A Functional Analysis of the Role of *IGF2* in *IDDM2*-Encoded Susceptibility to Type 1 Diabetes

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Genetic studies have identified a number of loci demonstrating linkage to type 1 diabetes. One of the largest single contributors to genetic susceptibility, after the major histocompatability complex, is the *IDDM2* locus, which maps to a nontranscribed variable number of tandem repeats (VNTR) minisatellite upstream of the insulin (*INS*) and insulin-like growth factor 2 (*IGF2*) genes. In a progression from population to functional studies, recent reports have shown that VNTR susceptibility alleles (class I) have different transcriptional effects on *INS* than protective VNTR alleles (class III) in thymus and pancreas, two tissues important in the pathogenesis of the disease. Similar VNTR transcriptional effects on *IGF2* have also been proposed as a mechanism by which the *IDDM2* locus confers susceptibility in addition to, or instead of, effects on *INS*. We evaluated this hypothesis by comparing *IGF2* expression levels from chromosomes with the protective class III alleles to those with class I alleles in tissues relevant to type 1 diabetes pathogenesis. In thymus, class III alleles were associated with an *IGF2* mRNA level of 4.7 ± 0.9 (mean ± SE, arbitrary units, *n* = 12) compared with 4.7 ± 1.3 for class I alleles (*n* = 17). The same absence of a significant difference was found in pancreas, where class III alleles were associated with a level of 28.4 ± 4.2 (*n* = 7) and class I alleles with a level of 29.5 ± 5.2 (*n* = 6). There was a significant correlation between fetal age and *IGF2* in both tissues, but fetal ages were not different in the genotype groups compared. We therefore did not detect any significant difference in *IGF2* mRNA levels associated with the protective class of VNTR alleles as compared with the predisposing class. This is evidence against the hypotheses that have suggested *IGF2* is a mediator of *IDDM2*-encoded susceptibility and corroborates previous studies suggesting insulin is the gene involved. *Diabetes* 47:831–836, 1998

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bp, base pair; PCR, polymerase chain reaction; Q-C-RT-PCR, quantitative competitive reverse transcription–polymerase chain reaction; VNTR, variable number of tandem repeats; UTR, untranslated region.

Type 1 diabetes is characterized by a breakdown in cellular immune tolerance to the insulin-producing pancreatic β-cells (1). It is a polygenic disorder, linked to several genetic loci (2,3). The *IDDM2* susceptibility locus maps to a variable number of tandem repeats (VNTR) minisatellite on 11p15.5, upstream of the insulin (*INS*) and insulin-like growth factor 2 (*IGF2*) genes (4–11). The individual repeat sequences are 14–15 base pairs (bp) long with the consensus sequence ACAGGGGT-GTGGGG (7,12–15). The VNTR alleles fall mostly into two common size classes (10,16). Class I alleles (26–63 repeats) are predisposing to type 1 diabetes, while class III alleles (140–210 repeats) have a dominant protective effect.

The molecular and pathophysiologic mechanisms by which *IDDM2* determines risk are not known. Since the VNTR is not transcribed, its allelic forms must be exercising their effect by regulating gene transcription. We have, therefore, begun a systematic search in pancreas and thymus, tissues known to be of importance in the pathogenesis of type 1 diabetes, for VNTR allelic effects on mRNA levels of the nearby *INS* and *IGF2* genes. The products of these genes have well-characterized functions that suggest likely hypotheses for their contribution to *IDDM2*-encoded susceptibility.

The insulin gene is a target of tissue-specific VNTR transcriptional effects, and its product is the only known β-cell–specific autoantigen in type 1 diabetes. In vitro, two studies have shown that class III VNTR alleles are associated with higher gene expression than class I alleles in β-cells (17,18), while a third reports association of class III alleles with lower expression (19). The reason for the discrepancy is not clear. Studies performed in vivo on human pancreas confirm that class III VNTR alleles are associated with a statistically significant but marginal decrease in insulin expression (10,20,21). Insulin is also expressed in human thymus (22,23), with a marked VNTR allele effect on *INS* transcription levels. Most class III VNTR alleles are associated with two- to threefold higher levels of insulin expression than class I alleles (22,23). We and others have proposed that alleles that enhance thymic insulin expression will help induce better immune tolerance to the autoantigen. Such a gain of function could explain the dominant protective effect of the class III VNTR alleles.

VNTR allelic effects on *INS* do not exclude the possibility that other genes are involved in *IDDM2*-encoded susceptibility instead of, or in addition to, *INS*. The *IGF2* gene is transcribed from four promoters located 4–27 kilobases downstream of the VNTR, a distance compatible with transcriptional control. VNTR transcriptional effects on

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IGF2 expression have been shown in human placenta (24), indicating the potential for similar transcriptional effects to occur in other tissues.

In most tissues, *IGF2* is expressed only from the paternally inherited copy (25,26). The maternally inherited copy is transcriptionally silent. This is the phenomenon known as genomic imprinting (27). Therefore, only the paternal VNTR alleles can act on *IGF2* expression. This may explain the parent-of-origin effects seen in some populations for transmission of VNTR alleles (5,10,21,28,29).

There are also biological roles of IGF-II that suggest possible primary functions in the pathogenesis of type 1 diabetes (30). IGF-II is a ubiquitously expressed mitogenic peptide with important autocrine/paracrine signaling actions (31). Regulation of local IGF-II levels may thus modulate aspects of the process leading to type 1 diabetes. In pancreas, alleles associated with higher *IGF2* expression may enhance paracrine signaling to T-cells and thus promote progression of the immune assault on β-cells. In transgenic mice, overexpression of *IGF2* in thymus resulted in decreased thymocyte apoptosis (32,33) via an autocrine/paracrine mechanism. Alleles that increase *IGF2* expression in thymus would, therefore, increase the probability that potentially autoreactive thymocytes will reach maturity. Alternatively, it has been proposed that thymic IGF-II may act as a selecting peptide against insulin-reactive T-cells (34,35), given its high homology to proinsulin.

In this study, we directly address these hypotheses by comparing *IGF2* transcription levels from chromosomes with paternal class III VNTR alleles to those with paternal class I alleles in human fetal thymus and pancreas.

RESEARCH DESIGN AND METHODS

Samples. Human fetal pancreas and thymus samples, between 12.3 and 22 weeks' fetal age, and maternal blood were obtained at the time of pregnancy termination with written maternal consent and approval by the Institutional Review Boards of the Maisonneuve-Rosemont and Montreal Children's Hospitals. Tissues were immediately frozen, and nucleic acids were extracted using phenol-chloroform.

Genotyping. All samples were genotyped for VNTR status by polymerase chain reaction (PCR) as previously described (20,22). Individual class I alleles differing in length by a single repeat could be resolved by PAGE and designated by a three-digit number representing size in mobility units, following previously established terminology (10). DNA from maternal blood was also typed for VNTR. Stable inheritance of a single maternal VNTR allele was confirmed in most fetal samples studied, and the other allele was designated the paternal VNTR allele.

Quantitation of *IGF2* in thymus and pancreas. *IGF2* expression in thymus (30) and pancreas (20) is exclusively from the paternally inherited allele. Therefore, steady-state mRNA levels of samples with a paternal class III VNTR allele were compared with those with a paternal class I allele, using a quantitative competitive reverse transcription–PCR (Q-C-RT-PCR) method. The RNA concentration of each sample was determined by optical density, and an equal amount of each was reverse transcribed to cDNA using random hexamer primers and Superscript RT (Gibco). Parallel samples to which Superscript RT was not added were assayed by PCR to confirm the absence of genomic DNA contamination or PCR carryover. An equal volume of cDNA from every sample was then added to each of three different amounts of an *IGF2* PCR competitor and amplified by a previously described PCR method (36) for a 236-bp segment of exon 9 of *IGF2* in the 3′ untranslated region (UTR). This segment is shared by transcripts from all four *IGF2* promoters. The competitor sequence is identical to the endogenous 236-bp segment except for a 39-bp internal deletion made by PCR-based in vitro mutagenesis (37). Because both templates are amplified in the same PCR tube with the same PCR primers, the efficiency of amplification of the two templates is equal. The ratio of PCR product obtained for each template is thus proportional to the ratio in the starting template. The PCR products were labeled internally with ^{32}P-dATP, resolved by PAGE, and quantitated using a phosphorimager (Fuji) to obtain three ratios for each sample representing the amount of endogenous *IGF2* relative to a known amount of *IGF2* competitor.

To confirm equal loading, the amount of starting RNA in each RT reaction described above was determined by Q-C-RT-PCR on the cyclophilin housekeeping gene, which can be used as an indicator for total amount of RNA, using commercially available primers and an RNA cyclophilin PCR-competitor construct, as per the manufacturer's instructions (QuantumRNA Module; Ambion). The RNA cyclophilin competitor is identical to the endogenous cyclophilin sequence, except for a 25-bp internal deletion. An equal number of competitor copies was added to each RT reaction and amplified along with the endogenous cyclophilin cDNA. The PCR products were resolved using PAGE, and the bands corresponding to endogenous cyclophilin and cyclophilin competitor were quantitated to obtain a ratio of endogenous to competitor.

The *IGF2* ratios for each sample were normalized for amount of starting RNA, using the cyclophilin measurements as an indicator of total RNA content, and averaged. This mean represents the amount of *IGF2* mRNA for a set amount of total RNA. The calculations for comparing *IGF2* mRNA levels across samples are as follows:

- (endogenous *IGF2*/competitor *IGF2*) = amount of endogenous *IGF2* transcripts relative to a known amount of PCR competitor (n = 3 per sample);
- 1/(endogenous cyclophilin/competitor cyclophilin) = normalizing factor for amount of RNA in each sample (n = 1 per sample);
- (normalizing factor) × (endogenous *IGF2*/competitor *IGF2*) = normalized ratio for the amount of endogenous *IGF2* relative to a known amount of competitor for a set amount of total RNA (n = 3 per sample);
- (normalized ratio) × (moles of competitor added per reaction) = amount of *IGF2* mRNA (n = 3 per sample); and
- for each sample, average the amount of *IGF2* mRNA measured at each concentration of competitor assayed (n = 3 per sample).

Establishment of the Q-C-RT-PCR method. To verify that the relative amount of endogenous and competitor PCR product is proportional to the amount of each starting template and to determine the appropriate competitor concentrations to be used, a small number of samples were titrated against varying concentrations of competitor. For the *IGF2* mRNA quantitation, various amounts of competitor (1.25, 2.5, 5, 10, 20, 40, 50, 80, 100, 125, 200, and 400 amol) were added to an equal amount of cDNA from the same pancreas or thymus sample and amplified by PCR. This was repeated several times for each tissue using various numbers of PCR cycles. The competitor concentration at which the ratio of endogenous to competitor product was approximately 1, the next higher and the next lower concentrations were chosen to assay each sample in triplicate competitor concentrations. Similarly for cyclophilin, a known amount of competitor, ranging from 1×10^6 to 1×10^{10} copies, was added to 5 reverse transcription reactions, each with the same amount of starting RNA from the same pancreas or thymus sample. The concentration of competitor at which the ratio of endogenous to competitor product was closest to 1 was chosen to assay all samples.

RESULTS

Establishment of the Q-C-RT-PCR. For competitive RT-PCR to be quantitative, the ratio of endogenous to competitor PCR product must be proportional to the ratio in the starting template. Proportional amplification of the two templates was verified by titrating an equal amount of total RNA with varying amounts of competitor. For the cyclophilin Q-C-RT-PCR, the ratio of endogenous cyclophilin to copies of cyclophilin competitor added was shown to correlate with the proportion of each starting template with addition of between 1×10^8 and 1×10^{10} copies of competitor (pancreas R^2 = 0.9931, thymus R^2 = 0.9951) (Fig. 1); lower concentrations resulted in the endogenous transcripts completely outcompeting the competitor. An amount of 5×10^9 copies of cyclophilin competitor was added to each sample assayed because this was the concentration at which the endogenous to competitor ratio was near 1, indicating an approximately equal starting concentration of the two templates. The endogenous to competitor cyclophilin ratios obtained using a single competitor concentration covered a very tight range of values between the samples assayed (thymus: 0.4–2.6, with 76% of samples between 0.4 and 1.5; pancreas: 0.5–2.5, with 85% of samples between 0.5 and 1.5), indicating that the level of cyclophilin expression as a percentage of total RNA does not

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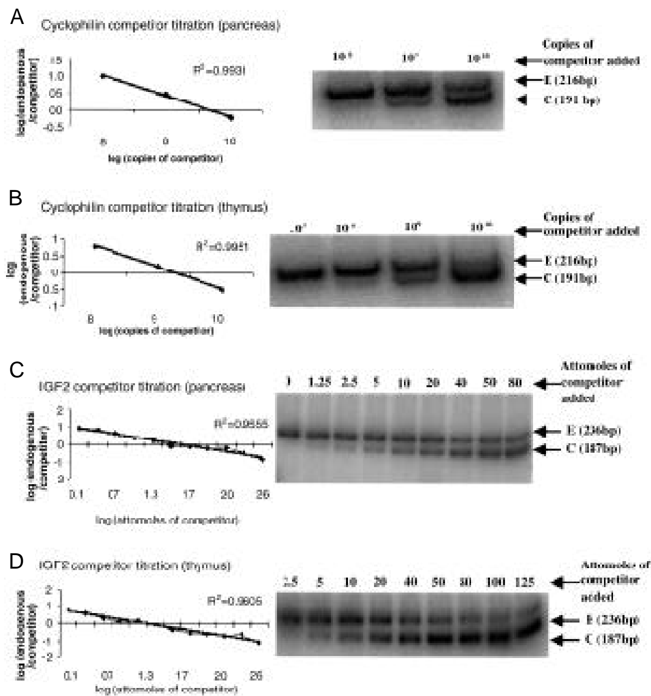

FIG. 1. An identical amount of endogenous template was co-amplified with varying amounts of PCR competitor to confirm that the endogenous and competitor templates for (*A*) cyclophilin in pancreas, (*B*) cyclophilin in thymus, (*C*) *IGF2* in pancreas, and (*D*) *IGF2* in thymus were amplified with equal efficiency and, thus, that the relative amount of final PCR product is proportional to the relative amount of starting template. The results using 22 PCR cycles are shown.

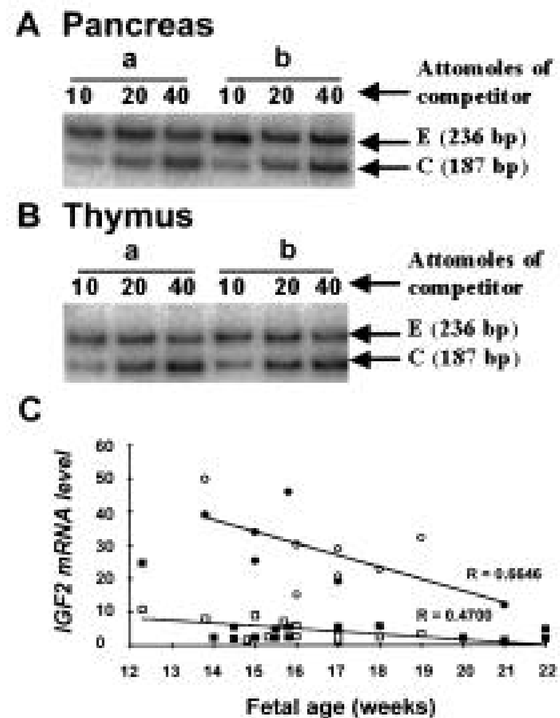

FIG. 2. Representative autoradiographs of the *IGF2* quantitation assays, carried out in triplicate using 10, 20, or 40 amol of *IGF2* PCR competitor, are shown for two samples of human fetal pancreas (*A*) and thymus (*B*). The upper band (E) is the 236-bp endogenous *IGF2* RT-PCR product, and the lower band (C) corresponds to the 187-bp *IGF2* competitor product. *C*: The correlation of fetal age (weeks) with *IGF2* expression level for human fetal pancreas with a paternal class I VNTR allele (●), pancreas with a paternal class III allele (○), human fetal thymus with a paternal class I allele (■), and thymus with a paternal class III allele (□). The *IGF2* expression level for each sample is taken as the mean of measurements made using 10, 20, and 40 amol of *IGF2* competitor (Tables 1 and 2). Class I and III alleles are not associated with significantly different *IGF2* mRNA levels. There is a significant negative correlation of fetal age with *IGF2* mRNA level in both pancreas (R = 0.6646, P < 0.0086) and thymus (R = 0.4700, P < 0.005), independent of VNTR class.

vary greatly between individuals with fetal age between 12.3 and 22 weeks.

Similarly for the *IGF2* Q-C-RT-PCR, an equal amount of total RNA was titrated with concentrations of *IGF2* competitor by adding between 1.25 and 400 amol for both thymus and pancreas, with 18, 20, 22, 24, and 26 PCR cycles. The ratio of endogenous to competitor PCR product correlated with the proportion of starting template, indicating that the two templates are amplified with equal efficiency, regardless of cycle number (pancreas R^2 between 0.9137 and 0.9704; thymus R^2 between 0.9605 and 0.9779) (Fig. 1). While the absolute intensity of PCR product varied with the number of cycles, the ratio of endogenous to competitor product was not significantly affected. We thus chose to use 22 PCR cycles for all samples assayed because this was the minimal number of cycles at which strong bands were consistently attained. These experiments also establish the precision of the method, which was able to detect changes in competitor concentrations of less than twofold. All samples were assayed in triplicate by addition of 10, 20, or 40 amol of competitor.

***IGF2* expression levels in pancreas.** In pancreas, only the paternal *IGF2* gene copy is expressed (20). To compare the effects of class III VNTR alleles with class I alleles on *IGF2* expression level, we identified seven pancreas samples with a paternal class III VNTR allele and six with a paternal class I VNTR allele. The average *IGF2* mRNA level expressed from paternal chromosomes with a class III VNTR was 28.4 ± 4.2 (mean ± SE, arbitrary units), compared with 29.5 ± 5.2 from paternal chromosomes with a class I VNTR, as determined by Q-C-RT-PCR (NS) (Table 1, Fig. 2*A*). Replication among the three competitor concentrations at which each sample was assayed was very good (mean coefficient of variation 0.1 [range 0.03–0.23]) (Table 1). There was a significant negative correlation of *IGF2* levels with fetal age (R = 0.6646, P < 0.0086, n = 13), but fetal ages were not different between the class I (median fetal age 15.4 weeks [13.8–21]) and class III (median fetal age 17 weeks [13.8–19]) groups (Table 1, Fig. 2*C*). These results demonstrate the absence of a significant VNTR allele effect on *IGF2* expression in pancreas.

***IGF2* expression levels in thymus.** As in pancreas, *IGF2* is imprinted in the thymus, resulting in exclusive expression from the paternal gene copy (30). Unlike insulin, which is expressed only in a rare subset of thymus cells (38), *IGF2* is ubiquitously expressed. To determine if there are VNTR allele effects on *IGF2* expression in thymus, *IGF2* mRNA levels were quantitated in 12 samples with a paternal class III VNTR allele and in 17 with a paternal class I VNTR allele. The

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TABLE 1
IGF2 mRNA Q-C-RT-PCR results in human fetal pancreas

Maternal genotype	Fetal genotype	Paternal VNTR	Fetal age (weeks)	*IGF2* mRNA level (amol)		
				10	20	40
683/683	683/728	728	21.0	13.6	11.3	11.0
NA	655/742	I	17.0	19.3	19.7	18.3
800/III	814/III	814	15.0	26.3	24.3	25.2
828/843	655/843	655	15.0	33.6	37.0	30.4
NA	669/828	I	13.8	42.2	36.1	38.7
669/III	814/III	814	15.8	58.1	43.9	35.3
728/814	814/III	III	16.0	18.6	14.0	12.8
756/770	756/III	III	17.0	24.5	17.4	19.8
655/828	828/III	III	18.0	24.1	15.6	28.0
698/756	756/III	III	17.0	24.8	33.0	28.5
843/843	843/III	III	16.0	33.8	27.1	29.2
800/976	976/III	III	19.0	34.9	30.1	31.0
786/III*	786/III*	III	13.8	48.5	49.2	51.7

The maternal genotype, fetal genotype, paternally inherited VNTR allele, and the fetal age of each sample are shown with the corresponding *IGF2* mRNA levels obtained using three different competitor concentrations, in arbitrary units, after correcting for the amount of starting RNA and amount of competitor. Class I alleles are represented either by a three-digit number representing size in mobility units (10) or by an "I" when the size of the paternal allele could not be determined. *The maternal and fetal class III alleles were distinguishable by size. NA, not available.

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average *IGF2* mRNA level associated with the class III VNTR alleles was 4.7 ± 0.9, compared with 4.7 ± 1.3 in those associated with the class I alleles (NS) (Table 2, Fig. 2*B*). Replication of the quantitated *IGF2* level was very good among the three competitor concentrations used (mean coefficient of variation 0.24 [0.04–0.54]) (Table 2). There was a significant negative correlation of *IGF2* levels with fetal age (R = 0.4700, P < 0.005, n = 29), but fetal ages were not different between the class I (median fetal age 15.8 weeks [12.3–22]) and class III (median fetal age 15.8 weeks [12.3–19]) groups (Table 2, Fig. 2*C*). Clearly there is no significant VNTR allele effect on *IGF2* transcription in thymus.

DISCUSSION

The *IDDM2* locus is the only type 1 diabetes susceptibility locus that has been mapped to a specific genetic element besides *IDDM1*. This has permitted a functional analysis of the physiologic mechanism(s) by which *IDDM2* contributes to disease susceptibility. One such mechanism may be the tissue-specific modulation of *INS* expression in the thymus by VNTR alleles (22,23). A non–mutually exclusive hypothesis is that *IDDM2*-encoded susceptibility is mediated through *IGF2* (30,34). To directly test this hypothesis, we compared *IGF2* expression levels in vivo from chromosomes with a class III VNTR allele to those with a class I allele. There were no significant differences in *IGF2* mRNA levels between the predisposing and protective classes of VNTR alleles in either pancreas or thymus. Small effects of VNTR class on *IGF2* expression have been described in placenta (24). Thus, as with insulin, the effects of VNTR alleles on *IGF2* expression are tissue specific.

Functionally, *IGF2* has been considered a candidate for the *IDDM2* effect because of its well-characterized properties as a mitogenic peptide (30), with important autocrine/paracrine effects on thymus, lymphocytes, and pancreas (31–33). Its candidacy would also be supported by its potential role as a selecting peptide in the thymus, which would promote tolerance to proinsulin, to which it has a high homology (34,35). The absence of differential VNTR allele effects on *IGF2* expression in thymus and pancreas argues strongly against these considerations.

The preferential transmission of VNTR-susceptibility alleles at *IDDM2* from fathers to diabetic offspring has been described in some populations (5,10,21,28,29), but not in others (10,11,21,39). The absence of a differential effect between VNTR class I and III alleles on expression of the imprinted *IGF2* gene suggests that if there is a parental effect in transmission of VNTR alleles, it is not mediated through *IGF2*. However, the parental effect may be mediated through the insulin gene, which shows polymorphic silencing of insulin transcription from chromosomes with a class III VNTR in thymus (22,23). This may represent allele-specific genomic imprinting where insulin transcription is silenced only when located *cis* to specific class III alleles and transmitted from a parent of a specific sex, resulting in a parent-of-origin effect.

Our analysis has assumed that the VNTR acts in *cis*, but it is conceivable that it may also modulate *IGF2* transcription in *trans*. Action in *trans* by nontranscribed sequences would require postulating an unknown mechanism, as opposed to the well-known effect of enhancers in *cis*. Physical association of the two copies of the imprinted Prader-Willi locus on 15q12 has been reported, which might suggest such a mechanism (40). Even more intriguing, statistical evidence has recently been presented that the VNTR may act in *trans* in the paternal germ line (41). Paternally inherited 814 class I alleles do not predispose to type 1 diabetes when the untransmitted paternal allele is a class III, but they are as predisposing as all other class I alleles when the untransmitted allele is a class I. No evidence of such *trans* effects was found for other specific class I alleles. However, this phenomenon does not have a large effect on *IDDM2*-encoded susceptibility because only about 5% of individuals inherit an 814 allele from an 814/III father. It is therefore

TABLE 2
IGF2 mRNA Q-C-RT-PCR results in human fetal thymus

Maternal genotype	Fetal genotype	Paternal VNTR	Fetal age (weeks)	*IGF2* mRNA level (amol)		
				10	20	40
683/683	683/728	728	21.0	1.1	1.1	1.6
NA	786/814	I	22.0	1.4	1.5	3.0
814/814	814/814	814	14.5	1.3	1.6	2.7
814/III	641/814	641	20.0	1.4	1.8	2.9
742/814	786/814	786	15.5	1.7	2.1	3.1
828/843	655/843	655	15.0	1.6	2.2	3.0
800/800	800/800	800	14.0	1.8	2.3	2.8
669/III	814/III	814	15.8	2.1	2.0	3.2
NA	800/843	I	17.0	1.7	2.4	3.4
NA	800/828	I	22.0	4.6	5.1	—
NA	786/843	I	15.5	5.2	5.1	4.7
814/814	728/814	728	17.0	3.2	5.2	6.8
NA	683/770	I	17.0	4.0	6.2	6.1
NA	714/786	I	14.5	5.6	5.8	5.0
NA	786/843	I	15.8	4.0	6.9	—
NA	641/843	I	18.0	4.1	7.0	6.1
NA	669/843	I	12.3	17.9	31.4	24.1
814/828	828/III	III	14.8	1.3	1.8	1.8
698/756	756/III	III	17.0	1.0	1.4	2.4
669/814	669/III	III	15.3	1.4	1.9	3.8
655/828	828/III	III	18	1.7	2.3	3.3
728/814	814/III	III	16.0	2.2	2.5	3.0
756/770	756/III	III	17.0	1.9	2.6	3.6
800/976	976/III	III	19.0	1.9	2.1	5.7
786/III	III/III	III	16.0	4.7	6.2	6.8
III/III	III/III	III	15.7	7.1	7.6	6.7
786/III*	786/III*	III	13.8	5.8	7.1	11.2
756/756	756/III	III	15.0	7.1	10.6	—
843/III	III/III	III	12.3	9.1	7.8	15.3

The maternal genotype, fetal genotype, paternally inherited VNTR allele, and the fetal age of each sample are shown with the corresponding *IGF2* mRNA levels obtained using three different competitor concentrations, in arbitrary units, after correcting for the amount of starting RNA and amount of competitor. Class I alleles are represented either by a three-digit number representing size in mobility units (10) or by an "I" when the size of the paternal allele could not be determined. *The maternal and fetal class III alleles were distinguishable by size. NA, not available.

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unlikely that *trans* effects are obscuring a VNTR allelic affect on *IGF2* expression in this study.

Our results do not entirely eliminate *IGF2* as the gene involved in *IDDM2*. The VNTR may have tissue-specific transcriptional effects on organs other than thymus and pancreas that are involved in the pathogenesis of diabetes in less obvious ways. These results are important, however, for the most efficient planning of future research on the *IDDM2* locus, which must now focus on the insulin gene.

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