0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(3):794–803

doi: 10.1210/me.2004-0119

Increased Insulin Translation from an Insulin Splice-Variant Overexpressed in Diabetes, Obesity, and Insulin Resistance

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Type 2 diabetes occurs when pancreatic β-cells become unable to compensate for the underlying insulin resistance. Insulin secretion requires β-cell insulin stores to be replenished by insulin biosynthesis, which is mainly regulated at the translational level. Such translational regulation often involves the 5′-untranslated region. Recently, we identified a human insulin splice-variant (SPV) altering only the 5′-untranslated region and conferring increased translation efficiency. We now describe a mouse SPV (mSPV) that is found in the cytoplasm and exhibits increased translation efficiency resulting in more normal (prepro)insulin protein per RNA. The RNA stability of mSPV is not increased, but the predicted secondary RNA structure is altered, which may facilitate translation. To determine the role of mSPV in insulin resistance and diabetes, mSPV expression was measured by quantitative real-time RT-PCR in islets from three diabetic and/or insulin-resistant, obese and nonobese, mouse models (BTBR*ob*/*ob*, C57BL/6*ob*/*ob*, and C57BL/6*azip*). Interestingly, mSPV expression was significantly higher in all diabetic/insulin-resistant mice compared with wild-type littermates and was dramatically induced in primary mouse islets incubated at high glucose. This raises the possibility that the mSPV may represent a compensatory β-cell mechanism to enhance insulin biosynthesis when insulin requirements are elevated by hyperglycemia/insulin resistance. (*Molecular Endocrinology* 19: 794–803, 2005)

TYPE 2 DIABETES ensues when peripheral insulin resistance can no longer be compensated by increased insulin secretion and biosynthesis. The ability of the pancreatic β-cell to increase insulin secretion requires continuous replenishment of the insulin stores by insulin biosynthesis. Previous studies have shown that glucose-induced insulin biosynthesis is mainly regulated at the translational level (1–5). However, our knowledge of the mechanisms involved in translational regulation of insulin biosynthesis is limited.

Translational regulation of a particular mRNA often involves the 5′-untranslated region (5′UTR) of the gene (6–9). The insulin gene consists of three exons and two introns, and the 5′UTR includes exon 1 and part of exon 2, because the coding region starts in exon 2. Both introns are flanked by canonical splice sites, but in addition, intron 1 was found to contain a cryptic 5′ splice site (10). Because this splice site lies within the 5′UTR region, alternative splicing using this site gives rise to a splice-variant (SPV; GenBank accession no. AY899304) without affecting the coding region. This SPV is expressed in normal human islets and demonstrates increased translation efficiency both *in vitro* and *in vivo* (10). In addition, glucose increases SPV expression in a time-dependent manner. This suggests that enhanced SPV expression may represent a compensatory mechanism in response to prolonged hyperglycemia or increased insulin requirements by generating more insulin protein from less mRNA in times of increased demand (10). We therefore hypothesized that SPV expression may be involved in β-cell adaptation to the increased insulin requirements of obesity, insulin resistance, and diabetes.

The goal of the present study was to test this hypothesis and to gain a better understanding of the role of this recently described insulin SPV. To this end we characterized a mouse SPV (mSPV; GenBank accession no. AY899305) and by analyzing its expression, structure, and translational efficiency found it to be functionally very similar to the human SPV. This enabled us to study insulin SPV expression in obese and nonobese mouse models of insulin resistance and diabetes.

First Published Online November 18, 2004

Abbreviations: mNAT, Mouse native insulin; mSPV, mouse insulin splice-variant; SPV, splice-variant; 5′UTR, 5′-untranslated region; WT, wild-type.

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

RESULTS

Expression of a mSPV Retaining Intron 1 in the 5′UTR

Similar to the human insulin gene, the mouse homologue (mouse insulin 2 gene) is also alternatively spliced, as demonstrated by RT-PCR using mouse insulin 2 cloning primers and cDNA derived from healthy mouse pancreata (Fig. 1B). Compared with the human SPV (10), the mSPV is significantly larger, and subcloning and sequencing revealed that the mSPV is generated by retention of the whole 102-bp intron (Fig. 1A), in contrast to the small 26-bp intronic sequence retained in humans. This suggests that although a putative 5′ splice site sequence (AGGT) is present in intron 1 (Fig. 1A), it is not used in mice. No other SPVs could be detected, and using nonselective primers outside any exon-exon or exon-intron boundaries, only native insulin (mNAT) and the described mSPV were amplified (Fig. 1C). The expression of the predicted mSPV was also confirmed by Northern blotting (Fig. 2). In addition, sequence analysis demonstrated that intron 2 is correctly spliced out and that this mSPV is derived from the mouse insulin 2, not insulin 1, gene (Fig. 1A).

Insulin SPV Is Translated More Efficiently into Native (Prepro)Insulin Protein

To compare the translation efficiency of the newly identified mSPV with mNAT, we used *in vitro* translation assays, as described in *Materials and Methods*. The results demonstrated that the SPV yielded 2.4-fold more (prepro)insulin protein than mNAT (Fig. 3, A and B). In addition, we generated reporter constructs to analyze the effects of the mSPV 5′UTR *in vivo*. In these transfection experiments, the presence of the 102-bp intronic sequence in the mSPV 5′UTR led to a small, but highly significant, increase in translation efficiency ($P = 0.0002$) compared with the mNAT 5′UTR and determined by luciferase activity (Fig. 3C).

Cytoplasmic Localization of the Insulin SPV

To address the question of whether, despite retention of intron 1, the mSPV is transported out of the nucleus into the cytoplasm and thereby becomes accessible for translation *in vivo*, we measured the mSPV/mNAT ratio by quantitative real-time RT-PCR in the cytoplasmic fraction of Min6 β-cells. Interestingly, we found an almost 2-fold higher mSPV/mNAT ratio in the cytoplasm (39 ± 3%; $P = 0.05$; Fig. 4A) compared with

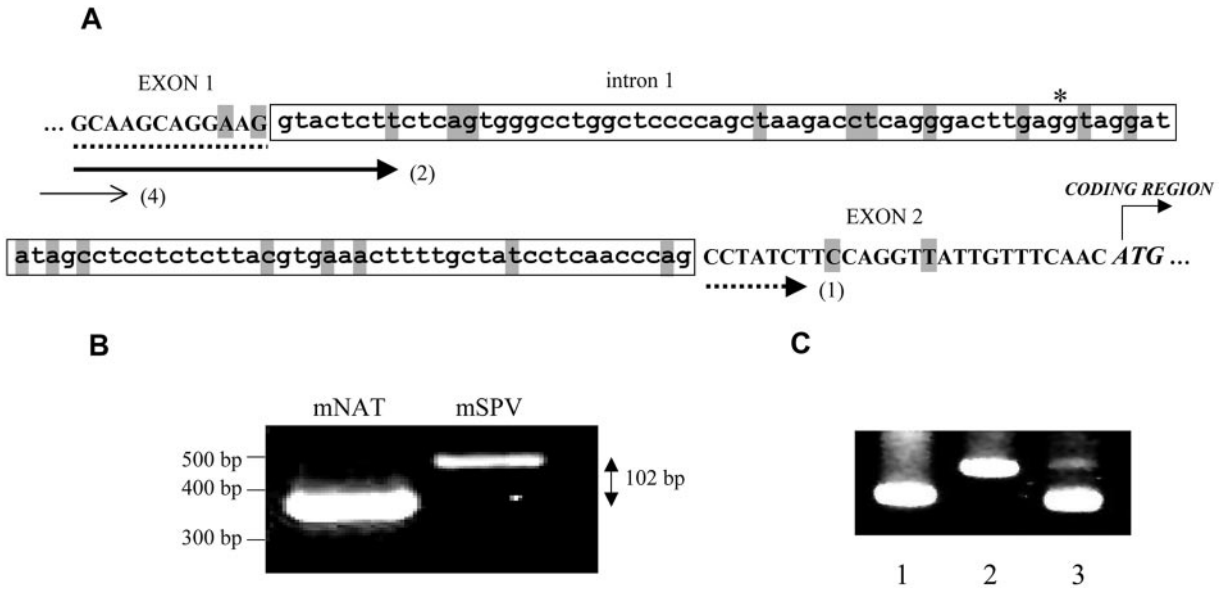

Fig. 1. Mouse (Prepro)Insulin 2 Gene SPV

A, Sequence of intron 1 retained in the 5′UTR of the mouse insulin 2 gene SPV. Exonic sequences are in *capital letters*, the 102 bp of intron 1 are framed, and the beginning of the coding region is symbolized by the ATG start codon. *Gray boxes* mark mouse insulin 2 gene-specific base pairs (distinct from insulin 1 gene). The position of the primers used for the RT-PCRs demonstrated in B and C are shown by *arrows*, and numbers in *brackets* correspond to the primer numbers of Table 1. The *asterisk* marks a potential AGGT splice site within intron 1. B, RT-PCR of mNAT and mSPV, using RNA of normal mouse pancreas as a template. The exon 1-exon 2-spanning primer (1) was used for mNAT, and the exon 1-intron 1-spanning primer (2) was used for mSPV; the 3′ primer (3) in exon 3 (not shown) was used for both reactions. Bands represent 10 μl PCR product run on an ethidium-stained 3% agarose gel. No additional bands were seen, and sequencing confirmed that the larger size of the mSPV was due to retention of the whole 102-bp intron 1. C, Simultaneous RT-PCR of mNAT and mSPV in the same reaction using the nonselective exon 1 5′ primer (4) (lane 3). As a comparison, mNAT (lane 1) and mSPV (lane 2) RT-PCR products generated as described in B are shown. The one larger and fainter band in lane 3 corresponds to the mSPV in lane 2. For all three reactions, RNA extracted from Min6 β-cells was used.

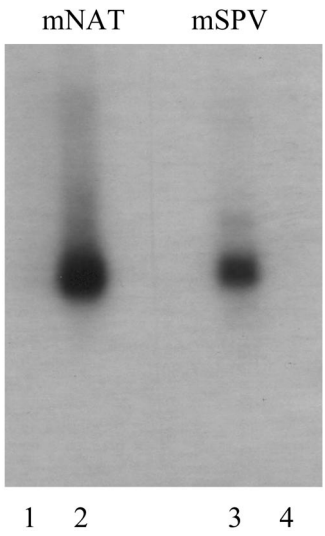

Fig. 2. Northern Blot of mSPV

Northern blotting was performed as described in *Materials and Methods*. RNA from Min6 β-cells was run in lanes 2 and 3, and lanes 1 and 4 represent negative controls using RNA from the 293 non-β-cell line; 20 μg total RNA were loaded per lane. Half the membrane was probed with the mNAT-specific probe spanning exon 1-exon 2, and half was probed with the mSPV-specific probe within intron 1. In lane 3, the slightly larger and fainter band compared with the band in lane 2 is consistent with the predicted mSPV RNA.

cDNA derived from Min6 whole cell extract (22 $\pm$ 5%) (11), demonstrating that the mSPV does reach the cytoplasm. Although these findings also suggest that the mSPV may even be transported preferentially out of the nucleus and could thereby enhance insulin translation and synthesis, additional studies are necessary to prove this hypothesis. [The overall higher mSPV/mNAT values in these insulinoma-derived Min6 cells compared with normal primary mouse islets are consistent with our previous finding of insulin SPV overexpression in insulinomas (11).]

RNA Stability of the Insulin SPV

To investigate whether increased RNA stability of the SPV was contributing to the increased amount of insulin protein generated, we analyzed the rate of RNA decay of both endogenous mNAT and SPV insulin mRNA using the mouse β-cell line, Min6. Although the mNAT mRNA was found to be quite stable, with 97 $\pm$ 7% remaining after 24 h of actinomycin D treatment, the SPV was rapidly degraded, dropping to 43 $\pm$ 12% after 1 h and to 30 $\pm$ 1% after 24 h (Fig. 4B). These findings make any contribution from RNA stability very unlikely and suggest that the major mechanism leading to more insulin synthesis from the SPV is protein translation. Because this increased translation efficiency occurred in wheat germ extract in the absence of any mammalian proteins, involvement of RNA-binding proteins seemed less likely, and *cis*-acting factors within the 5′UTR sequence of the SPV appeared to be the most plausible mechanism.

Increased Translation Efficiency Is Associated with Alterations In Secondary RNA Structure

A potent mechanism of translational regulation by *cis*-acting factors is based on the secondary RNA structure of the mRNA itself (6, 12). To assess the potential involvement of secondary RNA structure in the observed differences in translation efficiency, we compared the predicted secondary RNA structures of mNAT and mSPV using m-fold. We found that retention of intron 1 dramatically altered the structure compared with that of the native RNA (Fig. 5), which was paralleled by the described increase in translation efficiency (Fig. 3).

Expression of the Insulin SPV Is Increased in Diabetes and Insulin Resistance

Having identified this insulin mSPV and shown that it exerts increased translation efficiency, we wanted to study its roles in diabetes, obesity, and insulin resistance. We also tested for potential expression of an insulin 1 mSPV (m-1-SPV) retaining intron 1 using specific primers (Table 1), but found only an extremely low expression level, with an m-1-SPV/m-1-NAT ratio of 0.01% even in BTBR *ob*/*ob* mice (data not shown); therefore, we did not further pursue this m-1-SPV.

Focusing on mouse insulin 2, we analyzed mSPV expression in obese (*ob*/*ob*) and nonobese (*azip*) mouse models of diabetes and insulin resistance on different strain backgrounds (BTBR and C57BL/6) (13–16). Using specific primers for mNAT and mSPV, the mSPV/mNAT ratio was determined by quantitative RT-PCR in isolated islets of mutant/transgenic mice and compared with the ratio in wild-type (WT) littermates (Fig. 6). The mean mSPV/mNAT ratio in WT mice was 4.9 $\pm$ 0.7% (Table 2). (In contrast to the other control mice, the C57BL/6 *azip* controls were all females, which may explain the higher mSPV/mNAT ratio observed.) Interestingly, the mSPV/mNAT ratio was 3.7-fold elevated in diabetic BTBR *ob*/*ob* animals compared with lean WT controls (Fig. 6A). In the insulin-resistant, nondiabetic, C57BL/6 *ob*/*ob* and C57BL/6 *azip* mice significantly higher mSPV/mNAT values were found compared with healthy controls (Fig. 6, B and C).

These results suggest that although hyperglycemia is clearly contributing to mSPV overexpression, insulin resistance in the presence and absence of obesity also causes an increase in mSPV expression. Interestingly, despite some differences in the absolute mSPV/mNAT ratios in the different mouse models, overall insulin-resistant transgenic or mutant animals had a significantly higher mSPV/mNAT ratio than WT mice, as assessed by two-way ANOVA (Table 2).

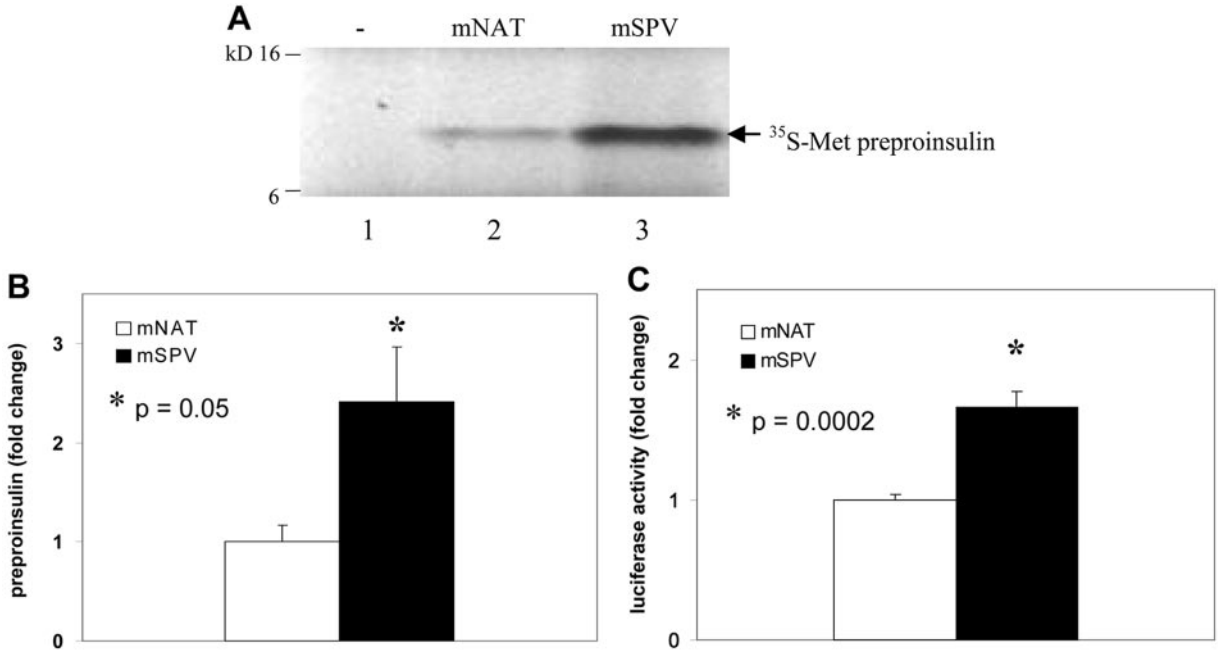

Fig. 3. Translation Efficiency of mSPV

A, Radiograph of *in vitro* translated [^{35}S]methionine-labeled (prepro)insulin using exactly the same amounts of RNA, mNAT (lane 2), and mSPV (lane 3) or no RNA (lane 1). The 12.5-μl translation reactions were incubated for 40 min at 30 C, and 5 μl were used for electrophoresis. B, Quantification of translation efficiency of mNAT (□), set at 1, and mSPV (■). *Bars* represent the mean fold change ± SEM of six independent experiments using *in vitro* transcribed RNA from two different batches. C, Transfection study assessing mNAT and mSPV translation efficiencies *in vivo*. The mNAT and mSPV 5′UTRs were cloned into the pGL3 control vector between the simian virus 40 promoter and luciferase reporter gene, yielding two constructs differing only in the absence or presence of the intronic 102 bp. Transfections were performed as described in *Materials and Methods*, and luciferase activity was assessed as a measure of translation efficiency. *Bars* represent fold changes in luciferase activity in cells transfected with the mSPV 5′UTR construct (■) compared with the mNAT 5′UTR (□). The mean ± SEM of two independent experiments performed in triplicate are shown.

Glucose Is Sufficient to Increase mSPV Expression in Primary Mouse Islets

To address the question of whether glucose alone could induce mSPV expression outside the context of any additional factors present in insulin-resistant or diabetic animals, we measured mSPV/mNAT ratios in primary mouse islets isolated from lean WT mice. Incubation of these islets at high glucose for 70 h led to a dramatic 15-fold increase in the mSPV/mNAT ratio (Fig. 7), suggesting that glucose is sufficient to induce mSPV expression in healthy mouse islets.

DISCUSSION

In the present study we demonstrate that islets of diabetic and insulin-resistant mice overexpress an insulin gene SPV that can be translated more efficiently into normal (prepro)insulin protein. Interestingly, we previously showed that normal human islets also express a similar insulin gene SPV conferring increased translation efficiency (10). Although we found that the expression of this human SPV was up-regulated by glucose in a time-dependent manner (10), its pathophysiological significance under conditions of altered glucose homeostasis remained unknown. We therefore used the mSPV described here to gain insight into the role of this newly identified phenomenon of alternative splicing of the insulin gene in diabetes, obesity, and insulin resistance.

The importance of alternative RNA processing in the endocrine system has recently began to emerge (17), and the fact that alternative splicing of the insulin gene is conserved between species suggests that it plays an important physiological role. Human and mouse insulin SPVs differ, in that in humans only the first 26 bp of intron 1 are retained, whereas the 5′UTR of the mSPV includes the whole intron 1 (Fig. 1). The first question was therefore whether this mSPV retaining all 102 bp of intron 1 would also confer increased translation efficiency in analogy to the human SPV (10).

We found high levels of mSPV in the cytoplasm of β-cells (Fig. 4A), confirming that the mSPV does not represent pre-mRNA trapped in the nucleus and suggesting that it contributes to insulin translation *in vivo*. Furthermore, our *in vitro* translation assays demonstrate that compared with mNAT mRNA, the mSPV is

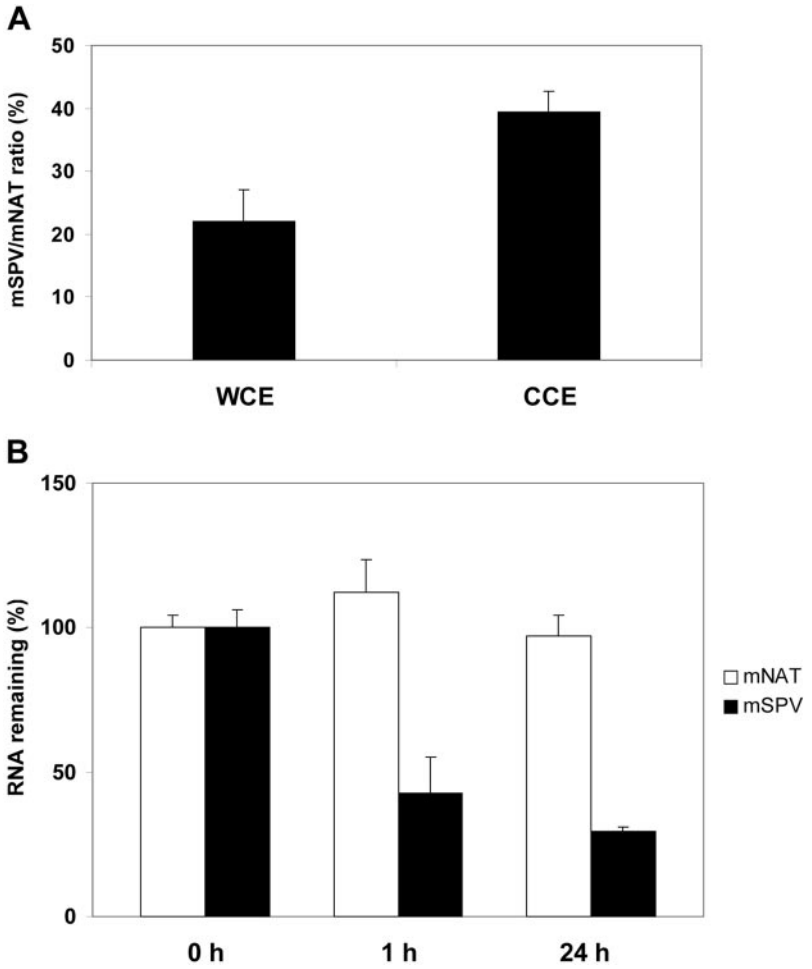

Fig. 4. Cytoplasmic Localization and RNA Stability of mSPV

A, mSPV expression in cytoplasm. Total RNA was extracted from Min6 β-cells, either straight from whole cell extract (WCE) or from the cytoplasm after cell fractionation (CCE) performed as described in *Materials and Methods*. mSPV and mNAT were measured by quantitative real-time RT-PCR. *Bars* represent the mean mSPV/mNAT ratios as a percentage ± SEM of triplicate measurements. B, RNA stability of mNAT and mSPV. Transcription in Min6 β-cells was blocked with actinomycin D (5 μg/ml) at 0 h, and the remaining endogenous mNAT (□) or mSPV RNA (■), as a measure of RNA stability, was quantified by real-time RT-PCR using the 18S ribosomal subunit as an internal standard. *Bars* represent the percentage of remaining RNA after 1 and 24 h of actinomycin D treatment compared with that at 0 h; the mean ± SEM of at least two independent experiments analyzed in triplicate are shown.

translated more efficiently into normal mouse (prepro)-insulin (Fig. 3, A and B). In addition, we show that the mSPV 5′UTR confers increased translation efficiency to a heterologous reporter gene in *in vivo* transfection studies (Fig. 3C). This suggests that the species difference lies solely at the sequence level and that functionally, in terms of increased translation efficiency, the two insulin SPVs are the same. Moreover, we found that translation efficiency correlated with changes in the predicted secondary RNA structure of the 5′UTRs (Fig. 5). This is again consistent with our findings in human islets (10) and studies of RNA structure affecting translation in other systems (6, 12) as well as with the hypothesis that the altered RNA structure may facilitate ribosomal binding and thereby enhance translation.

The discovery of this mSPV functioning similar to that of the human SPV provided a model to investigate the role of the insulin SPV in diabetes, obesity, and insulin resistance. Human studies are limited, because diabetic or insulin-resistant organ donors are typically excluded, and their islets are not isolated. In addition, severe confounding effects from premortem diabetes treatment and from the lengthy process of human islet isolation have to be expected if such islets are to be analyzed. To avoid these problems, we used three different mouse models to study the expression of the mSPV and now demonstrate that mSPV is significantly overexpressed in diabetes (BTBR *ob*/*ob*; Fig. 6A) and insulin resistance (C57BL/6 *ob*/*ob*; Fig. 6B) of obese mice as well as in nonobese, insulin-resistant mice (C57BL/6 *azip*; Fig. 6C). In addition to these single

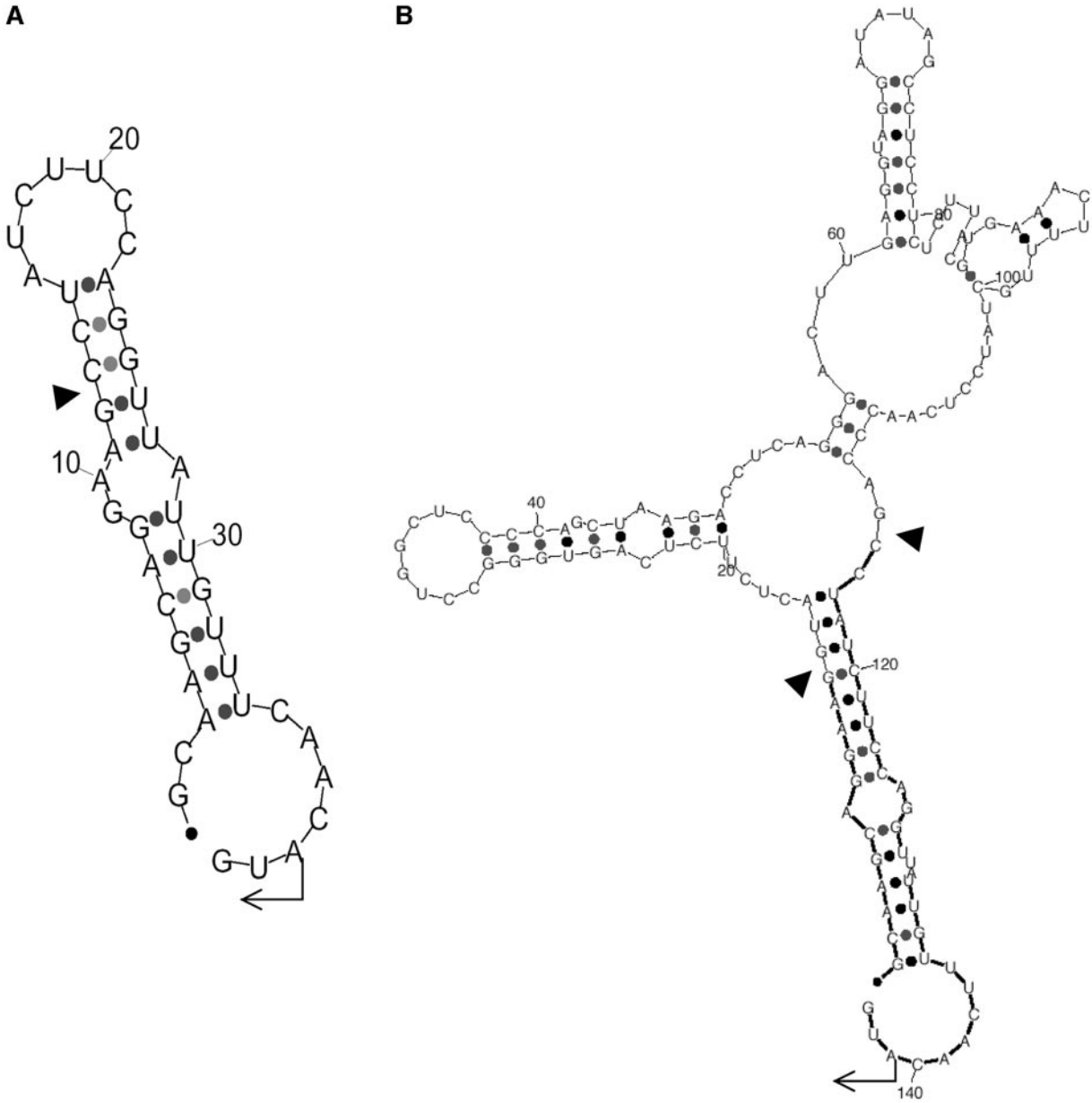

Fig. 5. Predicted Secondary RNA Structures of mNAT (A) and mSPV (B) 5′UTRs

Sequences were analyzed by m-fold as described in *Materials and Methods*. Calculated energies (dG) were −7 for mNAT and −26 for mSPV. *Open arrows* mark the AUG translational start sites. *Arrowheads* point to the sites of the spliced out intron (A) and the beginning and end of the retained intron 1 (B); the exonic sequence is shown with *bold lines*.

comparisons within each mouse model (Fig. 6) and despite some variability in absolute mSPV/mNAT numbers between them, taking all mouse models together revealed that overall, insulin-resistant mice have a significantly higher mSPV/mNAT ratio than WT controls (Table 2).

Because alternative splicing allows for more insulin biosynthesis (via increased translation efficiency of the mSPV) without the need for more transcription of the insulin gene, it is tempting to speculate that mSPV expression may represent a compensatory mechanism of the β-cell at times of increased insulin requirements, such as hyperglycemia and/or insulin resistance. Because there are currently no tools available to artificially overexpress the mSPV *in vivo* without otherwise altering the metabolism, we are unable to assess at this point whether mSPV expression is truly able to improve insulin production and glucose homeostasis in insulin-resistant or diabetic animals, and the compensatory mechanism discussed remains hypothetical. It also remains to be determined whether mSPV overexpression is reversible and whether it occurs in other forms of obesity, insulin resistance, or diabetes.

Table 1. Oligonucleotides Used in this Study

Sequence 5′-3′	Use (Position)
1. GCAAGCAGGAAGCCTATCT	mNAT 5′ cloning primer (exon 1-exon 2 spanning)
2. GCAAGCAGGAAGGTACTCTT	mSPV 5′ cloning primer (exon 1-intron 1 spanning)
3. GTGGGTCTAGTTGCAGTAGT	mNAT & mSPV 3′ cloning primer (exon 3)
4. AGCCCTAAGTGATCCGCTACAA	mNAT & mSPV 5′ taqman primer (exon 1)
5. ATCCACAGGGCCATGTTGAA	mNAT 3′ taqman primer (exon 2)
6. AGCAGGAAGCCTATCT	mNAT taqman probe (exon 1-exon 2 spanning)
7. TCAAGTCCCTGAGGTCTTAGCTG	mSPV taqman 3′ primer (intron 1)
8. CTCTTCTCAGTGGGCCT	mSPV taqman probe (intron 1)
9. AGCCCTAAGTGATCCGCTACAA	mNAT 5′ primer for Northern probe
10. ATCCACAGGGCCATGTTGAA	mNAT 3′ primer for Northern probe
11. GGTACTCTTCTCAGTGGGCCTG	mSPV 5′ primer for Northern probe
12. AGGCTGGGTTGAGGATAGCA	mSPV 3′ primer for Northern probe
13. CCCAAGCTTGCAAGCAGGAAGCCTATCT	5′UTR mNAT 5′ cloning primer for reporter construct
14. CCCAAGCTTGCAAGCAGGAAGGTACTCTT	5′UTR mSPV 5′ cloning primer for reporter construct
15. CCCAAGCTTGTTGAAACAATAACCTGGAAGATA	5′UTR mNAT & mSPV 3′ cloning primer for reporter construct

Nevertheless, to date our findings establish that β-cells of three distinct diabetic/insulin-resistant mouse models respond with increased mSPV expression. Although we cannot rule out the possibility that the genetic background or the leptin deficiency present in both *ob/ob* and *azip* mice may have contributed to the observed effects, our findings of induced SPV expression in isolated primary mouse islets incubated at high glucose concentration (Fig. 7) suggest that such factors are not required. Instead, these findings imply that elevated glucose levels are sufficient to induce SPV expression, which is consistent with our previous observation of increased human SPV in response to glucose (10). In contrast, insulin did not affect SPV expression in human islets (10), making hyperinsulinemia or an autocrine insulin effect very unlikely as the cause of increased mSPV expression in insulin-resistant mice.

To investigate the possibility that increased RNA stability of the mSPV may be involved in some of the observed findings, we measured the RNA decay of endogenous mouse mNAT and mSPV and found that mSPV RNA is degraded faster than mNAT (30% *vs.* 97% remaining after 24 h, respectively; Fig. 4B). Therefore, the increased amount of (prepro)insulin protein generated from mSPV seems to be due to increased translation efficiency and not to increased mSPV RNA stability. In fact, due to the rapid mSPV RNA degradation within 1 h (43% remaining), we may have underestimated the effect of mSPV on translation efficiency in our translation assays.

The reduced RNA stability of mSPV compared with mNAT also suggests that the observed increase in mSPV expression in diabetic and insulin-resistant mice (Fig. 6) results from enhanced alternative splicing of the insulin gene rather than selectively decreased degradation of mSPV RNA. Because the diabetes/insulin resistance of the studied mice is genetically determined, and no acute intervention was performed to induce mSPV expression in these animals, it seems reasonable to assume that the mSPV/mNAT measurements obtained take the relative mSPV instability into account and reflect a steady state.

Taken together, the results of these studies suggest a novel mechanism by which alternative splicing of the 5′UTR leads to enhanced translation and protein synthesis in response to *in vivo* changes in glucose homeostasis. This pathway confers metabolic control of protein synthesis without the need for increased transcription and thereby represents a powerful biological mechanism that may also regulate the production of other proteins. However, if not actively sought, it can easily remain unappreciated, because it involves only subtle changes in the noncoding region of the target gene.

Even more importantly, our findings of mSPV overexpression in diabetic and insulin-resistant mice conferring increased insulin translation provide new insight into the regulation of insulin biosynthesis as well as into β-cell biology in the face of insulin resistance and diabetes. Increased β-cell mass through replication and neogenesis has been known as the main compensatory mechanism of the β-cell in response to insulin resistance (18–20). Defects in this pathway and loss of functional β-cell mass by apoptosis are thought to underlie the progression to overt diabetes (20–22). These and other findings have made the importance of the β-cell in the development of type 2 diabetes more apparent over the last years (23–25). However, regulatory steps of insulin synthesis and compensatory mechanisms within the β-cell have remained largely unknown, and a better understanding of the processes involved is critical to improve diabetes treatment and identify novel therapeutic targets. The results presented here shed new light on these aspects as they raise the possibility that overexpression of the newly identified insulin SPV may represent an attempt of the β-cell to maintain adequate insulin biosynthesis (a prerequisite of insulin secretion) when insulin requirements are increased by obesity, insulin resistance, and diabetes.

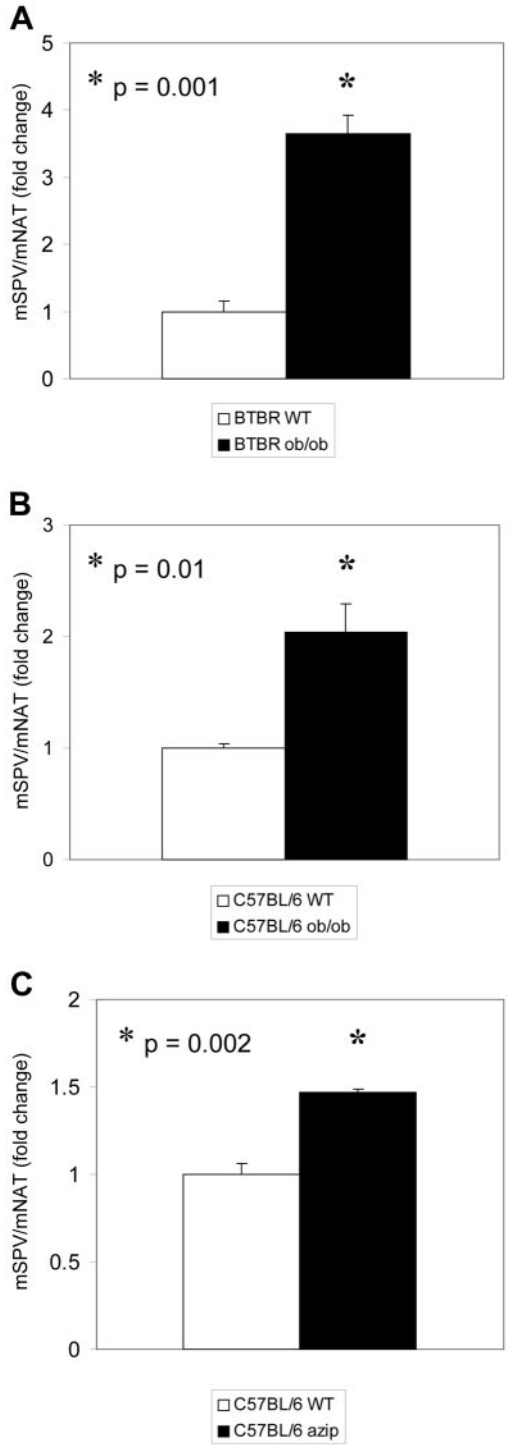

Fig. 6. mSPV Overexpression in Diabetic and Insulin-Resistant Mice

The mSPV, expressed as the mSPV to mNAT ratio, was measured by quantitative real-time RT-PCR in islets from A) obese, insulin-resistant, and diabetic BTBR *ob/ob* mice; B) obese and insulin-resistant C57BL/6 *ob/ob* mice; and C) nonobese, lipoatrophic, insulin-resistant C57BL/6 *azip* mice as described in *Materials and Methods*. *Bars* represent the fold change in the mSPV/mNAT ratio in transgenic mice compared with WT controls set at 1. Data are presented as the mean ± SEM of triplicate determinations.

MATERIALS AND METHODS

Cloning of the mSPV and Plasmid Construction

Pancreatic RNA was extracted from healthy BL/6 mice (gift from Dr. Klaus Pechhold, NIDDK, NIH) and reverse transcribed with the First Strand cDNA synthesis kit (Roche, Indianapolis, IN). A standard 35-cycle PCR was performed with the Elongase kit (Invitrogen Life Technologies, Inc., Carlsbad CA) using specific primers 1 and 3 for mNAT and primers 2 and 3 for mSPV (Table 1). The mSPV and mNAT PCR products, including the 5′UTR from position −38 with and without the 102 bp of intron 1, respectively, were cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen Life Technologies, Inc.) under the control of the T7 promoter. To generate reporter constructs, the mouse insulin 5′UTR was amplified from Min6 cDNA using the Elongase kit and primers 13 and 15 for the mNAT 5′UTR and primers 14 and 15 for the mSPV. Primers contained the *Hin*dIII restriction sites, and PCR-generated amplicons were ligated into the *Hin*dIII site of the pGL3 control vector (Promega Corp., Madison, WI) between the simian virus 40 promoter and the firefly luciferase reporter gene. All constructs were confirmed by sequencing.

Northern Blotting

Total RNA was isolated from Min6 β-cells and 293 cells using TRIzol (Invitrogen Life Technologies, Inc.) according to the manufacturer's instructions and run on a denaturing agarose gel containing 2% formaldehyde and 1× 4-morpholinepropanesulfonic acid. RNA was transferred to a nylon membrane in 10× standard saline citrate overnight and cross-linked to the membrane. Probes were generated by PCR using primers 9 and 10 for mNAT and primers 11 and 12 for mSPV. Products were purified by gel extraction and labeled with Redivue P-32 (Amersham Biosciences, Piscataway, NJ) using the Rediprime II Random Prime Labeling system (Amersham Biosciences). Membranes were incubated with the probes overnight at 65 C in a rotating incubator. After washing three times with decreasing concentrations of SCC (2× to 0.1×) with 0.1% sodium dodecyl sulfate, bands were visualized by radiography using BioMax light film (Eastman Kodak Co., Rochester, NY) and a 2-h exposure at −80 C.

In Vitro Translation Assays

Plasmid DNA was linearized with the restriction enzyme *Stu*I and transcribed using the RiboMAX large-scale RNA production system according to the manufacturer's instructions (Promega Corp.). Transcribed RNA was deoxyribonucleased and purified, and 1.5 μg uncapped RNA were added to a 12.5-μl *in vitro* translation reaction using the TNT wheat germ extract system (Promega Corp.) and ^{35}S-labeled methionine (Amersham Biosciences). Due to the absence of a ubiquitination system, the wheat germ extract is superior to the reticulocyte lysate system for the production of small proteins such as insulin. Samples were run on a 10–20% Tris-glycine gel, the *in vitro* translated ^{35}S-labeled preproinsulin was visualized by radiography, and the amount of translated protein was quantified with ImageQuant 5.2 (Amersham Biosciences).

Transfection Studies

293 Cells were plated in 12-well plates, grown overnight to 60% confluence, and transfected with the reporter constructs containing the 5′UTR of mNAT or mSPV (0.5 μg DNA/well) using FuGene 6 (Roche) according to the manufacturer's instructions. To control for transfection efficiency, cells were cotransfected with 5 ng/well pRL-thymidine kinase (Promega Corp.) control plasmid expressing the *Renilla* lucif-

Table 2. mSPV/mNAT Ratios in the Three Mouse Models Studied (Absolute Numbers)

Mouse Strains	Phenotype	Gender	mSPV/mNAT (%)[a]	±SEM
WT controls				
BTBR	Lean	Male	3.3	± 0.5
C57BL/6	Lean	Male	3.6	± 0.2
C57BL/6	Lean	Female	7.8	± 0.5
Mean			4.9	± 0.7
Insulin-resistant mice				
BTBR *ob/ob*	Obese, insulin resistant, and diabetic	Male	12	± 0.9
C57BL/6 *ob/ob*	Obese and insulin resistant	Male	7.4	± 0.9
C57BL/6 *azip*	Lipodystrophic and insulin resistant	Female	11.4	± 0.1
Mean			10.3[b]	± 0.8

[a] *Numbers* represent means of triplicate measurements.
[b] $P < 0.001$ (insulin-resistant *vs.* WT mice), calculated by two-way ANOVA.

erase reporter gene. Three hours after transfection, medium was changed to DMEM containing 25 mM glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin. Cells were harvested 24 h after transfection, and firefly and Renilla luciferase activities were quantified with the Dual Luciferase Assay Kit (Promega Corp.).

Cell Fractionation

To determine whether the mSPV is properly transported out of the nucleus and accessible for ribosome binding and translation in the cytoplasm *in vivo*, we obtained cytoplasmic fractions by performing cell fractionation as described previously (26, 27) using the mouse β-cell line, Min6. Cells were grown in high glucose DMEM (Invitrogen Life Technologies) with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.05 mM β-mercaptoethanol. Cells were trypsinized, washed, and resuspended in 2 ml ice-cold lysis buffer containing 50 mM HEPES, 3 mM $MgCl_2$, 0.3 M sucrose, and 10 μl RNasin. Cells were disrupted by passing them through a 20-gauge needle, and cell extract was centrifuged at 4 C for 1 h at approximately 100,000 × *g*. The supernatant representing the cytoplasmic fraction was used for RNA extraction with TRIzol reagent according to the manufacturer's instructions.

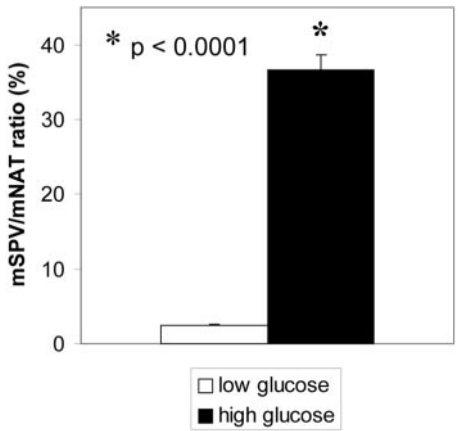

Fig. 7. Glucose Induction of mSPV in Primary Mouse Islets

Islets were isolated from six WT C57BL/6 mice and incubated at low (2.5 mM) or high (25 mM) glucose for 70 h before total RNA was extracted. The expression of mNAT and mSPV was measured by quantitative real-time RT-PCR. *Bars* represent mSPV/mNAT ratios (mean ± SEM of triplicate determinations) at low (□) and high (■) glucose concentrations.

RNA Stability Assay

To assess and compare the stabilities of the two mRNA species, the amounts of endogenous mNAT and mSPV mRNA were quantified after overall transcription was blocked by actinomycin D in Min6 β-cells. Cells were grown to 70–80% confluence as described above, the medium was changed, and 18 h later, cells were treated with actinomycin D (5 μg/ml). RNA was extracted at different time points using TRIzol reagent, and samples were analyzed for mNAT or mSPV by quantitative real-time RT-PCR using primers 4 and 5 and probe 6 or primers 4 and 7 and probe 8 (Table 1), respectively. The 18S ribosomal subunit was used as an internal standard, as described previously (10).

Secondary RNA Structure

The predicted secondary RNA structures were analyzed using the Michael Zucker algorithm and the m-fold program as described previously (28, 29).

Mouse Models and Islet Isolation

To study the expression of the mSPV in diabetes and insulin resistance, the islets of three mouse models were studied. BTBR *ob/ob* mice are severely insulin resistant and overtly diabetic (13), and C57BL/6 *ob/ob* mice are insulin resistant, but not diabetic (13, 15). As a nonobese model of insulin resistance, A-ZIP/F_1 lipoatrophic mice on the C57BL/6 background (C57BL/6 *azip*) were used (14, 16). All mouse studies were performed in accordance with the guidelines for the use and care of laboratory animals of University of Wisconsin or the NIH. Mouse islets from 6- to 14-wk-old diabetic and/or insulin-resistant mice as well as from the corresponding WT littermates were isolated and hand-picked as described previously (13, 30). All *ob/ob* mice and their controls were males, all *azip* mice and their littermates were females; islets from this latter group were a gift from Drs. O. Gavrilova and C. Vinson, NIH. Islets from three to six mice were pooled per sample. Total RNA was extracted from mouse islets with TRIzol reagent according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR

RT was performed with the First Strand cDNA synthesis kit (Roche), and quantitative real-time PCR was performed on a PRISM 7000 TaqMan apparatus (Applied Biosystems, Foster City, CA). Primers 4 and 5 and probe 6 (Table 1) specifically detected mNAT, whereas primers 4 and 7 and probe 8 (Table 1) selectively measured mSPV. For analysis of the mouse insulin 1 gene, primers 9–12 (Table 1) were used. All measurements were performed in triplicate.

Statistical Analysis

Data are presented as the mean ± SEM. Unless stated otherwise, *P* values were calculated using two-tailed *t* tests.

Acknowledgments

Received March 19, 2004. Accepted November 9, 2004.

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This work was supported by Grant 7-03-JF-37 from the American Diabetes Association (to A.S.) and National Institute of Diabetes and Digestive and Kidney Diseases Grant 58037-01 (to A.D.A.).

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