SHORT COMMUNICATION

Genomic Structure and Promoter Sequence of the Insulin-Dependent Diabetes Mellitus Autoantigen, IA-2 (PTPRN)

Jingping Xie, Baowei Zhang, Michael S. Lan,[1] and Abner Louis Notkins[2]

Experimental Medicine Section, Oral Infection and Immunity Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892-4322

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IA-2 is a transmembrane protein tyrosine phosphatase, expressed in neuroendocrine cells, and a major autoantigen in insulin-dependent diabetes mellitus. In the present study we elucidated the structure of the IA-2 gene (HGMW-approved symbol PTPRN) and its promoter sequence. A 40-kb genomic clone covering the whole IA-2 coding sequence and 4 kb proximal 5′-upstream sequence was isolated and mapped. The IA-2 gene encompasses approximately 20 kb with 23 exons ranging from 34 bp to more than 650 bp. The extracellular domain is encoded by exons 1–12, the transmembrane region by exon 13, and the intracellular domain by exons 14–23. The transcriptional start site(s) of the IA-2 gene was mapped by 5′ rapid amplification of cDNA ends to 97 bp upstream of the translational start site. A 3-kb 5′-upstream region was sequenced, revealing a GC-rich region and TATA-less sequence containing several potential transcription-regulating sites (i.e., Sp1, CREB, GATA-1, and MZF). Functional promoter activity was confirmed by transient transfection of U87MG cells with deletion mutants linked to a luciferase reporter gene.

IA-2 is a new member of the transmembrane protein tyrosine phosphatase (PTP) family (17). Its cDNA encodes a protein of 979 amino acids with a large extracellular domain, a transmembrane domain, and an intracellular domain (8). The gene is located on the long arm of human chromosome 2 (2q35) (9). Tissue distribution studies revealed that it is expressed in pancreatic islets and other neuroendocrine cells and is processed as a transmembrane protein in secretory granules (21).

Clinical studies revealed that 65 to 75% of new-onset insulin-dependent diabetes mellitus (IDDM) patients have autoantibodies to IA-2 compared to 1% or less of the normal population (4, 10, 17). Taking into account another IDDM autoantigen, glutamic acid decarboxylase (GAD-65), up to 90% of IDDM patients have autoantibodies to one or both of these antigens (17). Of particular interest is the observation that the autoantibodies to IA-2 and GAD-65 appear years before the onset of IDDM and have become important markers for identifying individuals at high risk of ultimately developing clinically apparent IDDM (17). In the general population it is estimated that about 1 in 600 children will ultimately develop IDDM. However, in the individuals who have autoantibodies to both IA-2 and GAD-65 the likelihood of developing IDDM within 5 to 10 years is in the 40 to 60% range (17).

Although autoantibodies to IA-2 are clearly a marker for IDDM, what triggers the immune response to this protein and the role that the immune response actually plays in the pathogenesis of IDDM remain unclear. Comparison of the protein sequence of IA-2 with other members of the PTP family showed 35–45% sequence similarity in the PTP domain, but little similarity in the extracellular domain (8). Epitope mapping revealed that the major autoantigenic determinants are located at the carboxyl-terminus of the intracellular domain (26, 7). Further, studies revealed that sera from IDDM patients that reacted with IA-2 showed no reactivity with other members of the PTP family (16) and that the epitopes on IA-2 are largely conformationally dependent (24). Also in contrast to other members of the PTP family, the intracellular domain of IA-2 is resistant to proteolytic cleavage by trypsin. To understand this molecule better, its relationship to other PTPs, and its role in IDDM, knowledge of its genomic structure and transcription regulatory elements is required. In the present study, we isolated the IA-2 gene[3] from a human cosmid library, determined the intron–exon junctions, sequenced the 5′-upstream region, and demonstrated promoter activity using transient transfection analysis.

To determine the genomic structure of IA-2, we ini-

[1] Current address: Research Institute for Children, Department of Pediatrics, Biometry and Genetics, Louisiana State University Medical Center, New Orleans, LA 70123.

[2] To whom correspondence should be addressed at Experimental Medicine Section, National Institute of Dental Research, National Institutes of Health, Building 30 Room 121, 30 Convent Drive MSC 4322, Bethesda, MD 20892-4322. Telephone: (301) 496-4535. Fax: (301) 402-4163. E-mail: notkins@yoda.nidr.nih.gov.

[3] The HGMW-approved symbol for the gene described in this paper is PTPRN.

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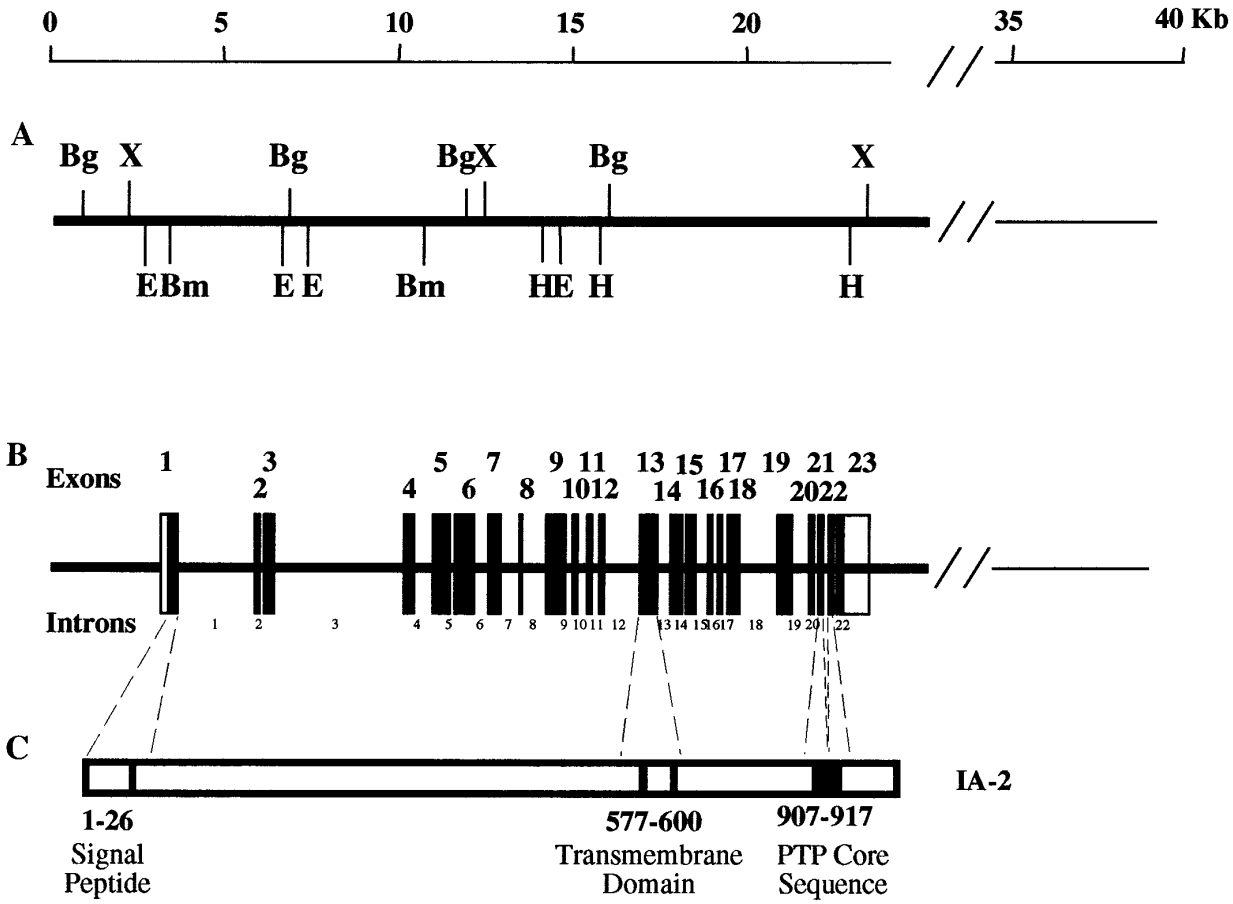

FIG. 1. Schematic diagram of the genomic structure of IA-2. (**A**) Restriction map of Cos 6 for endonucleases *Bam*HI (Bm), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), and *Xho*I (X). (**B**) The approximate positions and relative sizes of the exons (vertical bars) and introns (horizontal bars) are indicated in relationships to Cos 6 and (**C**) IA-2 protein. The open bars in exons 1 and 23 represent, respectively, 5′ and 3′ untranslated regions.

tially screened a human genomic DNA library in λ phage (Stratagene, La Jolla, CA; insert size 9 to 15 kb) with randomly radiolabeled (Life Technologies, Gaithersburg, MD) IA-2 cDNA as a probe. Restriction mapping and Southern blot analysis revealed four clones containing inserts of approximately 13.5 kb. T3 and T7 RNA polymerase-generated RNA probes used for chromosome walking, however, failed to identify overlapping clones. Therefore, we switched to a cosmid library (Stratagene; average insert size 38 kb). By screening of duplicate filters, representing at least 10^6 colonies, with the IA-2 probe over 100 clones were isolated. One clone (Cos 6) was found to contain the largest 5′ sequence and therefore was characterized in depth (Fig. 1A). PCR analysis of Cos 6 with primers close to both ends of IA-2 cDNA (antisense primer at cDNA position 80 and sense primer at cDNA position 3540) revealed that the clones encompassed the full length of the IA-2 cDNA (data not shown). Clone Cos 6 then was mapped from both ends with restriction endonucleases (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Xho*I) by a FLASH nonradioactive gene mapping kit (Stratagene). The first *Bam*HI site at 3.9 kb corresponded to the *Bam*HI site of the IA-2 cDNA located at position 107 in the 12th codon. The authenticity of the clone was confirmed by Southern blot analysis. Genomic DNA, isolated from the lymphocytes of three individuals, was restriction digested and probed with full-length IA-2 cDNA. Southern blots revealed bands (3.5, 5, and 18 kb for *Eco*RI digestion and 1.4, 4.7, and 23 kb for *Hin*dIII digestion) consistent with the sizes predicted from the mapped cosmid genomic clones (data not shown). The sum of band sizes from different restriction enzymes indicates that the IA-2 gene is less than 30 kb.

To determine the intron–exon junctions of the IA-2 gene, cloned cosmid DNA (Cos 6) was amplified by PCR with 12 pairs of primers spread out over the full-length of the IA-2 cDNA. The sequence of the PCR product from the genomic DNA then was compared with the IA-2 cDNA. By this approach, the locations of the exons and their relation to IA-2 cDNA and protein were determined (Figs. 1B and 1C). Several introns were completely sequenced; to estimate the sizes of those unsequenced introns, primers pairs annealed to the flanking regions of each exon were designed for PCR amplification. Intron size was calculated by subtracting the size of the cDNA sequence between primers from the estimated size of the PCR products. The sum of all the introns and exons of the IA-2 gene was approximately 20 kb, which is consistent with the data of

TABLE 1

Intron–Exon Junction of Human IA-2

Exon	Exon size (bp)	Intron	Intron size (kb)	Sequence at exon–intron junction: 5′ splice donor		3′ splice acceptor		AA at junction: AA	Pos.	Type
1	213						GGTGGG	—	—	—
2	50	1	1.4	CCCACG	**gt**cagg	ctac**ag**	GCTGTC	G	39	1
3	114	2	0.26	TTCAGG	**gt**cagg	cctt**ag**	ATGGCT	D	56	1
4	97	3	3.4	CCCAAG	**gt**aagg	gatc**ag**	GATTGT	G	94	1
5	262	4	0.8	GGACAG	**gt**aggc	ctac**ag**	GTCTGG	R	126	2
6	355	5	0.09	CACCAG	**gt**aagt	ccac**ag**	TTTGGC	Q/F	213	0
7	133	6	0.4	AGCCAG	**gt**taac	cagc**ag**	ATGCGG	D	332	1
8	34	7	0.6	ATCCGG	**gt**aagt	tcgc**ag**	AGGGGT	G	376	1
9	275	8	0.6	AAGAAA	**gt**gagt	gaac**ag**	ACAATG	K/T	387	0
10	87	9	0.22	TCAGAA	**gt**aagg	ctcc**ag**	GCCCCT	K	479	2
11	80	10	0.3	CATCAG	**gt**gggg	tggc**ag**	TGTGGT	S	508	2
12	65	11	0.18	AAGCAG	**gt**acct	ctgc**ag**	GGCTGG	G	535	1
13	219	12	0.9	GGACAG	**gt**atgc	ccac**ag**	AGGGAG	Q/R	556	0
14	201	13	0.5	TACCAG	**gt**gtgc	ccac**ag**	GACCTG	Q/D	629	0
15	148	14	0.1	ATTCTG	**gt**cagg	ccac**ag**	GCATAC	L/A	696	0
16	74	15	0.54	TGCCCT	**gt**cagt	gtgc**ag**	ATGACC	Y	746	1
17	78	16	0.232	CCCATT	**gt**gagt	ctcc**ag**	ATTGAG	I/I	770	0
18	119	17	0.093	TGGCAG	**gt**gggc	ccct**ag**	ATGGTG	Q/M	796	0
19	167	18	1.2	TATGAG	**gt**cagc	gggc**ag**	GTGAAC	E/V	836	0
20	54	19	0.5	CCGCAG	**gt**tggc	tccc**ag**	GAAGGT	R	892	2
21	65	20	0.576	CTGCAG	**gt**gggt	tttc**ag**	TGATGG	S	910	2
22	74	21	0.214	CAAAAG	**gt**aggg	ctcc**ag**	GAGTGA	G	932	1
23	>650	22	0.213	TCTAAG	**gt**gacc	ctcc**ag**	GACCAG	K/D	956	0

Note. Nucleotide sequences indicated at the intron (lowercase letters) and exon (uppercase letters) junctions. Exons are numbered from the 5′ end as illustrated in Fig. 1. The sizes of introns and exons are indicated in basepairs. The amino acid interrupted by an intron is indicated by a single letter and its position is shown. For type 0 introns, the 2 amino acids on each side of the intron are given.

restriction enzyme mapping and Southern blot analysis. Table 1 shows the size of each exon and intron, the junction sequences, and the location and phasing of each intron in the IA-2 gene. The sizes of the exons range from the shortest with 34 nucleotides (exon 8) to the longest (exon 23) with more than 650 nucleotides in the 3′ UTR region. A long exon in the 3′ UTR also is found in other members of PTP family (3, 5, 23). Exon 1 contains the entire 5′ untranslated region and the beginning of the coding region to amino acid residue 39. There are 12 exons in the extracellular domain, 1 exon (No. 13) in the transmembrane region, and 10 exons in the intracellular domain. The introns vary in the size from 90 bp (intron 5) to 3.4 kb (intron 3), and all of the splice acceptor and donor sequences agree with the GT/AG rule (15). Among introns in the IA-2 gene, 9 were type 0, where introns occur between codons (i.e., introns 5, 8, 12, 13, 14, 16, 17, 18, and 22), 8 were type 1, where introns interrupt the first and second base of the codon (i.e., introns 1, 2, 3, 6, 7, 11, 15, and 21), and 5 were type 2, where introns interrupt the second and third base of the codon (i.e., introns 4, 9, 10, 19, and 20). We have sequenced more than 6 kb of introns. During characterization of the IA-2 genomic structure, no major microsatellite sequence was found except for a 14-TG repeat in intron 18. We also failed to detect any polymorphism of the IA-2 gene by Southern analysis (data not shown).

Based on its genomic structure, it is now possible to localize certain important features of the IA-2 molecule to specific exons. The tryptic cleavage site located immediately after the arginine at position 653, which when cleaved results in a 40-kDa fragment (17, 24), is encoded by exon 15. Consecutive lysines, which are thought to be involved in the natural processing of IA-2 into a 64-kDa fragment, located at position 386–387 (8), are encoded by exons 8 and 9. A second lysine pair at position 447–448 is encoded by exon 9, and the PTP core sequence at position 907–917 is encoded by exons 20 and 21.

The transcription start site was determined by primer extension and 5′ RACE with RNA prepared from a cultured glioblastoma cell line (U87MG) in which the IA-2 gene is highly expressed. Primer extension with primer at position 80 of IA-2 cDNA produced three major dots in a highly smeared background. This suggested random termination of reverse transcription and potential multiple transcription start sites. Good primer extension data were not obtained with primers annealed to different regions of the 5′ terminus of IA-2 cDNA, perhaps due to the extremely high GC content (more than 80%) in the upstream region. To overcome this problem we attempted to use 5′ RACE. By regular dG-tailing this also was unsuccessful. Therefore the reversed-transcribed cDNA ends were dA-tailed with terminal deoxynucleotidyltransferase. DNA polymerase I then was used to synthesize the second-strand DNA. Thereafter, two consecutive PCRs were per-

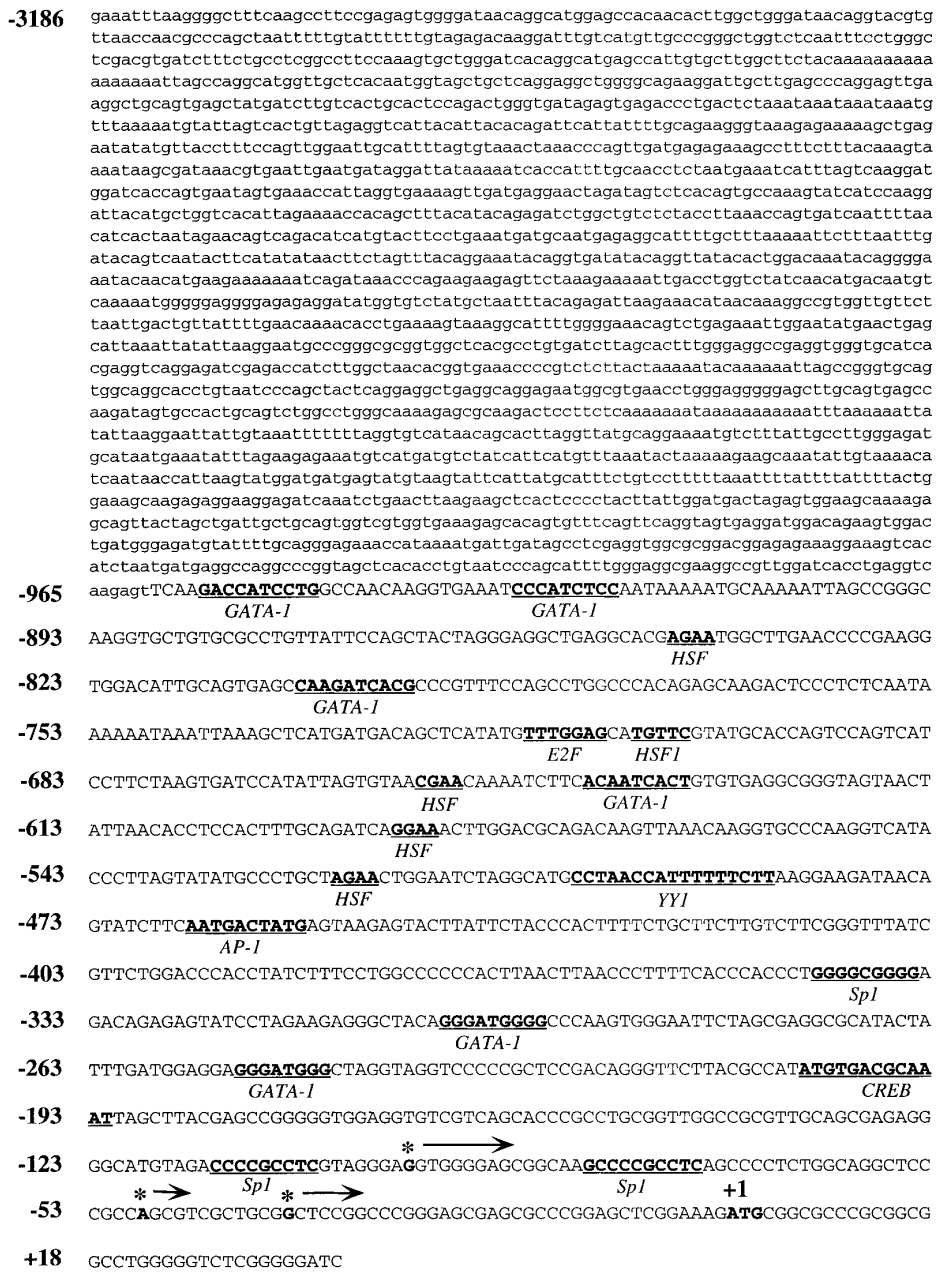

FIG. 2. Nucleotide sequence at the 5′-end of the human IA-2 gene (GenBank Accession No. AF042285). Three potential transcription start sites are indicated by an asterisk followed by an arrow. The potential binding sites for transcription factors, such as Sp1, GATA-1, c-Myb, CREB, and HSF-1, in the proximal 1 kb of the promoter are indicated in bold and underlined. The adenosine of the translated initiating codon is assigned position +1.

formed with two nested sets of primers. This modified procedure resulted in a distinct band approximately 300 bp in length (not shown). The band was gel purified and subcloned into pCR2.1 vector (Invitrogen), and plasmid DNA from 20 randomly picked clones was subjected to DNA sequencing and aligned with the genomic sequence. The data showed that six clones terminated at position −97, seven clones at position −49, and five clones at position −37. These three positions represent the most likely transcription start sites. Comparison of the RACE data with the genomic sequence of IA-2 indicates that there is no intron in the 5′ UTR of the IA-2 gene.

The 5′-upstream DNA sequence extending 3186 bp upstream of the transcription start site (Fig. 2) was determined by automated DNA sequencing and compared with known sequences in the GenBank DNA database. Examination of the 5′-upstream region failed to detect either a TATA or CAAT box. The region between the transcription start site and the translation start site, similar to other transmembrane PTPs (3, 5, 23), was found to be highly GC rich. In addition, this region contained two Sp1 binding sites near the transcription start site. Sp1 binding sites are thought to be critical for initiation of transcription in TATA-less genes (1, 19). In addition there are a number of poten-

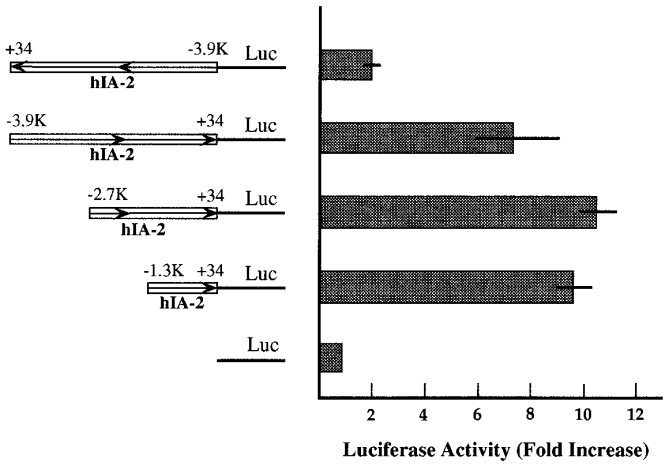

FIG. 3. IA-2 gene 5′-upstream deletion mutants and their relative promoter activity. The 3.9-kb *Bam*HI fragment was fused to a promoterless reporter vector (pGL2 basic) in the sense or antisense orientation relative to the luciferase gene and transiently transfected into U87MG cells. Number (K) on each construct indicates the end of the promoter fragment relative to the first translation codon. Arrows indicate the orientation of the promoter relative to the luciferase reporter gene. Luciferase activity is expressed as fold increase over the promoterless vector. Values represent averages of three independent experiments.

tial GATA factor binding sites (14) as well as binding sites for other transcriptional factors such as AP1, CREB (cAMP response element binding protein), E2F, HSF (heart shock factor), and YY1 (20, 25).

To see whether the 5′-upstream region possessed promoter activity, deletion mutants of the IA-2 gene, fused to the luciferase reporter gene in pGL2 basic (Promega), were constructed. Deletion mutants ranging from −3.9 kb to +34 bp were prepared in the forward and reverse orientations and assayed for promoter activity by transient transfection in U87MG cells. As seen in Fig. 3, the 3.9-kb construct, in sense orientation, showed a 7-fold increase in activity over the promoterless luciferase construct. The 3.9-kb construct, in reverse orientation, showed approximately the same degree of activity as the promoterless control. Deletion of the sequence between −3.9 and −2.7 kb increased reporter expression from 7- to 10-fold above the promoterless control, suggesting that the deleted sequence contained negative regulatory elements. Further deletion from −2.7 to −1.3 kb had little effect on reporter expression. The exact location and nature of the functional elements contributing to IA-2 promoter activity are under further investigation.

Both protein tyrosine kinases and PTPs play important roles in cellular signaling, including proliferation, differentiation, and transformation. Over 50 different PTPs have been identified. PTPs are divided into three broad categories: class I, cytosolic (e.g., PTP1b); class II, transmembrane with one phosphatase domain (e.g., HPTPβ); and class III, transmembrane with two phosphatase domains (e.g., CD45 and LAR). The latter class is thought to have evolved by capturing a transmembrane peptide and extracellular domain or duplicating a cytoplasmic PTP domain (6). In this context it is of interest that the PTP core sequences (VHCSAGVCRTC) of CD45 and LAR (3, 5, 18, 23) and IA-2 are all interrupted by a type 2 intron within the serine codon. Based simply on structural domains, IA-2 is a class II PTP. However, IA-2 is distantly related to the other members of the PTP family. The extracellular domain of IA-2 shows only 27% identity and the intracellular domain only 35% identity to its closest neighbors (8, 17). Moreover, IA-2 from mice, rats, and humans show no enzyme activity with conventional PTP substrates. This is due to the substitution of 2 amino acids in the highly conserved PTP catalytic domain. Enzyme activity can be restored by replacing the aspartic acid at position 911 with alanine and the alanine at position 877 with aspartic acid (13; P. M. Li and A. L. Notkins, unpublished data). The function of IA-2 in its native configuration thus remains unclear. Either IA-2 acts on a highly specific, but still unidentified substrate or it has an entirely different function. Recently, another transmembrane PTP, designated IA-2β (also known as phogrin or PTP-NP), was isolated (2, 11, 12, 22). IA-2β is most closely related to IA-2 and shows 27% identity in the extracellular domain and 76% identity in the intracellular domain. IA-2 and IA-2β have many properties in common, including lack of enzymatic activity with conventional PTP substrates, restricted tissue distribution, localization to membranes of secretory granules, and reaction with autoantibodies in sera of IDDM patients (17). Based on these findings, it is possible that IA-2 and IA-2β belong to a new subclass within the PTP family with different function and/or substrate specificity. The elucidation of the genomic structure of IA-2β, which is now under way, might provide further support for this argument.

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