Complete molecular scanning of the human Fas gene: mutational analysis and linkage studies in families with Type I diabetes mellitus

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Abstract

Aims/hypothesis. The human Fas gene (*FAS*) on chromosome 10q24.1 encoding a cell surface receptor involved in apoptosis was evaluated as a candidate susceptibility gene for Type I (insulin-dependent) diabetes mellitus. Apoptosis mediated by Fas is important in maintaining peripheral self-tolerance and in down-regulating the immune response and could have a role in immune-mediated beta-cell destruction.

Methods. We did a molecular scan of the entire human *FAS* (promoter, exons 1–9 including exon-intron boundaries and the 3' UTR) using single strand conformational polymorphism-heteroduplex analysis.

Results. We identified 15 mutations, of which 11 are new. Of these a *g-1194A→T* and a *g-295A* give rise to alterations of transcription-factor-binding consensus sequences for c-Myb, SP-1 and NF-κB, respec-

tively. A total of 1068 people from a Danish family collection comprising 138 Type I diabetic sib-pair families (289 affected and 121 unaffected offspring) and 103 Type I diabetic parent-offspring multiplex families (103 affected and 112 unaffected offspring) were typed for the three most frequent polymorphisms with high heterozygosity indices and for a *FAS* microsatellite. Haplotypes were established and data analysed using the extended transmission disequilibrium test, ETDT.

Conclusion/interpretation. We found no overall evidence for linkage of the *FAS* polymorphisms to Type I diabetes. We conclude that it is unlikely that the Fas gene does contribute to genetic susceptibility for Type I diabetes. [Diabetologia (2000) 43: 800–808]

Keywords Genetics, candidate gene, apoptosis, tolerance, microsatellite, ALPS, c-Myb, SP-1, NF-κB.

Type I diabetes mellitus (insulin-dependent) is a multifactorial polygenic autoimmune disease for which susceptibility is determined by environmental and ge-

netic factors. The major histocompatibility complex (MHC), *IDDM1*, is the strongest genetic determinant [2]. At least 14 putative additional loci with minor effects have been reported [3]. The difficulty in confirming loci that have been identified and identifying new loci conferring minor susceptibility to complex diseases, including Type I diabetes, was highlighted by two recent large genome-wide screenings. Apart from *IDDM1* and *IDDM10* [4, 5] these failed to support any of the putative Type I diabetes susceptibility loci. Association studies and linkage disequilibrium mapping that use candidate genes could be a more powerful approach [6]. In this study we have evaluated *FAS* as a candidate susceptibility gene for Type I diabetes.

Fas is a type I transmembrane protein belonging to the tumour necrosis factor receptor superfamily. It is

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Abbreviations: 3' UTR, 3' Untranslated region; GAS, gamma-activation site; IFN, interferon; NF-κB, nuclear factor-κB; SSCP, single strand conformational polymorphism; TDT, transmission disequilibrium test; ECL, enzyme-derived chemiluminescence; ETDT, extended transmission disequilibrium test.

expressed on the surface of many types of cells, such as lymphocytes, fibroblasts, epithelial and certain endothelial cells [7]. Signalling by Fas leads to apoptotic cell death. Apoptosis can be induced by the T-cell effector ligand, FasL, through binding to Fas. Apoptosis mediated by Fas/FasL has a pivotal role in peripheral lymphocyte homeostasis both in activation induced cell death in the down regulation of an immune response and in peripheral clonal deletion of autoreactive T-lymphocytes [8].

The human *FAS* has nine exons spanning 26 kb [9, 10] and maps to chromosome 10q24.1 [11]. A number of rare heterozygous mutations in the human *FAS* associated with autoimmune lymphoproliferative syndrome (ALPS) (a disorder of lymphocyte homeostasis and immunological tolerance) have been described in which the genotype influences the penetrance of ALPS [12, 13].

Apoptosis mediated by Fas could be altered in Type I diabetes both on the T-cell and beta-cell level and have a role in the development of human Type I diabetes. In the immune system, the expression of Fas on T-lymphocytes and B-lymphocytes is reduced in humans with preclinical and clinical Type I diabetes [14]. Human pancreatic beta cells do not constitutively express Fas, but after treatment with IL-1 β in vitro, Fas expression is observed on isolated human beta cells [15, 16]. Pancreas biopsy specimens from recent-onset Type I diabetic patients showed Fas expression on beta cells and FasL expression on islet infiltrating T-cells [17]. Beta cells from newly diagnosed diabetic patients expressed Fas on their surface, showing extensive apoptosis possibly mediated by FasL-positive T-lymphocytes [18].

Further, gene variations with quantitative effects on Fas expression could contribute to the breakdown of peripheral tolerance and Type I diabetes.

We therefore screened the entire human *FAS* for mutations by single strand conformational polymorphism (SSCP)-heteroduplex analysis. We tested the polymorphisms found with a high heterozygosity index for linkage in a Danish Type I diabetic family collection. We also tested a *FAS* microsatellite marker [19] localised 48.5 kb within the human *FAS* for linkage as an additional marker.

Subjects and methods

Subjects. Material for the SSCP-heteroduplex analysis was provided by 20 unrelated Danish Caucasian Type I diabetic patients and 10 healthy Danish Caucasian males.

The material for the linkage analysis was obtained from 1068 people of which 647 came from 138 Danish Caucasian sib-pair Type I diabetes mellitus multiplex families, with 289 Type I diabetic offspring (133 females), and 121 (62 females) non-affected offspring. Both parents in 99 families and 1 parent in 39 families were available for genotyping. All index cases, defined as the first affected offspring (68 females/

70 males), had onset of Type I diabetes before age 30 years (mean $\pm$ SD: 10.0 $\pm$ 5.5 years). The other 421 subjects were from 103 Danish Caucasian parent-offspring multiplex families, characterised by at least 1 parent and only 1 offspring affected with Type I diabetes. Both parents, all 103 affected (53 females) and 112 unaffected (55 females) offspring were genotyped. All affected offspring had onset of Type I diabetes before age 30 years (mean $\pm$ SD: 10.0 $\pm$ 4.8 years). All families were unrelated.

The material for the determination of allele frequencies for the new promoter mutations comprised the 240 Danish Caucasian Type I diabetes index cases and 77 healthy Danish Caucasian males. Ethical approval was obtained from the ethics committee of Copenhagen County.

Screening for and identification of mutations in the human *FAS*. Oligonucleotide sequences for PCR amplification of the human *FAS* promoter, exons 1–9 with adjacent intron borders and the 3' untranslated region (3' UTR) of the human *FAS* were designed from the genomic human *FAS* sequence [10] (GenBank AC X82279-X82286) to generate 26 overlapping PCR products 212–300 bp long (Table 1) with an average overlap of 52 bp between segments.

Polymerase chain reaction (PCR) amplification of genomic DNA was done with the primers listed in Table 1 and 150 ng DNA as a template. The assay conditions were as follows: primers 0.5 μ mol/l each, deoxyribonucleoside triphosphates (dNTPs) 100 μ mol/l, MgCl₂ 0.5–2.0 mmol/l, *Taq* DNA polymerase (Promega, Madison, Wis., USA) 0.5 U/sample with 1 $\times$ reaction buffer, all in final concentrations in a reaction volume of 40 μ l.

The cycling conditions were 94°C for 3 min, followed by 30–35 cycles: 95°C for 45 s, annealing (varying between 48 and 64°C for different primers) for 45 s and extension at 72°C for 2 min 30 s, followed by a final extension at 72°C for 10 min.

For the SSCP-heteroduplex analysis [20, 21] of the human *FAS* we used the 26 overlapping PCR amplicons listed in Table 1, and the Gene Gel Excel 12.5/24 Kit / GeneGel Clean 15/24 Kit with the PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, after the PCR amplification 4 μ l of the reaction mixture from each sample were mixed with 4 μ l of denaturing buffer (99% formamide, 1% xylene cyanol, 20 mg bromophenyl blue). The samples were denatured at 95°C for 5 min, chilled on ice for 10 min to allow heteroduplex formation and then loaded onto non-denaturing polyacrylamide gels (15% and 12.5%, respectively) with or without 1% glycerol. Samples were run on the GenePhor Electrophoresis Unit (Amersham Pharmacia Biotech) with 15 W, 25 mA at 5°C for the excel gels and 9 W, 15 mA at 15°C for the clean gels containing 1% glycerol. Gels were stained using the Plus One DNA Silver Staining Kit (Amersham Pharmacia Biotech) on a Hoefer Automated Gel Stainer (Pharmacia Biotech, San Francisco, Calif., USA). Samples showing mobility shifts or heteroduplex formation were sequenced using cycle sequencing.

For cycle sequencing 30 μ l of PCR product from samples showing mobility shifts or heteroduplex formation were sequenced using the PCR product pre-sequencing kit and the Thermo Sequenase Cycle Sequencing Kit (both Amersham Pharmacia Biotech, Cleveland, Ohio, USA) according to the manufacturer's instructions. Primers (Table 1) were labelled with γ^{32} P using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions.

Samples were run on 6% polyacrylamide gels. Gels were dried and exposed to x-ray films.

Restriction enzymes for PCR-based RFLP. The PCR amplicons (Table 1) were digested with the following restriction enzymes:

Table 1. Primers For human *FAS* and the *FAS* microsatellite

Primers		Area	GenBank AC	Basepairs	Amplicon size (bp)	Primer sequences	Annealing temperature	MgCl ₂ concentration (μmol/l)		
22	Forward	Promoter	<i>X82279</i>	5' : 30		5'CCTTATCCCACITCTTTTGT				
23	Reverse			3' : 250	239	5'TTTTCTTCTGGAGCCTGTT	52 °C	1.5		
24	Forward			5' : 167		5'GGTGGTAAGTGCAGTGACAGA				
25	Reverse			3' : 396	249	5'TCAGTATTCCTTTGGCATCG	54 °C	1.0		
26	Forward			5' : 364		5'AGGTTGCAGAGTGAGGTG				
27	Reverse			3' : 567	224	5'TCTTGTAATTCTTTCGTGCTC	52 °C	1.0		
28	Forward			5' : 525		5'CTACCGTTCCAAAGCAATAGT				
29	Reverse			3' : 748	242	5'TACAGATGTTGCGCCATAG	54 °C	1.5		
30	Forward			5' : 693		5'AGCTTTGTTTTCTCTTGAGA				
31	Reverse			3' : 911	234	5'CAGAGCGCAGAGGTCC	56 °C	1.5		
32	Forward	Exon 1 ^a		5' : 855		5'CGCAAGTCTTTCTCTGAGTGA				
33	Reverse			3' : 1084	249	5'GTGAGGAAGGAGTCAGGGTT	58 °C	1.0		
36	Forward			5' : 1054		5'CCCGGGACAGGAATGC				
37	Reverse			3' : 1264	231	5'CCCAGAAGCGTCTTTGAACAC	56 °C	0.5		
38	Forward			5' : 1180		5'CCCAGCGAGGCTTCCTTCC				
39	Reverse			3' : 1403	244	5'CGTACTGAGCGGGTCCACCAA	60 °C	1.5		
34	Forward			5' : 1338		5'TGGCACGGAACACACCCTGAG				
35	Reverse			3' : 1553	231	5'GCGCCCCGCCCCACTTT	64 °C	1.5		
70	Forward			Exon 2 ^a	<i>X82280</i>	5' : 158		5'GGGTTACACTTCTTTACCAC		
71	Reverse					3' : 349	212	5'TGATTACTATGTGCTACTCCT	50 °C	1.0
72	Forward	Exon 3 ^a	<i>X82281</i>	5' : 181		5'GCCCCGTGTCCTGTTCAAAC				
73	Reverse			3' : 433	273	5'CACAGTAGGCCCAATTTCAA	54 °C	2.0		
74	Forward	Exon 4 ^a	<i>X82282</i>	5' : 111		5'CACCATTTTCATAGTCTGC				
75	Reverse			3' : 348	258	5'CTCTCAGTCAGTGTACTTCC	50 °C	1.0		
76	Forward	Exon 5 ^a	<i>X82283</i>	5' : 211		5'CCCCTAATTTACAAAGTGC				
77	Reverse			3' : 415	225	5'TCAAGAGTGATTGGTTTTTCT	50 °C	1.0		
78	Forward	Exon 6 ^a	<i>X82283</i>	5' : 369		5'TTACGGTTATATCCTCCTTTC				
79	Reverse			3' : 600	249	5'CCCCCAAGTTATTTCAAT	60 °C	1.0		
80	Forward	Exon 7 ^a	<i>X82284</i>	5' : 20		5'TCTCACATGCATTCTACAAGG				
81	Reverse			3' : 270	271	5'GCAAGACTCCATCTCAAACAA	50 °C	1.5		
82	Forward	Exon 8 ^a	<i>X82285</i>	5' : 48		5'TTTTATACATCAAGCAACTGA				
83	Reverse			3' : 240	212	5'TGAAATTGGCCTATTACTCT	48 °C	1.0		
40	Forward	Exon 9 ^a and the 3'UTR	<i>X82286</i>	5' : 144		5'TTTCACTAATGGGAATTTCAT				
41	Reverse			3' : 367	242	5'AGTTGATGCCAATTACGAA	50 °C	2.0		
42	Forward			5' : 337		5'AAGACACAGCAGAACAGA				
43	Reverse			3' : 561	245	5'AATTGCATATACTCAGAACTG	50 °C	1.5		
44	Forward			5' : 497		5'CTCAGAAAATTCAAACCTTCA				
45	Reverse			3' : 715	239	5'CCCAACTAGATTTTAAACATC	50 °C	1.0		
46	Forward			5' : 668		5'GCCAACATATTTGTAGATTTT				
47	Reverse			3' : 895	247	5'GATTCATAGAGTGGGGTTAG	50 °C	1.5		
48	Forward			5' : 852		5'TGAATGTAATCAGTGTATGTT				
49	Reverse			3' : 1078	246	5'AGAGCCTTATTTAAATTCTG	50 °C	1.0		
60	Forward			5' : 1023		5'CTTTGTTTATAACTCTGAGAA				
61	Reverse			3' : 1239	232	5'CTTTTGATGCCTGAGA	46 °C	1.0		
62	Forward			5' : 1215		5'CTGGCTCAAAACTACCTACTT				
63	Reverse			3' : 1494	300	5'GGGGGAAAAATAAGAATCTAT	50 °C	1.0		
64	Forward			5' : 1402		5'AATTCTAGCCTGGTTTGGAGA				
65	Reverse			3' : 1608	227	5'TGAGGGTATGACAAGAGCAAT	50 °C	1.0		
66	Forward			5' : 1578		5'CAATGGTAAAATCATCATCT				
67	Reverse			3' : 1819	262	5'TTTTATAATTCCAAACACAAG	48 °C	1.5		
68	Forward			5' : 1759		5'ACTGAAGCAGATACCTGGAAC				
69	Reverse			3' : 2038	298	5'TTCTTGGAATAGCAACCT	50 °C	1.5		
Fas microsatellite										
1	Forward	CA repeat polymorphism within 48.5 kb of <i>FAS</i>					5'GATCATACAGAGAC			
2	Reverse				192–212	5'Bio tCATTCTCACATACAATGAG	52 °C	1.5		

^a Including flanking exon-intron boundaries

Fig. 1. Human *Fas* gene; distribution of mutations. Exons are represented by boxes and numbered in a 5' to 3' direction. Exons 1–5 encode the signal peptide and three extracellular cysteine-rich domains responsible for binding FasL. Exon 6 encodes the transmembrane domain. Exons 7–9 encode the intracellular portion. Exon 9 encodes the death domain. *mutations described previously

(1) Primers 24/25; *Apa*I 2 U/sample in buffer A for 16 h and *Acc*I 2 U/sample in buffer G for 16 h. (2) Primers 30/31; *Nde*I 20 U/sample in buffer D for 4 h and *Mva*I 4 U/sample in buffer H for 16 h. (3) Primers 36/37; *Ava*I 2 U/sample in buffer B for 16 h. (4) Primers 80/81; *Dra*I 5 U/sample in buffer B for 16 h. All reactions were carried out in a reaction volume of 20 μ l at 37°C. All the restriction enzymes and the buffers except *Mva*I and buffer H (Boehringer Mannheim Biochemica, Mannheim, Germany) were from Promega.

***FAS* microsatellite.** Primer sequences (primers 1/2) are shown in Table 1 [19]. Using biotinylated primers we typed the *FAS* microsatellite by PCR of 40 ng genomic DNA template followed by PAGE, blotting and made visible with a streptavidin-peroxidase catalysed enzyme-derived chemiluminescence (ECL) reaction. Cycling conditions were: 94°C for 3 min, 30 cycles of: 95°C for 30 s, 52°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 10 min. We added 20 μ l stop buffer (98% formamide) to 20 μ l of the PCR product, heated it for 2 min at 94°C and placed it on ice. We ran 6 μ l of this mixture on a 6% polyacrylamide gel at 65 W. The PCR products were then transferred to a positively charged nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, USA) and UV cross-linked (UV Stratalinker 2400; Stratagene, La Jolla, Calif., USA). The membranes were developed using the Streptavidin-HRP, S-5512 (Sigma, St. Louis, Mo., USA) and ECL gene detection reagents (Amersham Pharmacia Biotech, USA) and exposed to x-ray films for 1–5 min [22].

Samples homozygous for the different alleles were confirmed by sequencing on an ABI PRISM 310 DNA sequencer system (Perkin Elmer, Foster City, Calif., USA).

MatInspector. To assess for potential DNA binding sites in the human *FAS* promoter for various transcription factors we used the MatInspector program [23].

Statistical analysis. Transmission disequilibrium test (TDT) for linkage [24] and intrafamilial association [25] and extended transmission disequilibrium test (ETDT) for linkage with mul-

tiallele markers were carried out [26]. We considered differences significant at p less than 0.05. Linkage disequilibrium was calculated as the disequilibrium coefficient, D [27].

Results

Molecular scanning of the human FAS by SSCP-heteroduplex analysis

A complete molecular scanning of the entire human *FAS* (promoter region, exons 1–9, exon-intron borders and the 3' UTR) was carried out using SSCP-heteroduplex analysis and direct sequencing in 20 Type I diabetic patients and 10 healthy control subjects. We identified 15 mutations (Table 2, Fig. 1), of which 4 have been described previously [28, 29].

In the promoter region we found eight mutations. Of these a $g-1194A \rightarrow T$ and a $g-295A$ ins give rise to alterations of transcription factor binding consensus sequences for c-Myb, SP-1 and nuclear factor- κ B (NF- κ B), respectively. We confirmed the silent $g250A \rightarrow G$ in codon 58 (ACA) and the silent $g154C \rightarrow T$ in codon 198 (ACC) described in [28].

Linkage analysis

FAS. The TDT [24] was applied to analyse linkage between the $g-690T \rightarrow C$, the $g-670G \rightarrow A$ polymorphisms in the *FAS* promoter and the $g154C \rightarrow T$ polymorphism in codon 198 and Type I diabetes. The parental heterozygosity indexes were 0.26, 0.51 and 0.39, respectively. We found no evidence for linkage between any of the three polymorphisms and Type I diabetes ($p = 0.55$, $p = 0.91$ and $p = 0.84$, respectively, Table 3).

The ETDT for multi-allele marker loci [26] was used to analyse for linkage between haplotypes established from the two *FAS* promoter polymorphisms, haplotypes established from the above three loci in the *Fas* gene and Type I diabetes. The heterozygosity indexes of the parents were 0.62 and 0.72 for the *FAS* promoter haplotypes and the three loci haplotypes, respectively. There was no evidence for linkage to Type I diabetes, [allele-wise TDT: *FAS* promoter

Table 2. Mutations in the human *FAS*; promoter, exons 1–9 including exon-intron boundaries and the 3'UTR region

Region	Mutation	Allele frequencies ^b	Heterozygosity index	Remarks
Promoter <i>X82279</i>	<i>g-1377 G → A^a</i>	A allele: 0.2		SP-1 Silencer region
Promoter <i>X82279</i>	<i>g-1221 C → G</i> <i>ApaI</i> RFLP	G allele: D: 0.006 ^c /C: 0		
Promoter <i>X82279</i>	<i>g-1194 A → T</i> <i>AccI</i> RFLP	T allele: D: 0.006 ^c /C: 0		c-Myb
Promoter <i>X82279</i>	<i>g-690 T → C</i> <i>NdeI</i> RFLP ^{c,d}	C allele: D: 0.2/C: 0.15	0.26	Enhancer region
Promoter <i>X82279</i>	<i>g-670 G → A^a</i> <i>MvaI</i> RFLP ^{c,d}	A allele: D: 0.62/C: 0.55	0.51	GAS Enhancer region
Promoter <i>X82279</i>	<i>g-397 C → T</i>	T allele: D: 0.025/C: 0		
Promoter <i>X82279</i>	<i>g-295 Ains</i> <i>AvaI</i> RFLP	mu: D: 0.01 ^e /C: 0		Composite SP-1/NF-κB
Promoter <i>X82279</i>	<i>g-129 Cins</i>	mu: D: 0.025/C: 0		
Exon 3 <i>X82281</i>	<i>g250 A → G</i>	G allele: D: 0.025/C: 0		Codon 58: ACA ^a , silent
Intron 3 <i>X82281</i>	<i>g435 Gdel</i>	mu: D: 0.05/C: 0.10		
Intron 5 <i>X82283</i>	<i>g439 G → C</i> <i>BfaI</i> RFLP	C allele: 0.65		
Exon 7 <i>X82284</i>	<i>g154 C → T</i> <i>DraI</i> RFLP ^d	T allele: D: 0.26/C: 0.33	0.39	Codon 198 ACC ^a , silent
3'UTR <i>X82286</i>	<i>g1107 A → T</i>	T allele: D: 0.13/C: 0.05		
3'UTR <i>X82286</i>	<i>g1307 T → C</i>	C allele: D: 0.02/C: 0.05		
3'UTR <i>X82286</i>	<i>g1626 T → C</i> <i>Sml</i> IRFLP	C allele: 0.9		

D, diabetic patients; C, healthy control subjects; mu, mutant
^a Mutations described previously, ^b in the screening population, ^c two loci haplotypes: promoter, ^d three loci haplotypes: promoter and codon 198, ^e allele frequencies in 240 diabetic index cases.

Promoter mutations are numbered relative to the start codon. Various RFLPs refer to typing methods

Table 3. *FAS* promoter (*MvaI* and *NdeI*) and codon 198 (*DraI*) polymorphisms: transmission frequencies of alleles from heterozygous parents to offspring

	<i>MvaI</i> ^a		<i>NdeI</i> ^b		<i>DraI</i> ^c	
	G	A	T	C	C	T
Affected	150	152	64	71	117	116
Index cases	93	105	40	53	72	77
Not affected	99	83	49	44	67	72

p (TDT), ^a ≥ 0.24, ^b ≥ 0.18, ^c ≥ 0.67

haplotypes: *p* = 0.4; *FAS* three loci haplotypes, *p* = 0.35 (Tables 4, 5)].

For the *FAS* three loci haplotypes, we also tested for intrafamilial association [25] by considering the transmission of different haplotypes to index cases and observed skewed transmission of the CAC haplotype (Table 5) with a transmission frequency of 61 % of this haplotype to index cases. This was not, howev-

er, statistically significant (*p* value, uncorrected = 0.08, *p* value, corrected = 0.75).

***FAS* microsatellite.** We also typed the Danish family collection for a *FAS* microsatellite (CA repeat polymorphism) located within 48.5 kb of the *Fas* gene on chromosome 10q24.1 (Table 1; primers 1/2) [19]. The microsatellite marker was highly polymorphic, with 10 alleles observed, ranging from 17 to 26 CA repeats and a heterozygosity index of 0.83 in the parents (Table 6). The extended transmission disequilibrium test (ETDT) for multi-allelic marker loci [26] was used to analyse for linkage between the *FAS* microsatellite and Type I diabetes. All affected offspring were considered. There was no evidence for linkage to Type I diabetes, (allele-wise TDT *p* = 0.53, Table 7).

Linkage disequilibrium. The disequilibrium coefficients, *D*, for the *g-690T→C* and *g-670G→A*, for the *g-690T→C* and the *g154C→T*, for the *g-670G→A*

Table 4. *FAS* promoter polymorphisms haplotypes, *Nde*I and *Mva*I. Transmission frequencies of haplotypes from heterozygous parents to offspring

	Haplo-type	Trans-mitted	Non trans-mitted	χ^2	<i>p</i> value ^a
Affected	TG	131	140	0.3	0.59
	CG	2	6		
	TA	142	136	0.13	0.72
	CA	59	52	0.44	0.51
Index cases	TG	55	56	0.01	0.92
	CG	1	3		
	TA	58	62	0.13	0.72
	CA	31	24	0.89	0.35
Not affected	TG	90	72	2.0	0.16
	CG	3	5		
	TA	79	90	0.72	0.40
	CA	35	40	0.3	0.56

^a Uncorrected *p* values. *p* (ETDT) ≥ 0.4 **Table 5.** *FAS* haplotypes, three loci: *Nde*I, *Mva*I and *Dra*I. The three loci haplotypes consist of: *Nde*I, *Mva*I and *Dra*I RFLP, respectively. Transmission frequencies of alleles from heterozygous parents to offspring

	Haplo-type	Trans-mitted	Non trans-mitted	χ^2	<i>p</i> value ^a
Affected	TGC	69	82	1.1	0.3
	TGT	88	79	0.49	0.49
	CGC	2	1		
	CGT	0	3		
	TAC	119	119	0	1.0
	TAT	24	26	0.08	0.78
	CAC	56	48	0.6	0.43
Not affected	TGC	45	39	0.43	0.51
	TGT	59	48	1.13	0.29
	CGC	3	4		
	CGT	1	2		
	TAC	67	77	0.7	0.4
	TAT	15	22	1.3	0.25
	CAC	35	33	0.06	0.81
Index cases	TGC	41	54	1.78	0.18
	TGT	54	51	0.09	0.76
	CGC	1	1		
	CGT	0	2		
	TAC	73	79	0.24	0.62
	TAT	17	14	0.29	0.59
	CAC	43	28	3.169	0.075

^a Uncorrected *p* values. *p* (ETDT) ≥ 0.35

and the *g154C*→*T*, for the *g-690T*→*C* and the *FAS* microsatellite, and for the *g154C*→*T* and the *FAS* microsatellite were −0.04, −0.03, 0.11, 0.03 and 0.02, respectively. Between the *g-690T*→*C* and the *FAS* microsatellite and between the *g154C*→*T* and the *FAS* microsatellite, 5 % recombinatory events occurred.

Table 6. Distribution of *FAS* microsatellite alleles in the 240 Type I diabetic index subjects

Allele size bp	Repeat No.	<i>n</i>	Allele frequency
194–196	17–18	4	0.008
198	19	25	0.052
200	20	98	0.204
202	21	152	0.317
204	22	117	0.244
206	23	18	0.038
208	24	61	0.127
210–212	25–26	5	0.01
Total		480	1.0

Table 7. *FAS* microsatellite; transmission frequencies of the five most frequent alleles from heterozygous parents to offspring

	Allele size (repeat units)	Trans-mitted	Non trans-mitted	χ^2	<i>p</i> value ^a
Affected	19	32	45	2	0.14
	20	122	108	0.85	0.36
	21	157	165	0.2	0.65
	22	145	122	2	0.16
	24	70	75	0.17	0.68
Index cases	19	23	24	0.02	0.89
	20	76	67	0.57	0.45
	21	94	105	0.61	0.43
	22	86	78	0.39	0.53
	24	46	47	0.01	0.92
Not affected	19	26	28	0.07	0.79
	20	63	73	0.74	0.39
	21	111	90	2.19	0.14
	22	84	87	0.05	0.82
	24	33	42	1.08	0.30

^a Uncorrected *p* values. *p* (ETDT) ≥ 0.53

Discussion

We have completed a molecular scanning of the entire human *FAS* identifying 11 new mutations and confirming four described previously. We found eight mutations in the promoter region where several of these could affect interesting transcription factor binding sites. The promoter mutations are numbered relative to the start codon. The *g-1377G*→*A* polymorphism in the silencer region has been described previously [29] and could affect the SP-1 binding site [30]. We tried unsuccessfully to establish a mutagenically separated PCR assay [31] to type the family collection for this polymorphism. In the enhancer region of the human *FAS* we identified a new *g-690T*→*C* polymorphism and confirmed a *g-670G*→*A* polymorphism [29]. The new *g-690T*→*C* polymorphism results in a missing restriction site for *Nde*I. The *g-670G*→*A* polymorphism, resulting in a missing restriction site for *Mva*I, abolishes the consensus binding site, ATTCCAGAAA [32], for the nuclear tran-

scription element gamma activation site (GAS) important in interferon (IFN) α and IFN γ signalling.

We identified new mutations only present in the Danish Type I diabetic families and not in healthy control subjects. Of the mutations two, a *g-1221C→G* and a *g-1194A→T*, were in complete linkage disequilibrium with each other. The *g-1194A→T* is in a c-Myb binding site sequence, **A**AC(G/T)G, where the **A** is essential for binding of the transcription factor [33] and results in a new restriction site for *AccI*. It has been shown that c-Myb is a direct transcriptional activator of the human *FAS* promoter [10]. The allelic frequency in 240 unrelated Type I diabetic patients for the mutant allele was 0.006. The mutant allele was not present in 77 healthy control subjects. We also identified a new *g-295A* ins which occurs at a composite binding site for both the SP-1 and NF- κ B transcription factors at -295 to -286 required for activation-driven Fas up regulation [34]. These two transcription factors compete for this binding site. In resting peripheral blood lymphocytes Fas expression is low and SP-1 is bound to this site. In activated lymphocytes, as a response to NF- κ B heterodimer binding, Fas expression is up regulated. The allelic frequency in 240 unrelated Type I diabetic patients for the mutant allele was 0.01. The mutant allele was not present in 77 healthy control subjects.

We found no evidence of linkage between any of the polymorphisms identified in the human *FAS* and Type I diabetes.

Haplotypes were established from the two polymorphisms in the promoter (the *g-690T→C* and the *g-670G→A*) and the *g154C→T* polymorphism in exon 7. As a test for intrafamilial association [25] we considered the transmission of haplotypes to index cases in the diabetic families and found a skewed transmission of the CAC haplotype with 61 % transmitted to index cases.

The risk that we might have overlooked mutations in the human *FAS*, which could be linked to Type I diabetes, is minimal. Thus, the sensitivity and the specificity of the SSCP-heteroduplex analysis in our laboratory was high, 93 % and 92 %, respectively compared with a sensitivity in the range of 70–90 % reported by others [20]. We have also sequenced 15 out of the 26 PCR amplicons equal to approximately 58 % of the human *FAS* in a minimum of four subjects. The remaining nine amplicons showed no polymorphic patterns in the SSCP-heteroduplex analysis. Minor susceptibility genes are expected only to modify risk, hence they are likely to be frequent in the general population. In the power estimates we have assumed a 12.5 % deviation [35]. The probability of making a type II error of the 5 % probability level in our statistical analysis of the *MvaI* and the *DraI* polymorphisms is low (~ 14 % and 24 %, respectively) because the power is relatively high due to the many transmissions observed. Based on the percentage of

parents heterozygous for the *NdeI*, the *MvaI* and the *DraI* polymorphisms it was expected that about 161, 316 and 241 transmissions to affected offspring could be observed for the polymorphisms, respectively. The risk for a type II error for the *NdeI* polymorphism was about 43 %.

Finally, we also tested a *FAS* microsatellite, localised within 48.5 kb of the human *FAS*, for linkage to Type I diabetes in the Danish families. The *FAS* microsatellite is close to the human *FAS* and was a priori considered a good marker for *FAS* unless there was a mutational “hotspot” situated between them. The microsatellite was highly informative with ten alleles observed and a heterozygosity index in the parents of 0.83. We found no evidence for linkage to Type I diabetes for the *FAS* microsatellite which correlates well with results for the polymorphisms in the gene proper. Linkage disequilibrium analysis showed, however, that the *FAS* microsatellite is not in linkage disequilibrium with the polymorphisms identified in *FAS*. Hence, this microsatellite is possibly not a good marker for *FAS*. Furthermore, no evidence for linkage between the *FAS* microsatellite and Type I diabetes was found in an Australian sib-pair family data set [19].

A genome-wide linkage study showed linkage between chromosome 10p13-p11 and Type I diabetes [5], a region not including *FAS*. *IDDM17* (a Type I diabetes susceptibility locus described in a Bedouin Arab pedigree) maps to chromosome 10q25 [36]. *FAS* maps just outside this region (personal communication, C.F. Verge).

In murine models of Type I diabetes, pancreatic beta-cell death mediated by T-cells during the course of diabetes is mainly by apoptosis [37–39]. There is conflicting evidence for the role of Fas-mediated apoptosis in the development of diabetes in the nonobese diabetic (NOD) mouse [40–45].

We have scanned the entire human *FAS* (promoter region, exons 1–9, exon-intron borders and the 3' UTR region) by SSCP-heteroduplex analysis and have identified a total of 15 mutations, of which 4 have been described previously. In the promoter region we identified several mutations, which could alter the consensus sequence for the SP-1, c-Myb, GAS and NF- κ B transcription factors. These polymorphisms could be relevant for the understanding of genetic susceptibility to other immunological disorders. We found, however, no evidence for linkage between individual polymorphisms or constructed haplotypes from polymorphisms from three loci in the human Fas gene or a *FAS* microsatellite and Type I diabetes in a large Danish, Caucasoid Type I diabetic family collection.

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