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Originals

Genetic analysis of obesity-induced diabetes associated with a limited capacity to synthesize insulin in C57BL/KS mice: evicence for polygenic control

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Summary. Expression of obesity-induced diabetes associated with the diabetes or *db* mutation in mice varies in inbred strains. This study utilized a genetic analysis to evaluate the number of genes responsible for the difference in diabetes responses between mice of the susceptible C57BL/KsJ (BL/Ks) and resistant 129/J inbred strains. BL/Ks (*db*/+) males and 129/J (+/+) females were bred to generate F_1 hybrids, and the F_1 females (*db*/+ and +/+, distinguished by progeny testing) were backcrossed to BL/Ks (*db*/+) males. A total of 252 backcrossed males were obtained, of which 31 were *db/db* and obese. While the plasma glucose of all the fed backcrossed mice was greater than 22 mmol/l, the expression of diabetes varied considerably, as measured by fasting plasma glucose, fed plasma insulin, and pancreatic insulin and proinsulin mRNA content. That proinsulin mRNA content was a good indicator of diabetes severity and islet dysfunction was seen in the inverse correlation between proinsulin mRNA content and fasting plasma glucose ($r=0.69$, $p<0.001$), and a direct correlation between proinsulin mRNA and plasma insulin ($r=0.86$, $p<0.001$), and pancreatic insulin content ($r=0.61$, $p<0.01$). If a single gene were responsible for severe islet dysfunction, one-half of the backcrossed mice would develop low proinsulin mRNA levels like the BL/Ks parent, and one-half would be resistant to islet destruction. Statistical evaluation (SKUMIX) of the distribution of these parameters in backcrossed mice rejected with a high degree of probability a bimodal distribution. Thus, it was concluded that while a dominant gene (or genes) is responsible for diabetic (>22 mmol/l) unfasted plasma glucose in all backcrossed mice, allelic differences at two or more genetic loci are responsible for the differences between the two strains in diabetes severity measured by fasting plasma glucose, pancreatic insulin, and proinsulin mRNA content.

Key words: C57BL/KsJ *db/db* mice, genetic analysis, proinsulin mRNA, polygenic control.

The diabetes (*db*) mutation on chromosome 4 occurred spontaneously in C57BL/KsJ (BL/Ks) mice [1, 2]. In the homozygous condition (*db/db*), this mutation produces hyperphagia, progressive obesity, and insulin resistance. The initial adaptation to the insulin resistance is one of islet hyperplasia resulting in marked hyperinsulinaemia, but ultimately the islets develop B-cell necrosis, and the mice become insulinopoenic, severely hyperglycaemic and lose weight. The response to the obesity-induced insulin resistance of the *db* mutation in BL/Ks mice, however, differs from that in other inbred strains, with a milder diabetes noted for example in C57BL/6J (BL/6) and 129/J mice [3-8]. The diabetes resistant strains appear to compensate for the insulin resistance by developing pronounced islet cell hyperplasia and hyperinsulinaemia without islet destruction.

In previous studies, *db/db* mice of the diabetes susceptible BL/Ks strain were observed with longitudinal measurements of proinsulin mRNA to assess insulin biosynthesis [9]. At five weeks of age, the *db/db* mice had a four-fold increase in serum insulin and a comparable increase in proinsulin mRNA above that in the lean littermates. Progressive hyperglycaemia developed, and at 10 and 13 weeks a marked loss of insulin biosynthesis was demonstrated, as evidenced by parallel decreases in serum insulin and proinsulin mRNA. The measurements of proinsulin mRNA permitted a quantitative assessment of total B-cell function in vivo, and allowed estimation of changes in insulin synthesis that occurred with obesity and with age. The loss of insulin synthesis was related to a worsening of hyperglycaemia, and insufficient insulin synthetic capacity was associated with the ultimate development of severe diabetes.

In the present experiments, the number of background modifying genes responsible for determining a limited capacity to synthesize insulin has been assessed in BL/Ks mice. The influence of genetic background on the expression of the *db* mutation in mice has been evaluated by Coleman and associates [10, 11].

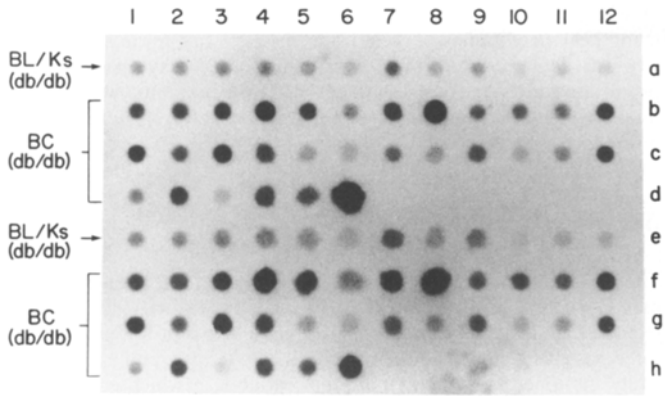

Fig. 1. Autoradiograph of dot-blot hybridization of pancreatic RNA with ^{32}P-rat proinsulin (PI) cRNA demonstrating the relative concentration of proinsulin mRNA in total RNA in *db/db* mice of the BL/Ks parental strain ($n=12$) compared to that in *db/db* backcross (BC) mice ($n=30$) at the same age (14 weeks). The proinsulin mRNA concentration in *db/db* backcross mice varied from <1 ng to >20 ng/pancreas, while the concentration in BL/Ks parental mice was <7 ng/pancreas. PI mRNA was measured in total pancreatic RNA (10 µg) relative to a synthetic rat I proinsulin cRNA standard as described in Methods. Rows a–d are duplicated in rows e–h

Blood glucose, plasma insulin, and islet histology were assessed in F_1 hybrids of the BL/Ks and BL/6 strains and in backcrosses of F_1 hybrid mice to each parental strain. Lack of segregation of these parameters in the backcrossed (BC) mice into two classes (F_1 like vs BL/Ks or BL/6 like), led to the conclusion that expression of the diabetes syndrome was under multigenic control. Moreover, the genes responsible for the more severe diabetic syndrome of the BL/Ks mice were recessive to those in the BL/6 genome. More recently, in the course of development of a BL/Ks.BL/6-H-2B congenic stock, Leiter et al. [12] re-evaluated diabetes susceptibility in BL/Ks and BL/6 strains. The results were surprisingly different from those initially reported, with all the *db/db* mice of both the F_1 and F_2 generations exhibiting severe hyperglycaemia and atrophic islets. These results were difficult to explain, but consistent with multiple diabetes susceptibility genes in the BL/Ks strains which now appeared to be dominant.

Because of the discrepancy of previous reports, crosses of mice of the 129/J inbred strain, previously shown to be resistant to obesity induced-diabetes associated with the *db* mutation [7, 8], with KsJ mice were evaluated. If a single gene were responsible for severe loss of proinsulin mRNA and diabetes in BL/Ks mice, expression of the diabetes syndrome in F_1 hybrid mice backcrossed to the BL/Ks parental strain might segregate into diabetic and non-diabetic populations, defined by their capacity to synthesize insulin. This analysis has been completed, and the results are consistent with the interpretation that multiple genes regulate the expression of diabetes and the capacity to synthesize insulin following induction of obesity by the *db* mutation in BL/Ks mice.

Materials and methods

Animals

Male C57BL/KsJ mice, heterozygous for the *db* mutation (*db/+*), and female 129/J mice, homozygous non-diabetic at the *db* locus (+/+), were obtained from the Jackson Laboratories (Bar Harbor, Me, USA) after weaning at roughly four weeks of age. Mice were given free access to tap water and chow (Purina Rodent Chow, Ralston Purina, St. Louis, Mo, USA) throughout the experiment.

Breeding protocol

BL/Ks (*db/+*) male mice were crossed with 129/J (+/+) females. Half of the F_1 mice would be heterozygous for the *db* mutation (*db/+*) and the other half would be homozygous non-diabetic (+/+). The F_1 female mice (*db/+* and +/+) were backcrossed with BL/Ks (*db/+*) males, and the BC mice would be expected to produce *db/db*, *db/+*, and +/+ progeny in the ratio 1/8:4/8:3/8. The homozygous diabetic BC mice (*db/db*), determined by the progeny test for obesity and characteristic shape at five weeks of age [1–3] were maintained to measure plasma glucose and insulin, pancreatic insulin and pancreatic proinsulin mRNA contents. Male mice were analysed exclusively to avoid gender differences in the development of diabetes [5, 15]. By breeding BL/Ks males (*db/+*) with 129/J females (+/+), and then backcrossing F_1 females with parental BL/Ks males, all of the male BC mice had only the BL/Ks Y-chromosome.

Measurements of plasma glucose and insulin concentrations

Whole blood (300 µl) was collected between 09.00 and 11.00 hours in the fed or fasted state (18 h) from the retro-orbital sinus of ether anaesthetized animals at 5, 10, and 14 weeks of age using a heparin-treated microhaematocrit capillary tube. Plasma glucose was measured using the Beckman Glucose Analyzer II, and plasma insulin was assayed by a double antibody radioimmunoassay (RIA) method with purified rat insulin standards (Novo Inc., Copenhagen, Denmark). All assays were done in duplicate.

Determination of pancreatic insulin

Mice were killed in the fed state at 14 weeks of age. Animals were injected intraperitoneally with 15 mg pentobarbital sodium/100 g body weight. Before cardiorespiratory arrest, the abdomen and thorax were dissected, then the pancreas was excised. Pancreatic tissue was rapidly cut into small pieces and these were divided into two aliquots of approximately equal weight, weighed, then one was homogenized immediately for isolation of pancreatic RNA, and the other was frozen in liquid nitrogen. The frozen aliquots of pancreas for insulin determination were stored at $-80°$C until all specimens were available; then each specimen was homogenized in 2 ml of acid ethanol (75% ethanol, 0.15 mol/l HCl) with a Polytron homogenizer (Brinkman, Westburg, NY, USA). Homogenates were incubated at 4°C for 48 h, then the suspension was centrifuged for 10 min at 12,000 x *g*. The pellet was resuspended in 1 ml of acid ethanol. After incubation at 4°C for 24 h, the suspension was centrifuged as mentioned above. The second supernatant was pooled with the first and diluted 1:3000 in 0.1 mol/l phosphate-buffered saline solution, pH 7.4., containing 0.25% bovine serum albumin. Insulin was measured by double antibody RIA. All assays were performed in duplicate.

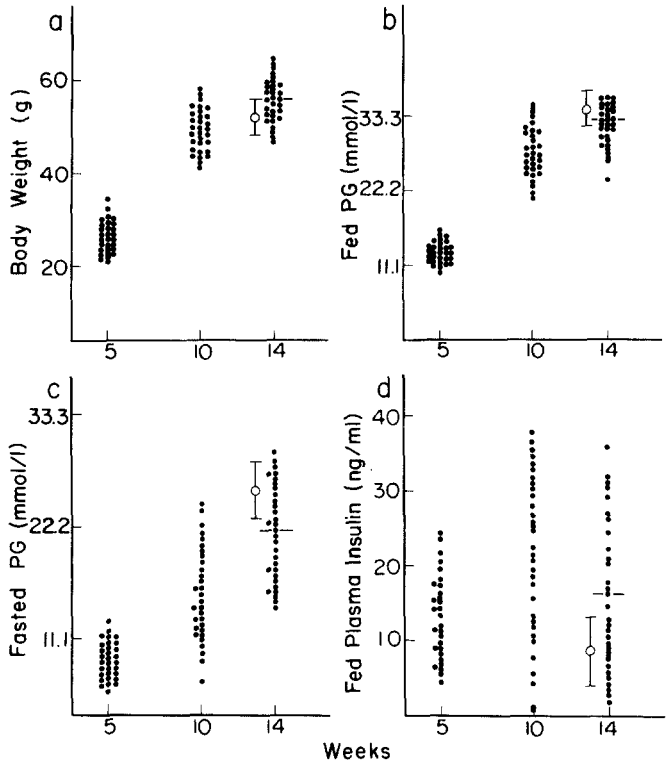

Fig. 2. **a** Body weight, **b** fed plasma glucose (PG), **c** fasted PG, and **d** fed plasma insulin were determined as described in the Methods on 31 BC male *db/db* mice which were obtained from 252 progeny of BL/Ks (*db*/+) X F_1 (BL/Ks [*db*/+] X 129J [+/+]). The open circles and error bars represent the mean ± S.D. of determinations of *db/db* mice ($n=12$) of the BL/Ks parental strain for comparison at 14 weeks of age. The mean of the values in backcross mice at 14 weeks of age is indicated by the dotted horizontal lines

Determination of pancreatic proinsulin mRNA

Immediately after dissection, aliquots of pancreas were homogenized at high speed with the Polytron homogenizer in 2.5 ml of 4 mol/l guanidine thiocyanate solution containing 25 µl of 2-mercaptoethanol. RNA was purified by serial ethanol precipitation using the method described previously [9]. Total RNA yield was assessed by measuring optical density at 260 nm, and purity of RNA was estimated from ratios of absorbance at 260 and 280 nm; in all samples the ratios were >2.05. Intactness of RNA was assessed by electrophoresing 5 µg aliquots of total RNA on 1.2% agarose formaldehyde gels as described [9]. Integrity was assessed in stained gels by a ratio of 28S and 18S rRNA bands greater than 1.0 in all samples.

Pancreatic RNA (10 µg) in 15 µl of sterile water containing 6.7% formaldehyde and 10 X SSC was denatured at 50°C for 15 min, and applied directly to nylon membranes (Biotrans, ICN Biomedicals, Inc., Cleveland, Oh, USA) using a 96-well Hybri-Dot apparatus (Bethesda Research Laboratories, Gaithersburg, Md, USA). RNA from all mice was applied to a single membrane in duplicate. Serial dilutions of an unlabelled mouse proinsulin 1 cRNA (synthetic 311 bases) and 20 µg aliquots of mouse liver and kidney RNA were also applied. Blots were air dried and baked at 80°C for 2 h.

Proinsulin mRNA was assessed by its hybridization to the mouse proinsulin I single-stranded cRNA labelled with α-^{32}P-CTP (> 600 Ci/mmole, Amersham Corporation, Arlington Heights, Ill, USA) with a kit according to the manufacturer's protocol (Riboprobe Gemini System, Promega, Madison, Wis, USA). Blots were exposed to Kodak XAR-5 film overnight at −70°C with an intensifying screen (Fig. 1). Densitometric analysis of autoradiographs was made with a Hoefer GS300 scanning densitometer (San Francisco, Calif, USA) utilizing peak height to assess relative intensity. A linear range of intensity was demonstrated among serial dilution of a synthetic mouse proinsulin I cRNA. Comparison of the cRNA standards with mouse total RNA hybridization allowed an estimation of proinsulin mRNA by weight.

Statistical analysis

The SKUMIX model: Consider any quantitative trait, X (such as glucose levels) which is influenced by a single major locus with two alleles, A and a. We denote the mean value of the trait for each of the three possible genotypes AA, Aa and aa, by μ_{AA}, μ_{Aa}, and μ_{aa}, respectively. Assume that there is a random variable, ξ, which is normally distributed with mean 0 and variance E so that for each individual with genotype G,

$X=\mu_G+\xi$.

Under this model, the distribution of X in a population will be a mixture of three normal distributions, one for each genotype, with some overall mean, μ. If the probability of having allele a is q, then the probability of having genotypes AA, Aa, and aa, are $(1-q)^2$, $2q(1-q)$, and q^2, respectively, which correspond to the areas under the three component distributions. Since the *relative* distances between genotypic means are what are important here, it is convenient to reformulate the model in terms of parameters t and d, such that

$\mu_{aa}=\mu_{AA}+t$, and $\mu_{Aa}=\mu_{AA}+dt$.

If there is a "single major gene effect", then the commingled distribution at the population level will be non-normal, have non-zero higher moments and in general be skewed, as t (and/or d) will be non-zero.

To add robustness to the model and to afford some protection against non-genetic sources of skewness, MacLean et al. [13] suggest using a power transformation of X. Under this approach, the commingling analysis is performed on the *transformed* scale, Y, where for constants r and p

$$Y=\begin{cases}\dfrac{r}{p}\left[\left(\dfrac{X}{r}+1\right)^{p}-1\right] & \text{for } p\neq 0\\ r\ln\dfrac{X}{r}+1 & \text{for } p=0\end{cases}$$

$p\to 0$

(Note that this is a continuous function for all p, since the lim of the first line in the definition of Y is the second line.) Therefore, the full three component model has 7 parameters: the overall mean, μ; the within component variance, E; the probability of having the a allele, q; the distances between genotypic means, as governed by t and d, and the skewness transformation parameters, p and r. In our experi-

Table 1. Fed plasma glucoses (PG), fasted plasma glucose, fed plasma insulin, pancreatic insulin and proinsulin (PI) mRNA at 14 weeks of age

	BL/Ks [db/db]		*Backcross [db/db]*	
Fed PG (mmol/l)	34.1 ± 0.7[a]	(12)	31.4 ± 0.6	(31)
Fasted PG (mmol/l)	25.6 ± 0.8[a, c]	(12)	21.6 ± 0.8	(31)
Fed plasma insulin (ng/ml)	8.0 ± 1.4[a, d]	(12)	15.8 ± 1.9	(31)
Pancreatic insulin (mg/pancreas)	2.7 ± 0.5[a, d]	(12)	6.1 ± 1.1	(30)
PI mRNA (ng/pancreas)	2.7 ± 0.4[b, d]	(12)	7.1 ± 1.0	(31)

Each value indicates mean ± SEM. Number of mice are in parentheses. [a] $p<0.01$ and [b] $p<0.001$ vs Backcross mice for t-test. [c] $p<0.05$ and [d] $p<0.01$ vs Backcross mice for variance test

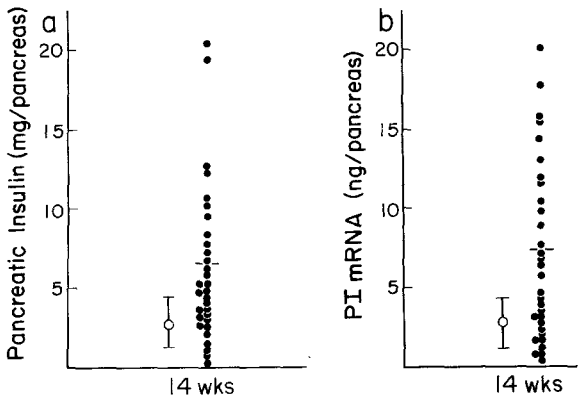

Fig. 3. Pancreatic insulin (**a**) and proinsulin (PI) mRNA (**b**) was determined in *db/db* mice ($n=31$) at 14 weeks of age (closed circles) and compared to that in *db/db* mice of the BL/Ks parental strain ($n=12$, $X \pm S.D.$, open circles), at the same age. The means of values mean in backcrossed mice at 14 weeks of age are represented by the dotted horizontal lines

ment one homozygous distribution is missing by design, but the same model may be used in this case by simply fixing $d=0$, so that only two commingled distributions remain. The model may be fit to the data by the method of maximum likelihood, and nested submodels tested through the likelihood ratio criteria. The data were analysed using this model as implemented in the FORTRAN program SKUMIX [13, 14].

Results

Body weight, plasma glucose, and insulin of the backcrossed db/db mice

F_1 hybrid female (*db/+* and *+/+*) mice from BL/Ks (*db/+*) and 129/J (*+/+*) parents were backcrossed to BL/Ks (*db/+*) male mice and a total of 252 BC male mice were produced. At five weeks of age, 31 of the BC mice were identified as *db/db* which were maintained until 14 weeks of age. The *db/db* mice were readily distinguished from lean littermates (*db/+* or *+/+*) by obesity and their characteristic shape at five weeks of age [1-3]; the body weight of the BC (*db/db*) was significantly higher than that of the lean littermates (26.1 ± 2.8 g and 21.3 ± 1.0 g at 5 weeks of age, mean $\pm$ SEM, $p<0.001$). The changes from 5-14 weeks of age in body weight, fed and fasted plasma glucose, and fed plasma insulin of the BC (*db/db*) mice are shown in Figure 2. All BC (*db/db*) mice showed an increase in body weight (Fig. 2a). The mean body weight of the BC mice at 14 weeks of age was no different from that of *db/db* mice of the BL/KsJ parental strain.

Fed plasma glucose showed a marked elevation from 5 to 14 weeks of age (Fig. 2b); all *db/db* BC became diabetic at 14 weeks of age with fed plasma glucose concentrations > 22 mmol/l. The mean value of the BC *db/db* mice, however, was lower than that of the BL/Ks (*db/db*) mice (Table 1, $p<0.01$).

The fasted plasma glucose and the fed plasma insulin concentrations at 10 or 14 weeks of age ranged in a continuum of values (Fig. 2c and 2d). The fasted plasma glucose at 14 weeks ranged from 13.9 mmol/l to 30.5 mmol/l. Some BC mice were still markedly hyperinsulinaemic relative to the BL/Ks parental strain at 14 weeks. The mean fasted plasma glucose was significantly lower and the mean fed plasma insulin significantly higher in BC (*db/db*) mice compared to that of the BL/Ks (*db/db*) parental mice (Table 1, $p<0.01$ for the fasted plasma glucose, and $p<0.01$ for fed plasma insulin).

Table 2. SKUMIX Analysis

		E	U	T	Q^2	$-2 \ln L + c$	X^{2a}	df	*p* value
Fed	A	0.789	0.064	1.67	0.5	80.92	13.90	3	0.003
PG	B	0.203	0.110	1.38	0.67	67.02			
	C	0.625	0.110	-	-	71.07	4.05	2	N.S.
Fasted	A	0.666	0.082	2.03	0.5	93.90	8.42	3	0.038
PG	B	0.643	−0.00	2.07	85.48				
	C	0.969	0.00	-	-	86.96	1.48	2	N.S.
Plasma	A	0.377	0.037	1.54	0.5	88.44	1.95	3	0.583
insulin	B	0.618	−0.001	1.332	0.27	86.49			
	C	0.969	0.00	-	-	86.59	0.10	2	N.S.
Pancreatic	A	0.202	0.184	1.152	0.5	113.11	40.76	3	7×10^{-6}
insulin	B	0.402	0.000	3.001	0.07	72.35			
	C	0.468	0.000	-	-	84.12	11.77	2	0.002
PI mRNA	A	0.202	0.145	1.630	0.5	94.65	10.03	3	0.018
	B	0.549	0.000	1.814	0.15	84.62			
	C	0.469	0.000	-	-	86.96	2.34	2	N.S.

A general transformation power (P) which allows that some levels of skewness could not be differentiated from 1 (normal distribution). A: Parameters were fitted by the method of maximum likelihood for the expected single gene model (2 distributions) using information from the BL/KsJ (*db/db*) parental strain. B: Parameters for the general model (2 distributions) estimating the backcross (*db/db*) mice data alone. C: Parameters for a polygenic model (unimodal distribution). [a] X^2 = difference between A ($-2 \ln L + c$) and B ($-2 \ln L + c$), or B ($-2 \ln L + c$) and C ($-2 \ln L + c$)
PG = plasma glucose

Pancreatic insulin and proinsulin mRNA of the backcross mice (db/db)

The BC (*db/db*) mice were killed at 14 weeks, since a previous study [9] demonstrated a severe reduction in proinsulin mRNA in BL/Ks (*db/db*) mice at 12 weeks of age, and the diabetes was so severe that some of the mice died. As shown in Figure 3a, pancreatic insulin of the BC (*db/db*) mice at 14 weeks ranged in a continuum of values. Total pancreatic proinsulin mRNA content in each *db/db* mouse also ranged widely from 0.5 ng–20 ng (Fig. 1 and 3b). A considerable number of mice showed a markedly higher insulin value or proinsulin mRNA content compared with that of *db/db* mice of the parental BL/Ks strain.

Estimation of the number of the genes determining the expression of diabetes

In an attempt to use all of the information available in the experiment, the BC *db/db* data was tested to determine whether it was significantly better explained as a bimodal mixture of two distributions (as expected there was a single gene control) or whether a single, unimodal, possibly skewed distribution would fit the data better (as expected if the phenotype were under polygenic control or a single dominant gene). The program used was SKUMIX (see Methods), and the results of this analysis are shown in Table 2 and Figure 4. Five parameters are fit by the method of maximum likelihood: the overall mean (U) of the mixed distribution, the variance (E) within each component distribution, the distance (T) between the means of the two distributions, the proportion of data in the rightmost distribution (Q^2), and a power transformation parameter (P) which allows some levels of skewness. Finally for each model, -2 times the log-likelihood (-2 ln L+c) is given, and by the likelihood ratio test we can contrast nested hypothesis. There are two major hypotheses to test.

The first test is whether two distributions fit the data significantly better than a single distribution. Rejection of a single distribution would be evidence against polygenic (and/or a dominant major gene) control. The second test is whether the parameters of two distributions are consistent with that predicted under a single gene model. Rejection here would argue against just one other locus besides the *db* locus. For the latter test, there are two approaches which could be taken. First, any information on the BL/Ks (*db/db*) mice can be ignored, and the distribution of the phenotype in the backcross mice alone simply used. In that case, if there is only one more locus, the two distributions would have equal proportions, so that $Q^2=1/2$. A two-distributional model with Q^2 fixed to 0.5 could then be tested, against the alternative of a general two distribution one. This would provide a X^2 with one degree of

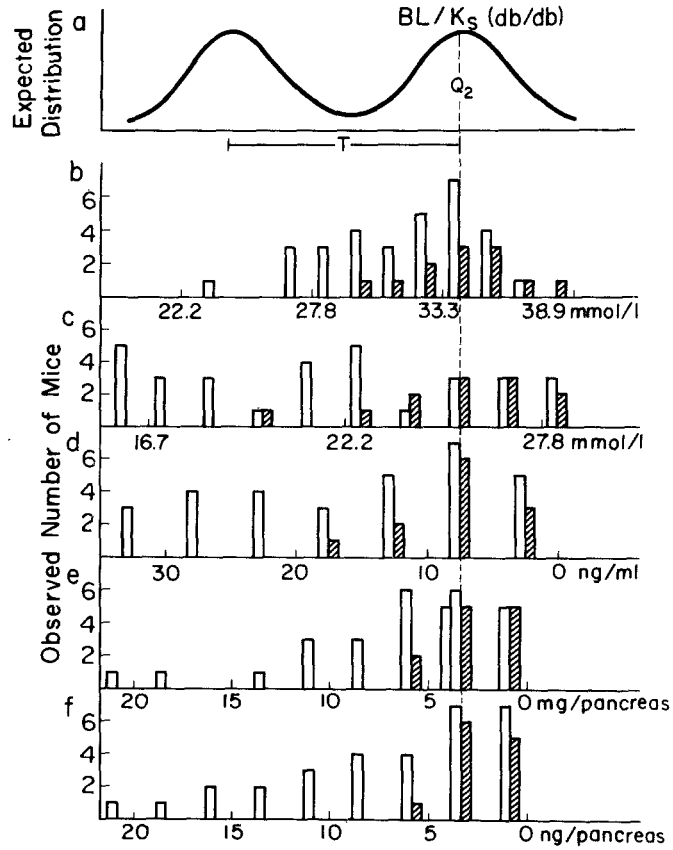

Fig. 4. The expected distribution (**a**) predicted from the SKUMIX analysis (Table 2), and the observed distributions of fed plasma glucose (**b**), fasted plasma glucose (**c**), fed plasma insulin (**d**), pancreatic insulin (**e**), and proinsulin mRNA (**f**) in *db/db* mice of the parental BL/Ks strain (hatched bars) compared to that in the *db/db* BC mice (open bars). For each histogram the dotted vertical line represents the median value of *db/db* mice of the parental BL/Ks strain

freedom. On the other hand, the BL/Ks (*db/db*) distribution could be used as well. If the distribution of the BC (*db/db*) mice is standardised to mean 0 and variance 1, then since the overall mean is the sum of the individual distribution means weighted by the proportion of area under each curve, under a single gene model, $0=(\mu_1+\mu_2)/2$, so that $\mu_1=\mu_2$. If the BL/Ks (*db/db*) data is standardized to this same mean and variance, then the expected distance between the two means of the backcross (*db/db*) data is twice the BL/Ks (*db/db*) mean. For that matter, the variance within each of the component distributions for the BC (*db/db*) should be the same as the variance of the BL/Ks (*db/db*) on this scale (Fig. 4a). Table 2 shows SKUMIX parameters in the presence of information on the BL/Ks (*db/db*). A two-distributional model of each phenotype was rejected with a high probability except for the phenotype for plasma insulin level, regardless of the presence or absence of the information for the BL/Ks (*db/db*) data (compare lines A and B, Table 2). On the other hand, a single distribution of all phenotypes including plasma insulin in the BC (*db/db*) mice could not be rejected (line C, Table 2). These results provide strong evidence

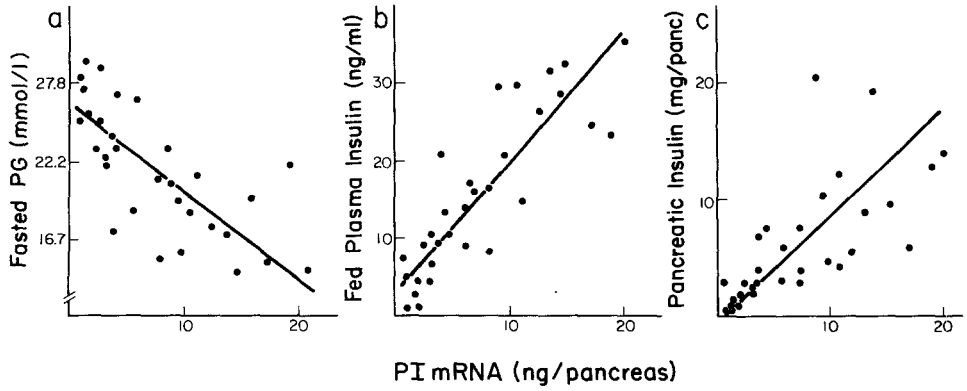

Fig. 5. Correlation between pancreatic proinsulin (PI) mRNA and fasted plasma glucose (**a**), fed plasma insulin (**b**), and pancreatic insulin (**c**) in 31 BC *db/db* mice at 14 weeks of age

to support the hypothesis of control by multiple genes or by a dominant single major gene. If a single dominant gene determined the phenotypes observed, distribution of each phenotype in the BC (*db/db*) mice must be consistent with that in the BL/Ks (*db/db*) mice. As shown in Figure 4 and Table 1, however, the distribution pattern of each phenotype in the BC mice was significantly different from that in *db/db* mice of the BL/Ks parental strain, rejecting a single dominant major gene hypothesis.

Our results concluded that the diabetes phenotype associated with this *db* mutation is under polygenic control in the BL/Ks mice. In addition, a marked hyperglycaemia (> 22 mmol/l) in all BC (*db/db*) mice in the fed state also suggested the presence of a dominant factor(s) in the BL/KsJ background.

Discussion

This study looked at the expression of diabetes induced by the *db* mutation after generating F_1 hybrid mice from a diabetes susceptible (BL/Ks) and diabetes resistant (129/J) strain, and backcrossing the F_1 hybrids to the diabetes susceptible strain. The recent finding of high blood glucose in all F_1 and F_2 progeny of BL/Ks and BL/6 crosses (12) suggested that either the genes controlling diabetes susceptibility in the BL/Ks strain had become dominant to those in BL/6 mice, or that the BL/6 strain had become less diabetes resistant. We had recently observed higher blood glucose in BL/6 *db/db* males at one month of age (indistinguishable from that of BL/Ks *db/db* males) than previously reported [15], further suggesting that the BL/6 strain had become less diabetes resistant. The effect of the *db* mutation in 129/J mice has been extensively evaluated [7, 8]. The 129/J *db/db* males were found to be hyperphagic, obese, and severely hyperinsulinaemic, with plasma glucose peaking at 2–4 months of age at approximately 15 mmol/l. Histologic examination of pancreata from the 129/J *db/db* mice revealed massive hyperplasia of pancreatic B-cells, but no B-cell necrosis or atrophy. The degree of hyperplasia in the islets was more extensive than had been observed in other *db* carrying stocks, and this strain appeared to be well suited for evaluating the diabetes susceptible background modifiers in the BL/Ks strain.

For genetic analysis, knowing that the *db* mutation is necessary, but not sufficient, to induce obesity and diabetes, we examined the possibility of exactly one other modifying gene in linkage disequilibrium with the *db* locus which works together to develop diabetes. Now each parental strain is assumed to be homozygous at each locus. If the BL/Ks (*db/+*) is "aa" at this other locus, then the 129/J (+/+) must be "AA". The F_1 generation are half *db/+* and half +/+, and all heterozygous at the A locus. Backcrossing the female F_1 with the male BL/Ks (*db/+*), and selecting out the BA *db/db*, we expect half to be Aa and half to be aa. If "a" is dominant for the trait of interest, we would not be able to tell phenotypically between the genotypes Aa and aa, and there would be a single unimodal distribution. If "a" is recessive, or at least far from dominant, then this would produce a mixture of two distributions, creating a bimodal distribution of the phenotype in the 31 *db/db* backcross mice with one mode near each parental mean. At the other extreme, if diabetes is under polygenic control, then in this BC *db/db* progeny there would be a single unimodal distribution for the phenotype as well as in a single dominant gene hypothesis. In a classic BC experiment, where we look for a single gene only, we would also have the phenotype measured independently on the parental strains, which under the single gene model would have the same relevant genotypes. We could use this independent information in addition to the bimodality in the BC mice data to test the single hypothesis. In this experiment, however, since we are dealing with the *db* locus as well as the A locus, we would need to have information on the *db/db*-aa *and db/db*-Aa genotypes. The BL/Ks strain homozygous for *db* locus exists and is *db/db*-aa genotype. The *db* mutation is no longer maintained on the 129/J background, however, so it was necessary to breed BL/Ks (*db/+*) with 129/J (+/+) mice, and so no F_1 generation homozygous for the *db* mutation (*db/db*-Aa) mice could be obtained. Therefore, we can only get independent information on the phenotype distribution of one of the two relevant genotypes. Even in the absence

of F_1 *db/db* data however, we have tested whether two distributions fit the data significantly better than a single distribution for the various parameters measured in the BC mice (Table 2). Either using the distribution of the parameters in *db/db* mice of the parental strain BL/Ks, or simply using the distribution of the phenotype in the BC mice alone, all of the parameters fit a unimodal distribution better than a bimodal, and thus the evidence strongly favours multiple genes involved in diabetes susceptibility.

The genes involved in diabetes susceptibility in BL/Ks mice are unknown. Recently responses to dexamethasone administration in *db/db* BL/Ks and BL/6 mice [15] suggested greater insulin resistance in BL/Ks mice, In addition, dexamethasone failed to induce proinsulin mRNA in the BL/Ks *db/db* mice suggesting that a limitation of insulin production at the level of insulin synthesis might explain the enhanced diabetes susceptibility of this strain. Type C retrovirus induction in pancreatic B-cells of some *db*-sensitive strains has been noted [16, 17], while other studies have suggested differences in immune responsiveness [18–20]. One study noted a limited capacity for B-cell DNA replication [21]. Finally, when Leiter crossed obesity-induced diabetes susceptible BL/Ks mice with NOD mice which develop spontaneous autoimmune diabetes, none of the offspring developed diabetes, thus indicating that different genes are responsible for the two types of diabetes [22].

Background modifying genes in the *db* model have been shown to be strongly gender related, as the diabetes syndrome occurs only in males in some *db* congenic stocks, and can be prevented by female sex steroids [7, 22]. Leiter has accumulated evidence suggesting that the sex differences are produced by sex-linked differences in the metabolism of androgens and their precursors in these inbred strains. The BL/Ks mice appear to be more sensitive than the BL/6 mice to endogenous androgens. The current study avoided this gender related genetic contribution by evaluating only male BC mice with Y-chromosomes from the BL/Ks parental strain.

As seen in the current analysis, previous studies had suggested that multiple genes determine expression of diabetes in BL/Ks mice with obesity induced by the *db* mutation. The initial study of BL/Ks and BL/6 BC mice [11] failed to show a clear segregation of glucose, insulin, or islet histology into F_1-like and BL/Ks-like, but observations were pooled on 10 male and 10 female BC mice. In the more recent study [12] there was no segregation of fed plasma glucose in the F_2 generation, and diabetes susceptible genes in BL/Ks mice were dominant to resistance genes in BL/6 mice. The current analysis in BL/Ks and 129/J mice clearly indicated that diabetes susceptible genes in BL/Ks mice are dominant to resistant genes in the 129/J strain as well. The current study further extends the previous observations. While fed plasma glucoses in all of the BC animals were clearly in the diabetic range (> 22 mmol/l), the severity of diabetes in these mice varied, as determined by the spectrum of fasting plasma glucose, fed plasma insulin, pancreatic insulin content, and proinsulin mRNA. That proinsulin mRNA content, previously shown to reflect the degree of insulin synthesis in vivo [23], is a good indicator of the severity of diabetes and degree of islet dysfunction, is reflected in the inverse correlation (Fig. 5 A) between proinsulin mRNA and fasting plasma glucose ($r = 0.69$, $p < 0.001$), and the direct correlation (Fig. 5 b) between proinsulin mRNA and fed plasma insulin ($r = 0.86$, $p < 0.001$), and (Fig. 5 c) pancreatic insulin content ($r = 0.61$, $p < 0.01$). Using proinsulin mRNA as one measure of functional B-cell mass, the distribution of values in the BC mice could not be accounted for by a single gene controlling islet destruction.

It was initially anticipated that if a single gene were responsible for severe islet destruction, one-half of the BC mice would develop low proinsulin mRNA levels like the BL/Ks parent, and one-half would be resistant to islet destruction. We could then look for linkage of the locus responsible for islet necrosis in the BC mice and attempt to define the locus by the process of reverse genetics. Finding that allelic differences at more than a single locus are responsible for this islet destruction makes this type of analysis far more difficult. The implications of these findings in closely related species of mice, for analysis of genetic susceptibility to obesity-induced diabetes in man, is obvious.

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