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Analysis of the human GDNF gene reveals an inducible promoter, three exons, a triplet repeat within the 3′-UTR and alternative splice products

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Glial cell line-derived neurotrophic factor (GDNF), a distant member of the TGF-β superfamily, is a survival factor for various neurons, making it a potential therapeutic agent for neurodegenerative disorders. Here we present the genomic structure and characterization of the promoter of the human GDNF (hGDNF) gene. It contains three exons coding for a cDNA of 4.6 kb including large 5′- and 3′-untranslated regions (UTRs). The 3′-UTR contains a polymorphic AGG repeat that appears not to be expanded in patients suffering from different neurodegenerative disorders. RT–PCR results in at least three different hGDNF transcripts including one that lacks exon 2. Transient expression experiments reveal that exon 2 is essential for proper cellular processing to yield a secreted form of hGDNF, whereas expression of exon 3 alone is sufficient to code for a mature form of hGDNF retained within the cell. Our data show that the hGDNF gene is driven by a TATA-containing promoter preceding exon 1. A second promoter element has been mapped to a region 5′ of exon 2. Both promoters are in close proximity to CpG islands covering exons 1 and 2. Using luciferase as a reporter gene, the TATA-containing hGDNF promoter facilitates a 20- to 40-fold increase in transcription when compared with a corresponding promoterless construct, whereas the second promoter confers only weak activity. Furthermore, fibroblast growth factor 2, tetradecanoyl 12-phorbol acetate, an inflammatory agent, and cAMP increase promoter activity, suggesting that GDNF transcriptional regulation is a target of exogenous signals.

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is an important neurotrophic and potential differentiation factor for embryonic midbrain dopaminergic, spinal motor, cranial sensory, sympathetic and hindbrain noradrenergic neurons (1–3). During mammalian development, it is most highly expressed in the metanephric kidney and gastrointestinal tract, with lower levels in the embryonic nervous system (4–6). In the GDNF null mouse model, the absence of uteric bud induction and enteric neurons is manifested clinically by renal agenesis and severe malformation of the kidney. Deficits also occur in the dorsal root ganglia, sympathetic and nodose neurons, whereas the noradrenergic and midbrain dopaminergic neurons are apparently normal (7–9). *In vitro* GDNF promotes the survival and morphological differentiation of human dopaminergic neurons and increases dopamine uptake in embryonic midbrain cultures. The observation that rhesus monkeys with chemically induced parkinsonism show functional recovery after intracerebral GDNF injection (10) suggests a role for GDNF as a neuroprotective agent and therefore its possible therapeutic use against neurodegenerative diseases.

GDNF is a distantly related member of the transforming growth factor β (TGF-β) superfamily, as indicated by a cystine knot motif common to all its members (3). Processed GDNF shares <20% amino acid sequence homology with other members of the TGF-β family, considerably lower than the 42–92% amino acid homology amongst the other mature proteins within the five TGF-β subfamilies. Neurturin, a recently described neurotrophic factor, shares 42% sequence homology with GDNF and the two are thus likely to form a new subfamily.

GDNF signalling is mediated through a membrane-bound receptor comprised of two subunits. One of these is a glycosyl-phosphatidylinositol-anchored, ligand-binding component, designated GDNF family receptor α (GFR-α1), the other the tyrosine kinase RET. Together they form a functional receptor unit upon GDNF binding (11,12). Through complex formation of the GFR-α1 and RET proteins, GDNF induces RET-mediated tyrosine phosphorylation in target cells.

Mutations in the *ret* gene have been described for patients with Hirschsprung's disease, a hereditary disorder caused by the malformation of neural crest-derived enteric neurons. Because GDNF is one activator of RET, mutation analysis has been performed on the GDNF gene for those patients with diseases previously associated with the GDNF/RET signalling system

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who did not carry demonstrable mutations in the *ret* gene. The only GDNF gene mutation discovered thus far was in a patient with Hirschsprung's disease (13), while investigations for GDNF mutations in RET receptor-associated diseases and dopaminergic neurodegenerative disorders have been negative (14–17).

The GDNF gene, mapped to chromosome 5p12–p13.1 (18), was previously reported to contain two exons, the second of which codes for the entire mature GDNF protein as well as for the cleavage site used in processing the GDNF proprotein. One alternatively spliced GDNF transcript is an isoform of the initially described precursor protein with an in-frame deletion of 78 bp at the 3′-end of the until now named exon 1, initially described by Cristina *et al.* and Springer *et al.* (19,20). Information about the genomic organization and the flanking sequences of the hGDNF gene are essential prerequisites for both gene therapy experiments with large genomic constructs as well as for the genetic analysis of hereditary disorders potentially associated with GDNF function.

Here we present the genomic structure of the hGDNF gene locus, covering a 28 kb interval at 5p12–p13.1, found by analysis of a PAC clone containing the hGDNF gene. Sequence analysis of the promoter region and of the exon flanking intronic sequences provides the basis for mutation analysis of the hGDNF gene on the level of genomic DNA. Furthermore, our data show that the hGDNF gene in fact contains three exons, rather than two as reported previously (3). Three CpG islands, one covering the untranslated exon 1, another part of the 5′-end of the first intron and the third island covering all of exon 2 were identified. The gene is driven by at least one inducible promoter containing a proximal putative TATA box 5′ of exon 1. In addition, we have mapped and characterized a second, weaker promoter lying immediately before exon 2. Comparative analysis of the two promoters showed a much higher activity for promoter 1. Our data provide evidence that this promoter is sensitive to inflammatory as well as to neurodifferentiating stimuli. This may indicate a requirement for increased physiological GDNF expression within neuronal repair and survival after inflammation and nerve injury, as well as within development.

RESULTS

Identification of a PAC clone containing the GDNF gene locus

We screened a human PAC library (21) with probes specific to the coding sequence of exon 2 [according to the previous definition (3)] of the hGDNF gene. The PAC clone 24B12, containing a 167 kb insert DNA (data not shown), showed positive hybridization signals with exons defined until now as exons 1 and 2 (old exon nomenclature) of the hGDNF gene. Restriction analysis with *Bam*HI and *Bbr*PI revealed that these exons now defined as exons 2 and 3 (new exon nomenclature) lie in the centre of the 167 kb PAC insert (Fig. 1A). To exclude deletion of the PAC clone, we analysed the GDNF gene structure based on genomic DNA. *Bam*HI- or *Bbr*PI-digested genomic DNA on Southern blots was hybridized with labelled probes derived from exons 1 and 3. The same fragment lengths were obtained for both PAC and genomic DNA.

Genomic structure of the GDNF gene

The genomic organization was determined by fine mapping of the *Bam*HI and *Bbr*PI fragments (Fig. 1B).

Additional sequences are present in the 5′-part of the mouse GDNF cDNA (accession no. U37459) but absent in the previously published hGDNF cDNA (accession no. L15306) sequence. This sequence was amplified from mouse genomic DNA using HPm1.f/HPm1.r as primers and used in Southern hybridization experiments with PAC 24B12 (data not shown). The results indicated the presence of an additional exon (newly designated as exon 1 in this study; the previous exon 1 will be referred to as previous) 5′ of the previously published exons. Sequence analysis of the 1.1 kb *Pst*I–*Bbr*PI fragment containing exon 1 demonstrated a perfect splice donor site at its 3′-end (22; Fig. 2A, in italic, boxed). No splice acceptor was found at the 5′-end of exon 1, suggesting this exon to be the first. Flanking intronic sequences for exon 2 were determined by inverse PCR experiments (23) using *Sau*3A-digested and religated PAC DNA as a template for exon 2-specific PCR primers IP2.f and IP2.r, revealing a splice acceptor site 5′ of exon 2 and a second, less canonical, splice acceptor within the open reading frame (ORF) (Fig. 3A, in italic, boxed). The sequence 5′ of exon 3 was analysed by subcloning the 0.7 kb *Eco*RI fragment (Fig. 1B). Inverse PCR for exon 3 flanking intronic sequences using *Sau*3A-digested and religated PAC DNA as a template and exon 3-specific primers did not show a conserved splice donor site as far as 198 bp downstream of the stop codon of exon 3, thus suggesting exon 3 to be the last.

Sequence analysis of the promoter region, the 5′-UTR, exon 1 and intron 1

Sequence analysis of the promoter region and exon 1 was performed by subcloning the corresponding genomic DNA fragments *Eco*RI–*Pst*I (1.0 kb) and *Pst*I–*Bbr*PI (1.1 kb) (Fig. 1B). The possibility of two adjacent *Pst*I sites positioned next to each other was excluded by sequencing a 120 bp PCR product primed in the flanking sequences of the *Pst*I site. Intron 1 was sequenced using a 4.8 kb long range PCR product primed in the coding sequences of exons 1 and 2 (Figs 2A and 3A and data not shown).

The Proscan program (NIH http://bimas.dcrt.nih.gov/molbio/proscan/) was used to search for a possible promoter. Essential promoter elements, including a TATA box, a GC box, a CREB site and two putative NF-A1-like binding sites, were localized in a 340 bp region upstream of exon 1 (Figs 1B and 2A).

XGRAIL 1.3c analysis (24) was performed for a 7.9 kb genomic region including the 5′ sequence of exon 1, intron 1 and exon 2. Three CpG islands, with GC contents of 70, 59 and 71%, were found (Figs 1B, 2A and 3A). The CpG island prediction for the second and third islands was supported by detection of four and two *Bss*HI sites, respectively, which were confirmed by sequence analysis (Figs 1B, 2A and 3A). Furthermore, a possible promoter was indicated in the region lying at the 5′-end of exon 2 (Figs 1B and 3A).

Both the mouse and human exon 1 contain ORFs which are in-frame with exon 2 (Fig. 2A and B). Mouse exon 1 contains a start codon not conserved in the human gene (Fig. 2A and B). In the overlapping section of mouse and human ORFs the nucleotide homology was found to be 82%. The similarity for amino acids

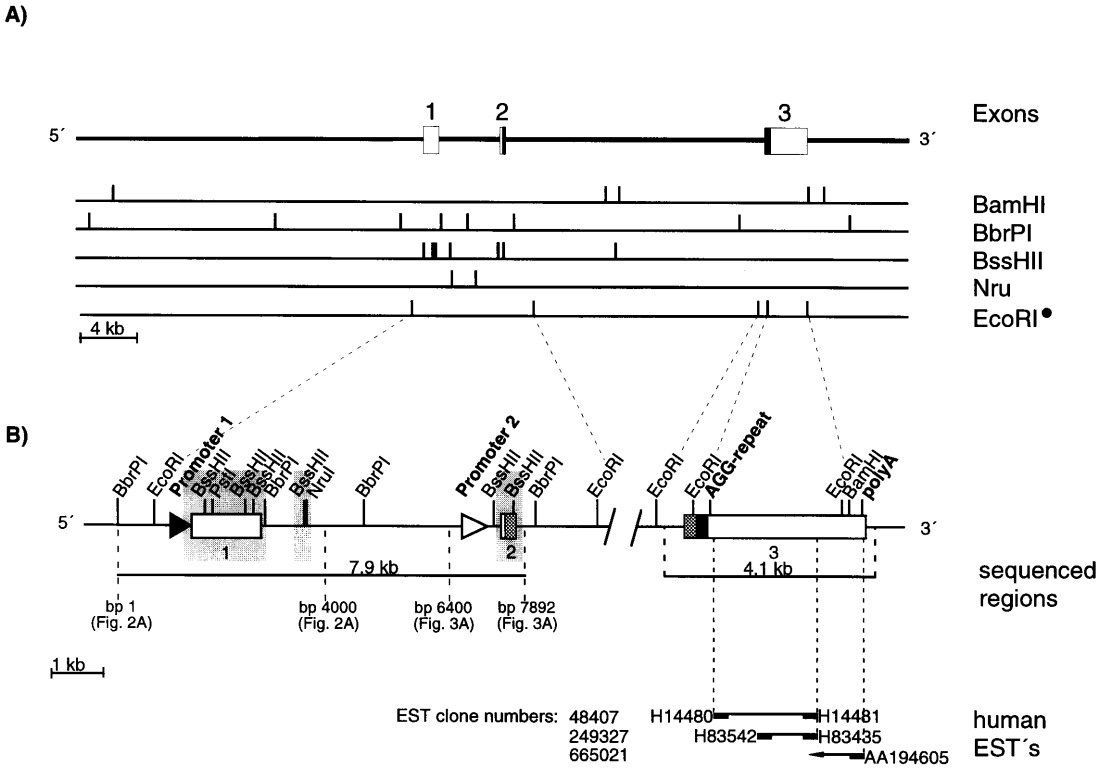


Figure 1. Organization of the human GDNF gene. (**A**) Restriction enzyme map for the GDNF locus derived from genomic sequence and Southern blot analyses (for further details see Materials and Methods). Exons are indicated as boxes. Solid boxes correspond to sequences coding for the GDNF protein, open boxes correspond to UTRs. EcoRI•, those *Eco*RI fragments hybridizing with exon 1, 2 or 3. Vertical lines show restriction sites of the corresponding enzyme marked on the right hand side. (**B**) Detailed map of the GDNF gene with features highlighted as follows. Exons are indicated as described for (A); dark grey boxes, the coding sequence for prepro-GDNF; light grey boxes, CpG islands predicted by the XGRAIL program; filled triangle, position of the promoter predicted by the Proscan program; open triangle, position of the promoter predicted by XGRAIL. Sequenced regions are indicated by black lines; poly A, polyadenylation site; AGG repeat, polymorphic repeat. Below is a schematic representation of human EST clones derived from the 3′-part of the GDNF gene. Clone numbers are marked on the left hand side, GenBank accession nos H14480, H14481, H83542, H83545, AA194605 and black boxes indicate the sequenced parts of the EST clones.

varied between 59 and 85%, with the highest degree 3′ of the mouse start codon (Fig. 2B).

Characterization of the putative transcription start point

Two initiator consensus sequences, an adenosine flanked by pyrimidines (minimal version) or Py_2CAPy_5 (maximal version), were located 33 [transcriptional initiation site (ins)–33] and 47 (ins–47) bp downstream of the putative TATA box, respectively (Fig. 2A), in agreement with the proximity of initiation sites to TATA boxes described for other promoters (22). Comparison of the human genomic sequence with the recently published transcription start site of the mouse GDNF cDNA showed the first initiation site (ins–33) to be the most probable (25). A 5′-RACE experiment was done to determine the transcription initiation site. A 104 bp PCR product was amplified using cDNA from adult retina. Subsequent sequencing showed the start of the PCR product at position 1492 of the genomic sequence, 26 bp downstream of the supposed initiation site calculated for the human gene in comparison with the mouse (25; Fig. 2C). Comparison of the genomic sequences showed 95% homology between human and mouse for the 115 bp upstream of the transcription start site, including the TATA box (Fig. 2C).

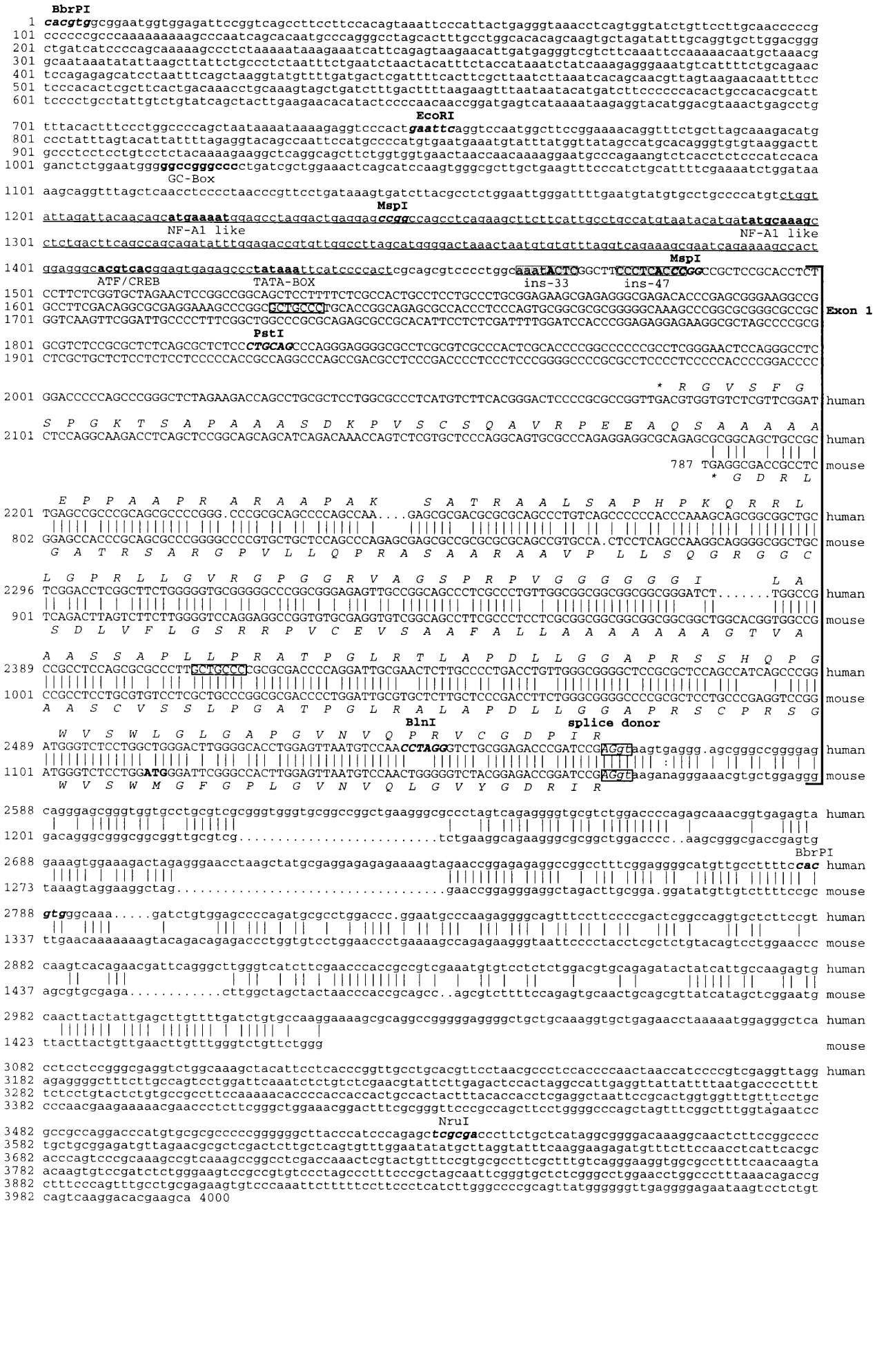
cDNA sequences of the GDNF gene obtained from database entries

A search of the public databases using the published 3′-untranslated region (UTR) of the mouse cDNA revealed three human EST database entries (hs480165.em_est4, hs542236.em_est4 and hsaa16717.em_est6). Sequences of these EST clones and the coding region of exon 3 were used for primers HP3.f and EST1.r, EST2.r and EST3.r for XL-PCR to amplify and analyse the human sequence encompassing 3.3 kb (Figs 1B and 3A). A polyadenylation signal and an additional 220 bp of genomic sequence were found within a product amplified by inverse PCR using *Eco*RI-digested PAC DNA as template and primers IPX.f/r derived from the 3′-most EST clone (Fig. 3A). A comparison of the mouse GDNF cDNA (accession no. U37459) with the genomic sequence of human GDNF localized downstream of the stop codon revealed a variable degree of homology between 87 and 42% compared with the human sequence (Fig. 3B).

Analysis of alternative spliced transcripts

RT–PCR experiments with cDNAs from fetal brain, heart and liver, rat glioma cell line B49 and human embryonic kidney tumour cell line HEK 293 demonstrated PCR products of three different lengths (Fig. 4A). These PCR products were cloned in pGEM-T and sequenced for further characterization. The first

A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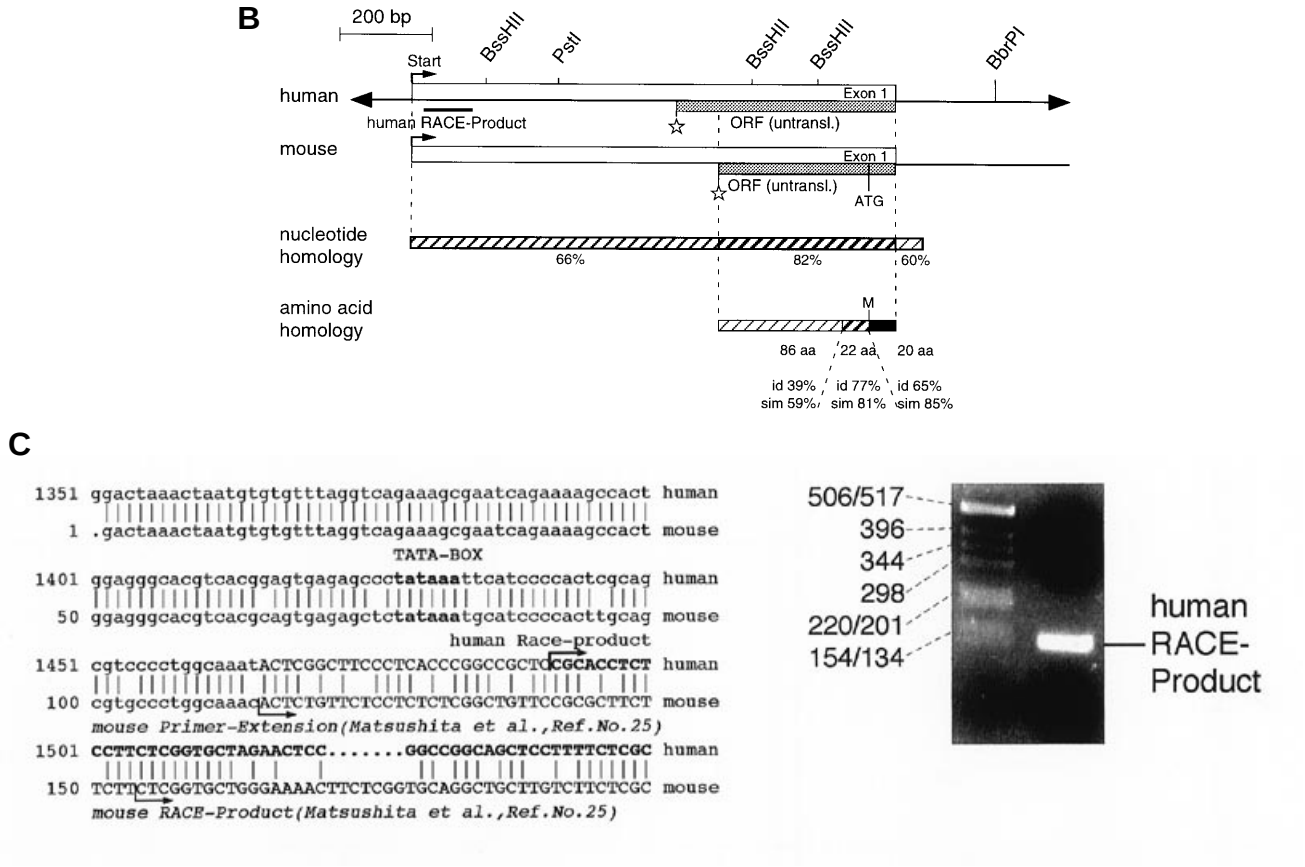

Figure 2. Sequence of hGDNF exon 1 and comparison of the human and mouse sequences. (**A**) Comparison of the sequenced region (numbered as in Fig. 1B) around hGDNF exon 1 with the corresponding genomic mouse sequence. Vertical lines show sequence identity. The 3′ splice donor of exon 1 is shown in italic and boxed; intronic sequences are shown in lower case letters; the grey boxes show the putative translation initiation site. The deduced amino acid sequences of the non-translated ORFs within human and mouse exon 1 are shown above or below the nucleotide sequence in italic; the mouse sequence shows an additional start codon (bold, positions 1114–1116); this region was sequenced for both hGDNF genomic DNA and for cDNA without revealing an ATG. Boxed sequences show a 7 bp repeat around positions 1630–1636 and 2409–2415. The sequence between these boxes was deleted in PCR product XL (primer pair XL-p.f/XL-p.r). The underlined region shows the promoter region predicted by computer analysis using the Proscan program. Restriction sites used for cloning of fragments into the luciferase vector pAH1409 are depicted in bold italic. (**B**) Schematic representation of nucleic acid and amino acid homology between the human and mouse GDNF exon 1. GDNF exon 1 is indicated as an open box; deduced human and mouse GDNF ORFs are shown as grey boxes; asterisks mark in-frame stop codons; ATG represents a start codon. The degree of identity and homology is expressed in per cent. (**C**) Comparison of the mouse and human core promoter and the transcription initiation site. The depicted promoter region 5′ of the putative transcriptional starts show a sequence identity of 95%. The mouse data were obtained from Matsushita *et al*. (25). Thin arrows mark the 5′-ends of the mouse RACE product and the mouse primer extension product. The thick arrow and bold capital letters mark the human RACE product, obtained with cDNA from adult retina, ending 26 bp 3′ of the corresponding mouse transcription start site. Lower case letters show the promoter region. The human RACE product is shown on the right hand side with a product length of 140 bp, including the RACE linker and primer sequences.

two, amplified from all cDNAs except liver, were the full-length transcript and a previously reported transcript (19,20) missing 78 bp at the 3′-end of the previous exon 1 (now exon 2). This is due to an alternative splice site in the exon 2 coding region at bp 1342 (Fig. 3A). The third transcript, lacking the entire exon 2 sequence, was amplified from 293 cells only (Fig. 4A).

Transient expression of the GDNF protein in 293 cells

Fusion of an artificial start codon to hGDNF exon 3 resulted in a GDNF protein lacking the protein prepro sequences (Fig. 4B). Transient expression of this fusion construct leads to an exclusively intracellular accumulation of GDNF protein; no GDNF could be detected in the supernatant of transfected 293 cells (Fig. 4C, left, and data not shown). In contrast, a hGDNF that includes the endogenous ATG followed by the prepro sequences of the precursor protein encoded by exon 2 and the very 5′-part of exon 3 (Fig. 4B) was normally processed, as shown by the presence of mature secreted GDNF protein in the supernatant of transiently transfected cells (Fig. 4C, middle). Western blot analysis of the latter protein resulted in detection of two bands of ~15 and 18 kDa (Fig. 4C, right), the 18 kDa signal probably corresponding to a more glycosylated form of GDNF (3). The apparent molecular weight of the GDNF homodimer is ~36 kDa, which was not unambiguously detectable under denaturing conditions. Western blot analysis of supernatants taken after 24 and 48 h transfection may suggest a longer half-life for the highly glycosylated hGDNF protein, which in relative terms is more abundant in the cell supernatant conditioned for 48 h (Fig. 4C, right).

Analysis of the polymorphic AGG repeat in patients with neurodegenerative disorders

A polymorphic AGG repeat 129 bp downstream of the stop codon of the human GDNF gene is not present in the corresponding mouse cDNA (Fig. 3A; mouse sequence not shown here). Screening of 27 patients with Parkinson's disease, eight patients with idiopathic parkinsonian syndrome, eight patients with ALS, three patients with Alzheimer's disease, six patients with spinocerebellar ataxia and six patients with DOPA-responsive

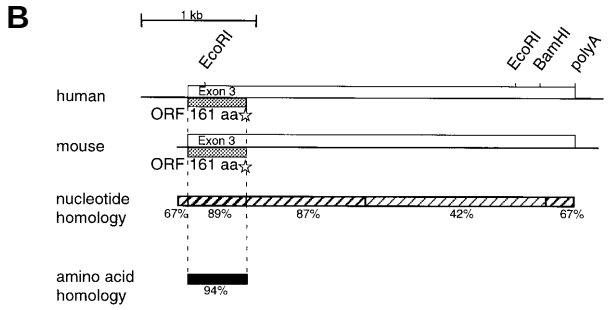

Figure 3. Sequenced region around exons 2 and 3 including the 3′-UTR. (**A**) The coding regions of exons 2 and 3 (numbered as in Fig. 1B) are shown in bold capital letters; intronic sequences are shown in lower case letters; splice donor/acceptor consensus sequences are boxed and in italic. Black arrows show the position of the primers used for the PCR-amplified promoter constructs 5+ and 5–. The AGG repeat in the 3′-UTR of the GDNF gene is marked in bold italic. IMAGE clone numbers and GenBank accession numbers are shown on the right hand side; the polyadenylation signal is underlined and in bold. (**B**) Nucleotide homology of the 3′-UTR of the GDNF gene; amino acid homology depicted for the translated part of exon 3 is shown as a black box.

dystonia showed no significant elongation of the polymorphic AGG repeat in the 3′-UTR. Eight control samples obtained from healthy donors were also evaluated. Allele lengths in patients and healthy donors varied between eight and 18 AGG repeats, with 70% of the analysed chromosomes showing 11 AGG repeats. Forty four per cent of the investigated individuals were heterozygous for this repeat length (Table 1).

Table 1. Analysis of the polymorphic AGG repeat in patients with neurodegenerative disorders

	Alleles (no. AGG repeats)							
	8	9	10	11	12	14	15	18
Absolute frequency in 66 screened individuals	5	6	18	92	1	7	2	1
Frequency (%)	3.8	4.5	13.6	69.7	0.8	5.3	1.5	0.8

Functional analysis of the GDNF promoters in eukaryotic cells

PCR amplification (primers XL-p.f/XL-p.r) of a 2.3 kb fragment containing the promoter and most of exon 1 resulted in a reproducible 790 bp deletion within this region. This deletion is situated 3′ of the TATA box, exactly flanked on both sides by a repeated sequence 5′-GCTGCCC-3′ (Fig. 2A, boxed) and characterized by a high GC content of 74% (Fig. 2A). In order to analyse a potential promoter activity within the 2.1 kb *Eco*RI–*Bbr*PI region, different restriction fragments thereof were fused to the luciferase gene in expression vector pAH1409 (26; Fig. 5A). All constructs were transiently transfected into 293 cells together with a construct constitutively expressing green fluorescent protein (GFP) at a 1:5 ratio and the expression levels integrated by counting GFP-positive cells.

The levels of luciferase activity shown in Figure 5B were obtained from two out of 10 independent experiments with two identically transfected duplicates. Using this approach, an ~8-fold increase in expression compared with the promoterless pAH1409 vector was observed for the predicted core promoter fragment 4+ that includes the TATA box of the hGDNF promoter in the 5′→3′ orientation (Fig. 5B, 4+). The 3′→5′ antisense orientation of fragment 4 (4–) resulted in a reduction in activity, indicating that the orientation, as anticipated for promoters, is important for full induction of transcription activity (Fig. 5B, 4–). Even greater increases in luciferase activity (~20-fold) than seen for fragment 4+ could be monitored from construct 1 (Fig. 5B, 1), including a wider array 5′ and 3′ of the TATA box. Transfection of a *luc* expression construct driven by fragment 2 not containing the TATA box but rather a large part of the 5′-UTR within exon 1 yielded comparably low luciferase activity (Fig. 5B, 2).

In order to analyse the second promoter, which had been indicated by XGRAIL computer analysis, promoter constructs 5+ and 5– were created by PCR amplification of the region bp 6407–7260 (Fig. 3A) using primer pairs Pro2+.f/Pro2+.r for the sense and Pro2-.f/Pro2-.r for the antisense direction, which contained restriction sites for enzymes situated in the MCS of pAH1409 (Fig. 5A). A weak increase in luciferase activity in comparison with promoterless pAH1409 could be seen (Fig. 5B, 5+ and 5–).

Activation of the GDNF promoters

In order to analyse regulation of the GDNF promoter preceding exon 1, luciferase reporter assays were performed with constructs 1 and 4+ after stimulating the transfected 293 cells with various agents. The phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), as well as fibroblast growth factor (FGF) 2, tumour necrosis factor α (TNF-α), retinoic acid (RA), TGF-β, interferon γ (IFN-γ), cyclic adenosine monophosphate (cAMP), lipopolysaccharide (LPS), interleukin 1β (IL1β) and interleukin 10 (IL10) were applied as stimuli. Treatment of the cells with cAMP resulted in an ~2-fold increase in luciferase activity relative to that of the unstimulated construct 1, which contains the largest part of the promoter region, and a lower increase for construct 4+ (Fig. 5B, cAMP), which contains the core promoter only. TPA and FGF2 showed lower relative induction rates with an ~1.6-fold increase in activity (Fig. 5B, TPA and FGF2).

TPA treatment was most effective with construct 4+; minor effects were seen with promoter construct 1. Stimulation of 293 cells transfected with constructs 2, 3 and 4– showed minimal induction of luciferase activity compared with the unstimulated sample (data not shown). TNF-α, TGF-β, IFN-γ, LPS, IL1β and IL10 did not increase promoter activity (data not shown). The constructs 5+ and 5–, which represent the promoter preceding exon 2, showed no response to TPA, nerve growth factor (NGF) or FGF2 (Fig. 5B, right).

DISCUSSION

GDNF is a survival factor for a variety of neuronal types. The neurotrophic activity of GDNF may therefore have therapeutic implications for gene therapy of neurodegenerative disorders such as Parkinson's disease. As our study reveals, the GDNF gene is organized within 28 kb of genomic DNA at 5p12–p13.1. Analysis of the GDNF gene locus predicts a mature GDNF RNA of ~4.6 kb. The amino acid sequence for the human prepro-GDNF is encoded by two exons with an intron within the prodomain.

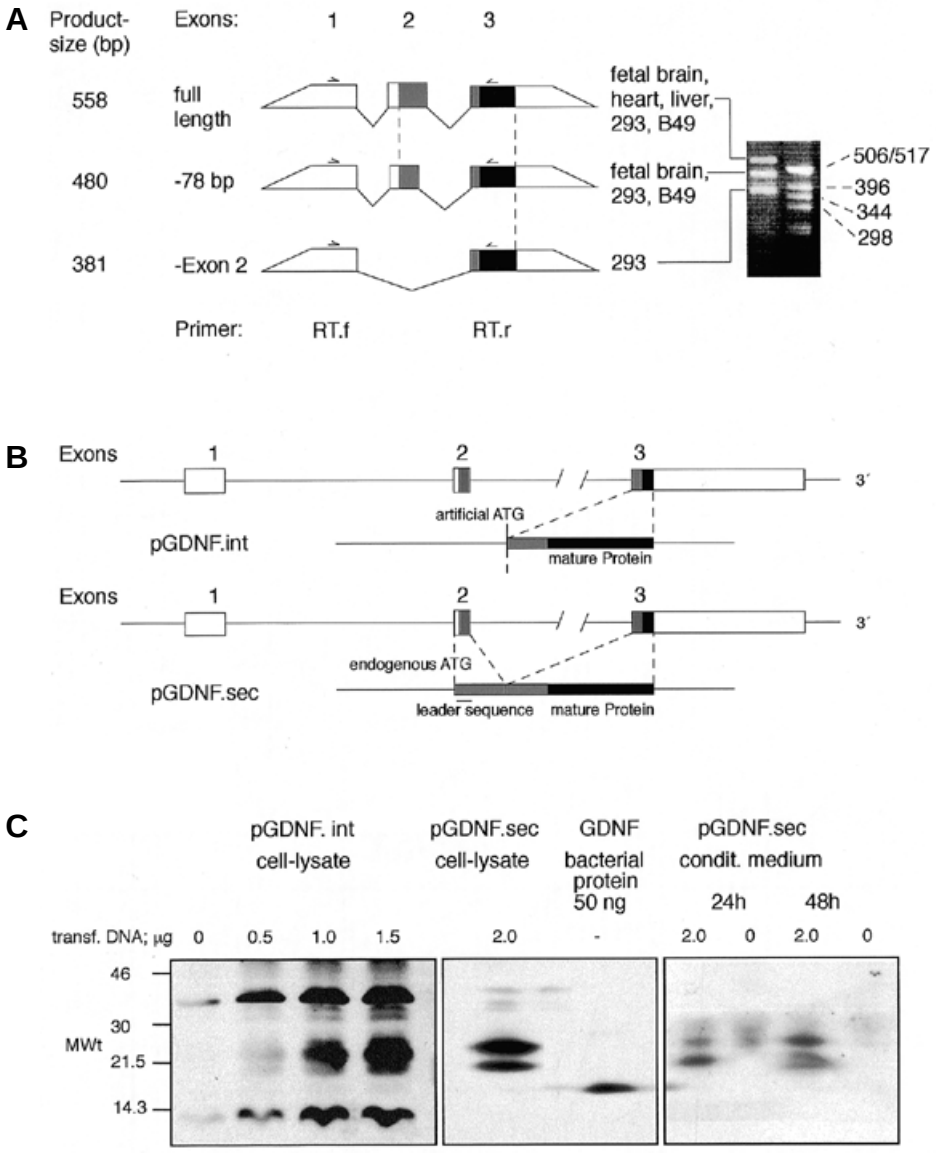

Figure 4. Analysis of GDNF expression. (**A**) Schematic representation of RT–PCR products obtained from different GDNF transcripts. Exons are indicated as described for Figure 1A. (Top) The complete GDNF transcript. (Middle) A splice variant lacking 78 bp in exon 2. (Bottom) A splice variant that is missing the entire exon 2. Products obtained by RT–PCR are represented on the right hand side and show three different fragments. (Left) GDNF-specific RT–PCR products from 293 cells. (Right) Size markers (Gibco BRL). (**B**) hGDNF expression plasmids used for *in vitro* transfection assays. The construct pGDNF.int contains hGDNF exon 3 and an artificial start codon introduced through PCR (primer pair GD.f/GDX.r) giving rise to an intracellular form of GDNF; the construct pGDNF.sec contains the complete sequences of exons 2 and 3 of hGDNF, including the endogenous start codon, giving rise to a secreted form of hGDNF. (**C**) GDNF protein expression detected by western blot analysis. pGDNF.int was transfected at 0.5, 1.0 and 1.5 mg DNA/well and total cell extracts analysed after 48 h. The first lane in this box shows a mock-transfected sample; the weak GDNF signal is caused by endogenous GDNF expression of 293 cells (left). pGDNF.sec was transfected at 2.0 mg DNA/well and protein expression analysed likewise. As a control, purified recombinant GDNF protein, expressed in *E.coli*, was loaded at 50 ng/lane (middle). With pGDNF.sec expression, concentrated conditioned medium of transfected cells was analysed likewise for secreted GDNF after 24 and 48 h (right). For further details see Materials and Methods and Results.

Sequence analysis of 7.9 kb of genomic DNA leads to detection of an additional exon 4.8 kb upstream of the previously published exons with high homology to the mouse cDNA sequence. In accordance with the exon nomenclature of the mouse GDNF gene, we designate the new GDNF exon ‘exon 1’. Sequence analysis revealed an appropriate splice donor site for exon 1, but no 5′ splice acceptor site, indicating that this exon is indeed the first for this gene. The translational start codons for both the human as well as the mouse prepro-GDNF is localized in exon 2. However, the ORF extends to exon 1 for both GDNF cDNAs. For the mouse cDNA there is an additional ATG within the ORF in exon 1, which is not present in the human GDNF cDNA. This mouse ATG is not in accordance with the consensus sequence for translation start codons (27), so it probably does not represent an actual start codon. Furthermore, the nucleic acid sequence of this ORF is more conserved (82%) than the corresponding amino acid sequence (39–65%). Thus, it appears likely that GDNF exon 1 represents the 5′-UTR of the human GDNF gene. The high degree

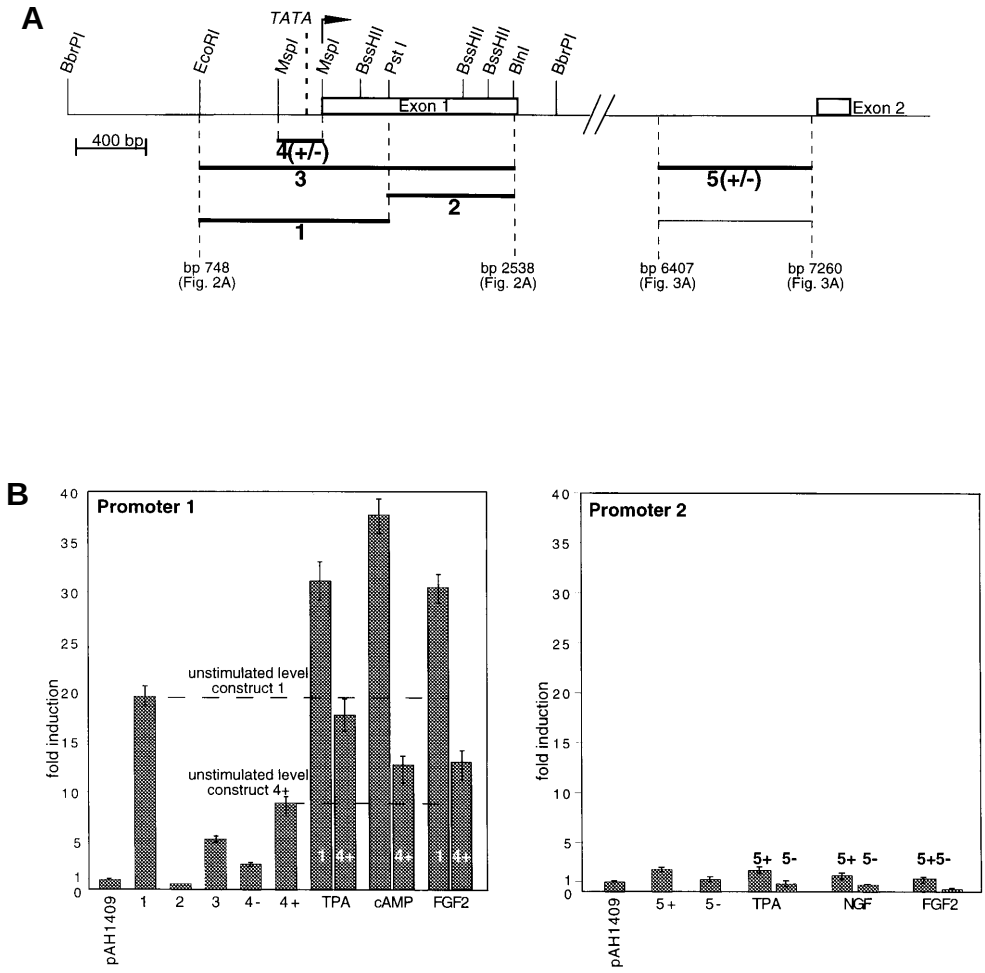

Figure 5. Luciferase promoter–reporter assays using promoter fragments of the hGDNF gene. (**A**) Seven different constructs with the following DNA fragments were used for luciferase assays: 1, containing the 1.1 kb *Eco*RI–*Pst*I fragment; 2, containing a 0.7 kb *Pst*I–*Bln*I fragment including transcribed sequences of exon 1 under exclusion of the splice donor site of this exon; 3, containing a 1.8 kb *Eco*RI–*Bln*I fragment, obtained by partial digestion of the 2.1 kb *Eco*RI–*Bbr*PI fragment; 4+, containing the 0.24 kb *Msp*I core promoter fragment (Fig. 2A); 4–, containing the 0.24 kb *Msp*I fragment cloned in the antisense orientation; 5+ and 5–, containing promoter 2 in the sense and antisense orientations, respectively (Fig. 3A). (**B**) Luciferase activity of the corresponding constructs after transfection into 293 cells. After transfection, cells were either left untreated or incubated with TPA (100 ng/ml), 8-bromo-cAMP (1 mM), RA (1 mM), FGF2 (10 ng/ml) or NGF (40 ng/ml). CMV–LTR–Luc was used as a positive control resulting in an ~800-fold higher activity when compared with the promoterless construct pAH1409.

of sequence conservation between the human and mouse 5′-UTRs and the lack of an in-frame start codon point towards possible regulation of both human and mouse GDNF expression at the transcriptional or translational level through this region. Genomic PCR amplification of the region around exon 1 including the 5′-UTR resulted in a reproducible deletion of 790 bp in the 5′-UTR sequence, possibly due to secondary structures (data not shown). This assumed complex structure of the conserved 5′-UTR might be involved in regulation of GDNF expression. Untranslated first exons likely serving regulatory functions have been described for other genes, such as *PLC*γ2 (28) and c-*myc* (29).

Near exon 1 we found a promoter marked by a potential TATA box. Several findings suggest that this promoter and its TATA box are functional. About 33 and 47 bp 3′ of the TATA sequence two motifs facilitate transcription initiation. The location of these motifs relative to the TATA box matches with the positions of transcription initiation sites in the majority of characterized vertebrate promoters (30). The mouse transcription start site was determined by primer extension and found to be situated in the same location (25). Promoter comparison between mouse and human shows that the 115 bp regions around the TATA box share 95% sequence identity. The remarkably high conservation of both mouse and human sequences would suggest the existence of important regulatory elements in this region. Three CpG islands, the first in the promoter region, the second including partial sequences of intron 1 and the third covering exon 2 and flanking sequences, are predicted by XGRAIL analysis. Fusion of this promoter to a luciferase reporter gene either with or without inclusion of exon 1 results in efficient expression of luciferase in HEK293 cells relative to a promoterless construct. Antisense positioning of the putative core promoter sequence 5′ of the luciferase gene results in decreased expression from this construct, indicating that the promoter and most likely the TATA box need proper localization 5′ of the gene for transcriptional activity. Construct 2, which contains most of the putative 5′-UTR contained in exon 1 of the GDNF gene, did not show promoter activity. This underscores the assumption that these sequences

belong to the 5′-UTR of the GDNF gene. RT–PCR with primers located within the first exon reveals this exon as being transcribed from a 5′ promoter in human fetal brain, human and rat adult brain, human heart and liver as well as in the human embryonic kidney (HEK) cell line 293 and in the rat glioma cell line B49.

A second putative promoter was detected within the first intron by XGRAIL analysis. Two studies have described intragenic promoters within intron 1, one by Suter-Crazzolara *et al*. (31), equivalent to the second promoter described here, and one by Woodbury *et al*. (32), which is not yet fully characterized. Several different promoters for the TGF-β superfamily have been described (33–35). There is great variability within these promoters regarding the presence of promoter elements such as TATA boxes or possible binding sites for transcription factors. Intragenic promoters have been described for the cystine knot neurotrophin, brain-derived neurotrophic factor (36).

Further genomic sequence analysis of 4.1 kb of genomic DNA including GDNF exon 3 revealed a polyadenylation signal 2.9 kb distal of the stop codon. Sequence comparison of the human and mouse 3′-UTR reveal homology of 87% for the extreme 5′-end of the 3′-UTR, a relative low degree of conservation (42%) for the central region and 67% for the region containing the polyadenylation signal at the 3′-end. Most strikingly, the polymorphic AGG repeat found in the human 3′-UTR sequence is divergent and very short in the corresponding mouse sequence. Several neurodegenerative diseases are caused by significant elongation of polymorphic trinucleotide repeats (37). In Huntington's disease, the most prominent example, the elongation of triplet repeats affects the coding region of the disease-causing gene, generating a mutated huntingtin protein that contains a possibly toxic polyglutamine stretch (for a review see ref. 38). In order to investigate a possible expansion of a triple repeat within the GDNF gene, we screened 27 patients with six different neurodegenerative phenotypes. We found no significant elongation of the AGG repeat in the 3′-UTR of the GDNF gene which could point to a role for GDNF within the corresponding disease. Focusing on the coding regions of the GDNF gene, the work of others has supplied no evidence for relevance of mutational load in neurodegenerative diseases (15–17,39).

Comparison of the exon–intron structure of other members of the TGF-β superfamily show that neurturin and inhibin α-chain (INHA), a gonadal hormone involved in the non-steroidal regulation of follicle stimulating hormone secretion, are the nearest relatives of GDNF (40,41). Two exons have been described for the inhibin gene. The mature GDNF protein is encoded by a single exon, as is the case for neurturin, which shows 49% DNA sequence homology to the GDNF gene within the coding sequence for the mature protein. Only the two exons corresponding to the coding sequence have been published. As with GDNF, the prepro sequences are encoded by parts of the same exon and the preceding exon.

We do not yet know whether or not the neurturin gene also contains an additional exon coding for a 5′-UTR. This requires further experimentation.

Northern blot analysis of RNA from several tissues did not result in a clear hybridization signal for the different GDNF transcripts, neither in our hands nor in the hands of others (42), suggesting that GDNF transcripts are present in only minute amounts. RT–PCR experiments show alternative splicing for exon 2 in different tissues. The cDNA derived from 293 cells contains transcripts lacking the entire exon 2 sequence and therefore lacking the corresponding start codon. Two other in-frame ATGs are, however, found 6 and 42 bp downstream of the 5′-end of exon 3, of which at least the second one is homologous to the Kozak leader sequence motifs (27), raising the possibility of a second translation initiation site. Products started from here could result in a form of GDNF which is retained within the cell, serving perhaps as an intracellular ligand provided the cell co-expresses RET and GFR-α1. Such an intracellular form of GDNF could initiate an intracellular autocrine loop, as is true for some growth factors and cytokines (43), thus enhancing the potential of these cells to profit from GDNF-mediated action. The transcript lacking the prepro sequences encoded by exon 2 has not yet been found in other tissues. This transcript might be specific for this tumour cell line or it may be developmentally specific. In embryogenesis, small clusters of cells or even single cells must survive and position themselves within the developing organism. Single migrating neural crest-derived cells, for example, might require autocrine stimulatory mechanisms. We have exogenously expressed an intracellular form of GDNF in 293 cells by artificially introducing an ATG within exon 3. As expected, this results in an ~14–20 kDa glycosylated protein which is intracellularly retained. We are currently investigating whether the intracellular form of GDNF, when ectopically expressed in GDNF-responsive neurons, can enhance their survival. GDNF exogenously expressed in 293 cells with the endogenous ATG, including the leader, results in a secreted protein detectable in conditioned medium. Notably, during the time course after transfection a putatively more glycosylated form of GDNF seems to accumulate over a less glycosylated form. This suggests different degrees of protein stability dependent upon the degree of glycosylation.

Another alternative splice product lacking 78 bp at the end of exon 2 is present in a variety of tissues (19,20). This is likely to result in a divergent prepro sequence lacking 26 amino acids which might be differently processed or secreted.

Several recent reports have described the regulation of GDNF expression. GDNF mRNA levels are increased *in vivo* in response to kainate-induced epileptic seizures (44). Treatment of mouse astrocytes and C6 glial cells with bacterial LPS and inflammatory cytokines such as TNF-α, IFN-γ and IL1β results in increased GDNF expression in these cells (45). Others report that the induction of axonal dopaminergic sprouting in parkinsonian mice is mediated by IL1β; however, IL1β application was not reported to increase levels of acidic FGF or GDNF gene expression (46).

In our promoter stimulation experiments we used 10 different factors, including TPA, FGF2 and cAMP. TPA, which showed a significant induction of promoter activity in the transient expression assay, is a potent tumour-promoting phorbol ester and, more importantly, a strong inflammatory agent that activates protein kinase C (PKC), resulting in activation of Raf and the MEK/MAPK signal cascade (47; for a review see ref. 48). This finally leads to activation of transcription factors and alterations of gene expression. However, no conserved transcription factor binding sites could be detected in our inducible promoter constructs. Interestingly, endogenous expression of GDNF can be suppressed using PKC inhibitors in human astrocytes (49). Astrocytes have been shown to respond to brain injury, ischemia and inflammation with up-regulation of neurotrophic factor expression (50–53). PKC and Raf expression is highest in brain and is expected to play a role in brain functioning, learning and neuronal survival (54). FGF2 and cAMP produced a moderate

increase in expression, suggesting other possible signal transduction pathways involving Ras and protein kinase A, respectively (55,56). Lack of induction using the other stimuli does not necessarily mean that they have no function *in vivo*. Receptors, intracellular signal transducers and transcription factors potentially present in certain cell types relevant for GDNF function could be absent in the 293 cells used for the promoter assays.

In conclusion, we have determined the structural organization of the GDNF gene and characterized some of its functional features. Two (or more) distinct promoters and at least three alternative splice products suggest spatiotemporal regulation of GDNF expression, which would be consistent with the many different functions associated with GDNF.

We suggest an intrinsic potential of the gene to respond to distinct physiological stimuli leading to protein isoforms with possibly divergent functions. The further elucidation of regulation of this gene, protein expression and protein diversity should advance our understanding of the signalling and function of this very important neurotrophic factor within human genetics, development, physiology and disease. A profound knowledge of GDNF functions will aid the developement of specific applications in therapeutic and gene therapeutic approaches.

MATERIALS AND METHODS

DNAs and cDNAs

Genomic DNA for PCR reactions or Southern blots was extracted from human or mouse whole blood using the QIAamp Blood Kit (Qiagen) according to the manufacturer's protocol.

293 cDNA was obtained by preparing total RNA from the embryonic kidney tumour cell line 293 using RNA STAT-60 (Tel-Test). cDNA synthesis was primed with random hexamers using the First Strand cDNA Synthesis Kit (Pharmacia). Tissue-specific cDNAs (fetal brain, heart and liver) were purchased from Clontech. PAC DNA was isolated by the alkaline lysis method described recently (57) for either PCR or PFGE and Southern analysis.

Gel electrophoresis

PFGE was performed in 1.5% agarose in 0.5× TAE (20 mM Tris base, 10 mM acetic acid, 0.5 mM EDTA). Pulsing frequency was a 10 s switch time for 30 h at 140 V at a constant temperature of 15°C. Horizontal gel electrophoresis was carried out on 0.7% agarose gels in 1× TBE. Southern blots were generated using nylon transfer membranes (Qiagen). PCR products for analysis of the AGG repeat were run on an ABI sequencer and detected with Gene Scan Analysis 2.0.2 (ABI).

Hybridization and probes

Exon-specific hybridization probes were obtained by PCR amplification with primers of human GDNF exon 2 (HP2.f/r) and exon 3 (HP3f./r) using human genomic DNA as template. For identification of human exon 1, a PCR product was assessed using mouse Gdnf exon 1-specific primers (HPm1.f/r) and mouse genomic DNA as template. The PCR products were purified with the QIAquick PCR purification kit (Qiagen).

For mapping of the PAC insert, *Bam*HI and *Bbr*PI fragments were separated on a 0.7% agarose gel and purified with the QIAgel extraction kit (Qiagen). Probes were labelled with [α-^{32}P]dCTP using the random hexamer primer method. Repetitive DNA of PAC fragments was blocked with 1 mg human placenta DNA per 150 µl labelling reaction at 65°C 1 h before hybridization.

Hybridization was overnight at 65°C in 1.5× SSPE, 1% SDS, 0.5% Blotto, 1% dextran sulfate and 1 mg salmon sperm DNA. Washing was performed in 3× SSC, 0.1% SDS three times for 15 min at 65°C.

Identification and characterization of PAC clones

A human PAC library (21) from total genomic DNA was screened using an [α-^{32}P]dCTP-labelled PCR product of exon 3 (primers HP3.f/r) of the human GDNF gene as probe. Positive PAC clones were cultured on kanamycin–agarose plates. Single colonies were picked and grown in 5 ml overnight cultures in LB with a kanamycin concentration of 25 mg/ml. PAC inserts were analysed by hybridization experiments with overlapping *Bam*HI and *Bbr*PI fragments. The localization of GDNF exons on different PAC fragments was determined by hybridization with exon-specific PCR products using primer pairs HP1.f/r, HP2.f/r and HP3.f/r. *Bbr*PI fragments hybridizing with exon 1, 2 or 3 were fine mapped with further restriction enzymes.

PCR, RT–PCR and 5′-RACE

PCR amplifications were carried out with genomic DNA or PAC DNA as template according to standard protocols using 100 ng DNA, 30 pmol each primer, 250 mM each dNTP, 15 mM $MgCl_2$, 0.5 U Tfl DNA polymerase and 1× buffer (Epicentre Techniques) under the following conditions: initial denaturation for 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at a primer-specific annealing temperature, 1 min at 72°C and a last extension step at 72°C for 5 min.

Long range PCRs were performed with the XL-PCR kit (Perkin Elmer) and PCR programmes recommended by the distributor using a two step cycle programme consisting of 1 min at 94°C and 5 min at 60°C.

Templates for RT–PCR were single-stranded cDNAs obtained by reverse transcription of 293 and B49 total RNA or purchased cDNA (Clontech). Primers used for PCR are listed in Table 2.

The RACE experiment was performed with the Marathon-Ready cDNA amplification kit (Clontech). The touchdown PCR programme recommended by the distributor was modified using five cycles of 30 s at 94°C and 45 s at 72°C, five cycles of 30 s at 94°C and 45 s at 70°C and 25 cycles of 30 s at 94°C and 45 s at 68°C. The nested PCR was with the two GDNF-specific RACE primers listed in Table 2.

Inverse PCR

In order to obtain exon flanking sequences, PAC DNA was digested overnight with *Sau*3A or *Eco*RI. Restriction enzymes were inactivated by heating at 60°C for 20 min followed by ethanol precipitation. The precipitated DNA was resuspended in 10 µl TE and religated overnight. The resulting random circular DNA fragments were used as template DNA for a long range PCR using primer pairs IP2.f/r and IP3.f/r, pointing towards the intron sequences. The resulting PCR products underwent sequence analysis.

Table 2. GDNF-specific PCR primers

Primer		Sequence	Annealing temperature (°C)	Product size
Genomic long range PCR				
Exon 1/exon2	XL-1.f	5′-GAACTCTTGCCCCTG-3′	58	4.8 kb
	XL-1.r	5′-GGTGTGGAGCAGCACCAG-3′		
Promoter region/exon 1	XL-p.f	5′-GAACATTGATGAGGGTCGTCTTC-3′	58	1.5 kb[a]
	XL-p.r	5′-GATGGCTGGAGCGCGGAG-3′		
Exon 3/EST sequences	HP3.f	5′-TATGCCAGAGGATTATCCTGATCAG-3′	62	
	EST1.r	5′-CCTTTCCAGGGTATGTCTCCAC-3′		1.0 kb
	EST2.r	5′-CGAGCACATATACTTTGGCACCT-3′		1.5 kb
	EST3.r	5′-GATCAGAGAAGTCAGAACTTCCAA-3′		3.3 kb
Hybridization probes				
Mouse exon 1	HPm1.f	5′-CCTGCCCGAGGTCCGG-3′	58	
	HPm1.r	5′-CTCGGATCCGGTCTCCG-3′		
Human exon 1	HPh1.f	5′-GAACTCTTGCCCCTGACC-3′	58	130 bp
	HPh1.r	5′-CTCGGATCGGGTCTCCG-3′		
Exon 2	HP2.f	5′-AAGATGAGGTTATGGGATGTCGTGG-3′	58	150 bp
	HP2.r	5′-CACTGCTCAGCGCGAAGGGCG-3′		
Exon 3	HP3.f	5′-TATGCCAGAGGATTATCCTGATCAG-3′	58	246 bp
	HP3.r	5′-TAGCCCAGACCCAAGTCAGTGAC-3′		
RT–PCR				
Exon 1	RT.f	5′-GAACTCTTGCCCCTGACC-3′	58	381–558 bp
Exon 3	RT.r	5′-TAGCCCAGACCCAAGTCAGTGAC-3′		
3′-UTR of AGG-repeat	IP3.f	5′-ATGTTGCAGACCCATCGCC-3′	60	291–318 bp
	REP.r	5′-CCAGGCTCCCATGATGGC-3′		
Inverse PCR				
Exon 2	IP2.f	5′-GGCGAAGACCGCTCCCTC-3′	60	Lengths
	IP2.r	5′-GGTGTGGAGCAGCACCAG-3′		not
Exon 3	IP3.f	5′-ATGTTGCAGACCCATCGCC-3′	60	defined
	IP3.r	5′-TTGAGTCTTTTAATGGTGGC-3′		
PCR for expression experiments				
Exon 3/stop codon	GD.f (***Xho*I site Start**)	5′-ACA**CTCGAG**A**ATG**CCAGAGGAATTATCCTGATCA-3′	60	498 bp
Exon2/stop codon	GDL.f (***Xho*I site Start**)	5′-ATGGCCGC**CTCGAGATG**AAGTTATGGGATGTCGTGG-3′		658 bp
	GDX.r (***Xho*I site**)	5′-GAG**CTCGAG**TCAGATACATCCACACC-3′		
Promoter fragments	Pro2+.F (***Bam*HI site**)	5′-**AAGCTT**GACAAATGCAGTCTTTGCCTAACAG-3′	66	854 bp
	Pro2+.R (***Bam*HI site**)	5′-**GGATCC**CGTCCGGCGGCGGCACC-3′		
	Pro2-.F (***Eco*RI site**)	5′-**GGATCC**GACAAATGCAGTCTTTGCCTAACAG-3′	66	854 bp
	Pro2-.R (***Bam*HI site**)	5′-**AAGCTT**CGTCCGGCGGCGGCACCTG-3′		
RACE experiment				
1st round	RACE-R1	5′-CTTTCCTCGCGCCTGTCGAAGGC-3′		
2nd round	RACE-R2	5′-TTCCCGCTCGGGTGTCTCGCCCT-3′		104 bp

[a]This PCR product shows a shorter length than expected from mapping and sequencing data.

DNA sequencing

Fragments or PCR products were subcloned in pSL1180 (Pharmacia) and pGEM-T (Promega), respectively, for sequence analysis. Nucleotide sequences were determined by the dideoxynucleotide chain termination method using a Taq Dye-Deoxy Terminator Cycle sequencing kit (ABI). The reactions were run on an Applied Biosystems 377 automated sequencer.

Cell culture

The human embryonic kidney cell line 293 and rat glioma B49 were used for preparation of total RNA. In addition, 293 cells were used for promoter analysis. Cells were grown in RPMI 1640 on 140 × 20 TC dishes with 10% L-glutamine and 10% fetal calf serum (B49) or calf serum (293) at 37°C and 7% CO_2 (all media from Life Technologies). The medium was supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml; Life Technologies).

Transient expression experiments

Two different GDNF expression constructs were designed. Construct 1 contains the sequence coding for the mature protein beginning at amino acid 78. An artificial start codon was introduced by the primer sequence GD.f. Construct 2 contains the endogenous GDNF start codon corresponding to amino acid position 1 and therefore including the prepro sequences of the GDNF protein. The corresponding cDNA fragments were obtained by RT–PCR of total RNA from 293 cells using primers GD.f and GDX.r for the intracellular form of GDNF (Fig. 4B, 1) and GDL.f and GDX.r for the secreted form of GDNF (Fig. 4B, 2). PCR primers included suitable restriction sites (*Xho*I) for ligation into expression vector pBC140.

Expression vectors, cell culture and promoter assays

GDNF promoter fragments were ligated 5′ of the luciferase reporter gene in vector pAH1409 (26), after a preceding cloning step into pBluescript to obtain suitable cloning sites. HEK293 cells were seeded in RPMI 1640 with high glucose (4.5 g/l), sodium bicarbonate (3.7 g/l) and 10% calf serum (Life Technologies) at a density of 1.5×10^6 cells/well and were transfected in six-well plates with 2 µg DNA of the promoter–luciferase constructs using Lipofectamine according to the manufacturer's instructions (Life Technologies). Individual experiments were performed with different DNA preparations to minimize artefacts due to a given DNA quality and thus ensure representative results. A construct giving rise to constitutive expression of GFP was co-transfected at a 1:5 ratio. All constructs were transfected in duplicate. Cells were harvested after 44–48 h. Luciferase assays were performed as described (50). Luciferase activity was normalized for transfection efficiency as determined by counting GFP-positive cells prior to harvesting.

Western blot analysis

Cells were seeded in six-well tissue culture dishes at a density of 1.5×10^6 cells/well 24 h prior to use. At 44–48 h after transfection cells were harvested, heated in H8 buffer (final concentrations 20 mM Tris–HCl, pH 7, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, 50 mM NaF, 100 mM Na_3VO_4), sonicated, run on 12% PAGE gels and blotted for 1 h in cooled blotting buffer (126 mM glycine, 24.7 mM Tris base) at 70 V. After blocking the membrane with blocking solution (1× PBS, 0.02% I-Block; Tropix), immune reactions were performed overnight at 4°C using a rabbit polyclonal α-GDNF antibody (Santa Cruz) in a 1:100 PBS dilution. After six washes for 5 min with PBS containing 1.5% Tween, the blot was incubated with a goat anti-rabbit second antibody (Tropix) for 2 h at 4°C. The detection reaction was carried out following the manufacturer's protocol (Tropix).

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ABBREVIATIONS

cAMP, cyclic adenosine monophosphate; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; GFR-α1, GDNF family receptor α; GFP, green fluorescent protein; HEK, human embryonic kidney; IFN-γ, interferon γ; IL10, interleukin 10; IL1β, interleukin 1β; ins, transcriptional initiation site; LPS, lipopolysaccharide; NGF, nerve growth factor; ORF, open reading frame; PKC, protein kinase C; RA, retinoic acid; RACE, rapid amplification of cDNA ends; TGF-β, transforming growth factor β; TNF-α, tumour necrosis factor α; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; UTR, untranslated region.

NOTE ADDED IN PROOF

A second GDNF promoter without CCAAT and TATA boxes but with binding sites for multiple transcription factors such as CREB, AP2, Zif/268, NFκB and MRE-BP has been described (C. Suter-Crazzolara, personal communication), which may account for the complex expression pattern of the gene.

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