Brief Genetics Report

Evidence That Insulin is Imprinted in the Human Yolk Sac

Gudrun E. Moore, Sayeda N. Abu-Amero, Gill Bell, Emma L. Wakeling, Amanda Kingsnorth, Philip Stanier, Eric Jauniaux, and Simon T. Bennett

Allelic variation in the size of the insulin (*INS*) variable number tandem repeat (VNTR) correlates with the expression of both *INS* in the pancreas and thymus and *IGF2* (the gene downstream of *INS*) in the placenta. In addition, the shorter, class I alleles are associated with type 1 diabetes, whereas the longer, class III alleles are associated with type 2 diabetes, polycystic ovary syndrome (PCOS), and size at birth. Parent-of-origin effects have been reported for type 2 diabetes and PCOS, thus implicating a role for genomic imprinting in these phenotypes. In mice, *Ins2* is imprinted and paternally expressed in the yolk sac. In humans, evidence for the imprinting of *INS* is circumstantial, with occasional monoallelic expression in the thymus. In the present study, we found evidence for the imprinted paternal expression of *INS* in the human yolk sac. Two other imprinted genes from the same cluster are also expressed monoallelically in the human yolk sac. *IGF2* was expressed solely from the paternal allele, and *H19* was expressed solely from the maternal allele. These data suggest not only further functional roles for the human yolk sac in early fetal growth, but also evidence for a potential causal link between the control of insulin expression during development and insulin/growth-related diseases in later life. *Diabetes* 50:199–203, 2001

Allelic variation at the insulin (*INS*) variable number tandem repeat (VNTR) is known to regulate the level of expression of both *INS* in the pancreas and thymus and *IGF2* in the placenta (1–5). The shorter, class I alleles correlate with levels of gene expression that are higher for *INS* in the pancreas, lower for *INS* in the thymus, and higher for *IGF2* in the placenta. Higher levels of *INS* expression associated with the longer, class III VNTR alleles in the thymus offer an explanation for the protective role of these alleles in type 1 diabetes susceptibility by inducing self-tolerance to preproinsulin peptides (1,2). The class I alleles are associated with type 1 diabetes, and the class III alleles are associated with type 2 diabetes, polycystic ovary syndrome (PCOS), and size at birth (5–10). In PCOS and type 2 diabetes, disease susceptibility is conferred in a parental sex-specific fashion (6,9), such that in nuclear families, the susceptible allele is transmitted preferentially from fathers. This suggests that the etiological effect is occurring when only the paternal allele is active, implicative of parental imprinting. Although the imprinting of *IGF2* is well established (11), evidence for monoallelic expression of insulin comes only from mice, in which *Ins2* (the mouse ortholog of *INS*) is imprinted in the yolk sac after 14.5 days (12,13). In mammals, the secondary yolk sac is regarded as being the primitive liver, involved in protein synthesis and nutrient transfer and acting as a stem-cell reservoir (14,15). The secondary yolk sac may also play a role as a primitive pancreas, because in the fourth week of development, the yolk sac forms the primitive gut, the endoderm of which gives rise to the epithelium and glands of the digestive tract.

In humans, there is only circumstantial evidence for the imprinting of insulin (e.g., the parent-of-origin effects and 5 of 23 cases of monoallelic expression in the thymus) (1,2). However, *INS* lies next to the paternally expressed *IGF2* gene and the maternally expressed *H19* gene in an imprinted cluster on human chromosome 11p15.5 (11,16). Therefore, we set out to test for *INS* expression in the human yolk sac and investigate whether it is imprinted. We present evidence that, similar to *IGF2* and *H19*, *INS* is expressed monoallelically and imprinted in the human yolk sac.

To investigate gene expression and imprinting status in yolk sacs, a maternal blood sample, a placental sample, and the yolk sac were collected from 38 subjects. The blood and placental samples were used to provide genomic DNA for genotyping, whereas the yolk sacs were used to extract total RNA. Each fetus was genotyped by polymerase chain reaction (PCR) for *INS*, *IGF2*, and *H19*, and the products were digested with the *Pst*I, *Apa*I, and *Rsa*I restriction enzymes, which are intragenic polymorphisms for *INS*, *IGF2*, and *H19*, respectively (Figs. 1–3) (4,11,16). This revealed 14 fetal samples that were heterozygous for *INS*, 20 for *IGF2*, and 14 for *H19* (Table 1). Once fetal heterozygosity was established, the respective maternal DNA

From the Molecular Biology Laboratory for Fetal Development (G.E.M., S.N.A.-A., G.B., E.L.W., P.S.), Division of Paediatrics, Obstetrics and Gynaecology, Queen Charlotte's and Chelsea Hospital, Imperial College of Science, Technology and Medicine; the Academic Division of Obstetrics and Gynaecology (E.J.), Royal Free and University College Medical School, University College, London; the Department of Medical Genetics (A.K.), Wellcome Trust Centre of Molecular Mechanisms in Disease, CIMR, University of Cambridge, Cambridge; and the Oxagen Limited (S.T.B.), Abingdon, Oxon, U.K.

Address correspondence and reprint requests to Dr. Gudrun Moore, Molecular Biology Laboratory for Fetal Development, Division of Paediatrics, Obstetrics and Gynaecology, Queen Charlotte's and Chelsea Hospital, Imperial College of Science, Technology and Medicine, Goldhawk Rd., London W6 OXG, U.K. E-mail:gemoore@ic.ac.uk.

Received for publication 19 April 2000 and accepted in revised form 25 September 2000.

PCOS, polycystic ovary syndrome; PCR, polymerase chain reaction; RT, reverse transcriptase; VNTR, variable number tandem repeat.

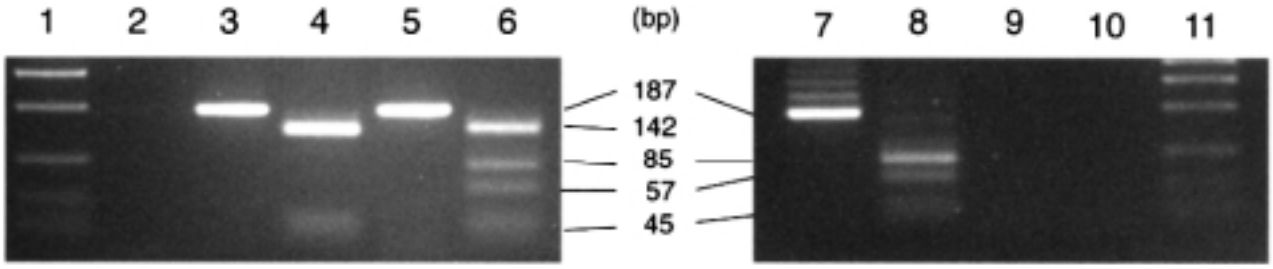

FIG. 1. Expression and imprinting of *INS* in human yolk sacs determined by 2% agarose gel electrophoresis. *Lane 1*, 100-bp ladder; *lane 2*, PCR blank; *lane 3*, undigested maternal genomic DNA; *lane 4*, maternal genomic DNA digested with *Pst*I (homozygous for the absence of the polymorphic site); *lane 5*, undigested fetal genomic DNA; *lane 6*, fetal genomic DNA digested with *Pst*I (heterozygous for the polymorphic site); *lane 7*, undigested yolk sac cDNA; *lane 8*, yolk sac cDNA digested with *Pst*I (only the paternally inherited bands of 85 and 57 bp are present); *lane 9*, RT-negative; *lane 10*, PCR blank; and *lane 11*, 100-bp ladder.

samples were analyzed for informativity in a similar manner. The criteria for a fully informative sample set for assessing imprinting status was a heterozygous fetal sample and a homozygous maternal sample. This would allow mono- or biallelic expression to be detected and the parental origin of the alleles to be determined. Of the heterozygous fetal DNA samples, five of the maternal samples were homozygous for *INS*, five for *IGF2*, and three for *H19*. Expression was investigated in all 38 yolk sacs, with gestational ages ranging from 6 weeks plus 5 days to 12 weeks plus 3 days (Table 1).

INS expression was detected in 16 of the 38 yolk sacs and expression was not seen before 8 weeks plus 4 days gestation. Between 8 weeks plus 4 days and 10 weeks gestation, 31% of the yolks sacs showed expression, whereas from 10 weeks to 12 weeks plus 3 days gestation, 65% expressed detectable levels of *INS*. The level of expression was ~1/5,000–1/10,000 of that seen in pancreas, but it was probably lower than the levels seen in the thymus because nested PCR was needed to visualize expression in the yolk sac (1,2). Both of the informative yolk sacs that expressed *INS* displayed only monoallelic paternal expression (Fig. 1). Monoallelic expression of *INS* was also seen in four other heterozygous yolk sacs, but these were not informative for parent-of-origin. It is also interesting to note that both informative yolk sac samples, based on linkage disequilibrium with the *Pst*I genotype, inherited the class III *INS* VNTR on the paternal chromosome. For the other four heterozygous samples, two alleles were class I and two were class III. The sample size is obviously not large enough to make any predictions about expression levels and class size association.

Monoallelic expression for *IGF2* was demonstrated in 18 yolk sacs and, within this group, all three fully informative sets showed monoallelic paternal expression (Fig. 2). The other two fully informative sets showed biallelic expression, giving further evidence of polymorphic expression for this gene.

H19 expression was also seen in all 38 yolk sacs. Monoallelic expression for *H19* was shown in 14 yolk sacs, with monoallelic maternal expression detected in three fully informative sets (Fig. 3). The difference in size between the *H19* reverse transcriptase (RT)-PCR product and the genomic PCR product is a result of the absence of two small introns in the yolk sac cDNA (17). This was confirmed by DNA sequencing (data not shown).

The class size of the *INS* VNTR has been associated with several phenotypes (6), most notably type 1 and 2 diabetes, PCOS, and size at birth (5–10). All of these phenotypes could be caused in part by an early developmental imbalance in the levels of insulin and other growth factors (e.g., IGF-2) that have an effect on fetal size, future insulin tolerance, or pancreatic development. The *INS* VNTR class size has also been shown to have a direct effect on the expression levels of *INS* and *IGF2* in relevant tissues (e.g., the pancreas, thymus, and placenta) (1–5). The early embryo depends on biological substrates from maternal tissue. These substrates cross the trophoblastic membrane and reach the embryo indirectly via the extracelomic cavity and the secondary yolk sac (15). We have shown that the secondary yolk sac is actively transcribing *INS*, *IGF2*, and *H19* mRNA. Although methylation analysis to further validate the existence of imprinting

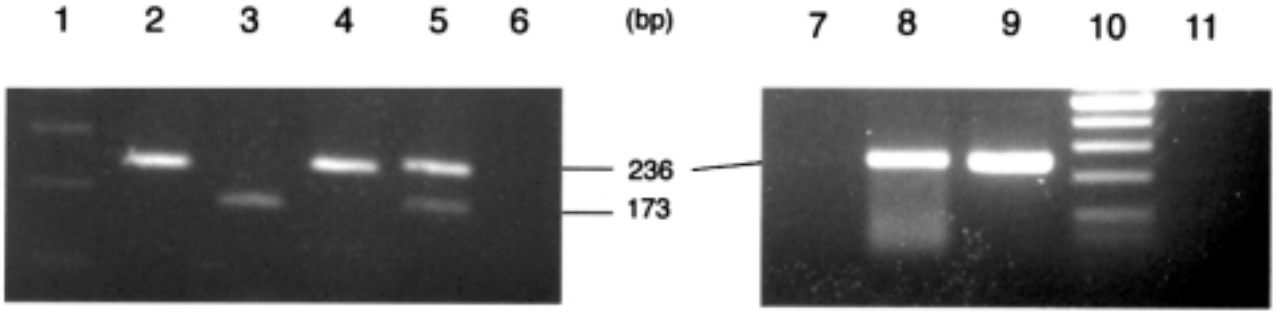

FIG. 2. Expression and imprinting of *IGF2* in human yolk sacs determined by 2% agarose gel electrophoresis. *Lane 1*, 100-bp ladder; *lane 2*, undigested maternal genomic DNA; *lane 3*, maternal genomic DNA digested with *Apa*I (homozygous for the polymorphic site); *lane 4*, undigested fetal genomic DNA; *lane 5*, fetal genomic DNA digested with *Apa*I (heterozygous for the polymorphic site); *lane 6*, PCR blank; *lane 7*, RT-negative; *lane 8*, undigested yolk sac cDNA; *lane 9*, yolk sac cDNA digested with *Apa*I (only the paternally inherited 236-bp band is present); *lane 10*, 100-bp ladder; and *lane 11*, PCR blank.

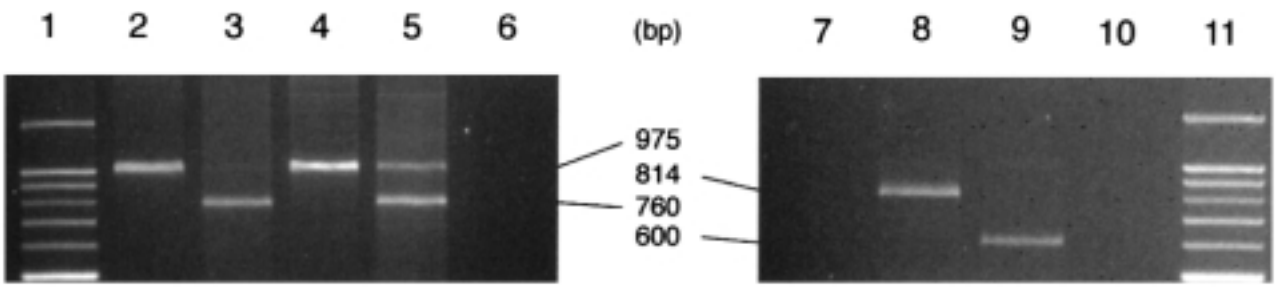

FIG. 3. Expression and imprinting of *H19* in human yolk sacs determined by 2% agarose gel electrophoresis. *Lane 1*, 100-bp ladder; *lane 2*, undigested maternal genomic DNA; *lane 3*, maternal genomic DNA digested with *Rsa*I (homozygous for the polymorphic site); *lane 4*, undigested fetal genomic DNA; *lane 5*, fetal genomic DNA digested with *Rsa*I (heterozygous for the polymorphic site); *lane 6*, PCR blank; *lane 7*, RT-negative; *lane 8*, undigested yolk sac cDNA; *lane 9*, yolk sac cDNA digested with *Rsa*I (only the maternally inherited 600-bp band is present); *lane 10*, PCR blank; and *lane 11*, 100-bp ladder.

was not technically feasible in our samples, our data provide evidence that *INS*, *IGF2*, and *H19* are all imprinted in human yolk sacs. Imprinting affected the maternal chromosome at the *INS* and *IGF2* loci, whereas the paternal chromosome was affected at the *H19* locus. However, we could not assess whether imprinting at the different loci we examined can affect opposite parental chromosomes in the same individual. Very little work has been reported on human yolk sac mRNA expression, and only two reports have been published on mouse yolk sacs detailing *Ins1* and *Ins2* expression (12,13). Considering the relatively short life span of the yolk sac in pregnancy, the complexity and maintenance of imprinting may appear superfluous. However, owing to the yolk sac's pivotal role as a substitute for the liver in early embryogenesis and its role in early nutrient transfer, regulated expression of crucial growth factors must be important to its own maintenance and could potentially influence control of fetal development and size. In the case of both *INS* and *IGF2*, expression is under the control of the *INS* VNTR (1–5). There is also the implication that these genes may be effectors of disease predisposition. This theory is greatly enhanced by newly emerging data on parent-of-origin effects that show the paternal transmission of the *INS* VNTR class III alleles is associated with both PCOS (9) and type 2 diabetes (17); there is also new data on the association of the *INS* VNTR with birth size (10). Moreover, a recent study demonstrated an under-representation of the class III *INS-IGF2* VNTR in the healthy offspring of heterozygous parents (18). Survival selection driven by variations of critical gene expression in the yolk sac could help explain this transmission-ratio distortion.

RESEARCH DESIGN AND METHODS

Samples. We studied 38 human yolk sacs, ranging from 6 weeks plus 5 days to 12 weeks plus 3 days gestation, which were collected at surgical termination for psychological reasons and stored at –80°C. Matched maternal blood samples, placentas, and yolk sacs were available for 31 sample sets, whereas only placentas and yolk sacs were available for the remaining 7 sets. Ethical approval for the collection of these samples was given by the Joint University College London/University College London Hospital Committee on the Ethics of Human Research (project no. 2538).

Extraction of DNA and RNA from the sample sets. DNA was extracted from maternal blood samples and fetal pancreatic and placental tissues by adapted standard methods (19). Total RNA was extracted from the yolk sacs using the standard guanidinium technique (20). Synthesis of cDNA from human yolk sac RNAs followed standard procedures using 0.2 µg random primers. All of the cDNAs from the yolk sacs were checked for integrity using control primers for *GADPH* (data not shown).

PCR detection of INS, IGF2, and H19. The sequence of the primers and PCR conditions were as follows: *INS* genomic DNA typing, forward primer TGGTGCAGGCAGCCTGCAG and reverse primer GTTCAAGGGCTTTATTC CATCTCTC, at conditions of 94°C for 4 min for 1 cycle; 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min for 35 cycles; and 72°C for 4 min for 1 cycle. The product size was 187 bp.

Fetal yolk sac cDNA typing, forward primer. The forward primer GAAGAG GCCATCAAGCAGATCACTG was used with the same reverse primer detailed above. Because of the extremely low copy number of insulin in the yolk sac, cDNA heminested PCR was carried out using primers that crossed two introns to avoid genomic contamination (4). The conditions were: 94°C for 4 min for 1 cycle; 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min for 35 cycles; and 72°C for 4 min for 1 cycle. The second-round nesting used the same primers as those used for the genomic DNA typing, but with only 25 cycles.

***IGF2*.** For both genomic DNA and yolk sac cDNA typing, the forward primer CTTGGACTTTGAGTCAAATTGG and reverse primer CCTCCTTTGGTCT TACTGGG were used.

Conditions. The conditions were: 94°C for 4 min for 1 cycle; 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 35 cycles; and 72°C for 4 min for 1 cycle. The product size was 236 bp (11). The primers for *IGF2* did not span an intron. Genomic contamination was ruled out by including a RT-negative sample in each PCR set as a control.

***H19*.** For both genomic DNA and yolk sac cDNA typing, the forward primer AACACCTTAGGCTGGTGG and the reverse primer GCTGAAGCCCTGGTGGG were used. The conditions were: 94°C for 4 min for 1 cycle; 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min for 35 cycles; and 72°C for 4 min for 1 cycle. The product size was 975 bp for the genomic product and 814 bp for the yolk sac cDNA (17). All PCRs were performed in 25-µl reaction volumes containing 67 mmol/l Tris-HCl, pH 8.8, 16.6 mmol/l $(NH_4)_2SO_4$, 1.5 mmol/l $MgCl_2$, 0.1% Tween-20, 100 ng of each primer, and 1 U Taq polymerase (Bioline, London, U.K.). Each PCR was cycled in an Omnigene thermal cycler (Hybaid, Ashford, U.K.).

Genotyping by restriction enzyme digestion of PCR products. Genotyping of maternal and fetal samples was necessary to find informative sample sets (e.g., a heterozygous fetus and a homozygous mother allows demonstration of imprinting as well as the parent of origin of the expressed fetal allele). When both maternal and fetal samples were heterozygous, monoallelic status was assigned if the fetal yolk sac showed expression of only one allele. A total of 17 µl PCR product was digested with 10 U enzyme (Promega, Southampton, U.K.) in a final volume of 20 µl. The PCR fragments were then analyzed by agarose gel electrophoresis.

The polymorphism used for *INS* altered a *Pst*I site where digestion of a 187-bp PCR product results in polymorphic fragments of 85 and 57 bp and constants of 142 and 45 bp (4). The polymorphism used for *IGF2* was at an *Apa*I restriction site where digestion cleaves the 236-bp PCR product into a 173- and 63-bp product (11). For the *H19* gene, the polymorphic *Rsa*I restriction site was used. In the yolk sac cDNA, *Rsa*I digests the 814-bp PCR product into 600-bp and 214-bp fragments, whereas in the genomic DNA, PCR products of 975 bp are cleaved into fragments of 760 and 215 bp (16). PCR products were electrophoresed on 2% agarose gels stained with 10 µg/ml ethidium bromide.

ACKNOWLEDGMENTS

S.N.A.-A. was a Dunhill Medical Trust Research Fellow and E.L.W. was an Action Research Fellow. The majority of the genotyping for the *INS* polymorphism was carried out in John

TABLE 1
Alleles plus imprinting status for *IGF2*, *H19*, and *INS* in 38 fetal yolk sacs

Sample no.	Ethnicity	Gestational age	*IGF2*				*H19*				*INS*			
			Mother	Baby	Yolk sac	Imprint status	Mother	Baby	Yolk sac	Imprint status	Mother	Baby	Yolk sac	Imprint status
1	AC	6+5	NA	1,2	2	MO	NA	2,2	2	NI	NA	1,1	NE	—
2	C	7+1	1,2	1,2	1	MO	1,2	1,1	1	NI	1,2	1,2	NE	—
3		7+5	2,2	2,2	2	NI	1,2	1,2	2	MO	F	F	NE	—
4	C	7+6	2,2	2,2	2	NI	2,2	2,2	2	NI	1,2	1,2	NE	—
5		8+1	1,2	1,2	2	MO	1,2	1,2	1	MO	F	F	NE	—
6	C	8+4	1,2	2,2	2	NI	1,1	1,1	1	NI	1,1	1,1	1	NI
7	C	8+6	1,1	1,2	2	I/P	1,1	1,1	1	NI	1,2	1,2	NE	—
8	AS	8+6	NA	1,2	2	MO	NA	1,2	2	MO	NA	1,1	NE	—
9	C	9	NA	1,1	1	NI	NA	1,2	2	MO	NA	1,1	1	NI
10	C	9	1,2	1,2	2	MO	1,2	2,2	2	NI	1,1	1,2	2	I/P
11	C	9+1	1,2	1,2	2	MO	2,2	1,2	2	I/M	1,2	1,2	NE	—
12	C	9+1	1,1	1,2	2	I/P	1,2	1,1	1	NI	1,1	1,1	NE	—
13	C	9+1	2,2	2,2	2	NI	2,2	1,2	2	I/M	1,2	1,1	NE	—
14	C	9+1	1,2	1,2	2	MO	1,2	1,2	1	MO	1,1	1,1	1	NI
15	AS	9+2	NA	2,2	2	NI	NA	2,2	2	NI	NA	1,1	NE	—
16	C	9+2	NA	1,2	2	MO	NA	2,2	2	NI	NA	1,1	NE	—
17	AS	9+2	1,2	1,1	1	NI	1,1	1,1	1	NI	1,1	1,2	NE	—
18	C	9+2	2,2	2,2	2	NI	2,2	2,2	2	NI	1,1	1,1	NE	—
19	C	9+2	1,2	1,2	2	MO	1,2	1,2	1	MO	1,1	1,2	NE	—
20	C	9+5	2,2	1,2	1,2	BA	2,2	2,2	2	NI	1,2	1,2	1	MO
21	C	9+6	1,1	1,1	1	NI	2,2	1,2	2	I/M	F	1,1	NE	—
22	C	10	NA	1,2	2	MO	NA	1,2	1	MO	NA	1,1	1	NI
23	C	10	1,2	2,2	2	NI	2,2	2,2	2	NI	1,1	1,2	2	I/P
24	C	10+1	2,2	2,2	2	NI	2,2	2,2	2	NI	1,1	1,1	1	NI
25	C	10+1	1,2	2,2	2	NI	1,2	2,2	2	NI	1,1	1,1	NE	—
26	C	10+1	1,2	2,2	2	NI	2,2	2,2	2	NI	1,2	1,2	2	MO
27	C	10+3	NA	1,2	2	MO	NA	1,2	2	MO	NA	1,2	NE	—
28	C	10+6	1,2	1,2	2	MO	2,2	2,2	2	NI	1,1	1,1	NE	—
29	C	10+6	1,2	2,2	2	NI	1,2	1,2	2	MO	1,2	1,1	1	NI
30	C	10+6	1,2	2,2	2	NI	2,2	2,2	2	NI	1,1	1,1	NE	—
31	C	11	1,1	1,2	1,2	BA	1,2	2,2	2	NI	1,1	1,1	NE	—
32	C	11	2,2	2,2	2	NI	1,2	2,2	2	NI	1,1	1,1	1	NI
33	C	11+2	1,2	1,1	1	NI	1,2	1,2	2	MO	1,2	1,1	1	NI
34	AF	11+4	2,2	1,2	1	I/P	2,2	2,2	2	NI	1,2	1,2	1	MO
35	C	12	1,2	1,2	1	MO	1,2	1,2	1	MO	1,1	1,2	NE	—
36	AF	12	1,2	2,2	2	NI	1,2	2,2	2	NI	1,2	1,2	2	MO
37	AS	12	1,2	1,2	1	MO	2,2	2,2	2	NI	1,1	F	1	NI
38	C	12+3	1,2	1,2	2	MO	1,2	1,1	1	NI	2,2	F	2	NI

Data for gestational age are expressed as weeks + days. AC, Afro-Caribbean; AF, African; AS, Asian; BA, biallelic expression; C, Caucasian; F, PCR failure; I, imprinted; M, maternally expressed; M, mother; MO, monoallelic but assignment of allele not possible; NA, DNA not available; NE, no expression; NI, noninformative; P, paternally expressed.

Todd's Wellcome Trust/Medical Research Council laboratory in the Cambridge Institute of Medical Research, Cambridge, U.K.

We are also grateful to Megan Hitchins for sequencing genomic and yolk sac *H19* PCR products. We would also like to thank Mark McCarthy for critical reading of the manuscript.

REFERENCES

1. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C: Insulin expression in human thymus is modulated by *INS* VNTR alleles at the IDDM2 locus. *Nat Genet* 15:289–292, 1997
2. Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD: The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the *INS* VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet* 15:293–297, 1997
3. Paquette J, Giannoukakis C, Polychronakos C, Vafiadis P, Deal C: The *INS* VNTR 5′ variable number of tandem repeats is associated with *IGF2* expression in humans. *J Biol Chem* 273:14158–14164, 1998
4. Vafiadis P, Bennett ST, Colle E, Grabs R, Goodyer CG, Polychronakos C: Imprinted and genotype-specific expression of genes at the *IDDM2* locus in pancreas and leucocytes. *J Autoimmun* 9:397–403, 1996
5. Bennett ST, Lucassen AM, Gough SCL, Powell EE, Undlien DE, Pritchard LE, Merriman ME, Kawaguchi Y, Dronsfield MJ, Pociot F, Nerup J, Bouzekri N, Cambon-Thomsen A, Ronningen KS, Barnett AH, Bain SC, Todd JA: Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat Genet* 9:284–291, 1995
6. Bennett ST, Todd JA: Human type 1 diabetes and the insulin gene: principles of mapping polygenes (Review). *Annu Rev Genet* 30:343–370, 1996
7. Ong KKL, Phillips DI, Fall C, Poulton J, Bennett ST, Golding J, Todd JA, Dunger DB: The insulin gene VNTR, type 2 diabetes and birth weight (Letter). *Nat Genet* 21:262–263, 1999
8. Waterworth DM, Bennett ST, Gharani N, McCarthy MI, Hague S, Batty S, Conway GS, White D, Todd JA, Franks S, Williamson R: Linkage and association of insulin gene VNTR regulatory polymorphism with polycystic ovary syndrome. *Lancet* 349:986–990, 1997

9. Bennett ST, Todd JA, Waterworth DM, Franks S, McCarthy MI: Association of insulin VNTR polymorphism with polycystic ovarian syndrome (Letter). *Lancet* 349:1771–1772, 1997
10. Dunger DB, Ong KKL, Huxtable SJ, Sheriff A, Woods KA, Ahmed ML, Golding J, Pembrey ME, Ring S, Bennett ST, Todd JA: Association of the *INS* VNTR with size at birth: ALSPAC Study Team: Avon Longitudinal Study of Pregnancy and Childhood. *Nat Genet* 19:98–100, 1998
11. Giannoukakis N, Deal C, Paquette J, Goodyer C, Polychronakos C: Parental genomic imprinting of the human *IGF2* gene. *Nat Genet* 4:98–101, 1993
12. Giddings SJ, King CD, Harman KW, Flood JF, Carnaghi LR: Allele specific inactivation of insulin 1 and 2, in the mouse yolk sac, indicates imprinting. *Nat Genet* 6:310–313, 1994
13. Deltour L, Montagutelli X, Guenet JL, Jami J, Paldi A: Tissue- and developmental stage-specific imprinting of the mouse proinsulin gene, *Ins2*. *Dev Biol* 168:686–688, 1995
14. Thomas T, Southwell BR, Schreiber G, Jaworowsky A: Plasma protein synthesis and secretion in the visceral yolk sac of the fetal rat: gene expression, protein synthesis and secretion. *Placenta* 11:413–430, 1990
15. Gulbis B, Jauniaux E, Cotton F, Stordeur P: Protein and enzyme patterns in the fluid cavities of the first trimester gestational sac: relevance to the absorptive role of the secondary yolk sac. *Mol Hum Reprod* 4:857–862, 1998
16. Zhang Y, Tycko B: Monoallelic expression of the human *H19* gene. *Nat Genet* 1:40–44, 1992
17. Huxtable SJ, Saker PJ, Haddad L, Walker M, Frayling TM, Levy JC, Hitman GA, O'Rahilly S, Hattersley AT, McCarthy MI: Analysis of parent-offspring trios provides evidence for linkage and association between the insulin-gene and type 2 diabetes mediated exclusively through paternally transmitted class III variable number tandem repeat alleles. *Diabetes* 49: 126–130, 2000
18. Eaves IA, Bennett ST, Forster P, Ferber KM, Ehrmann D, Wilson AJ, Bhattacharyya S, Ziegler A-G, Brinkman B, Todd JA: Transmission ratio distortion at the *INS-IGF2* VNTR. *Nat Genet* 22:324–325, 1999
19. Kunkel LM, Monaco AP, Middlesworth W, Ochs HD, Latt SA: Specific cloning of DNA fragments from the DNA of a patient with an X-chromosome deletion. *Proc Natl Acad Sci U S A* 82:4778–4782, 1985
20. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294–5299, 1979