Pancreatic β-cells—To examine whether the regulation of proinsulin biosynthesis was because of a β-cell-specific

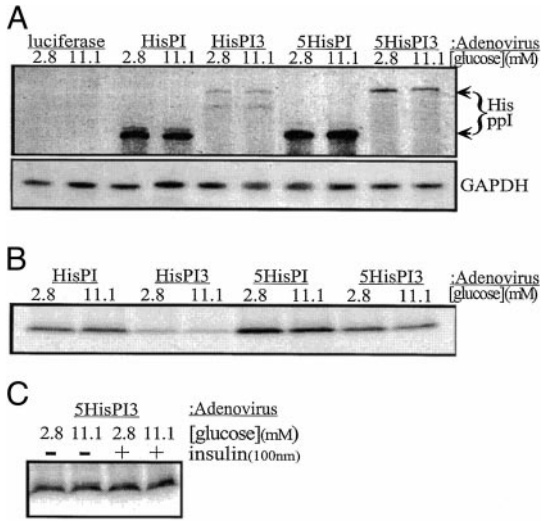

FIG. 7. **His-tagged proinsulin expression in primary hepatocytes.** Primary hepatocytes infected with the indicated adenovirus were cultured at 2.8 or 11.1 mM glucose. *A*, RNA levels of His-tagged proinsulin (*ppI*) were measured by RNase protection assay. *B*, [^{35}S]methionine-labeled His-tagged proinsulin levels from islets infected with the HisPI, HisPI3, 5HisPI, or 5HisPI3 adenoviruses and cultured at 2.8 or 11.1 mM glucose. *C*, [^{35}S]methionine-labeled His-tagged proinsulin levels from islets infected with the 5HisPI3 adenovirus cultured at 2.8 or 11.1 mM glucose in the absence or presence of 100 nM insulin.

mechanism, primary hepatocytes were infected with the His-tagged proinsulin-expressing adenoviruses. Primary hepatocytes were chosen as the liver is also a recognized glucose-sensing tissue. Analysis of the expression of the His-tagged preproinsulin mRNAs in primary hepatocytes showed no change in mRNA levels in response to glucose. However, an unexpected, yet intriguing observation of HisPI mRNA expression in primary hepatocytes was that the levels of the mRNAs carrying the rat preproinsulin 3′-UTR (HisPI3 and 5HisPI3) were much lower than those with the SV40 3′-UTR (HisPI and 5HisPI), despite equivalent GAPDH mRNA levels (Fig. 7*a*). This was in contrast to their expression in pancreatic islets, where the same titers of the purified recombinant adenoviruses gave similar levels for all four His-tagged proinsulin-expressing mRNAs (Fig. 4). Because the transcription of these mRNAs was regulated by the same CMV promoter sequences, it appeared that a pancreatic islet-specific mechanism exists for specific stabilization of the preproinsulin mRNA via an element within the 3′-UTR. The biosynthesis of the His-tagged proinsulin in hepatocytes tended to parallel HisPI mRNA expression levels. Therefore, although HisPI3 and 5HisPI3 biosynthesis was lower than that of HisPI and 5HisPI (Fig. 7*b*), no response was observed in His-tagged proinsulin biosynthesis to 11.1 mM glucose above that seen at 2.8 mM glucose from any of the virally expressed mRNAs (Fig. 7*b*). These hepatocytes were confirmed as glucose responsive in that, after a 6-h incubation at either 2.8 or 11.1 mM glucose, a significant ($p < 0.01$) increase in lactate output at 11.1 mM glucose (1.53 ± 0.09 mg/dl in the absence of insulin, 1.46 ± 0.53 in the presence of insulin) was observed compared with hepatocytes cultured at 2.8 mM glucose (0.45 ± 0.02 mg/dl in the absence of insulin, 0.42 ± 0.03 in the presence of insulin). Measurement of total protein synthesis in these primary hepatocytes showed that the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material was not stimulated in response to glucose, unlike isolated islets of Langerhans, where a stimulation of 2.23-fold (S.E. ± 0.14; $n = 26$) was observed. To stimulate total protein synthesis, primary hepatocytes were cultured in the presence or absence of 100 nM insulin, a known stimulator of general protein synthesis in hepatocytes (22). Indeed, total protein synthesis was stimulated by insulin (1.51-fold; S.E. ± 0.19; $n = 3$), but no specific stimulation of His-tagged proinsulin biosynthesis by insulin at either 2.8 or 11.1 mM glucose was observed (Fig. 7*c*). These results show that proinsulin translation was regulated by a β-cell-specific mechanism and reaffirmed that this was not because of a general glucose-sensing mechanism or an up-regulation of the general translation machinery.

DISCUSSION

Preproinsulin mRNA was one of the first mRNAs found to be specifically regulated at the translational level (4). However, over the subsequent 20 years the mechanism of this regulation has remained unknown. There are generic effects of glucose on up-regulating total protein synthesis in pancreatic islet β-cells, which in turn have a modest effect of increasing proinsulin synthesis. Glucose stimulates increases in general protein synthesis in pancreatic islet β-cells, most likely by the phosphorylation of certain general translation initiation factors (11, 12). Moreover, there is an additional general control mechanism applied to newly synthesized proteins destined for the β-cell secretory pathway, mediated via the nascent signal peptide, most likely through the signal peptide/SRP interaction and alleviation of SRP-mediated arrest of translation (5, 23). Both of these general mechanisms contribute to the up-regulation of proinsulin synthesis but are relatively minor and do not account for the specific nature of glucose-induced preproinsulin mRNA translation.

In this study, using a recombinant adenovirus-mediated expression of a His-tagged proinsulin reporter in isolated rat islets, we have revealed that the specific translational regulation of proinsulin biosynthesis by glucose was largely dependent upon elements that lie in the untranslated regions of preproinsulin mRNA. The 5′-UTR of preproinsulin mRNA encoded an element, most likely residing in a conserved stem-loop secondary structure (14, 15), that was necessary for the specific stimulation of proinsulin biosynthesis translation in response to glucose. The 3′-UTR of preproinsulin mRNA contained an element, most likely the conserved UUGAA sequence (Fig. 1), that had a tendency to suppress glucose-induced proinsulin biosynthesis but also to stabilize the preproinsulin mRNA in a pancreatic islet β-cell-specific manner. When expressed together, the 5′- and 3′-UTRs of preproinsulin mRNA acted synergistically to markedly stimulate the His-tagged proinsulin biosynthesis translation in a manner reminiscent of the endogenous proinsulin (Fig. 5). The finding of a cooperativity between the 5′- and 3′-UTRs of preproinsulin mRNA for translational control of proinsulin biosynthesis complements recent findings with the basal translational machinery that show an interaction between the cap binding complex subunit, eIF-4G, and the poly(A)-binding protein, PABP (24). Based on the findings in this study, we propose a model for the translation of the preproinsulin mRNA, illustrated in Fig. 8. Firstly, there are two generic effects; one is an effect of glucose on the β-cell translational machinery, mostly at the initiation phase (11, 12), resulting in an ~2-fold increase in total protein synthesis. The other is directed at a nascent signal peptide/SRP interaction (5–7), which is probably adaptive to an up-regulation in biosynthesis of proteins destined for the pancreatic β-cell secretory pathway, of which proinsulin is included. However, the major specific translational control of proinsulin biosynthesis by glucose is via the UTRs of preproinsulin mRNA. As the glucose concentration rises above 3 mM glucose, the 5′-UTR promotes a marked increase in translation of preproinsulin mRNA, and simultaneously there is an alleviation of suppressing preproinsulin mRNA translation mediated at the 3′-UTR,

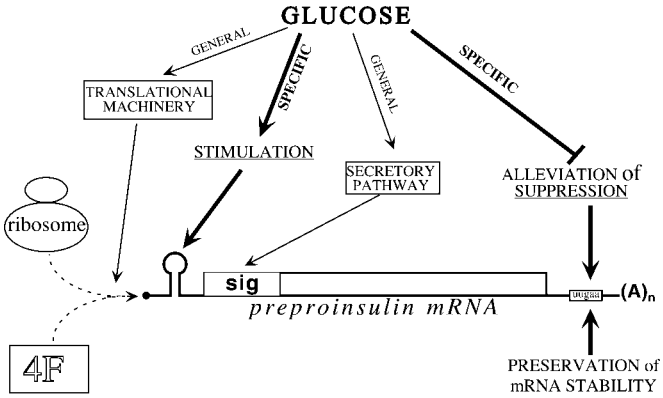

FIG. 8. **A model for the translational regulation of the preproinsulin mRNA in response to glucose.** Translation of the preproinsulin mRNA is regulated by glucose acting through general mechanisms (general translational factors and secretory pathway) and by specific mechanisms that require sequences in both the 5′- and 3′-UTRs. *4F* refers to the cap binding complex, eIF-4F. *sig* indicates the signal peptide encoded by the preproinsulin mRNA.

probably via an interaction with the 5′-UTR. Sequences in the 3′-UTR also specifically increase preproinsulin mRNA stability in β-cells, which will also contribute proinsulin biosynthesis at the translational level by preserving the amount of available preproinsulin mRNA template. Thus, there are multiple points for specific regulation of preproinsulin mRNA translation that, when operating coordinately, account for the fine control of proinsulin biosynthesis by glucose in pancreatic islet β-cells. It is likely that elements within the 5′- and 3′-UTRs of preproinsulin mRNA will interact with *trans*-acting factors, probably proteins, to confer their effects in glucose-regulated proinsulin biosynthesis translation and/or preproinsulin mRNA stability. Such *trans*-acting factors, and/or their regulation, are likely to be specific to the pancreatic β-cell, because the preproinsulin mRNA UTRs could not confer specific glucose-regulated translation of the preproinsulin mRNA in hepatocytes. Because glucose metabolism is required for glucose-induced proinsulin biosynthesis (1), one would predict that *trans*-acting factors should associate with the elements in the 5′- and 3′-UTRs of the preproinsulin mRNA in a manner that responds to secondary signals that arise from glucose metabolism and that these interactions should up-regulate preproinsulin mRNA translation. Future experimentation will be directed to identify such β-cell-specific preproinsulin mRNA-regulating *trans*-acting factors.

The untranslated regions of mammalian preproinsulin mRNAs contain conserved features that may be necessary for the regulation of proinsulin translation. Previous studies have shown that the translation of ~50 proteins in β-cells is stimulated greater than 10-fold in response to glucose (7). Intriguingly, a predicted stem-loop secondary structure in the 5′-UTR of the mRNA encoding two of these proteins, the prohormone convertases, PC2 and PC3, revealed a similar structure to that predicted to form in the 5′-UTR of the preproinsulin mRNA. It is likely that this secondary structure is involved in glucose-induced translational regulation of proPC2 and proPC3 biosynthesis in β-cells parallel to that of proinsulin (9, 20, 21). However, preproPC2 and preproPC3 mRNAs lack the conserved UUGAA element in the 3′-UTR that may explain their less robust translational response to glucose (15), as well as a shorter mRNA half-life relative to preproinsulin mRNA (25). Notwithstanding, it is quite likely that certain aspects of the mechanism for glucose-induced translation control of proinsulin biosynthesis unveiled in this study also apply to controlling the biosynthesis of other proteins destined for the insulin secretory granule compartment, particularly those that catalyze proinsulin sorting, processing, and regulated secretion (7). Moreover, the mechanisms that underlie nutrient-induced translational regulation of proinsulin biosynthesis are likely to be crucial in understanding wider aspects of β-cell physiology and metabolic homeostasis, because this is the major control of insulin production in mammals under normal physiological conditions (1–3, 18). Indeed, there is disregulation of proinsulin biosynthesis in an animal model of type II diabetes, which contributes to β-cell dysfunction and decreased availability of insulin (26). Finally, it is quite likely that the translational control mechanism for glucose-stimulated proinsulin biosynthesis in pancreatic β-cells may serve as a model for tightly regulating production of the major polypeptide product in other neuroendocrine cells. Neuroendocrine cells that produce a primary polypeptide product and store it intracellularly in secretory vesicles will have that store depleted by secretion of the polypeptide in response to an extracellular stimulus. Translational regulation of pre-existing mRNA of a polypeptide hormone/neurotransmitter by the same stimulus provides a means to rapidly and economically replenish intracellular stores lost by exocytosis and maintain them at optimal levels, as such preserving efficient secretory function of the neuroendocrine cell.

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Cooperativity between the Preproinsulin mRNA Untranslated Regions Is Necessary for Glucose-stimulated Translation

Barton Wicksteed, Terence P. Herbert, Cristina Alarcon, Melissa K. Lingohr, Larry G. Moss and Christopher J. Rhodes

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