The minisatellite DNA polymorphism consisting of a variable number of tandem repeats (VNTR) at the human INS (insulin gene) 5′-flanking region has demonstrated allelic effects on insulin gene transcription in vitro and has been associated with the level of insulin gene expression in vivo. We now show that this VNTR also has effects on the nearby insulin-like growth factor II gene (IGF2) in human placenta in vivo and in the HepG2 hepatoma cell line in vitro. We show that higher steady-state IGF2 mRNA levels are associated with shorter alleles (class I) than the longer class III alleles in term placentae. In vitro, reporter gene activity was greater from reporter gene constructs with IGF2 promoter 3 in the presence of class I alleles than from those with class III. Taken together with the documented transcriptional effects on the insulin gene, we propose that the VNTR may act as a long range control element affecting the expression of both INS and IGF2. The localization of a type 1 diabetes susceptibility locus (IDDM2) to the VNTR itself suggests that either or both of these genes may be involved in the biologic effects of IDDM2.

The INS 5′ Variable Number of Tandem Repeats Is Associated with IGF2 Expression in Humans*

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* This work was supported by the Medical Research Council of Canada (to C. D. and C. P.) and the Juvenile Diabetes Foundation International (to C. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: VNTR, variable number of tandem repeats; IGF, insulin-like growth factor; kb, kilobase pair(s); RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); CAT, chloramphenicol acetyltransferase.

of this minisatellite fall into three broad categories with the following size range: class I, 0.4–0.9 kb; class II, 1.2 kb; and class III, >2 kb. In human fetal and adult pancreas, class I alleles are associated with higher INS mRNA levels than class III (2, 12). In vitro, allelic effects are less clear as two studies showed higher reporter gene activity in the presence of class I alleles (13, 14), whereas another study showed lower reporter gene activity in the presence of class I alleles in pancreatic cells (15). The discrepancy between these studies may have been due to the choice of particular class I subtype used in the constructs or to the absence of genomic context required for the effects seen in vitro.

The VNTR lies 4.1 kb upstream of the first promoter of the human insulin-like growth factor II gene (16) (IGF2) on the short arm of chromosome 11 (11p15.5), and this physical proximity may allow the minisatellite to influence the expression of IGF2 as well as that of INS. IGF2 encodes an important fetal mitogen that is ubiquitously expressed; the placenta and the adrenal gland contain the highest levels of IGF2 transcripts among all fetal tissues (17–19). Besides its role as a growth factor, IGF-II promotes cell survival by preventing apoptosis (20) and has demonstrated immunomodulatory activity in a number of models (21). It is expressed by T-lymphocytes (22), and it has mitogenic and anti-apoptotic actions on these cells (21). The expression of IGF2 is regulated during development, and DNA sequence variants at or near the gene could conceivably affect its transcription. Primarily because of the close physical proximity of IGF2 to the VNTR and the allelic effects of the minisatellite on the expression of the adjacent insulin gene, we have begun an investigation into the association between the VNTR and IGF2 gene expression in vivo and in vitro.

EXPERIMENTAL PROCEDURES

This study was approved by the Institutional Review Board of the Ste-Justine Hospital, and informed consent was obtained for all tissues used.

Tissue Preparation and Nucleic Acid Purification—Human term placenta for DNA and RNA purification was obtained at the time of vaginal delivery or elective Cesaerian section. A 1 cm thick layer of placenta was first trimmed from the maternal side to ensure the removal of all the maternal decidual tissue. A narrow 0.5–1-g piece was then removed from the fetal side of the placenta, thoroughly washed in sterile phosphate-buffered saline, and immediately frozen on dry ice. Peripheral blood was collected in EDTA-containing Vacutainer collection tubes. The blood was aliquoted, and the aliquots were centrifuged in a benchtop centrifuge for 10 min at 800 rpm; the cell pellet was immediately frozen.

Placental DNA was recovered from phenol/chloroform/isomyl alcohol extractions following proteinase K and RNase A digestion of the tissue. Genomic DNA was obtained from blood cell pellets and from purified mononuclear blood cells with phenol extraction at neutral pH as described (23). Total RNA from placenta was isolated by acid guanidinium isothiocyanate followed by phenol/chloroform extraction (24). Genotyping—In all PCR reactions, 0.5 μg of genomic DNA was used. The reactions were, for the greater part and unless otherwise indicated,
carried out in the presence of 1 μCi of [α-32P]dATP, 75–150 pmol of PCR primer (sense and antisense), 0.1 m each dNTP, and commercially available buffers supplied with the thermostable DNA polymerase were used. The PCR can only amplify VNTR alleles whose products will be less than 1.5 kb, chiefly because of the large size of class II and class III alleles (greater than 1.2 kb) and the consequent increase in the GC content. All reagents were purchased from ID Labs (London, Ontario, Canada). An ammonium sulfate buffer was used in the PCR with 1 mM MgCl2 and 10% Me2SO with each dNTP at a final concentration of 1.5 mM and the supplier’s Taq polymerase. Following a heat denaturation step of 5 min at 94 °C, the PCR was performed for 30 cycles consisting of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C. The expected product size is 104 bp. Sense primer, 5′ CCCTACACGTCGGAAGACTTA 3′; antisense, 5′ GGCTTGTTTCAAGGGCTTAT 3′.

The radioactive VNTR PCR products were digested with 10–30 units of NcoI for 2–4 h at 37 °C, and the digests were electrophoresed in 6% polyacrylamide gels. The bands were visualized by autoradiography. Alleles were identified by the migration distance of the bands. Since P3 has two VNTR alleles (I and II) to be performed in the absence of radioactive dNTP precursor, thereby allowing us to visualize the products in 1% agarose gels after ethidium bromide staining. The PCR products derived from the 3′ INS region were digested for 2–4 h at 37 °C with 10–30 units of PstI, and the digests were visualized by autoradiography following electrophoretic separation in 8% polyacrylamide minigel.

cDNA Synthesis and Competitive RT-PCR—One μg of total RNA in diethyl pyrocarbonate-treated water was heated for 3 min at 80 °C and quickly cooled on ice. A mix of either 75 pmol of antisense polymerase chain reaction (PCR) primer or oligo(dT), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 1.3 mM dNTP mix, 6 units of human placental RNase inhibitor (Promega), and 400 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) was added to the RNA. Incubation was at 37 °C for 1 h followed by heating at 80 °C for 10 min.

The internal competitor standard was generated using PCR-based in vitro mutagenesis as indicated (25). The standard is 190 bp and consists of the identical sequence as that of the exon 9-derived IGF2 cDNA PCR product with an internal deletion of 46 bp. The competitor PCR product was subcloned into a T-Vector (Promega) using the supplier’s recommended protocol.

Following bacterial transformation and purification using the QIAamp kit (Qiagen, Mississauga, Ontario, Canada), the competitor plasmid was aliquoted in fractions of 0.01–1 × 10⁻⁶ mol (as determined spectrophotometrically). A typical assay included equal volumes of cDNA and competitor (1 μl) of a given concentration in the same buffer. The reaction was performed using the exon 9-specific IGF2 primers indicated below under the same PCR reaction and cycling conditions as described below.

The cDNA and competitor were mixed with sense and antisense primer (75 pmol each) in the buffer supplied by Life Technologies, Inc., or in a buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl2, and 0.1 μl each dNTP including 1 μCi of [α-32P]dATP (NEN Life Science Products) and 2 units of Taq polymerase (Life Technologies, Inc.). The cycling parameters were described in Tadokoro et al. (26) and consist of 30 cycles of 25 s at 94 °C, 25 s at 58 °C, and 90 s at 72 °C, followed by a final extension time of 5 min at 72 °C. Exon 9 sense primer, 5′ CCGAGCCTTGTAGCTCAATTGT 3′; antisense, 5′ CTCCTGCCTTGGTCAATTCCG 3′.

For GAPDH mRNA levels, the expected size of the PCR product (which derives from exons 4–8 of the glyceraldehyde-3-phosphate dehydrogenase gene, GAPDH) is 379 bp following the amplification of cDNA in the presence of 1.5 mM MgCl2. The PCR was carried out for 20 cycles consisting of 30 s at 94 °C, 1 min at 56.3 °C, and 2 min at 72 °C. Sense primer, 5′ CGGCAATGGCCAAGAGATGCT 3′; antisense: 5′ AGTCAACCTTACACCATCCA 3′.

Because of the risk of contamination of cDNA with genomic DNA during RNA isolation, we undertook different procedures to eliminate the possibility that genomic DNA would be amplified by PCR in cDNA amplifications. First, total RNA was treated with RNase-free DNase (Promega), and the RNA was then recovered by phenol/chloroform extraction followed by ethanol precipitation prior to cDNA synthesis. In addition, parallel PCR amplification of the reverse transcription (RT) reaction following incubation either with or without reverse tran-
To assess β-galactosidase activity, the cell lysate was incubated with a synthetic substrate supplied in a commercially available kit, and all the procedures of the supplier were followed (Promega). The end point measured was the spectroscopic analysis of the absorbance of the converted substrate at 420 nm. CAT activity was based on the counts/min obtained following scintillation counting and corrected for β-galactosidase activity.

**RESULTS**

The Association between the VNTR and IGF2 Expression in Human Term Placenta—The approach used to quantify insulin gene expression in vivo from class I and class III chromosomes in previous studies, in which the transcribed diallelic \( Pst I \) RFLP is in linkage disequilibrium with VNTR alleles (2, 3), was impossible for IGF2 in human placenta because of monoallelic expression (31, 32). We therefore used a competitive RT-PCR assay with internal competitor instead. We assessed IGF2 gene expression in normal human term placenta, where only the paternally transmitted gene copy is transcribed (31, 32) thereby allowing us to measure the expression associated with only one VNTR allele in each tissue sample. In heterozygotes, the paternal allele can be easily determined by genotyping the parents. Steady-state IGF2 mRNA levels among placentae were determined using a competitive reverse transcription-PCR (RT-PCR) assay that was reproducible and linear with negligible interassay variability. The cDNA and the competitor were coamplified in the same reaction. The competitor sequence was identical to that of the target minus an internal deletion of 46 bp to distinguish it from the target PCR product in a polyacrylamide gel. It is important to note that the PCR primers flank a sequence in exon 9 which is present in all of the IGF2 transcripts, irrespective of which promoter was used (20).

Following pilot experiments to determine a suitable amount that would titrate the specific IGF2 PCR product in a series of term placentae (data not shown), we coamplified the oligo(dT)-primed cDNA of all placentae with the same amount of internal competitor concentrations. The intensity of the PCR products was quantitated using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA). To control for the efficiency of the RT step, we performed PCR using the cDNA as template with primers flanking the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase gene (\( GAPDH \)) and terminated the PCR at the mid-exponential phase. The relative IGF2 mRNA levels were derived by correcting the intensity of the specific IGF2 PCR product with the intensity of the competitor PCR product and normalizing this value to the intensity of the \( GAPDH \) product corresponding to the same placenta.

In order to determine if the differences in mRNA levels were associated with VNTR classes, we genotyped the placentae at the VNTR by PCR using primers that flank the polymorphic region as well as for a \( Pst I \) RFLP which is in linkage disequilibrium with the VNTR (2, 13). The \( Pst I(+) \) allele is always transmitted together with class I alleles, whereas the \( Pst I(-) \) allele is most often transmitted with the class III alleles (2, 14) (about 20% of class III alleles are in cis to \( Pst I (+) \)). The \( Pst I \) genotypes were used to confirm the existence of a class III allele in samples in which only one class I PCR product could be detected. The genotypes of the parents (where available) were ascertained, and since IGF2 is expressed exclusively from the paternal chromosome in term placenta (31, 32), assignment of VNTR class to the chromosome from which IGF2 was expressed was straightforward in informative sets of samples. Figs. 1 and 2 show a sample of the genotypes observed at the VNTR and for the \( Pst I \) RFLP of INS. In Fig. 1, only the class I alleles are shown, and this is because the PCR can only amplify class I alleles (the large size and GC content of class III alleles have thus far been impediments in PCR amplification).

Matching IGF2 mRNA levels with paternally transmitted VNTR class, in all placentae studied, we discovered that the steady-state IGF2 mRNA levels from chromosomes with class I alleles was greater than from chromosomes with class III (Fig. 3, A and B). Furthermore, Fig. 3, C and D, shows the linearity of the competitive RT-PCR assay by comparing IGF2 mRNA levels among two placentae of class I, with the mRNA levels of two placentae of class III VNTR, using a similar range of internal competitor concentrations.

By comparing the expression of IGF2 among all placentae studied (13 class I and 9 class III, normalized for \( GAPDH \)), it is evident that, although the medians are statistically different (Fig. 3, B, \( p < 0.05 \), Mann-Whitney test), the range is variable. This could be due to differential effects of specific VNTR alleles within each class that may specifically affect IGF2 expression, and perhaps INS expression as well. Although this is only one of many possible explanations, it warrants further investigation.

Effects of the VNTR on IGF2 P1 and P3 Promoters in Vitro—We next examined the effects of the VNTR on reporter gene activity in vitro. Exploiting the genomic sequence from upstream of the VNTR to just downstream of the transcriptional start site of the first IGF2 promoter (P1) with the addition of the P3 promoter elements, we designed INS-IGF2 reporter gene constructs. The rationale for using these constructs was to test the in vitro effects of the VNTR on CAT expression in the natural genomic context, should secondary structure in the region be important.

In these constructs, the first and third IGF2 promoters (P1 and P3) were placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. To these constructs (shown in Fig. 4A), we fused either a class I VNTR (of the 683 subclass) or a class III allele upstream of the INS promoter without altering any defined minimal INS promoter region sequence (33). Transfection efficiency was monitored by cotransfection with a plasmid containing the β-galactosidase gene (pSVβ). Triplicate experiments were performed at three different transfection efficiencies. Results are shown normalized to CAT activity of a construct without a VNTR (pNCAT) following correction for β-galactosidase activity (Fig. 4B). As judged by the lack of a significant interaction in a two-way analysis of variance (\( p > \)
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DISCUSSION

Our results demonstrate that VNTR alleles are associated with differences in IGF2 mRNA levels in human placenta, similar to those already demonstrated for INS in fetal and adult pancreas (2, 12). Previous in vivo studies in fetal thymus showed that the VNTR effect on INS transcription is tissue-specific and opposite to that in fetal pancreas (3), the effect being much more subtle in the pancreas compared with that in the thymus. More recent in vivo studies in fetal pancreas and thymus (34) did not reveal a transcriptional effect of the VNTR on IGF2 such as we saw in placenta; however, whether this reflects a tissue or developmental stage specificity of VNTR action is not known. Our in vitro data suggest hepatocytes as another transcriptional environment in which VNTR alleles modulate IGF2 expression. Additionally, the effects, if any, of the VNTR on the postnatal expression of IGF2 as well as its association with human disease in addition to type 1 diabetes remain open for future investigation. It is interesting to note, however, that there appears to be a significant genetic contribution to the interindividual variability of circulating IGF-II levels in humans (35).

Type 1 diabetes (previously referred to as insulin-dependent diabetes mellitus, or IDDM) is an autoimmune disorder culminating in the destruction of the insulin-producing beta cells of the pancreas. The disorder is of a multifactorial nature with a significant polygenic component in the susceptibility (36). The INS VNTR minisatellite is 1 of 16 mapped type 1 diabetes susceptibility loci and has been designated IDDM2, where the class I alleles are associated with susceptibility and the class III with protection (2, 16). The preferential paternal transmission of susceptibility haplotypes at IDDM2 in some populations studied suggests the involvement of an imprinted gene in the predisposition to type 1 diabetes that could lie at or near the VNTR, which may be under its transcriptional effects (2, 16, 37, 38). An obvious human imprinted gene that could be a candidate for allelic effects of the VNTR is IGF2, because of its imprinted status (expressed from paternal chromosomes in most tissues studied) (31, 32, 38, 39) and its proximity to the VNTR (less than 4.1 kb).

As is the case for pancreatic INS expression, the class I alleles are associated with an increase in the levels of IGF2 mRNA relative to the levels from the protective class III alleles in vivo. The in vitro effects of the VNTR on IGF2 P3-driven CAT expression parallel those demonstrated in the study of Lucassen et al. (13) who report an enhanced transcription of INS in the context of a class I VNTR-based construct, compared with a class III construct in transiently transfected pancreatic cells with an INS reporter gene construct. In fact, the magnitude of the transcriptional effect of the class I allele compared with the class III allele on IGF2, in vitro, is comparable to that shown for INS (1.5–3.1 times higher INS expression from class I VNTR constructs than class III (13); 1.5 times higher CAT activity from IGF2 P3-based constructs with class I VNTR than class III, this report). This difference parallels our in vivo results as well.

Additionally, it must be noted that the magnitude of the effects of class I over class III alleles was not that different from what we report here, in the studies evaluating the allelic effects of the VNTR on INS mRNA levels in human thymus and pancreas (no higher than 3-fold with an average of 2.5-fold along with an intersample and an interassay variability) (2, 3, 40). Whereas transgenic mice deficient for the IGF-II gene display a growth retardation and are 60% the size and weight of their wild-type littermates (41), it is not known how more subtle differences in IGF2 expression will affect normal growth and development. That such small differences in mRNA levels are indeed physiologically relevant comes from the observation that relaxation of normally occurring monoallelic IGF2 expression (i.e. loss of imprinting, leading to a theoretical 2-fold increase in mRNA) is believed to underlie certain cases of fetal macrosomia (42, 43) and many tumors including Wilms’ (44, 45), rhabdomyosarcoma (46), choriocarcinoma (47), lung (48), glioma (49), and colorectal carcinoma (50). Finally, IGF2 transgenes introduced into mouse embryonic stem cells leading to a roughly 2-fold increase in Igf2 mRNA produced a parallel increase in the birth weight and organ weights of the chimeric fetuses (51).

The VNTR classes are composed of subclasses of specific alleles, which may in turn be polymorphic (2, 14) and may thus have potentially different allelic effects on the expression of IGF2. This may explain the range of transcript levels of placental IGF2 observed in this study and may also explain the variability observed in the studies on the insulin gene (3, 40). This is not without precedent as Green and Krontrira (5) have previously shown that specific VNTR alleles have different transcriptional effects in vitro in the context of the H1R1 minisatellite 3’ to the gene. The magnitude of the allelic effects in this latter study was variable among different subclones and not considerably different from the variability and magnitude observed in our study and in those on INS (2, 3, 13, 40). More importantly, our study was performed in the context of...
physiologically relevant promoters and not on strong viral promoters (Rous sarcoma virus) as was done by Green and Krontiris (5). It should be noted that since we compared mRNA levels across samples, we should also expect a non-VNTR-dependent variability among individuals based on nutritional status and stage in labor of the mother, as well as other unlinked genetic and epigenetic factors.

Our in vitro data reflect the situation observed in most fetal tissues, where IGF2 is expressed predominantly from P2, P3, and P4 but not from P1 (52). A similar pattern of promoter expression is seen in the HepG2 cell line where, as shown by Northern blot analysis (29) and more recently using an RNase protection assay (53), the major transcript is 6.0 kb and originates from P3. To preserve the natural genomic context as much as possible, we used reporter gene constructs containing the genomic sequence around the VNTR including the insulin

![Figure 3: Allelic effects of the VNTR on IGF2 expression in term placenta.](image)

**A** shows variable IGF2 expression among term placentae. Upper panel, the expression levels were determined by PhosphorImager analysis of the PCR products. An equal amount of internal competitor standard was used for all the placentae assayed (1 x 10^{-6} fmol). Equal volumes of placental cDNA and competitor were coamplified in the presence of radiolabeled dATP. In a separate experiment, the cDNA was amplified with primers specific for the GAPDH gene in the presence of radiolabeled dATP, and the PCR was terminated at the exponential phase (lower panel). The PCR products were resolved electrophoretically in an 8% polyacrylamide gel, and the bands were quantitated by PhosphorImager analysis. The numbers above each lane in both panels indicate different placentae. RT indicates the products of a PCR performed in the absence of the internal competitor using a mock reverse-transcribed total RNA preparation that had been treated with RNase-free DNase for each placental sample assayed. All experiments were performed at least three times.

**B** is a graphical and tabular representation of the results. The values are in arbitrary units and are expressed as the intensity ratio of the specific IGF2 PCR product to that of the internal standard (normalized to the intensity of the corresponding GAPDH PCR product). The bars in the graph indicate the median. The range is indicated in the accompanying table (bottom of B). Solid bar, class I VNTR; Open bar, class III VNTR. C, the expression levels among two paternally transmitted class I placentae were compared with those of two paternally transmitted class III placentae, using a range of internal standard amount to assess the linearity of the competitive RT-PCR assay. The relative concentration of IGF2 transcripts in each placenta is that concentration of internal competitor added at which the intensity of the specific IGF2 PCR product is near that of the internal standard (competitor amount in femtomoles: A = 1 x 10^{-5}; B = 5 x 10^{-5}; C = 1 x 10^{-4}; D = 5 x 10^{-4}; E = 1 x 10^{-3}). D, graphical representation of the competition curves. The x axis indicates the log_{10} of the concentration of internal standard added for coamplification, and the y axis indicates the log_{10} intensity ratio of the specific IGF2 PCR product to that of the internal standard. r^2 indicates the correlation coefficient, as a test of linearity of the assay.

**Figure 3**: Allelic effects of the VNTR on IGF2 expression in term placenta. A, variable IGF2 expression among term placentae. Upper panel, the expression levels were determined by PhosphorImager analysis of the PCR products. An equal amount of internal competitor standard was used for all the placentae assayed (1 x 10^{-6} fmol). Equal volumes of placental cDNA and competitor were coamplified in the presence of radiolabeled dATP. In a separate experiment, the cDNA was amplified with primers specific for the GAPDH gene in the presence of radiolabeled dATP, and the PCR was terminated at the exponential phase (lower panel). The PCR products were resolved electrophoretically in an 8% polyacrylamide gel, and the bands were quantitated by PhosphorImager analysis. The numbers above each lane in both panels indicate different placentae. RT indicates the products of a PCR performed in the absence of the internal competitor using a mock reverse-transcribed total RNA preparation that had been treated with RNase-free DNase for each placental sample assayed. All experiments were performed at least three times.

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**A**

![Diagram of pLCAT (class III VNTR) and pNCAT (no VNTR)]

**B**

![Graph showing Allelic effects of the VNTR on CAT expression in vitro](https://example.com/diagram.png)

**FIG. 4. Allelic effects of the VNTR on CAT expression in vitro.**

A, construction of the reporter gene constructs is described under “Materials and Methods.” The solid bar indicates the region containing the IGF2 promoters, P1 and P3. B, HepG2 cells were transiently transfected by the Lipofectin reagent with 0.3 μg of each construct, as described under “Materials and Methods.” For control of the efficiency of transfection, each construct was cotransfected with 1 μg of pSVβ, a plasmid encoding the β-galactosidase gene. Cells were harvested 48 h after cotransfection and assayed for CAT and β-galactosidase activity. All experiments were performed three times in triplicate, and the results of CAT activity, corrected by β-galactosidase activity, are presented normalized to pNCAT (mean ± S.E.). The normalized data are also shown in tabular form for only the class I (pSCAT-S) and class III (pLCAT) constructs, subdivided by relative transfection efficiency (based on β-galactosidase activity), where Medium transfection efficiency was 2.3-fold greater than Low transfection efficiency, and High transfection efficiency was 2.0-fold greater than Medium transfection efficiency (mean ± S.D., triplicate determinations).

**Table: Relative Transfection Efficiency**

| Relative Transfection Efficiency | Class I VNTR (pSCAT-S) | Class III VNTR (pLCAT) | Class I: Class III Transcription Ratio |
|----------------------------------|------------------------|------------------------|--------------------------------------|
| Low                              | 0.80 ± 0.14            | 0.55 ± 0.04            | 1.4                                  |
| Medium                           | 0.66 ± 0.03            | 0.47 ± 0.12            | 1.4                                  |
| High                             | 0.65 ± 0.05            | 0.38 ± 0.04            | 1.7                                  |

The effects of the VNTR on INS expression as well as on IGF2 suggest that the VNTR may be acting as a locus control region, whose transcriptional effects act globally on INS and IGF2. This also suggests possible VNTR effects on the other IGF2 promoters, which lie within 24 kb, a distance compatible with enhancer effects. One can argue that the allelic effects of the VNTR on insulin gene expression are more pronounced (3, 39) than on IGF2 (what we observe in this study) because INS lies closer to the VNTR (about 500 bp) than the placental IGF2 promoters (more than 11 kb downstream). It is remarkable that at this distance the VNTR still has allelic effects, but it is not without precedent; it should be noted that a VNTR contained in the sixth intron of the interleukin-1α gene (approximately 6 kb downstream from its promoter) has also been seen to influence interleukin-1α expression (11).

Functionally, the VNTR may be part of a nuclear matrix-attachment region and may influence the chromatin structure, modulating the accessibility of transcription factors to the nearby INS and IGF2 genes (54). Our speculation is that the shorter the number of tandem repeats, the greater the potential for the DNA not to be tethered to the nuclear matrix, thereby exposing a large chromatin loop (54) which facilitates transcription of the INS-IGF2 domain. One possibility is that the VNTR may act as a silencer of gene expression, whose effects could be proportional to the number of tandem repeats. Extrapolating from our results with the antisense VNTR construct, correct orientation may also be important for VNTR effects. As discussed above, the effects of the class I and class III alleles on INS gene expression are not the same in all tissues. For example, INS expression is higher from class I alleles in fetal and adult pancreas (2, 12), but in fetal thymus expression is higher from chromosomes with class III alleles (3, 40). Therefore, the regulation of gene expression modulated by alleles of this minisatellite may be more complex than initially thought and could therefore also involve allele-specific trans-acting factors whose effects on INS and IGF2 gene expression are tissue-specific and perhaps age-dependent. Finally, there is evidence that there may even be interactions or “cross-talk” between certain alleles since the preferential paternal transmission of diabetes susceptibility depends not only on the transmitted class I VNTR allele but also on the VNTR subclass of the untransmitted paternal allele (55).

It has been suggested previously (2, 21) that IGF2 may be a functional gene whose expression could be under transcrip-
tional effects of the VNTR at the IDDM2 locus. We have proposed (21) that possible mechanisms by which INS VNTR effects on IGF2 transcription could determine susceptibility to type 1 diabetes include a role of pancreatic IGF-II in islet regeneration, a role of thymic IGF-II in thymocyte selection by apoptosis, or a T-lymphocyte IGF-II autocrine loop amplifying cellular immune response. In view of the absence of any discernible effects of the VNTR on IGF2 in these tissues, these mechanisms appear unlikely. If IGF2 is involved in the IDDM2 effect in addition to (or instead of) INS, is must be doing so through a less direct mechanism, such as through effects on fetal nutrition or size, which have been found in some studies to be correlated with type 1 diabetes risk (56). Regardless of its possible relevance to diabetes, the effect we observe here appears to constitute an important part of the genetic background effects on INS transcription could determine susceptibility to diabetes, the effect we observe here appears to constitute an important part of the genetic background.