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Research Article

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Adaptation to Supraphysiologic Levels of Insulin Gene Expression in Transgenic Mice: Evidence for the Importance of Posttranscriptional Regulation

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Abstract

Insulin production was studied in transgenic mice expressing the human insulin gene under the control of its own promoter. Glucose homeostasis during a 48-h fast was similar in control and transgenic mice, with comparable levels of serum immunoreactive insulin. Northern blot and primer extension analyses indicated that more than twice as much insulin mRNA is present in pancreata from transgenic mice. Primer extension analysis using oligonucleotides specific for mouse insulins I and II or for human insulin, showed that the excess insulin mRNA was due solely to expression of the foreign, human insulin gene. The ratio of mRNA for mouse insulin I and II was unaffected by coexpression of human insulin. There were coordinate changes in the levels of all three mRNA during the 48-h fast, or after a 24-h fast followed by 24-h refeed. Despite the supraphysiologic levels of insulin mRNA in the transgenic mice, their pancreatic content of immunoreactive insulin was not significantly different from controls. The comparison of the relative levels of human and mouse insulin mRNAs with their peptide counterparts (separated by HPLC) indicates that the efficiency of insulin production from mouse insulin mRNA is greater than that from human, stressing the importance of posttranscriptional regulatory events in the overall maintenance of pancreatic insulin content. (*J. Clin. Invest.* 1993. 92:272–280.) Key words: transgenic mice • insulin • transcription • fasting

Introduction

Insulin production by the pancreatic B cell is regulated at the levels of transcription, translation, posttranslational processing and trafficking, and secretion (1–3). A number of physiological and pharmacological mediators affect such production. The best studied and certainly the dominant physiological signal is glucose (4), which, aside from its well-documented ability to stimulate insulin release (5), stimulates insulin mRNA translation (6) and stabilizes insulin mRNA (7), as well as possibly playing a role in the regulation of transcription of the insulin gene (8, 9). The modulation by physiological mediators of the various steps in the cascade of events implicated in insulin output from the pancreas results in the maintenance of a physiologic "set point" for insulin production. This set point is reflected in the pancreatic insulin content and circulating insulin levels encountered in given mammalian species and will be altered in response to a change in diet or physical activity, and in certain disease states including, most notably, diabetes.

Relatively little is known concerning the mechanism by which this set point for insulin production is established. Mice present a useful model for understanding the regulation of insulin production since, like rats (10–16), they possess two nonallelic insulin genes (insulin I and II) (17–19). In order to study further the relative contribution of transcriptional and posttranscriptional events in the establishment of the insulin set point, we have now examined this process in transgenic mice in which the human insulin gene is expressed in pancreatic B cells under the control of its own promoter (20). These mice are known to express significant levels of human insulin in a tissue-specific and regulated fashion (20). We have measured the relative levels of human and mouse insulin mRNAs and peptides in transgenic mice and compared them with those for mouse controls. The total insulin mRNA was more than twofold higher in the transgenic mice. Approximately one half of this insulin mRNA was derived from transcription of the human gene, yet, strikingly, total (mouse plus human) serum insulin and pancreatic insulin content were similar in transgenic and control animals. HPLC analysis of pancreatic insulin revealed the expected ratio of mouse insulin I:II in control mice, with no change in this ratio in transgenic animals. Human insulin accounted for some 28% of total insulin in the pancreas of the transgenic mice. Taken together, these findings strongly suggest that the establishment of a physiological set point for insulin production is dominated by posttranscriptional regulatory events.

Methods

Breeding and care of mice. Transgenic mice expressing the human insulin gene under the control of its own promoter were generated as described in detail by us previously (20). Mice were maintained ad libitum (unless stated otherwise for fasting and refeeding) on Prolab Rat, Mouse and Hamster 3000 (Agway, Waverly, NY). Positive male transgenic mice were mated with control females. To identify mice carrying the human insulin gene, small tail segments were taken, and total genomic DNA was purified and screened by Southern blot hybridization on nitrocellulose using standard procedures (20).

Serum insulin and glucose. Mice were bled periorbitally after ether anesthetization under the appropriate physiological regimen. Glucose assays were performed as recommended by the manufacturer of the assay kit (Sigma Immunochemicals, St. Louis, MO). Insulin was measured using a ^{125}I-insulin double-antibody radioimmunoassay kit according the manufacturer's instructions (Diagnostic Products Corp. Los Angeles, CA).

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Molecular biology

Materials. α-[^{32}P]dATP was purchased from New England Nuclear (Boston, MA); γ-[^{32}P]ATP was from ICN Radiochemicals, Div. ICN Biomedicals Inc. (Irvine, CA). All solutions were prepared in double-distilled water treated with 0.1% diethylpyrocarbonate (Sigma Immunochemicals). Restriction enzymes, avian myeloblastosis virus (AMV) reverse transcriptase, Klenow, and T4 polynucleotide kinase were purchased from Bio-Rad Laboratories (Hercules, CA), New England Biolabs, Inc, (Beverly, MA) Bethesda Research Laboratories, (Gaithersburg, MD), and Boehringer Mannheim Corp (Indianapolis, IN) and used under conditions recommended by the manufacturers.

Preparation of RNA. After the mice had been bled, they were killed by cervical dislocation. Pancreata were dissected and the tissue snap frozen in liquid nitrogen. Total cellular RNA was extracted and purified from the tissue samples, using the guanidinium isothiocyanite procedure as described (21). The purity of the RNA was assessed by determining the $A_{260/280}$ ratio (> 1.8) and by visualizing the ribosomal RNA after agarose gel electrophoresis with ethidium bromide.

Northern blot hybridization. Total insulin RNA was quantified using Northern blot hybridization as described (22). RNA samples (10 μg) were treated with formaldehyde (1.2 M formaldehyde, 50% formamide in Mops buffer) at 65°C for 15 min, and electrophoresed on horizontal 1% agarose gels containing 1.2 M formaldehyde (23). Additional samples were simultaneously electrophoresed and removed for staining with ethidium bromide. The RNA samples were then transferred overnight to nylon membrane (Biodyne; ICN Radiochemicals) via capillary action (20× SSC [3.0 M NaCl, 0.3 M Na citrate, pH 7.0]), and UV crosslinked according to the manufacturer's instructions (Stratagene, La Jolla, CA). For probing, the blots were prehybridized overnight at 42°C (50% formamide, 6× SSC, 5× Denhardt's solution [0.1% polyvinylpyrolidone, 0.1% BSA, 0.1% Ficoll], 0.1% SDS, 1 mg/ml denatured salmon sperm DNA, 50 mM $NaPO_4$, pH 6.5). The following day, the solution was replaced with hybridization buffer (prehybridization buffer containing 10% dextran sulfate) and 5×10^5 cpm/ml of the radioactive probe. The probe was generated by random-primer synthesis (Boehringer Mannheim) of a 1.1 kb AvaI fragment containing the human genomic insulin gene purified from plasmid pHINT 5 as previously described (20). Hybridization was carried out for at least 16 h at 42°C. The filters were washed (twice with 1× SSC, 0.1% SDS at room temperature for 15 min; once each with 0.1× SSC, 0.1% SDS at 37°C for 15 min; 0.1× SSC, 0.1% SDS at 55°C for 30 min), dried, and the total amount of insulin mRNA from each of the samples was directly quantified using a β-ray emission counter (Betascope 603; Betagen Inc., Framingham, MA).

Primer extension assays for quantification of insulin-specific RNA. Primer extension assays were used to quantify each of the insulin mRNAs as described (24–26). Each oligonucleotide (oligo) is listed in Table I and the corresponding region of the insulin gene depicted in Fig. 1. The oligos were synthesized on an Applied Systems (Mentor, OH) machine using phosporamidite chemistry and gel purified. After we determined the concentration, each oligo was optimized to yield saturation by T4 polynucleotide kinase and for primer extension reactions. Each oligo was tested for its specificity, using standard sequencing reactions. When the oligos were used in combination, there was no detectable interference or reduction of the primer-extended products. Briefly, 10 μg of total cellular RNA was resuspended in 5 μl of reverse transcriptase buffer (50 mM Tris-HCl, 60 mM NaCl, 10 mM dithiothreitol, pH 8.5) and hybridized with 1 pmol of primer that had been 5′-end labeled by treatment with polynucleotide kinase and γ-[^{32}P]-ATP at a specific radioactivity of ~ 5 μCi/pmol. The primer was extended using the AMV reverse transcriptase (2 U, Bio-Rad Laboratories), and the samples were subsequently electrophoresed on 6.0% acrylamide, 7.0 M urea sequencing gels. The gels were transferred to 3MM filter paper (Whatman, Inc., Clifton, NJ), dried, and the samples finally quantified directly using the Betascope 603 counter.

Biochemistry

Acid ethanol extraction of pancreas. Extraction was performed according to the method of Curry (27) with slight modifications. Pieces of pancreas (stored at −70°C) were weighed (~ 0.2–0.4 g wet wt), suspended in 4 ml acid ethanol (1:3 vol/vol of 0.7 M HCl/ethanol) containing 0.1% BSA and, after being cut into small fragments, homogenized on ice, with 4 × 10-s bursts with 1 min interval between each burst, using a Polytron Tissue Grinder (Kinematica, Lucerne, Switzerland). The blades of the grinder were rinsed with 1 ml acid ethanol/BSA and the pooled suspension incubated at 4°C overnight with gentle mixing. The homogenates were then centrifuged (3,000 *g*, 20 min, 4°C) and the supernatants set aside on ice. The pellets were resuspended in 4 ml acid ethanol/BSA and treated as above, but with only a 2-h incubation period at 4°C. This was repeated once. The three supernatants (total volume 15 ml) were pooled and centrifuged at 21,700 *g* for 25 min at 4°C to pellet all debris. The supernatant was split into 3 × 5 ml and transferred to a Speedvac Concentrator (Savant Instruments, Farmingdale, NY) and ethanol evaporated by connection to a water pump for 5 h.

After evaporation of ethanol, the volume of the extracts was adjusted to 7 ml with water, and the pH raised to 9.0 with 10 M NaOH. Half of each extract was then loaded onto C18 Sep-Pak cartridges (Waters Associates, Milford, MA) preactivated with 5 ml acetonitrile

Table I. Oligonucleotides Used for Primer Extension Analaysis

Oligo	Specificity	Sequence	Product length	Comparative sequences
			(bp)	
HINS	Human (H)	GCCATGGCAGAAGGACAGTG	63	MI: GCCATGTTGAAACAG(10/20) MII: GCCATGTTGAAACAA(10/20)
MINS1	Mouse I (MI)	GGGTAGGAAGTGCACCA	89	MII: GGGCAGGAAGCGCATCC(12/17) H: GGGCAGGAGGCGCATCC(12/17)
MINS2	Mouse II (MII)	GTGGGACTCCCAGAGGAAG	122	MI: TTTGGGCTCCCAGAGGGCA(13/19) H: GTCAGGTCCCCAGAGGGCC(11/19)
MINS3	MI, MII	CAGGGCCATGTTGAAACAAT	71	H: CAGGGCCATGGCAGAAGGA(13/20)
INS	H, MI, MII	TGGGTGTGTAGAAGAAGCC	215	100% homologous to all genes

This table represents each of the oligonucleotides used for primer extension (5′ to 3′), with the specificity, expected length of the primer extension product, and the comparative sequences for the other insulin genes. The numbers in the parentheses indicate the number of matching nucleotides of the oligo to the compared sequence. The region of the insulin gene corresponding to each oligonucleotide is shown in Fig. 1.

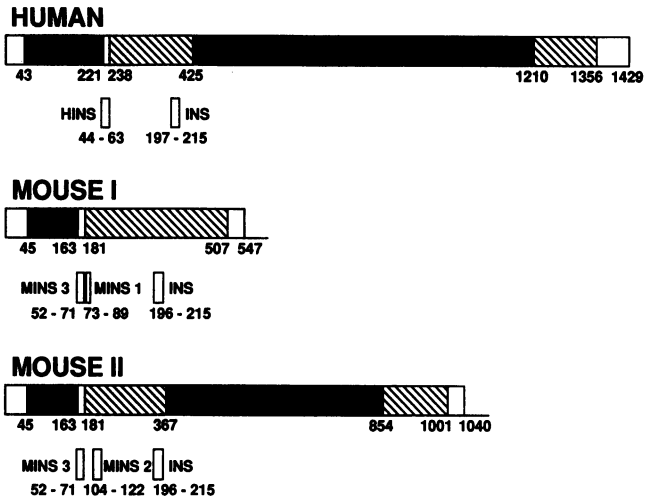

Figure 1. Line diagram of insulin genes with oligonucleotide hybridization sites. The three insulin genes are shown proportionately. Solid regions represent introns, the hatched regions represent coding sequences, and the white regions represent untranslated mRNA. The specific oligonucleotides utilized for primer extension analyses (for sequences see Table I) are shown beneath each gene. The numbering indicates the region of specificity on the mRNA transcript.

and 5 ml H_2O. Salts were removed by passing 5 ml 0.1% trifluoroacetic acid (TFA) through the cartridges, small hydrophilic peptides eluted with 5 ml 20% acetonitrile in 0.1% TFA and insulin finally eluted with 3 ml 45% acetonitrile in 0.1% TFA into a tube containing 100 μl 0.3% BSA. After evaporating acetonitrile (Speedvac connected to a water pump) the samples were frozen and then lyophilized. The dried product was redissolved in 250 μl 0.1% TFA and centrifuged at 17,800 *g* for 10 min to remove any particulate material that would otherwise have interferred with the subsequent analysis by HPLC. The supernatants were kept at −70°C pending analysis.

To attempt to quantify the extraction and Sep-Pak purification procedures, and in particular to ensure that there was no selective loss of just one of the three insulin species under study, the following control experiments were performed. To control for the efficiency of extraction of insulin from the pancreas, an aliquot of human ^{125}I-insulin mixed with ^{3}H-rat insulin I + II (prepared by biosynthetic labeling of rat islets followed by HPLC purification of the labeled insulin) was added to the acid ethanol before grinding the tissue. Recovery of all three radioactive insulin species was > 90%, with no detectable degradation as assessed by HPLC. It must be stressed, however, that this is not an ideal control experiment, since the exogenous, labeled, insulins cannot be added to the inside of the cells to be extracted. It can however be assumed that extraction of insulin from cells per se is essentially quantitative, with any potential losses due to degradation or adsorption in subsequent steps. The results are thus comforting in that they do not show any selective loss of just one of the three labeled insulin species.

Quantification of yield from the Sep-Pak purification step was achieved by loading pancreas extracts containing known amounts of immunoreactive insulin, and monitoring the recovery by immunoassay. For control mice, the yield was 86.8±6.6% (n = 27) and for transgenic mice 101±13% (n = 20). On occasion, there was an unexpectedly low yield (~ 20%) from a single Sep-Pak cartridge, reflecting the known intralot variability of these cartridges. Even when this occurred, there was no selective loss of just one insulin species, since if a second aliquot of the same extract was passed over a new Sep-Pak cartridge, and the HPLC profiles of immunoreactivity then compared for the two preparations, there was no change in the relative amounts of the three insulins.

Radioimmunoassay. Insulin was measured by radioimmunoassay using the charcoal-dextran separation method (28). Human and rat insulin standards (rat I and II insulin having the same amino acid sequence as their murine counterparts) were obtained from Novo-Nordisk (Bagsvaerd, Denmark). To confirm the precision of the insulin standards, both were subjected to partial amino acid sequencing (10 cycles) to establish their purity, followed by quantitative amino acid analysis (in triplicate) to allow for precise quantification independent of weight, immunoreactivity, or ultraviolet absorbance. Guinea pig anti-porcine insulin was the generous gift of Dr. Gordon Weir (Joslin Diabetes Center, Boston, MA). ^{125}I-porcine insulin was used as the tracer (Sorin Biomedica, Saluggia, Italy). When fractions eluting from HPLC were assayed using a dilution of less than 1:10, the standard curve was compensated by an appropriate volume of HPLC eluant handled in parallel with fractions eluting from the HPLC column. Since mouse and human insulins are recognized with different efficiency in the immunoassay, when the total insulin content of pancreas from transgenic mice was to be measured, a standard curve consisting of 25% human and 75% mouse insulin was used. For estimating the relative amounts of human or mouse insulins eluting from HPLC, fractions eluting with the mouse insulin peaks were measured using a mouse standard curve, and those eluting with human insulin using a human standard curve.

The pancreas extracts, both before and after Sep-Pak purification, clearly contained many products unrelated to insulin, which may have interfered in the radioimmunoassay. Samples were therefore subjected to serial dilution, and the immunoreactivity at each dilution measured. The samples were in all cases found to dilute in parallel with the insulin standard, thereby confirming that there was no nonspecific interference in the assay.

HPLC analysis of pancreas extracts. Separation of human insulin, and of the two mouse insulins, was achieved using a modification of our previously published methods (16, 19, 29, 30). In brief, an Ultrasphere 5-μm ODS column (4.6 × 250 mm) (Beckman Instruments, Fullerton, CA) was used attached to a Beckman System Gold HPLC System. Buffer A was TEAP (20 mM triethylamine, 50 mM phosphoric acid, 50 mM sodium perchlorate, pH 3.0) and buffer B 90% acetonitrile/10% H_2O. Insulins were eluted using a linear gradient of 32 to 35% buffer B over 80 min with a flow rate of 1 ml/min. Fractions of 0.5 ml were collected as from 35 min. Each fraction tube contained 50 μl 0.5-M borate, 1% BSA (RIA Grade, Sigma Immunochemicals, St. Louis, MO). The comparison of the amount of insulin eluting from HPLC with that injected confirmed our previous observations of quantitative recovery using similar HPLC systems (16, 19, 29, 30).

Results

Serum insulin and glucose levels during fasting. Of the many physiologic stimuli that affect levels of insulin production, we elected to characterize changes in insulin expression that occur during fasting (31). In particular, we wished to compare the effects of fasting on control mice with two expressed insulin species (mouse I and II), with those on transgenic mice with three expressed insulins (mouse I, mouse II, and human). Previous studies (20) revealed no demonstrable metabolic differences between these transgenic mice and controls, although detailed experiments characterizing the animals' responses to fasting had not been performed. Accordingly, we first looked at serum glucose and total insulin levels in the mice during a 48-h fast.

There was no apparent difference in the glucose or insulin values measured for control or transgenic mice, suggesting that glucose homeostasis in the transgenic mice was maintained despite the presence of the human insulin gene. During the

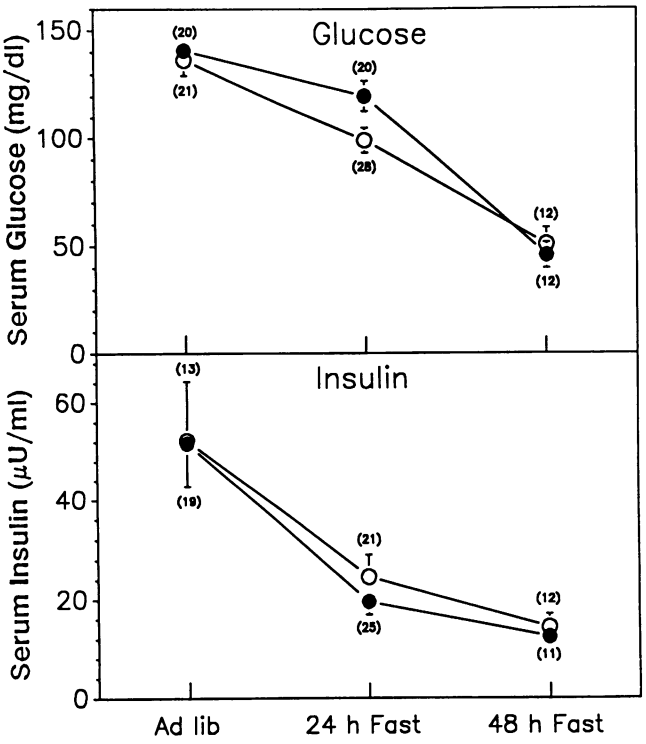

Figure 2. Serum glucose (*top*) and immunoreactive insulin (*bottom*) in transgenic and control mice during 48-h fast. Data are presented as mean±SEM with the number of mice in parentheses. Units for immunoreactive insulin, International Units, 1 U, 25 mg. O ---- O, control mice. ● ---- ●, transgenic mice.

48-h fasting period, levels of serum glucose (Fig. 2, *top*) in control mice fell by ~ 63% (from 136±7 to 51±8 mg/dl) and in transgenic mice by some 67% (from 141±4 to 46±6 mg/dl). Levels of serum immunoreactive insulin fell in parallel by 73% (from 52±12 to 14±3 μIU/ml) in control animals and by 77% in transgenic mice (from 52±9 to 12±2 μIU/ml). The absolute levels of serum insulin are somewhat elevated in both groups of mice. This most probably reflects mild stress due to ether anesthesia.

Relative amounts of mouse insulins I and II and of human insulin in the pancreas of control and transgenic mice. Previous characterization of the transgenic mice indicated that human insulin mRNA was present in islets and that human C-peptide (presumably derived from correctly processed human proinsulin) was secreted into the circulation (20). Having established that serum insulin levels in the transgenic mice (derived from the mouse I, mouse II, and human insulin genes) were almost identical to those in controls (derived from the two mouse insulin genes alone), it would have been interesting to be able to establish the contribution of each individual insulin peptide to the total insulin immunoreactivity measured in the serum. This would, however, have demanded accumulating serum from a prohibitive number of mice in order to have measurable amounts of insulin eluting in fractions from HPLC. We therefore elected to determine the relative contribution of human, mouse I, and mouse II insulin to pancreatic insulin content in the two groups of mice.

Pancreas extracts were prepurified and concentrated by passage through Sep-Pak cartridges before injection onto the HPLC system. Each fraction eluting from HPLC was subjected to radioimmunoassay using rat insulin standard for fractions eluting with mouse insulins, and human insulin standard for those eluting with human insulin. Note that rat and mouse insulins I and II have identical sequences and that insulins I and II display identical cross-reactivity in the radioimmunoassay. Representative elution profiles are shown in Fig. 3. Only two immunoreactive peaks were seen for control pancreas (*top*), eluting as mouse insulins I and II, with a third, minor peak eluting earlier than the two insulins. This material has been identified as mouse insulin II oxidized at methionine B29 (29), and for the purposes of calculating the relative amounts of the various insulin species is considered as mouse insulin II. As expected for the pancreas of a mouse (15, 19), there was more than twice as much insulin II than I.

The elution profile for an extract of transgenic mouse pancreas differed by the striking appearance of material coeluting with human insulin (Fig. 3, *bottom*). The elution profiles were remarkably constant from one HPLC run to the next, thereby allowing for full separation and quantification of all the insulin species from every sample injected to HPLC. Thus, for the 20 samples of transgenic mouse pancreas (all samples presented in Table II) the elution time for mouse insulin II was 54.2±0.9 min (mean±SEM). The elution times of mouse insulin I, human insulin, and oxidized mouse insulin II relative to that of mouse insulin II (R_f: mean±SEM) were 0.75±0.005, 0.48±0.006, and 0.24±0.01, respectively. In all cases the immunoreactivity returned to baseline levels between each of the identified peaks. The profile in Fig. 3 is thus truly representative.

In transgenic animals fed ad libitum, human insulin accounted for 28.2% of pancreatic insulin stores. There was no change in the ratio of mouse insulin I:II due to the expression of the human insulin gene (Table II). Thus, in transgenic mice, mouse insulin II accounted for 50% of immunoreactivity with the remainder divided almost equally between human insulin and mouse insulin I, and in both control and transgenic mice, the ratio of mouse insulin I:II was ~ 1:2.3. These relative amounts of pancreatic human, mouse I, and mouse II insulins were found to be unchanged during fasting and refeeding (Table II).

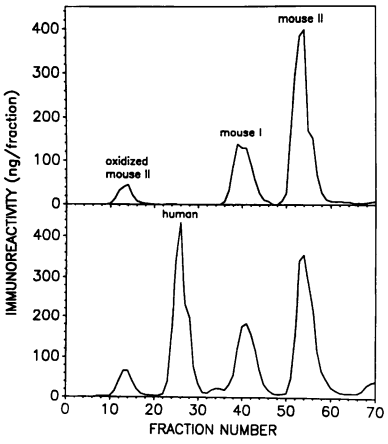

Figure 3. Analysis of pancreas extracts by reverse phase HPLC. Pancreas from a control (*top*) or transgenic (*bottom*) mouse fed ad libitum was extracted in acid ethanol and prepurified by Sep-Pak before injection onto HPLC. Fractions eluting from HPLC were subjected to radioimmunoassay using the appropriate standards for each peak (rat insulin for all mouse-related products and human insulin for the human insulin peak). The elution time of each product was established by injection of authentic standards.

Table II. Total (Mouse I + II + Human) Immunoreactive Insulin (IRI) Content of Control and Transgenic Mouse Pancreas and Percentage of Mouse I/II or Human Insulin Analyzed by HPLC

				Mouse I	Human
			Total IRI	Mouse I + II	Total
		n	*μg/g*	%	
Ad lib	Control	9	275±41	31±5	—
	Transgenic	5	256±23	30±3	28±3
24-h fast	Control	7	307±71	29±4	—
	Transgenic	5	324±110	32±2	31±7
48 h	Control	6	196±19	29±4	—
	Transgenic	4	253±71	34±4	31±3
24-h fast + 24-h refeed	Control	5	240±24	28±4	—
	Transgenic	6	327±72	32±2	30±4

Insulin was extracted in acid ethanol. Total IRI for controls was measured using a rat insulin standard (rat and mouse insulin being identical in sequence) whereas for transgenic mice the standard was composed of 25% human/75% rat insulin (since human and rat insulin do not cross-react in a parallel manner in the radioimmunoassay). The percentage of the individual insulin species was based on HPLC analysis (see legend to Fig. 3). The results are presented as the mean±SEM.

The total immunoreactive insulin measured in pancreas extracts of control and transgenic mice was not found to be markedly changed during the fasting period (Table II). There was, however, considerable interanimal variability that might have masked any minor changes in pancreatic insulin content upon fasting. Of greater significance was the finding that the immunoreactive insulin content of the pancreas of transgenic mice was not significantly different from that of the controls. For measuring the immunoreactive insulin content of the control mice pancreas, a standard consisting of a mixture of rat insulin I and II was used. Cross-reactivity in the radioimmunoassay for human insulin is, however, higher than for mouse (rat) insulin. For measuring insulin in the pancreas of transgenic mice, it was therefore necessary to use a standard consisting of 75% rat insulins mixed with 25% human insulin (this being close to the mean percentage of human insulin in the pancreas of transgenic mice, as shown above).

Quantification of total insulin mRNA by Northern blot analysis. The data showing similar levels of insulin in the circulation and in the pancreas of control and transgenic mice under all conditions studied suggested that similar levels of insulin mRNA would also be found in the pancreas of the two groups of mice. Surprisingly, such was not the case. When RNA purified from pancreas was quantified using Northern blot analysis, there was approximately two to three times more total insulin mRNA (mouse plus human) in the transgenic than in the control mice (Fig. 4).

Primer extension analysis

Total insulin mRNA. Since the apparent elevation of insulin mRNA in the transgenic mice pancreas seen by Northern hybridization analysis may have been artifactual due to an enhanced specificity of the probe for human rather than mouse insulin mRNA, primer extension analysis was performed to confirm and extend this finding. After comparison to the entire GenBank library to assure minimal homology to extrinsic nucleic acid sequences, INS, an oligonucleotide specific for all three insulin mRNAs, was generated (Fig. 1 and Table I). The oligonucleotide was 5′ radiolabeled, incubated with total pancreatic cellular RNA, and primer extended using reverse transcriptase. The primer extension products were separated on denaturing urea polyacrylamide gels and quantified directly with a β-ray emission counter.

Using the INS oligonucleotide for primer extension analysis of pancreatic RNA confirmed the results obtained by Northern blot hybridization, showing an ~ 2.2-fold increase in total insulin mRNA in the transgenic mice (Fig. 5; Table III). Control experiments using RNA extracted from tissue other than pancreas revealed no detectable insulin mRNA signal.

As expected from previous studies in the rat (31), there was a progressive decrease in insulin mRNA levels during the 48-h fast, with a pronounced rebound during the 24-h refeeding period following a 24-h fast (Table III).

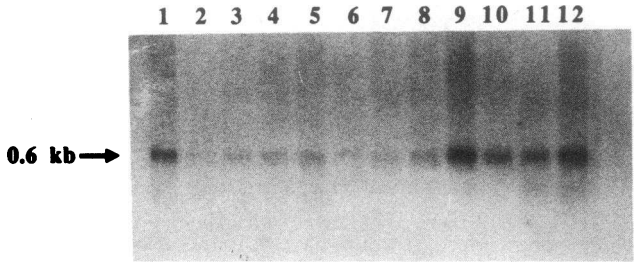

Figure 4. Northern hybridization analysis. Control and transgenic mice were sacrificed after feeding ad libitum. The samples were subjected to electrophoresis on 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized against a random-primer synthesized probe derived from human insulin cDNA. Control mice (lanes *1–6*); transgenic mice (lanes *7–12*). 10 μg total pancreatic RNA was loaded onto each lane. The arrow indicates the expected size for fully processed human, mouse I, or mouse II mRNA. Ethidium bromide staining of a duplicate gel suggested approximately equal amounts of RNA were loaded in each lane (data not shown).

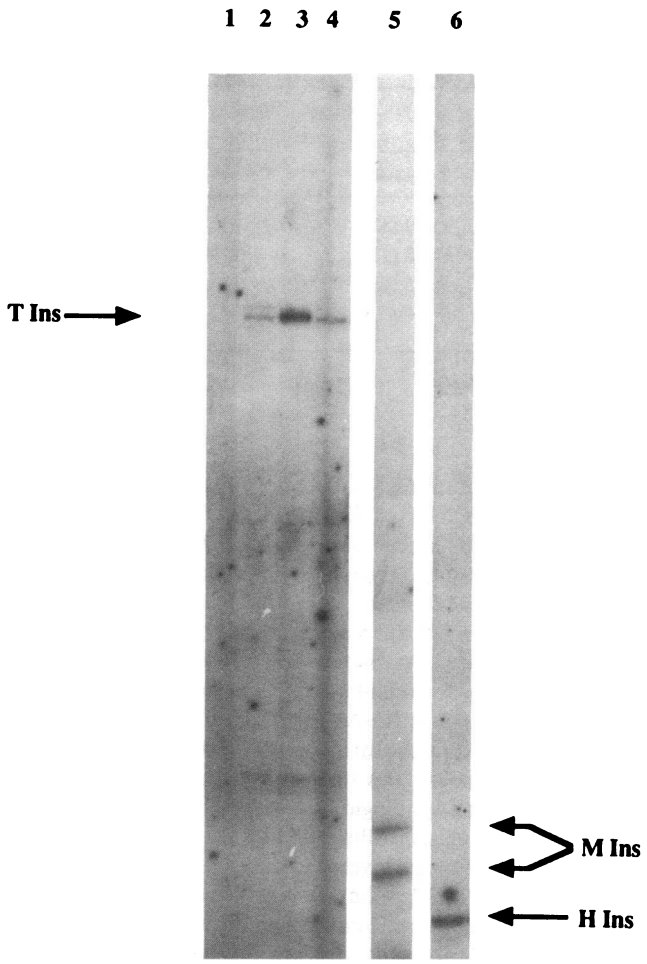

Figure 5. Primer extension analysis of human and mouse insulin mRNA. The specificity of the INS, MINS 3, and HINS oligonucleotide probes (see Fig. 1 and Table I) in detecting specific insulin mRNAs was initially evaluated using total pancreatic RNA derived from control and transgenic mice fed ad libitum. Lane *1*, INS oligonucleotide using transgenic total liver RNA (similarly, control mouse liver total mRNA did not result in a primer extension product); lanes *2* and *4*, INS oligonucleotide using control total pancreas RNA, generating a band of ~ 200 nucleotides (indicated by arrow marked *T Ins*). Lane *3*, INS oligonucleotide using transgenic total pancreas RNA; lane *5*, MINS 3 oligonucleotide using transgenic total pancreas RNA generating bands of ~ 70 and 75 nucleotides (mouse insulin II transcripts generate bands of both sizes using this oligonucleotide while the mouse I transcript results in a single band of 70 nucleotides; the two bands are indicated by the double arrowhead marked *M Ins*); lane *6*, HINS oligonucleotide using transgenic total pancreas RNA generating a band of ~ 63 nucleotides indicated by the arrow marked *H Ins*.

Total mouse (I + II) insulin mRNA. The increase in insulin mRNA in the pancreas of transgenic mice could have been due to the additional presence of human insulin mRNA and/or to changes in the levels of one or both of the endogenous mouse insulin mRNAs. To determine the relative amounts of total mouse insulin mRNA in the two groups of mice, MINS 3, an oligonucleotide specific for both mouse I and II insulin mRNA but significantly divergent from human, was prepared (Fig. 1, Table I). Primer extension analysis using this oligonucleotide revealed that there was no significant difference in the level of total mouse insulin mRNA in the pancreas of control or transgenic mice (Fig. 5, Table III). Under all conditions tested, total mouse insulin mRNA content in transgenic animals was within 20% of that of controls. This strongly suggested that the excess insulin mRNA in the transgenic pancreata was due to the presence of human insulin mRNA.

Human insulin mRNA. Primer extension analyses were also performed using HINS, an oligonucleotide specific for a sequence unique to human insulin mRNA (Fig. 1, Table I). The results (Fig. 5, Table III) confirm that the magnitude of both the decrease of human insulin mRNA during fasting of transgenic mice, as well as the increase upon refeeding, were similar to those observed for the mouse insulin mRNAs. No human insulin mRNA was detected by primer extension assay using total cellular RNA derived from control mouse pancreas, confirming the specificity of the HINS oligonucleotide.

Mouse I vs. II insulin mRNA. Previous reports concerning the ratio of mouse insulin I:II mRNA have been inconsistent with one another (17–19), possibly reflecting physiologic variability. To determine whether the coexpression of the human insulin gene in transgenic mouse B cells affected this ratio, two oligonucleotides were designed, each specific for a region unique to either mouse insulin I (MINS 1) or II (MINS 2) mRNA (Fig. 1, Table I). It was found that these oligonucleotides could be used for primer extension analysis either individually or in combination (Fig. 6), showing that the primers are specific and noninterfering. The data show that mouse insulin I mRNA is approximately twice as abundant (2.1 ± 0.1, $n = 6$) as insulin II mRNA. This value, however, may be influenced by the specific activity of the two radiolabeled oligonucleotide primers, and it is therefore not intended that this ratio be considered in quantitative terms. The ratio can, nonetheless, be used with confidence for comparative purposes, and it was found to be similar for control and transgenic mice, strongly suggesting that expression of human insulin does not affect the relative expression of the two mouse insulin genes.

Discussion

Expression of the human insulin gene under the control of its own promoter in transgenic mice has been described by other groups (32–36) as well as our own (20). These earlier studies showed that expression of the human gene was limited to the pancreatic B cells of such mice (20, 32–36), although more recently, it has been found that low levels of human insulin mRNA can also be detected in other tissues, including the brain, in one particular line of transgenic mice (S. J. Chan and D. Steiner, personal communication). Both the synthesis and the release of human insulin were regulated by glucose in much the same manner as the two endogenous mouse insulins. The expression of the foreign human insulin gene is presumed to be dependent upon the presence of a defined upstream region known to carry the *cis*-elements responsible for tissue-specific transcription (36). No attempt was made in these earlier studies, however, to compare the relative amounts of the three insulin mRNAs (mouse I, mouse II, and human) with those of the corresponding peptides. This comparative study has now revealed that the regulation of steady-state pancreatic insulin content is rather more complex than was previously supposed, with posttranscriptional events playing a crucial role.

Table III. Quantification of Primer Extension Analysis of Insulin mRNA in Pancreas of Control and Transgenic Mice

Primer mRNA species	Insulin mRNA levels as % control					
	INS Mouse I + II + Human		MIN 3 Mouse I + II		HINS Human	
	Control	Transgenic	Control	Transgenic	Control	Transgenic
Ad lib.	100±7 (6)	229±25 (12)	100±16 (6)	95±9 (12)	0	100±18 (6)
24-h fast	84±16 (6)	202±48 (11)	79±18 (6)	67±8 (12)	0	92±23 (6)
48-h fast	41±10 (6)	74±7 (6)	70±5 (6)	56±5 (6)	0	46±7 (6)
24-h fast + 24-h refeed	130±19 (6)	376±87 (6)	NM	NM	NM	NM

The data are presented as mean±SEM. NM, not measured.

Taken together, the data obtained from the analysis of insulin mRNA show that in the pancreata of transgenic mice there was ~ 2.2-fold more insulin mRNA than in those of controls, and that the increase was due to the additional presence of human insulin mRNA. Neither the total amount of mouse (I + II) insulin mRNA nor the relative amounts of the two individual mouse insulin mRNAs were affected by the expression of the human gene. During the fasting and refeeding there were parallel changes in mouse and human mRNA, thereby confirming the coordinate physiological regulation of the endogenous and exogenous insulin genes. In the present study, the steady-state levels of pancreatic insulin mRNA and peptides have been compared. As discussed in greater detail below, such data do not allow for quantification of rates of production or degradation of either mRNA or peptides. Despite this reservation it is apparent that the interplay between physiologic mediators and the various steps in insulin synthesis results in the maintenance of a physiologic set point for insulin content; that such a set point exists is illustrated by its maintenance in the face of supraphysiologic levels of insulin mRNA. Thus, despite the doubling of total insulin mRNA in the pancreas of transgenic mice, the pancreatic content of immunoreactive insulin was not significantly different from that of the control group regardless of the feeding regimen, and neither was glucose homeostasis affected.

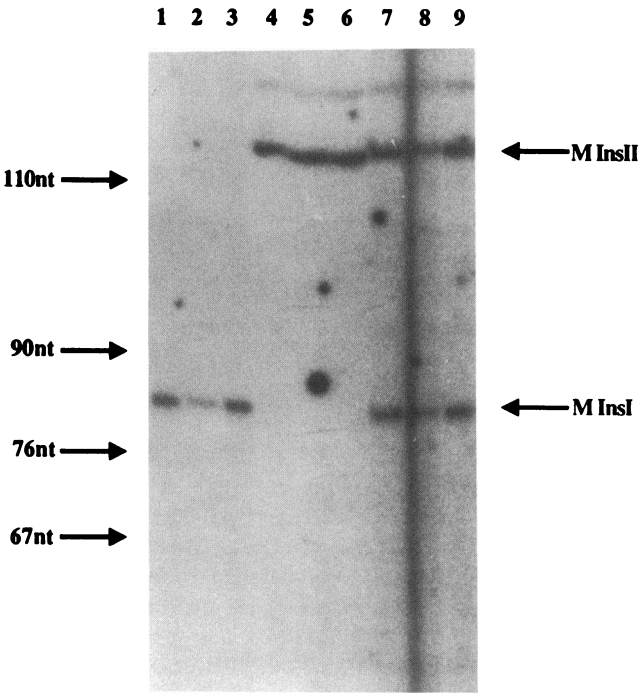

Figure 6. Primer extension analysis of mouse I and II insulin mRNA. The MINS 1 and MINS 2 oligonucleotides (see Fig. 1 and Table I) were used individually and in tandem to detect mouse insulin I and II mRNAs in total RNA samples derived from transgenic mice fed ad libitum. Primer extension experiments are as follows: lanes *1–3:* MINS 1 oligonucleotide (specific for mouse insulin I mRNA); lanes *4–6:* MINS 2 oligonucleotide (specific for mouse insulin II mRNA); lanes *7–9:* MINS 1 and MINS 2 oligonucleotides in tandem. The primer extension product derived from the mouse insulin I mRNA is predicted to contain approximately 89 nucleotides (see Fig. 1) and has a measured size of 84 nucleotides (arrow marked M InsI)). The product from the mouse insulin II mRNA is predicted to contain 122 nucleotides and has a measured size of 120 nucleotides (arrow marked M Ins II)

It is presumed that the set point is initiated in utero and can be changed; witness the alteration in insulin production that occurs soon after the birth of an infant of a diabetic mother. Other situations in which the set point can be profoundly disturbed may include certain disease states (including diabetes) and, of greater relevance to the present discussion, possibly other transgenic mice expressing human insulin. Thus, Marban et al. (35) have described two lines of transgenic mice expressing human insulin in their pancreatic B cells. These mice are both hyperinsulinemic and glucose intolerant. The authors attributed this condition to the expression of the transgene. This situation is quite different to that found in the present study, or with the extended series of transgenic mice studied by Bucchini and colleagues (32, 34, 36) that displayed apparently normal glucose homeostasis even in the face of a glucose challenge. Even though these fundamental differences between different transgenic lines cannot be explained at present, it must be presumed that the site of integration of the multiple copies of the human insulin gene in each line is at least one of the underlying factors. Regardless of the explanation, these differences exemplify the difficulties encountered when attempting to generalize any discussion based upon studies involving transgenic animals. In the particular case of transgenic mice expressing the human insulin gene, it is apparent that lines displaying disturbed glucose homeostasis, although interesting in themselves as models of type II diabetes, must be

considered apart from those that, as in the present study, display normal glucose sensitivity and insulin levels.

When the relative amounts of the insulin mRNAs in our transgenic mice are compared with those of the corresponding peptides, insulin production from mouse insulin mRNA is clearly more efficient than that from human. The interpretation of the data is somewhat complicated by the fact that we have simply measured steady state levels of both insulin mRNA and peptide rather than their rates of synthesis and disappearance. It is thus possible that human insulin is in fact produced more efficiently than we imagine, but that its rate of disappearance from the pancreatic B cells is more rapid than either of the two mouse insulins, leading to a lower steady state pancreatic content of the human peptide. Insulin disappearance from the pancreas is the sum of secretion and intracellular degradation (2, 37). Based upon current knowledge of human insulin production in transgenic mice, it appears likely that all three insulins are synthesized in the same B cells and, one must presume, packaged together in the same granules (36). If that is indeed the case, it is difficult to imagine that human insulin could be secreted in preference to the mouse insulins, the unit of secretion being a granule regardless of its content (2, 38). The degradation of insulin in the B cell arises by crinophagy (fusion of granules with lysosomes) (2, 39). Thus insulin degradation, like secretion, depends upon the granule as the basic operational unit rather than its contents.

Clearly, some posttranscriptional event must be relatively less efficient for human insulin than for mouse insulin. Since HPLC analysis revealed no accumulation of human proinsulin in the transgenic mouse pancreas (data not shown), it must be assumed that slow conversion of human proinsulin is not the explanation for the relatively low levels of human insulin peptide and that an event prior to formation of proinsulin itself, or the stability of newly formed proinsulin, must be responsible. When one considers the cascade of posttranscriptional events implicated in insulin production (1, 2), this limits the steps that may be rate limiting in the production of human insulin to translation itself, translocation of preproinsulin to the lumen of the rough endoplasmic reticulum, conversion of preproinsulin to proinsulin, and possibly the stability of newly synthesized proinsulin molecules within the rough endoplasmic reticulum or in transit to secretory granules.

From the data presented here, it can be concluded that the abundance of a given species of insulin mRNA cannot be taken as an index of the relative amount of insulin being produced and stored in the pancreas. This stresses the fundamental importance (and perhaps dominance) of posttranscriptional events in the overall regulation of pancreatic insulin production. It will be essential to understand each of these events, and above all their regulation, in the development of a strategy for insulin gene replacement therapy of diabetes. Such a therapy for diabetes will most likely utilize non-B cells for insulin production (40, 41). In this context, it will be critical to understand not only how the B cell regulates insulin production, but also how to recreate such regulation in an ectopic tissue.

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