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Immunology

Overexpression of β_2-microglobulin in transgenic mouse islet β cells results in defective insulin secretion

(class I heavy chain/nonimmune diabetes)

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ABSTRACT **Overexpression of heavy chains of the class I major histocompatibility complex in islet β cells of transgenic mice is known to induce nonimmune diabetes. We have now overexpressed the secretory protein β_2-microglobulin in β cells. Transgenic mice of one lineage had normal islets. Mice of another lineage did not become overtly diabetic but showed significant depletion of β-cell insulin. When mice were made homozygous for the transgene locus, they developed diabetes. Introduction of the β_2-microglobulin chain into class I heavy chain transgenic mice resulted in a significant improvement in their islet morphology and insulin content, and the female mice remained normoglycemic. These results suggest that different transgene molecules overexpressed in β cells can cause islet dysfunction, though not necessarily overt diabetes, and that this effect is mediated by the level of transgene expression. Evidence is provided to show that β-cell disruption by transgene overexpression occurs at the level of protein and involves a defect in insulin secretion.**

Experiments with transgenic mice have shown that extrathymic expression of foreign class I or class II major histocompatibility complex (MHC) molecules on islet β cells induced peripheral T-cell tolerance (1–3). An interesting and surprising outcome of these experiments was that abundant expression of class I or class II molecules, induced by linking these genes to the rat or human insulin promoter, did result in nonimmune insulin-dependent diabetes mellitus (IDDM).

These findings raise several questions. First, is the nonimmune diabetes induced specifically as a result of overexpression of the membrane-bound MHC molecules or can any transgenic protein abundantly expressed in β cells cause this effect? Several investigators have overexpressed non-MHC proteins in β cells; some reported diabetes, whereas others did not (for review, see ref. 4). So far only MHC molecules [with one exception (5)], calmodulin (6), and the Ha-*ras* oncogene (7) caused nonimmune IDDM in transgenic mice. Calmodulin, an intracellular calcium binding protein, and ras, a guanine nucleotide binding protein, might be expected to cause β-cell disruption through their effects on cell metabolism or cycle and so their properties may be different from those of MHC molecules.

Secondly, how are the MHC molecules mediating β-cell dysfunction? Is it through competition of transgene expression with insulin biosynthesis at transcriptional, post-transcriptional, translational, or post-translational levels? Lo *et al.* (2) suggested that transcriptional competition was unlikely since they found IDDM in a transgenic line with only two extra copies of the rat insulin promoter (RIP). Sarvetnick *et al.* (8) did not find IDDM in β-cell transgenic mice expressing either class II α or β mRNA but did when both chains were present, indicating that functional protein was required for IDDM to occur. They did, however, report a transient hyperglycemia in the class II α chain transgenic mice. Similarly, Miller *et al.* (9) reported hyperglycemia in class II β-chain transgenic mice. This suggests that individual class II chains can alone induce islet dysfunction but not always to the extent that overt diabetes occurs.

We have shown (1) that transgenic mice overexpressing the class I heavy chain H-2K^b in islet β cells became diabetic at a very early age without immune involvement. The class I heavy chain requires its associated light chain (β_2-microglobulin; β_2m) for cell surface expression, and in its absence the heavy chains remain at the endoplasmic reticulum (10). In our RIP–K^b heavy chain transgenic mice, the K^b chain showed low-level surface expression but very high-level cytoplasmic expression. This was presumably due to the fact that β cells normally express very low levels of heavy and light chain molecules and hence the concentration of light chain molecules was limiting.

In the absence of class I heavy chains, β_2m itself acts as a secretory protein (11). We have now expressed this molecule in islet β cells and shown it to have deleterious effects on β-cell function. We also provide evidence that the disruption of β cells by transgene overexpression occurs at the level of protein and involves a defect in insulin secretion.

MATERIALS AND METHODS

Transgenic Mice. RIP–K^b transgenic mice expressing allo–K^b in islet β cells have been described (1). The lineage used here (50-1) has not been described and differs from the transgenic mice previously described (1) in being of mostly CBA background (CBA × B10.BR. F_2 backcrossed onto CBA for at least three generations) and in having less-severe diabetes, so that the mice survive without insulin injections although they are diabetic by 28 days of age (blood glucose, >17 mM).

The RIP–β_2m mice were made as follows. The RIP–β_2m construct was made in pIC20R (12) by linking the rat insulin promoter, kindly provided by D. Hanahan (13), from position −695 to position +8 to a blunt-ended *Hae* II fragment covering part of the 5′ untranslated region, the first exon, and about 195 base pairs from the first intron of the β_2m^b allele (14). The *Hin*dIII fragment of the β_2m^b gene containing exons 2–4 and the 3′ downstream sequences was added so that the completed construct lacked a major portion of the first β_2m^b intron. The RIP–β_2m^b construct was removed from vector sequences and microinjected into CBA × B10.BR. F_2 fertilized eggs. Three transgenic founders expressed the transgene. One was sterile and the other two, called 78-3 and 88-4,

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Abbreviations: β_2m, β_2-microglobulin; IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex; RIP, rat insulin promoter.

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transmitted 8–10 and 20–30 copies of the transgene, respectively, to their offspring. These lineages were backcrossed onto CBA, which carries the endogenous β_2m^a allele differing by one amino acid from the β_2m^b transgene product.

Lineage 88-4 was made homozygous by brother × sister matings of transgenic mice. Presumptive RIP–β_2m/β_2m homozygotes were backcrossed to nontransgenic mice to ensure they were homozygous before establishing a lineage. Lineages 88-4 and 78-3 were mated to the RIP–K^b lineage 50-1 to obtain mice that were transgenic for K^b, K^b/β_2m, or β_2m or were nontransgenic.

Antibodies. Insulin, glucagon, and K^b (B8-24-3) antibodies have been described (1). Fluorescein-conjugated anti-insulin antibody was from Nordec and was diluted 1:5. The Ly-m11 monoclonal antibody, which is specific for β_2m^b when associated with the class I heavy chain, was used as supernatant (15) and detected with fluorescein-conjugated sheep anti-mouse immunoglobulin. The Gex-β_2m rabbit antibody was made as follows. A PCR product of the β_2m^b mature protein coding region (16) from Ile-21 to the "stop" codon was made by constructing oligonucleotides that spanned these two regions and that contained a *Bam*HI site in the 5′ oligonucleotide and an *Eco*RI site in the 3′ oligonucleotide. The PCR product was cloned into the *Bam*HI–*Eco*RI site of pGEX-2T and grown in HB101, and the β_2m^b protein product was recovered as described (17). A rabbit was immunized with 50 μg of protein in Freund's incomplete adjuvant and was given a booster injection after 6 weeks with 25 μg. Rabbit sera (1:150 dilution) was used for immunostaining. For double-staining experiments the Gex-β_2m antibody was protein A-purified, biotinylated, and detected with rhodamine-labeled avidin (Vector).

Isolation of Pancreatic Islets and Islet β Cells. Islets were isolated from the pancreas of 15- to 18-day-old transgenic mice as described (1) but were not cultured. Single β cells obtained by trypsin digestion were allowed to recover for 1 hr at 37°C before use. A sample was stained with anti-insulin antibody to check for β cells.

Insulin Content of Pancreas and Blood Glucose Measurements. Pancreata were removed from nonfasted transgenic mice at day 25, weighed, and homogenized in 5 ml of acid ethanol. Extracts were diluted 1:200 before the insulin radioimmunoassay was done. Transgenic status was determined by Southern blot analysis of tail DNA. Blood glucose was determined as before (1).

Immunostaining and Immunohistology. Immunostaining of isolated β cells and immunohistology were performed as described elsewhere (1).

Immunogold Electron Microscopy. Immunogold electron microscopy was performed on isolated islets by fixation in 0.5% glutaraldehyde for 10 min at 4°C and processing as described (18). Fixed islets were embedded in LR White and sectioned. Islets were stained with the Gex-β_2m antibody (diluted 1:800) or the anti-exon 8 antibody (diluted 1:400) and this was detected with protein A-gold. Sections were stained with uranyl acetate.

RESULTS

Abundant Expression of β_2m in RIP–β_2m^b Transgenic Mice. Transgenic mice expressing the b allele of β_2m (β_2m^b) in islet β cells were made. Two lineages, 88-4 and 78-3, were used for study. Like RIP–K^b transgenics (1), RIP–β_2m mice did not show any lymphocytic infiltration into the islets even though the β_2m^b transgene product was allogeneic in the CBA mouse, which carries the endogenous β_2m^a allele. Unlike the RIP–K^b transgenic mice, however, the RIP–β_2m transgenics did not become overtly diabetic at any age even though they expressed β_2m prenatally and throughout life (data not shown).

Normal mouse islet β cells express very low levels of class I heavy and light chain (β_2m) molecules. Therefore, overexpression of a β_2m transgene using the rat insulin promoter led to abundant β_2m light chains. Expression of isolated β_2m chains was detected in β cells of lineages 88-4 and 78-3 by using a rabbit polyclonal antibody to β_2m made with the pGex system (17). This antibody recognized individual transgenic β_2m light chains. Fig. 1 shows the expression pattern of isolated β_2m chains in islets of both transgenic lineages. Lineage 88-4 expressed β_2m in a patchy distribution whereas lineage 78-3 showed a confluent staining pattern. These islets were double stained for insulin and, in 88-4 mice, cells that stained brightly for β_2m also stained brightly for insulin. Cells empty of β_2m and insulin were likely to be β cells as they did not stain for glucagon or somatostatin (data not shown). Staining for insulin in islets of 78-3 mice was confluent. Thus transgenic mice overexpressing an allogeneic secretory protein, β_2m^b, showed no lymphocytic infiltration in their islets and no obvious diabetes but did show signs of insulin deficiency in one lineage though not in another.

Transgenic Mice Homozygous for the β_2m Locus Become Diabetic. Transgenic mice of lineage 88-4 were made homozygous. Many though not all of the RIP–β_2m/β_2m male and female homozygous mice became diabetic with blood glucoses exceeding 17 mM (data not shown). Thus the increased transgene expression was sufficient to cause enough β-cell disruption so that diabetes occurred in most animals. The level of overexpression of transgene molecules in β cells, therefore, appeared to contribute to the degree of islet damage.

Double-Transgenic Mice Expressing K^b and β_2m Molecules. The RIP–β_2m transgenic mice of lineage 88-4 were mated to RIP–K^b mice and offspring were tested for segregation of the two transgene loci by Southern blot analysis. Since β_2m is required for surface expression of K^b, it was expected that RIP–K^b/β_2m double-transgenic mice would have an increased surface expression of class I molecules compared to

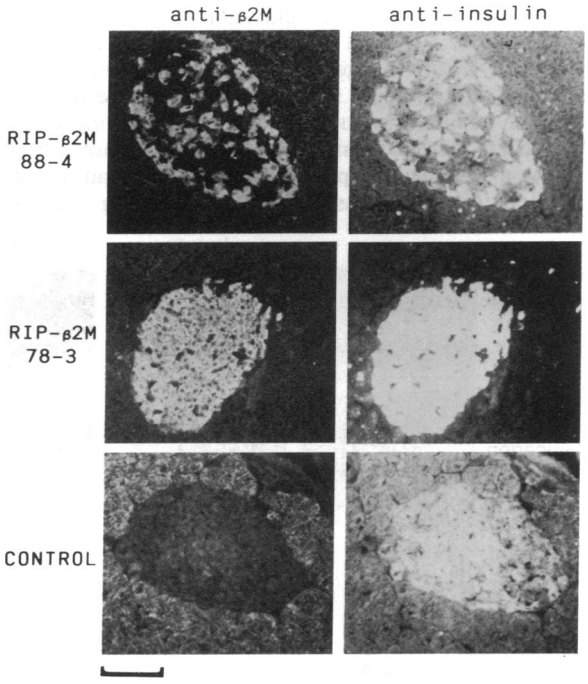

FIG. 1. Expression of β_2m and insulin in RIP–β_2m transgenic mice. Islets from 80- to 100-day-old mice were double-stained for β_2m and insulin by using biotinylated Gexβ2 and visualized with rhodamine-600 and fluorescein-conjugated anti-insulin antibody. Line 88-4 β cells show patchy costaining for both β_2m and insulin; line 78-3 β cells show confluent staining. (Bar = 15 μm.)

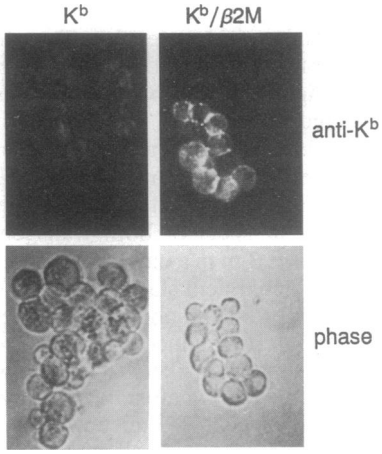

FIG. 2. Expression of H-2K^b on the surface of β cells from RIP-K^b single- and RIP-K^b/β_2m double-transgenic mice. β cells isolated from day-15 single- and double-transgenic mice were stained by fluorescence with the anti-K^b monoclonal B8-24-3, which only recognizes K^b associated with β_2m. Fluorescence (*Upper*) and phase-contrast (*Lower*) micrographs are shown. (×75.)

transgenic mice expressing RIP-K^b heavy chain alone (1). Fig. 2 shows β cells from RIP-K^b heavy chain transgenic and RIP-K^b/β_2m double-transgenic mice stained for surface class I K^b molecules. The RIP-K^b/β_2m double-transgenic mice showed increased surface expression of class I K^b molecules as expected. A similar result was found when islets were stained with the Ly-m11 monoclonal antibody, which recognizes β_2m^b in association with the class I heavy chain (data not shown). The increased surface expression of class I allo-K^b on islet β cells did not result in any lymphocytic infiltration into the islets (data not shown).

Pancreata from 23-week-old RIP-K^b, RIP-K^b/β_2m, RIP-β_2m and control mice were stained for insulin and glucagon. Control islets stained darkly for insulin and showed a characteristic staining pattern for glucagon (Fig. 3 *Upper*) and male and female mice had normal blood glucose levels (Fig. 3 *Lower*). RIP-β_2m transgenic islets from heterozygous mice of lineage 88-4 contained some β cells that were obviously deficient in insulin, although all mice were normoglycemic. RIP-K^b islets were extremely disorganized as shown by the distribution of glucagon-producing cells and had severely insulin-deficient β cells, as described before (Fig. 3 and ref. 1); these mice were diabetic by 28 days of age. Interestingly, islets from male and female double-transgenic RIP-K^b/β_2m mice had an improved morphology and insulin staining pattern compared to islets from RIP-K^b mice but were not as good as islets from RIP-β_2m heterozygous mice. Double-transgenic female mice were even normoglycemic for up to 23 weeks or more. Similar results were found when lineage 78-3 was crossed to RIP-K^b single-transgenic mice. Thus an increase in transgene expression in this case did not lead to greater islet dysfunction as it did in the homozygous RIP-β_2m/β_2m mice described above. This improvement in islet function was not the result of down-regulation of expression from the RIP-K^b transgene array by the presence of the RIP-β_2m array as determined by Northern blots of pancreas RNA (data not shown).

Our results show that the presence of both abundant heavy and light chains of the class I molecule allowed high-level surface expression of allo-K^b on islet β cells. This did not induce autoimmunity but caused a significant improvement in islet morphology when compared to that of RIP-K^b heavy-chain single-transgenic mice. Thus the ability of the β_2m molecules to combine with the K^b heavy chains and transport them away from the endoplasmic reticulum was associated with improved β-cell function. The ratio of K^b heavy chains to light chains was not determined.

Insulin Content. We have determined the insulin content of pancreata from young RIP-K^b, RIP-K^b/β_2m, RIP-β_2m, RIP-β_2m/β_2m, and control mice. At day 25, when RIP-K^b and RIP-β_2m/β_2m homozygous mice were diabetic, their insulin content was reduced to 30–60% that of control mice (Fig. 4). Since as little as 10% functioning β cells can keep a mouse normoglycemic, this result suggests that insulin was present in the islets but not secreted. The RIP-K^b/β_2m mice had marginally improved insulin content at day 25. As mice aged, the insulin content of RIP-K^b mice diminished to a greater extent than that of RIP-K^b/β_2m mice as shown by immunohistology in Fig. 3. RIP-β_2m (88-4) mice had near normal insulin content at this early age, which became impaired in older mice (Fig. 3).

Immunogold Electron Microscopy. We have attempted to localize the transgene products in the β cell by immunogold staining. Islets from young RIP-β_2m (88-4) transgenic mice were isolated and stained for insulin or β_2m. The β_2m protein was localized in the insulin secretory granules (Fig. 5). It was not possible from the morphology of the electron microscope sections to determine where else in the secretory pathway

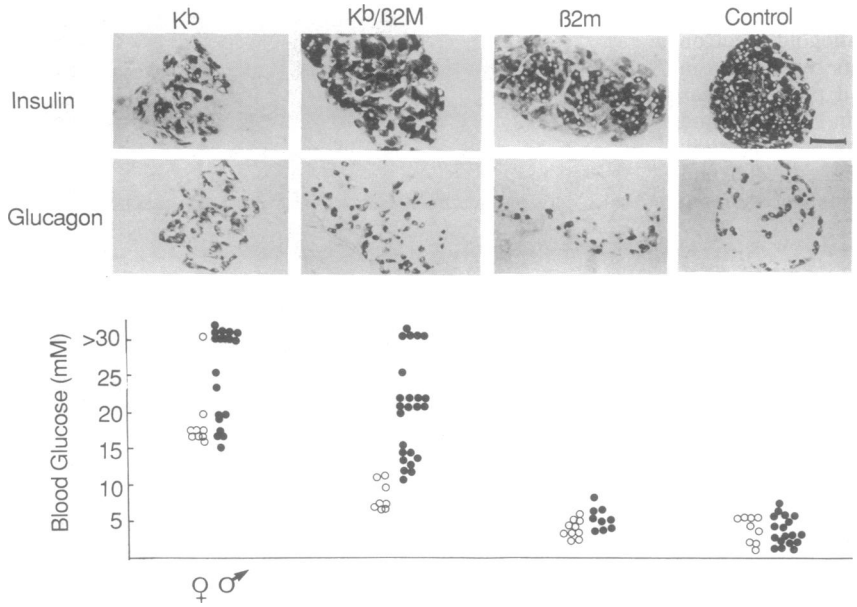

FIG. 3. Histopathology of pancreatic islets and blood glucose measurements in transgenic mice. (*Upper*) Bouin-fixed pancreas from control, RIP-β_2m (88-4 lineage), RIP-K^b/β_2m (88-4 lineage), and RIP-K^b male mice, aged 23 weeks, stained for insulin and glucagon by immunoperoxidase. (*Lower*) Random blood glucose measurements from male (solid circles) and female (open circles) mice, aged 15 to 23 weeks. (Bar = 100 μm.)

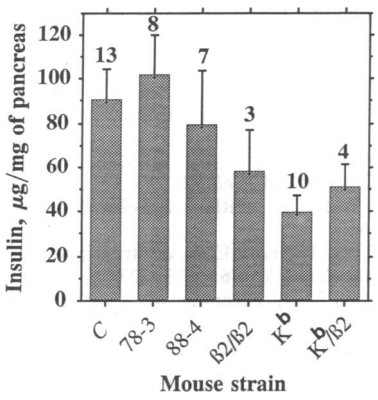

FIG. 4. Insulin content of pancreas from transgenic mice measured at day 25 after birth. Pancreas from control (C), β_2m(78-3), β_2m(88-4), β_2m/β_2m(88-4), K^b, and K^b/β_2m(88-4) nonfasted mice was analyzed for insulin content by radioimmunoassay. The numbers of mice in each group are given above the bars.

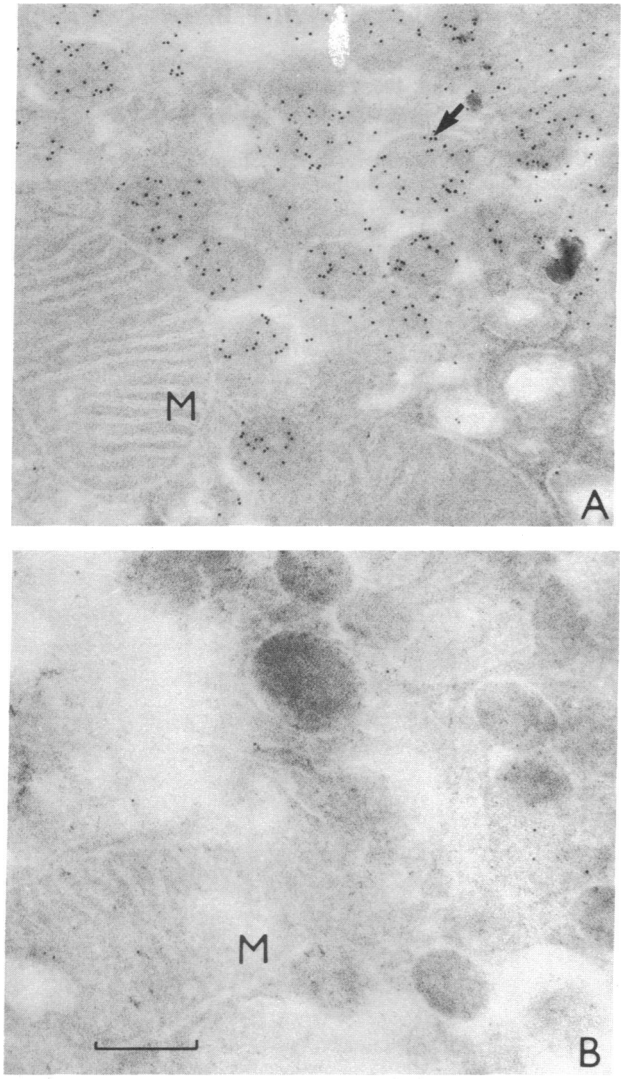

FIG. 5. Immunogold electron microscopy of β cells expressing β_2m. Sections of RIP-β_2m (88-4) islets were stained with the rabbit Gexβ2 polyclonal antibody (*A*) or with a control antibody (rabbit anti-exon 8 polyclonal) that does not stain RIP-β_2m islets (*B*), and 5-nm protein A-gold was used as the detector. The β_2m transgene product is seen in the insulin secretory granules (arrow). M, mitochondrion. (Bar = 0.2 μm.)

β_2m was localized. The secretory granules also stained for insulin (data not shown).

We were unable to detect the K^b heavy chain molecule by using an anti-exon 8 polyclonal antibody or to detect the functional K^b/β_2m molecule using a variety of monoclonal antibodies. This is because the fixation required to give sufficient cell morphology in the electron microscope destroyed the antigenicity of the K^b molecule.

DISCUSSION

We have found that a secretory protein β_2m, which is not known to regulate cell metabolism or cell cycle, can induce IDDM in transgenic mice when present in sufficient abundance. In the absence of IDDM one RIP-β_2m lineage showed insulin depletion in the islets by immunohistology, suggesting that β-cell dysfunction had occurred without progressing to diabetes. Another RIP-β_2m lineage showed no such islet abnormalities.

Increasing the amount of β_2m expression in the 88-4 transgenic line by homozygous matings resulted in IDDM in most male and female mice similar to that seen in RIP-K^b mice. Thus overproduction of the secretory β_2m molecules was almost as potent at causing β-cell destruction as the membrane-bound class I and class II molecules. It appears, therefore, that proteins other than MHC molecules can cause β-cell dysfunction, the extent of which relates to the level of transgene expression. This may explain why Böhme *et al.* (5) did not see IDDM in their MHC class II transgenic mice since they used cDNA constructs that usually express at much lower levels than genomic DNA constructs.

Increasing the amount of transgene expression in RIP-K^b/β_2m mice did not lead to greater islet destruction, as it did in homozygous RIP-β_2m/β_2m mice. In fact islets were improved in morphology when compared to class I heavy chain single-transgenic mice and female mice remained normoglycemic for more than 23 weeks. Males showed improved islet morphology but many were diabetic by 25 days of age. Likewise, in the ras-β-cell transgenic mice described by Efrat *et al.* (7), sex differences were noted in the onset of diabetes.

The improvement in islet morphology and insulin secretion in our double-transgenic mice may have been due to the transport of K^b heavy chain molecules by the β_2m light chains away from the endoplasmic reticulum where K^b may interfere with insulin translation and transport. Thus the damaging effect of transgene expression in islet β cells occurred at the translational or post-translational level and was not due entirely to the transgene array competing for transcriptional or post-transcriptional factors needed for insulin biosynthesis.

We have localized β_2m to the insulin secretory granules in RIP-β_2m mice but were unable to detect K^b heavy chains by immunogold electron microscopy. We did not expect to find β_2m in the insulin secretory granules because it has been shown that the regulated secretory pathway taken by insulin is normally separated from the constitutive secretory pathway followed by membrane proteins or secretory proteins such as β_2m (19, 20). Experiments with transfected cell lines, however, have shown that sorting of proteins to different pathways can be perturbed (21–23).

The presence of β_2m in the insulin secretory granules suggests an alternative explanation for β-cell dysfunction to that described above. Very abundant K^b heavy chains or β_2m light chains may be forced into the insulin secretory pathway where they influence the processing or regulated secretion of insulin. This hypothesis is supported by the fact that although the RIP-K^b and RIP-β_2m/β_2m mice were diabetic by day 25, they still contained significant amounts of insulin (or proinsulin) in their β cells. A similar effect was noted by Epstein

et al. (6) in their calmodulin β-cell transgenics. When K^b and β_2m were both present in our mice, the chains may have favored the constitutive secretory pathway with less impairment of insulin processing or secretion.

In summary, we have shown that β-cell dysfunction, but not necessarily overt diabetes, can be induced by a non-MHC transgene. This effect occurs at the level of protein and is likely to involve a defect in insulin secretion. We cannot, however, conclusively exclude transcriptional or post-transcriptional mechanism as party to the β-cell dysfunction, and it is possible that interference at these levels can also contribute to β-cell damage by transgenes.

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1. Allison, J., Campbell, I. L., Morahan, G., Mandel, T. E., Harrison, L. C. & Miller, J. F. A. P. (1988) *Nature (London)* **333,** 529–533.
2. Lo, D., Burkly, L. C., Widera, G., Cowing, C., Flavell, R. A., Palmiter, R. D. & Brinster, R. L. (1988) *Cell* **53,** 159–168.
3. Morahan, G., Allison, J. & Miller, J. F. A. P. (1989) *Nature (London)* **339,** 622–624.
4. Allison, J., Harrison, L. C., Campbell, I. L. & Miller, J. F. A. P. (1990) *Cur. Top. Microbiol. Immunol.* **156,** 121–135.
5. Böhme, J., Haskins, K., Stecha, P., van Ewijk, W., LeMeur, M., Gerlinger, P., Benoist, C. & Mathis, D. (1989) *Science* **244,** 1179–1183.
6. Epstein, P. N., Overbeek, P. A. & Means, A. R. (1989) *Cell* **58,** 1067–1073.
7. Efrat, S., Fleischer, N. & Hanahan, D. (1990) *Mol. Cell. Biol.* **10,** 1779–1783.
8. Sarvetnick, N., Liggitt, D., Pitts, S. L., Hansen, S. E. & Stewart, T. A. (1988) *Cell* **52,** 773–782.
9. Miller, J., Daitch, L., Rath, S. & Selsing, E. (1990) *J. Immunol.* **144,** 334–341.
10. Ploegh, H. L., Cannon, E. L. & Strominger, J. L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2273–2277.
11. Severinsson, L. & Peterson, P. A. (1984) *J. Cell Biol.* **99,** 226–232.
12. Marsh, J. L., Erfle, M. & Wykes, E. J. (1984) *Gene* **32,** 481–485.
13. Hanahan, D. (1985) *Nature (London)* **315,** 115–122.
14. Margulies, D. H., Parnes, J. R., Johnson, N. A. & Seidman, J. G. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2328–2331.
15. Tada, N., Kimura, S., Hatzfeld, A. & Hammerling, U. (1980) *Immunogenetics* **11,** 441–449.
16. Parnes, J. R. & Seidman, J. G. (1982) *Cell* **29,** 661–669.
17. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
18. Culvenor, J. & Crewther, P. E. (1990) *J. Protozool.* **37,** 59–65.
19. Orci, L., Ravazzola, M., Amherdt, M., Perrelet, A., Powell, S. K., Quinn, D. L. & Moore, H.-P. H. (1987) *Cell* **51,** 1039–1051.
20. Matsuuchi, L., Buckley, K. M., Lowe, A. W. & Kelly, R. B. (1988) *J. Cell Biol.* **106,** 239–251.
21. Moore, H.-P., Gumbiner, B. & Kelly, R. B. (1983) *Nature (London)* **302,** 434–436.
22. Carroll, R. J., Hammer, R. E., Chan, S. J., Swift, H. H., Rubenstein, A. H. & Steiner, D. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8943–8947.
23. Rosa, P., Weiss, U., Pepperkok, R., Ansorge, W., Niehrs, C., Stelzer, E. H. K. & Huttner, W. (1989) *J. Cell Biol.* **109,** 17–34.