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Rapid communication

Human endogenous retrovirus with a high genomic sequence homology with *IDDMK$_{1,2}$22* is not specific for Type I (insulin-dependent) diabetic patients but ubiquitous

A. Kim[1], H.-S. Jun[1], L. Wong[1], D. Stephure[2], D. Pacaud[2], R. A. Trussell[2], J.-W. Yoon[1,3]

[1] Julia McFarlane Diabetes Research Centre, Department of Microbiology and Infectious Diseases, Faculty of Medicine, The University of Calgary, Calgary, Alberta, Canada
[2] Department of Paediatrics, Faculty of Medicine, The University of Calgary, Calgary, Alberta, Canada
[3] Laboratory of Endocrinology, Institute for Medical Science, Department of Endocrinology and Metabolism, School of Medicine, Ajou University, Suwon, Korea

Abstract

Aims/hypothesis. It has been reported recently that a novel human endogenous retroviral gene, insulin-dependent diabetes mellitus (*IDDM*)*K$_{1,2}$22*, was expressed in the plasma of Type I diabetic patients but not in that of nondiabetic control subjects. This investigation was initiated to determine the specificity of the selective expression of *IDDMK$_{1,2}$22* in diabetic patients.
Methods. We isolated the total RNA from the plasma and lymphocytes of 13 new onset Type I diabetic patients and 10 normal control subjects and amplified it by reverse transcriptase polymerase chain reaction. We then determined the presence of *IDDMK$_{1,2}$22* with a specific primer set, U3/R-poly(A), used in a recent report and the 5′SAg/3′SAg primer set recognizing the putative superantigen encoding the region of the *IDDMK$_{1,2}$22* envelope (*env*) gene. In addition, we carried out nested PCR of the U3/R-poly(A) polymerase chain reaction product using U3N/R primers.
Results. We found no difference in the presence of the polymerase chain reaction products between diabetic patients and all nondiabetic subjects tested. Sequencing of the U3/R-poly(A) polymerase chain reaction products showed that the exact sequence of *IDDMK$_{1,2}$22* was not present in any of the samples tested, neither in the plasma of diabetic patients nor in that of nondiabetic control subjects. Endogenous retroviral sequences with 90–93 % sequence homology to *IDDMK$_{1,2}$22* were, however, equally present in both the diabetic and nondiabetic subjects.
Conclusion/interpretation. We conclude that a human endogenous retroviral gene with high sequence homology with *IDDMK$_{1,2}$22* is not specific for diabetic patients but, rather, is ubiquitous. [Diabetologia (1999) 42: 413–418]

Keywords A novel human endogenous retrovirus (*IDDMK$_{1,2}$22*), superantigen, autoimmune Type I diabetes, human endogenous retrovirus (HERV)-K10.

Type I (insulin-dependent) diabetes mellitus results from the destruction of pancreatic beta cells by a beta-cell-specific autoimmune process. Viruses have been considered as both primary agents injurious to the pancreatic beta cells and triggering agents of beta-cell-specific autoimmunity leading to Type I diabetes [1]. Retroviruses have been suggested as possible candidate viruses for the triggering of beta-cell-specific autoimmunity in nonobese diabetic (NOD) mice [2–7]. To date there is no unequivocal evidence that murine retrovirus can trigger autoimmune diabetes in NOD mice.

It has been reported previously that a preponderance of islet-infiltrating T-cells bearing a particular T-cell receptor β-chain segment (Vβ7) was attributable to the presence of a superantigen [8]. It was not

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Corresponding author: Dr. J.-W. Yoon, Laboratory of Viral Immunopathogenesis of Diabetes, Julia McFarlane Diabetes Research Centre, Faculty of Medicine, The University of Calgary; 3330 Hospital Dr. NW; Calgary, Alberta, Canada, T2N 4N1
Abbreviations: NOD, Nonobese diabetic; MMTV, mouse mammary tumor virus; HERV-K, human endogenous retrovirus; GADPH, glyceraldehyde 3-phosphate dehydrogenase; IL-2, Interleukin-2; PBMC, peripheral blood mononuclear cells; IVS, intervening sequence.

known, however, whether the superantigen was encoded by a bacterium or an endogenous retrovirus. A recent study showed that the superantigen was encoded by a novel human endogenous retroviral gene designated *IDDM* (insulin-dependent diabetes mellitus) *K$_{1,2}$22* [9]. *IDDMK$_{1,2}$22* was thought to be a novel member of the mouse mammary tumour virus (MMTV)-related family of human endogenous retrovirus (HERV)-K [9]. This newly identified retrovirus (*IDDMK$_{1,2}$22*) was reported to be expressed in the plasma of recent onset Type I diabetic patients but not in that of nondiabetic control subjects [9]. The report that a novel human endogenous retroviral gene encoding a superantigen was found exclusively in Type I diabetic patients was extremely intriguing. Therefore, we attempted to confirm the specificity of the selective expression of *IDDMK$_{1,2}$22* in the plasma and peripheral lymphocytes of Type I diabetic patients.

Our careful studies have shown that a sequence identical to that of *IDDMK$_{1,2}$22* was not present in either the plasma or peripheral lymphocytes from both Type I diabetic patients and nondiabetic control subjects. A related human endogenous retrovirus with 90–93 % sequence homology with *IDDMK$_{1,2}$22* was, however, equally present in both diabetic and nondiabetic subjects, indicating that this identified human endogenous retrovirus is unlikely to be associated with the development of autoimmune Type I diabetes in humans.

Subjects and methods

Subjects. We collected 3 to 7 ml of blood in vacutainer tubes, using K_3EDTA as an anti-coagulant, from recent onset Type I diabetic patients (n = 13) and normal control subjects (n = 10).

Isolation of genomic DNA, cytoplasmic RNA, and viral RNA. We reserved 200 µl of whole blood for isolation of genomic DNA using the QIAmp Blood Kit (Qiagen, Ontario, Canada) and the remainder was used for the isolation of lymphocytes using Ficoll-Paque centrifugation (Pharmacia Biotech, Uppsala, Sweden). Cytoplasmic RNA was isolated from the lymphocytes after separation from the nuclei, according to a RNeasy protocol (Qiagen). Viral RNA was isolated from 140 µl of plasma using the QIAmp Viral RNA kit (Qiagen).

Reverse transcription of cytoplasmic and viral RNA. Cytoplasmic RNA (10.5 µl) from the 50 µl of total RNA isolated from the lymphocytes and viral RNA (8 µl) from the 50 µl of isolate obtained from the plasma was reverse transcribed using a poly(A) or oligo(dT)$_{12–18}$ primer, as described elsewhere [10].

PCR amplification of IDDMK$_{1,2}$22 *from genomic DNA, cytoplasmic RNA, and viral RNA.* PCR was carried out on genomic DNA as well as cytoplasmic and viral cDNA. We used 4 µl of template for PCR in a buffer containing 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 10 mmol/l Tris-HCl (pH 9.0) (Pharmacia Biotech), 1 µl of 10 mmol/l dNTP mix, 1 µl each of 15 µmol/l 5′ and 3′ primer, and 0.5 µl of 5000 U/ml Taq polymerase (Pharmacia) in a final reaction volume of 50 µl. Three primer sets were used for the first round PCR: i) 5′SAg 5′-CTGCCAAACCTGAGGAAGAA-3′, 3′ SAg 5′-CACCACACTATTGGCCACAC-3′; ii) U35′-AGGTATTGTCCAAGGTTTCTCC-3′, R-poly(A) 5′-TTTTGAGTCCCCTTAGTATTTATT-3′; iii) U3 as above; R 5′-GTAAAGGATCAAGTGCTGTGC-3′. The primers U3 N 5′-GGGCAATGGAATGTCTCAGTA-3′, and R as above, were used for nested PCR of the U3/R-poly(A) PCR product. Cycle conditions for PCR were as follows: 10 cycles of 94 °C for 15 s, 68 °C for 30 s with –1.3 °C per cycle, and 72 °C for 45 s; 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 45 s. The PCR product was run on a 1 % agarose gel and detected by ethidium bromide staining.

Control PCR reactions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PCR was run on 4 µl of template in 50 µl of a reaction mix containing 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, and 100 mmol/l Tris-HCl (pH 9.0) (Pharmacia), 1 µl 10 mmol/l dNTP mix, 1 µl of 10 µmol/l 5′-GAPDH primer (5′-CGGAG TCAACGGATTTGGTCGTAT-3′), 1 µl of 10 µmol/l 3′-GADPH primer (5′-GTCTTCACCACCATGGAGAAGG CT-3′) and 0.5 µl of 5000 U/ml Taq polymerase (Pharmacia). The cycle conditions for the PCR were as follows: 35 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 2 min.

Interleukin-2 (IL-2) intervening sequence 3 (IVS-3) primers were used for the detection of genomic DNA in viral and cytoplasmic RNA isolates. The primer sequences were as follows: IL-2 IVS-3 5′ primer 5′-TAGCTCCTTTCAGCAGAG AAG-3′; IL-2 IVS-3 3′ primer 5′-GTCTTATAGGCCTGT TGCCTT-3′. Viral RNA (1.6 µl) or cytoplasmic RNA (2.2 µl) was added to the same reaction mix used for GADPH PCR with substitution of the appropriate primers. The cycle conditions were as follows: 40 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min.

Sequencing of the U3/R-poly(A) PCR product. Following PCR, the samples were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced using the U3 primer.

Results and discussion

A very attractive hypothesis for the involvement of an endogenous retrovirus in T-cell-mediated autoimmune Type I diabetes proposed by Conrad et al. [9] is that polyclonal activation of a Vβ-restricted T-cell subset, triggered by the expression of an endogenous retroviral superantigen in major histocompatibility complex (MHC) class II positive antigen presenting cells, initiates the destruction of pancreatic beta cells. Conrad et al. identified a novel human endogenous retroviral gene, *IDDMK$_{1,2}$22*, encoding a superantigen in plasma from 10 new onset Type I diabetic patients and 10 age-matched control subjects. *IDDMK$_{1,2}$22* was found only in the plasma of Type I diabetic patients and not in that of the control subjects.

This finding is very intriguing and could be invaluable for the understanding of the initiation of autoimmune Type I diabetes and for the early prediction of the disease if, in fact, the results are reproducible. Therefore, we carefully examined the expression of *IDDMK$_{1,2}$22* transcripts in the plasma of recent onset

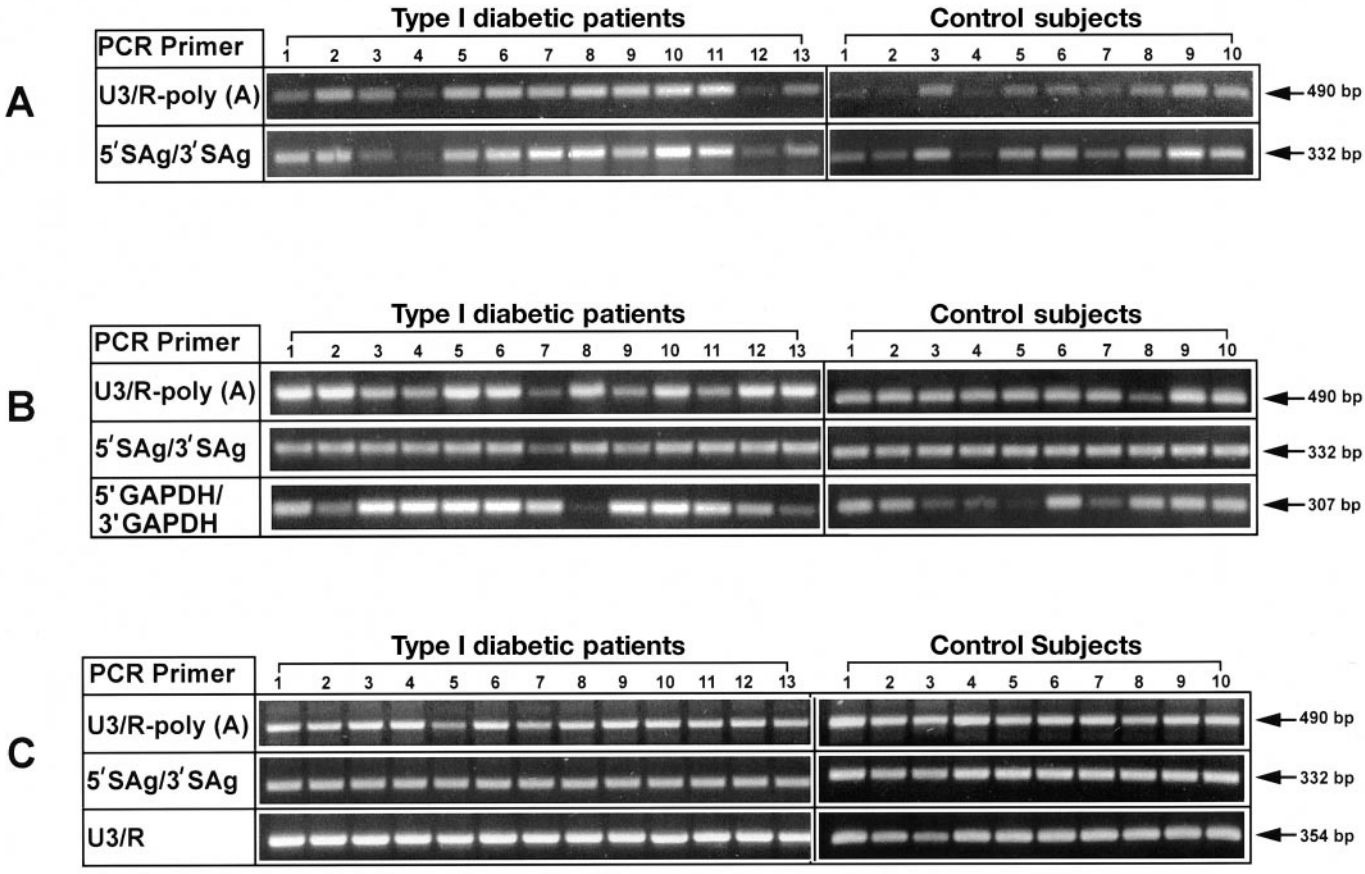

Fig. 1. A–C Retroviral signal detected in plasma viral RNA from both Type I diabetic patients and healthy control subjects. (**A**) Viral RNA was reverse transcribed using the R-poly(A) primer in place of oligo(dT). The amplified product was detected in all samples regardless of whether U3/R-poly(A) (490 bp product) or 5 ′SAg/3 ′SAg (332 bp product) primers were used for PCR. (**B**) A retroviral signal was detected in the cytoplasmic RNA from both Type I diabetic patients and normal control subjects. Cytoplasmic RNA was reverse transcribed using oligo(dT). The amplified product was detected in all samples using the U3/R-poly(A) primer set (490 bp product) or 5 ′SAg/3 ′SAg primer set (332 bp product) for PCR. The quality of cytoplasmic RNA isolation and reverse transcription was confirmed using primers specific for GAPDH for PCR amplification (307 bp product). (**C**) The U3/R-poly(A) primer set is capable of amplifying the appropriate product from genomic DNA. As expected the 5 ′SAg/3 ′SAg and U3/R primer sets amplified 332 bp and 354 bp PCR products, respectively. The U3/R-poly(A) primer set amplified a 490 bp PCR product, contrary to the finding of Conrad et al. [9]

Type I diabetic patients and healthy control subjects by RNA specific PCR using the U3/R-poly(A) primer pair specific for *IDDMK$_{1,2}$22*. In contrast to the mentioned study we detected PCR-amplified product uniformly in all the plasma viral RNA samples tested (Fig. 1A). To verify the specificity of the U3/R-poly(A) PCR product, we carried out nested PCR using the U3N/R primer set. We also detected the same PCR product in all the samples tested (data not shown). We found no difference in the detection of the U3/R-poly(A) PCR product between Type I diabetic patients and nondiabetic control subjects.

To determine whether there is any difference in the detection of the endogenous retroviral sequence encoding the superantigen region which is specific for *IDDMK$_{1,2}$22* between Type I diabetic patients and control subjects, we designed primers (5 ′SAg and 3 ′SAg) from the published sequence, amplified plasma cDNA using the newly designed primer pair, and examined the presence of the amplified products. We detected a 332 base-pair sized amplified product uniformly in all the tested samples (Fig. 1A). In addition to plasma samples, we also examined the expression of *IDDMK$_{1,2}$22* in the peripheral blood mononuclear cells (PBMC) from diabetic patients and control subjects by RT-PCR using the U3/R-poly(A) primer pair and the 5 ′SAg/3 ′SAg primer pair. We obtained the same results as with the plasma samples. The PCR product was detected uniformly in the tested samples (Fig. 1B).

Conrad et al. showed that the U3/R-poly(A) primer set amplified only the retroviral RNA transcript, but not genomic DNA, while the U3/R primer set amplified genomic DNA. To determine whether the U3/R-poly(A) primer set does not amplify genomic DNA, we did PCR on genomic DNA isolated from PBMC using the U3/R-poly(A), U3/R, and 5 ′SAg/3 ′SAg primer sets. We detected a PCR product of

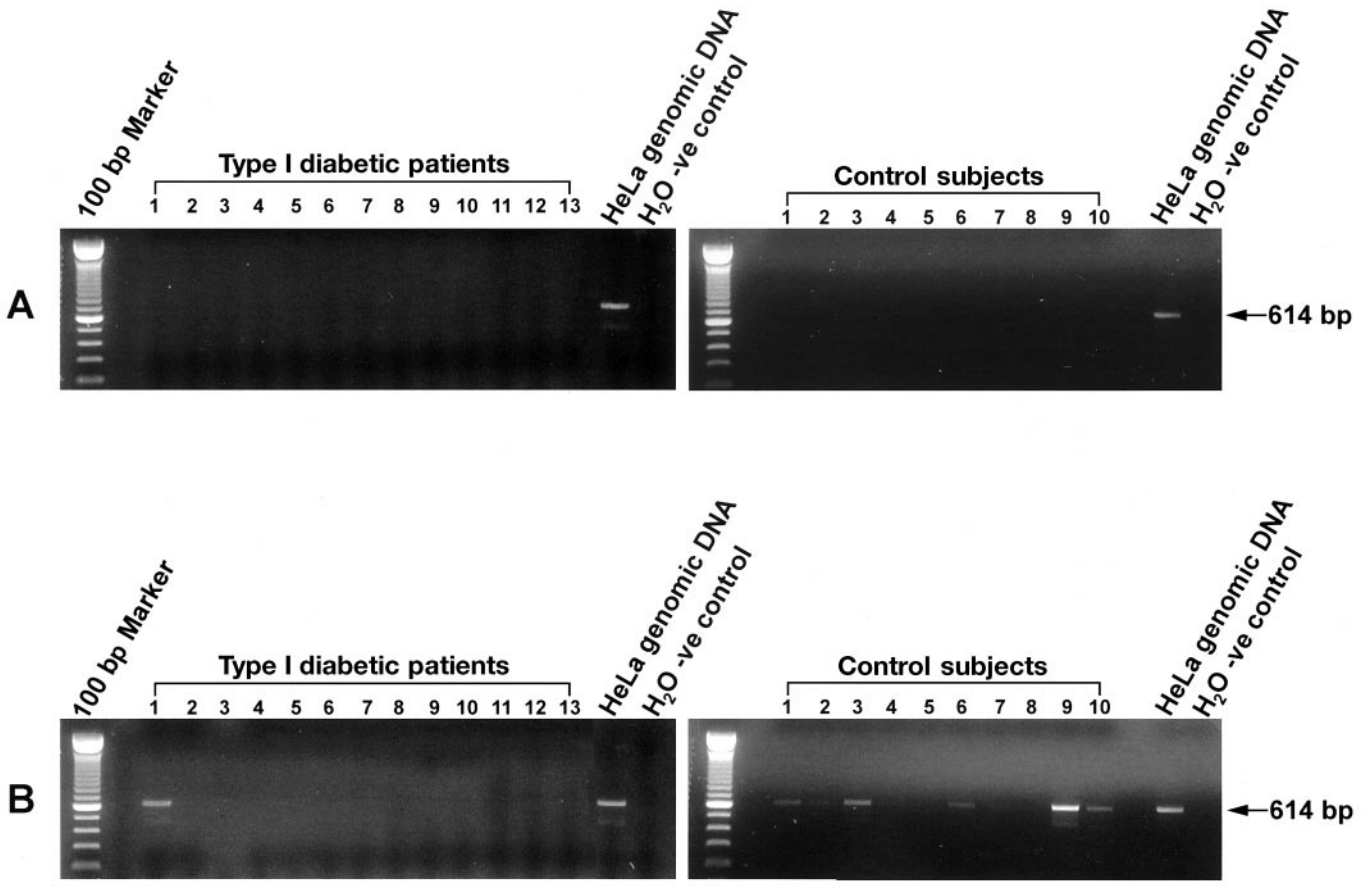

Fig. 2. No genomic DNA detected in the plasma viral RNA isolate from either Type I diabetic patients or healthy control subjects. (**A**) Plasma viral RNA; IL-2 intron 3 primers did not detect genomic DNA in the non-reverse transcribed viral RNA. (**B**) Lymphocyte cytoplasmic RNA; IL-2 intron 3 primers detected genomic DNA in the non-reverse transcribed cytoplasmic RNA from 1 of 13 Type I diabetic patients and 7 of 10 healthy control subjects. As a positive control, HeLa genomic DNA was amplified using the same primers. As a negative control, H_2O without template was amplified using the same primers

the expected size in all samples tested. This result suggests that the U3/R-poly(A) primer set efficiently amplified the same PCR product from both genomic DNA and RNA (Fig. 1C).

It is quite possible that genomic DNA could contaminate the plasma viral RNA preparation and that the contaminating genomic DNA may be amplified in the tested samples. To determine whether there was any DNA contamination in our plasma viral RNA and PBMC RNA preparations, we did PCR of plasma viral RNA and PBMC RNA (without reverse transcription) using IL-2 intron primers (IL-2 IVS-3 5′ and IL-2 IVS-3 3′). We did not detect any PCR product from the plasma viral RNA preparation, but we did detect a PCR product in 8 of 23 PBMC RNA samples (Fig. 2A and 2B). This result indicates that there was no genomic DNA contamination in the plasma viral RNA preparation but that some of the PBMC RNA samples did have genomic DNA contamination. There was, however, no difference in the detection of the amplified PCR product (retroviral RNA transcripts) from the PBMC samples (Fig. 1B), regardless of the genomic DNA contamination. Thus, the detection of the amplified PCR product in PBMC RNA samples was not due to the genomic DNA contamination alone.

In the different sets of experiments described above using different primers – the U3/R-poly(A) primer set and the 5′SAg/3′SAg primer set – our results show consistently that there is no difference in the expression of the endogenous retrovirus *IDDMK$_{1,2}$22* between Type I diabetic patients and nondiabetic control subjects. To determine whether the uniformly amplified PCR product did indeed come from *IDDMK$_{1,2}$22*, we sequenced the amplified PCR product using the U3 primer. We did not find a sequence identical to that of *IDDMK$_{1,2}$22*. We did, however, find similar sequences, sharing 90–93% sequence homology with that of *IDDMK$_{1,2}$22* (Fig. 3).

This study shows that an endogenous retrovirus, related to the HERV-K family, was certainly present equally in Type I diabetic patients and nondiabetic control subjects. Our finding is quite contradictory to the report by Conrad et al. [9]. A possible explana-

IDDMK$_{1,2}$22	AGGTATTGTC	CAAGGTTTCT	CCCCATGTGA	TAGTCTGAAA	TATGGCCTCG	TGGGAAGGGA	AAGACCTGAC	CATCCCCCAG	ACCAACACCC	GTAAAGGGTC	TGTGCTGAGG
DM 9	----------	----------	----------	-- -------	----------	----------	----------	-G--------	C--G------	----------	----------
DM 10	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
DM 11	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
DM 12	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
DM 13	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
NC 18	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
NC 21	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
NC 22	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------
NC 23	----------	----------	----------	----------	----------	----------	----------	-G--------	C--G------	----------	----------

IDDMK$_{1,2}$22	AGGATTAGTA	TAAGAGGAAA	GCATGCCTCT	TGCAGTTGAG	AGAAGAGGAA	GACATCTGTC	TCCTGCCCAT	CCCCTGGGCA	ATGGAATGTC	TCAGTATAAA	ACCCGATTGA
DM 9	----------	A--------G	-C--C-----	-------T--	-C------G-	AG---TC---	--------G-	--- ------	--T-------	--G-------	---------T
DM 10	----------	A--------G	-A--------	-------T-A	-C--------	AG----C---	--------G-	----------	----------	--G-------	---------T
DM 11	----------	A--------G	-A--------	-------T-A	-C----A-C-	AG----C---	--------G-	T-- ------	----------	C-G-------	---------T
DM 12	----------	A--------G	-A--C-----	----A--T-A	-C----A-G-	AG---TC---	--------G-	T-- ------	--T-------	--G-------	---------T
DM 13	----------	A--------G	-A--------	-------T-A	-C----A-G-	AG----C---	--------G-	T-- ------	----------	--G-------	---------T
NC 18	----------	A--------G	----C-----	-T-----T-A	-C----A-G-	AG---TC---	--------G-	T---------	--T-------	--G-------	----T---TT
NC 21	----------	A--------G	----C-A---	----A--T-A	-C------G-	AG---TC---	--------G-	T-- ------	--T-------	--G-------	---------T
NC 22	----------	A--------G	-A--C-----	----A----A	-C----A---	AG----C---	--------G-	T-- ------	----------	C-G-------	---------T
NC 23	----------	A--------G	----C-----	----A--T-A	-C--------	AG----C---	-----G--G-	--- ------	----G-----	C-G-------	----C----T

IDDMK$_{1,2}$22	ACATTCCATC	TACTGAGATA	TGTATATCTA	AAAGCACAGC	ACTTGATCCT	TTACCTTGTC	TATGATGCAA	ACACCTTTGT	TCACGTGTTT	GTCTGCTGAC	CCTCTCCCCA
DM 9	TTGC------	----------	--C-------	----------	----A-----	----------	----------	-G--------	----------	----------	----------
DM 10	TTGC------	----------	--C-------	----------	----A-----	----A-----	----------	-G--------	----------	----------	----------
DM 11	TTGC------	C-----A---	--C-------	----------	----A-----	----------	----------	-G--------	----------	----------	----------
DM 12	TTGC------	----------	--C-------	----------	----A-----	----A-----	----------	-G--------	----------	----------	----------
DM 13	TTGC------	------A---	--C-------	----------	----A-----	----A-----	----------	-G--------	----------	----------	----------
NC 18	TTCC----A-	------A---	--CC------	--------T-	----A-----	----------	----------	-G--------	----------	----------	----------
NC 21	TTGC------	------A---	A-C-------	----------	----A-----	----A-----	----------	-G--------	----------	----------	----------
NC 22	TTGC----A-	--A---A---	A-C-------	----------	----A-----	----------	----------	-A--------	----T-----	----------	----------
NC 23	TTGC------	--A---A---	A-C-------	----------	----A-----	----------	----------	-A--------	----T-----	----------	----------

IDDMK$_{1,2}$22	CTATTGTCTT	GTGACCCTGA	CACATCTCCC	TCA GGAGAA	ACACCCACGA	ATGATCAATA	AATACTAAGG	GGACTCAAAA	A
DM 9	-A--------	----------	------C---	--TTC-----	--------A-	----------	G---------	----------	-
DM 10	-A--------	----------	------C---	--T T-----	--------A-	----------	----------	----------	-
DM 11	-A--------	----------	------C---	--TTC-----	--------A-	----------	----------	----------	-
DM 12	-A--------	----------	------C---	--TTT-----	--------A-	----------	----------	----------	-
DM 13	-A--------	----------	------C---	--TTC-----	--------A-	----------	----------	----------	-
NC 18	-A--------	----------	------C---	--T ------	--------A-	----------	----------	----------	-
NC 21	-A--------	----------	------C---	--TTC-----	--------A-	----------	----------	----------	-
NC 22	-A--------	----------	------C---	--TTC-----	--------A-	----------	----------	----------	-
NC 23	-A--------	----------	------C---	TCT TC----	--------A-	----------	----------	----------	-

Fig. 3. Sequence of U3/R-poly(A) amplified PCR product. Row 1 is the published sequence of *IDDMK$_{1,2}$22* (Genbank Accession No. AF012335). Rows 2–6 are sequences obtained from Type I diabetic patients (DM = diabetes mellitus). Rows 7–10 are sequences obtained from normal control subjects (NC = normal control). 90 to 93% homology was observed between our sequences and that of *IDDMK$_{1,2}$22*. Only the nonmatching nucleotides are shown

tion for the distinct difference in the identification of *IDDMK$_{1,2}$22* between the Type I diabetic patients and the control subjects found by Conrad et al. could be two separate sets of experiments. In Conrad et al.'s report the schedule of blood collection and RNA preparation showed that all the Type I diabetic patients were investigated together on one occasion, while the control subjects were investigated together on another occasion. In such circumstances, an error in one set of experiments might account for the results found by Conrad et al. It is also conceivable that there was a significant genetic variability between the subject pools in the two different studies and/or significant differences in the specific exposure to other infectious agents, including certain viruses and bacteria. These diverse infectious agents possibly activate endogenous retroviral genes differently, which could be reflected in the differences in the detection of a retroviral gene in the samples collected from the disparate locations.

In conclusion, a sequence identical to that of *IDDMK$_{1,2}$22* was not detected in either the plasma or peripheral blood lymphocytes from Type I diabetic patients or nondiabetic control subjects. A related human endogenous retroviral gene with 90–93% sequence homology with *IDDMK$_{1,2}$22*, however, was equally present in both diabetic and nondiabetic subjects. Therefore, we conclude that an endogenous retroviral gene with high sequence homology with *IDDMK$_{1,2}$22* is not specific for Type I diabetic patients but is ubiquitous, indicating that this retrovirus is unlikely to be associated with the development of autoimmune diabetes in humans.

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