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Analysis of human neuropeptide FF gene expression

Johanna M. Nystedt,*,§ Annika M. Brandt,* Jami Mandelin,§ Ferdinand S. Vilim,† Edward B. Ziff‡ and Pertti Panula*,§

*Department of Biology, Abo Akademi University, Biocity, Turku, Finland

†Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, USA

‡Howard Hughes Medical Institute, Department of Biochemistry, New York University Medical Center, New York, USA

§Institute of Biomedicine/Anatomy, University of Helsinki, Helsinki, Finland

Abstract

As an initial step to study the function of the gene encoding the human neuropeptide FF (NPFF), we cloned a 4.7-kb sequence from the promoter region. Primer extension and 5′-rapid amplification of cDNA ends revealed multiple transcription initiation sites. Northern blot analysis of the mRNA expression revealed a specific signal only in poly(A) + RNA from medulla and spinal cord. Chimeric luciferase reporter gene constructs were transiently transfected in A549, U-251 MG, SK-N-SH, SK-N-AS and PC12 cells. The promoter activity was directly comparable with the level of endogenous NPFF mRNA as determined by real-time quantitative RT–PCR. The highest promoter activity was measured when a region from − 552 to − 830 bp of the 5′-flanking region was fused to the constructs, and a potential silencer element was localized between nucleotides −220 and −551. A twofold increase in NPFF mRNA was observed after 72 h of nerve growth factor stimulation of PC12 cells and the region between − 61 and − 214 bp of the 5′-flanking region was found to be responsive to this stimulation. We postulate that control of human NPFF gene expression is the result of both positive and negative regulatory elements and the use of multiple transcription initiation sites.

Keywords: antiopioid, neuropeptide, NPFF, PC12, promoter, RF-amide.

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Neuropeptide FF (NPFF), also referred to as morphine-modulating peptide or F-8-F-amide (Majane and Yang 1987; Panula *et al*. 1987), is a mammalian RF-amide neuropeptide originally identified as a mammalian peptide related to the molluscan cardioactive peptide FMRF-amide (Yang *et al*. 1985). Cloning of the cDNA encoding the NPFF precursor from human (Perry *et al*. 1997), mouse, rat and bovine (Vilim *et al*. 1999) revealed an evolutionally conserved structure of the gene, consisting of three exons and two introns and encoding the biochemically identified NPFF and also the related amidated bioactive peptides neuropeptide AF (NPAF or A-18-F-amide) and neuropeptide SF (NPSF). The NPFF peptides were the first characterized mammalian neuropeptides which belong to the family of RF-amide peptides, a group which today also comprises the peptide products generated from the prolactin-releasing peptide (PrRP) gene (Hinuma *et al*. 1998), the RFamide-related peptides (RFRPs) precursor gene (Hinuma *et al*. 2000) and the KiSS-1 gene (Lee *et al*. 1996).

NPFF, NPAF and NPSF play roles in mechanisms behind sensory and autonomic regulation (Panula *et al*. 1996, 1999; Roumy and Zajac 1998); in particular, pain modulation and opiate function. Additionally, these RF-amide peptides seem to have potent effects on the cardiovascular system (Roth *et al*. 1987) and neuroendocrine functions (Majane and Yang 1991; Majane *et al*. 1993; Aarnisalo *et al*. 1997). NPFF-like immunoreactivity has been observed in human plasma, which could also implicate hormonal functions for these peptides (Sundblom *et al*. 1995). Interestingly, NPFF and

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Address correspondence and reprint requests to Pertti Panula, Department of Biology, Abo Akademi University, Biocity, 2. floor, Artillerigatan 6 A, 20520 Turku, Finland. E-mail: ppanula@abo.fi

[1]The nucleotide sequence of the cloned promoter region described in this manuscript has been submitted to the Genebank™ with the following accession number: AF503515.

Abbreviations used: EST, expressed sequence tag; ISH, *in situ* hybridization; NPAF, neuropeptide AF (A-18-F-amide); NPFF, neuropeptide FF (morphine modulating peptide, F-8-F-amide); NPSF, neuropeptide SF; 5′-RACE, 5′-rapid amplification of cDNA clone ends; 3′-RACE, 3′-rapid amplification of cDNA clone ends; UTR, untranslated region; NGF, nerve growth factor.

NPAF seem to modulate human lymphocyte proliferation (Lecron *et al*. 1992) and binding sites for NPFF have been characterized on Jurkat T-cell lymphocytes (Minault *et al*. 1995).

After many years of intensive research to find receptors that deliver the intracellular effects of these potent ligands, several reports about functional NPFF receptors have now been published. Two G protein-coupled receptors (GPCRs), here called FFR1 and FFR2, have been identified as the main NPFF receptors (Bonini *et al*. 2000; Elshourbagy *et al*. 2000; Kotani *et al*. 2001; Liu *et al*. 2001). The receptors are encoded by separate genes and the human FFR1 and FFR2 share a 59% identity on an amino acid sequence level. The expression patterns of the receptors are distinct from each other, further clarifying the multiple roles of these RF-amide peptides. An acid-sensing, proton-gated DEG/ENaC channel has also been proposed to be activated by high concentrations of NPFF and potentiate acid-evoked currents from sensory neurones (Askwith *et al*. 2000) and very recently some receptors of a family of approximately 50 novel GPCRs called *mrgs*, expressed in specific subsets of nociceptive sensory neurones, were specifically activated by RF-amide neuropeptides such as NPFF and NPAF (Dong *et al*. 2001). The relevance of any of these characterized receptors in pain modulation is not yet known in detail, but the discovery of the receptors has certainly aided in defining and strengthening the physiological role(s) of these anti-opiate neuropeptides.

Currently, no published reports have described the expression pattern of the human NPFF gene. In rat CNS, NPFF precursor mRNA expression is restricted to neurones in the hypothalamus, medulla and the dorsal horn of the spinal cord as revealed by *in situ* hybridization (ISH) and an approximately 600 bp mRNA transcript of the NPFF gene has been detected by northern blotting in the corresponding areas (Vilim *et al*. 1999). Overall, similar neuroanatomical distribution of gene expression has been observed in mouse CNS (Brandt *et al*. 2000). Recently, the first thorough analysis of possible peripheral NPFF gene expression was published, revealing significant ISH signals only in rat spleen (Nieminen *et al*. 2000). The limited expression suggests the existence of molecular mechanisms by which the gene is specifically transcribed by distinct populations of cells. Very little is known about the transcriptional regulation of the NPFF gene. The only published report so far has shown by ISH that NPFF gene expression is up-regulated in an inflammatory pain state in rat, indicating mechanisms for inducible transcriptional regulation (Vilim *et al*. 1999). The inducible up-regulation of the mRNA transcripts clearly has a physiological function as it results in increased levels of mature peptides (Kontinen *et al*. 1997). Genes encoding neuropeptides are generally regulated by an interaction of multiple positive and negative transcriptional regulators, for example the gene encoding preprotachykinin A (PPT), and calcitonin gene-related peptide (CGRP; for review see Quinn 1996). The use of multiple transcription initiation sites has also been proposed as a regulatory mechanisms for some neuropeptide genes, e.g. the human prepro-orexin gene (Sakurai *et al*. 1999) and interestingly also for the rat PrRP gene (Yamada *et al*. 2001), another member of the RF-amide peptide family.

Because the NPFF system could be a potential novel target for therapeutics against opiate addiction and analgesia, a thorough understanding about the basic mechanisms underlying the function of the human NPFF gene is needed. In this report the use of transcriptional start sites and polyadenylation sites for the human NPFF gene is studied, the mRNA expression in the human brain is revealed, an initial functional characterization of the NPFF promoter is performed and evidence for an inducible transcriptional mechanism of the promoter by using nerve growth factor (NGF) stimulation of rat pheochromocytoma PC12 cells is presented. Additionally, a quantitative method to measure human and rat NPFF precursor mRNA is presented. This study represents an initial analysis of the NPFF promoter region and might also in part explain the mechanisms of neuronal-specific gene expression, still a central problem in modern molecular neurobiology.

Experimental procedures

Cells and cell culture

All cell culture reagents were purchased from Gibco, Life Technologies, Inc (Rockville, MD, USA) if not mentioned otherwise. Cell lines were kept in a humidified incubator at 37°C and with 5% CO_2. Human lung carcinoma A549 (ATCC) and rat pheochromocytoma PC12 (kindly donated by Dr L. Greene) were grown in Dulbecco's modified Eagle's medium. Human astrocytoma U-251 MG (kindly donated by Dr I. Virtanen), human neuroblastoma SK-N-SH (ATCC) and SK-N-AS (ATCC) were grown in RPMI-1640 medium. All cell culture media were supplemented with 10% fetal calf serum, 1 × Glutamax and 50 μg/mL penicillin and 50 IU/mL streptomycin, except for PC12 cells which were grown in 10% horse serum and 5% fetal calf serum. PC12 cells were cultured on collagen-coated dishes and stimulation of PC12 cells was accomplished by adding 50 ng/mL 7S-NGF (Sigma, St Louis, MO, USA) from mouse submaxillary glands in medium containing 1% fetal calf serum and 1% horse serum.

DNA sequence analysis

All oligonucleotides were synthesized by the Institute of Biotechnology (University of Helsinki, Finland) and Eurogentec (Bel S. A., Belgium) and the sequences of the oligonucleotides used in this study are presented in Table 1. Nucleic acid sequences were determined by automated sequencer (ABI Prism Automated Fluorescence Sequencer). Sequence assembly and analysis was accomplished by using Wisconsin Package Version 10.0, Genetics Computer Group (GCG; Madison, WI, USA). The nucleotide sequence of the 5′-flanking region of the human NPFF gene was analysed for

Table 1 DNA sequence of primers used in PCR and nested PCR

Name	Orientation	Sequence (5′- to 3′)
hWALK-DO	Antisense	GCTGCCACCACCTACCCTCCTAC
hWALKnest-DO	Antisense	GTGGATCCATCTAGAGCAGGCAAATG
hWALK2-DO	Antisense	GCAGACAGACCCCCATCCTCA
hWALK2nest-DO	Antisense	GACCTGCCTCATCGCCCTTCC
H-DO	Antisense	AGGATCCTGGACCTTGCATGCAGACAT
H-UP 0.2	Sense	GTGCTAGCTAATAAAGTCTGTAACTC
H-UP 0.5	Sense	GTGCTAGCCAAATCTGAGTAGCCTCC
H-UP 0.8	Sense	GTGCTAGCTTACAGGTGTGAGGCACT
H-DO#1	Antisense	AGCCTCACAGTAGCCTTA
H-DO#2	Antisense	ATAAATCCTGTCTCCAGA
hNPFF-UP	Sense	TGCTGCTGTTAATAGACGGG
hNPFF-DO	Antisense	TGGACCTTGCATGCAGACAT
5′-RACE_1	Antisense	TTGGGGGGCAAACATTGACAC
3′-RACE_2	Sense	GTCACTGTTGCACTACCTGCTC
hNPFF-PE	Antisense	CCCCGTCTATTAACAGCAGCAGC
hNPFF-TAQ-UP	Sense	CTGCTGGTGCTGCTGCTGTTAATAGA
hNPFF-TAQ-PROBE	Sense	ACCAGCTCTCCGCGGAGGAAGACA
hNPFF-TAQ-DO	Antisense	GACCCAGAGGTCTGGGCATCCT
hPBGD-TAQ-UP	Sense	GGGAAACCTCAACACCCGGCT
hPBGD-TAQ-PROBE	Sense	ATCCTGGCAACAGCTGGCCTGCA
hPBGD-TAQ-DO	Antisense	ACCCGGTTGTGCCAGCCCAT
rNPFF-TAQ-UP	Sense	TGCTACTGCTGCTGCTGAGGAACT
rNPFF-TAQ-PROBE	Sense	ACCAAGTCTTTGCAGAAGAAGATAAGGGACCC
rNPFF-TAQ-DO	Antisense	ATCCTGTCTGGAGTGTGGGCATACT
rGAPDH-TAQ-UP	Sense	TGATGCTGGTGCTGAGTATGTCGT
rGAPDH-TAQ-PROBE	Sense	TACTGGCGTCTTCACCACCATGGAGAA
rGAPDH-TAQ-DO	Antisense	GGGCGGAGATGATGACCCTTTT

promoter elements and potential consensus transcription factor binding sites with MatInspector V2.2 and MatInspector Professional computer software program via the publicly available World Wide Web server (http://193.175.244.40/TRANSFAC), the analysis programs available at Webgene server (www.itba.mi.cnr.it/webgene) and also with the GCG Wisconsin Package. Sequence assembly of the NPFF promoter was performed using Seqweb software package available at CSC web site (www.csc.fi). The search tool BLAST (Altschul *et al*. 1990) was used via the NIH server.

Cloning of the 5′-end of the human NPFF gene

The 5′-flanking region of the human NPFF gene was cloned using the Genome Walker kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The NPFF gene-specific oligonucleotide hWALK-DO (Table 1) was used in the primary PCR and hWALKnest-DO in the subsequent nested PCR. We were able to identify a single major PCR product in one of the human libraries (*Pvu*II) provided. Using this approach, 1.3 kb of the human NPFF promoter region was isolated, subcloned in pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced automatically in both directions. We continued the cloning of the human NPFF promoter region by using a second round of the same approach as above with the primary PCR primer hWALK2-DO and the nested primer hWALK2nest-DO (Table 1). We were able to clone an additional approx. 3.4 kb fragment of the human NPFF promoter (*Dra*I library), which was subcloned and sequenced.

RNA isolation and primer extension analysis

Total RNA was isolated using RNAwiz isolation reagent (Ambion, Austin, TX, USA). For some experiments poly (A) + RNA was selected from total RNA by using the Micro-FastTrack 2.0 kit (Invitrogen, Carlsbad, CA, USA). Primer extension was carried out as described previously (Carey and Smale 2000). Briefly, antisense oligonucleotides (see Table 1) hWALKnest-DO (for PE_1) or hNPFF-PE (for PE_2) were radiolabelled at the 5′-end with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (> 5000 Ci/mmol, Amersham Pharmacia Biotech). The radiolabelled primer (50 000–100 000 cpm) was added to total or poly(A) + RNA and the primer and RNA mix was precipitated. The pellet was resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) followed by an addition of 5 × PE buffer (1.25 M KCl, 50 mM Tris pH 7.5, 5 mM EDTA pH 8.0). Hybridization between the primer and RNA was done at 45°C or 68°C for 90 min. Reverse transcription was initiated by adding 100 U RNase H- Moloney murine leukemia virus (MMLV) reverse transcriptase (New England Biolabs, Beverly, MA, USA) in 40 μL of a mixture consisting of 1 × MMLV reverse transcriptase buffer (Biolabs), 0.5 mM dNTPs (each) and 100 μg/mL bovine serum albumin (BSA) and the reaction was carried out at 37°C for 1 h. The extension products were precipitated and pellets dissolved in a denaturing dye solution and analysed on a 8% polyacrylamide/7 M urea gel. The size was determined by comparison of a sequencing ladder

generated by using the same primers as for the primer extension with plasmids containing a genomic fragment corresponding to the 5′-region of the human NPFF gene. The T7 sequencing kit (USB Corporation, Cleveland, OH, USA) together with α-^{35}S-dATP was used for the sequencing reactions.

5′- and 3′-RACE assay

The Generacer kit was purchased from Invitrogen and was used according to the manufacturer's instructions. AMV reverse transcribed ligated human spinal cord poly A + RNA (250 ng) was used in RT–PCR with the following human NPFF-specific primer sets (Table 1): for 5′-RACE: 5′-RACE_1 in primary PCR and hNPFF-DO in nested PCR; and for 3′-RACE: hNPFF-UP in primary PCR and 3′-RACE_2 in nested PCR. The primary and nested PCR reactions were done according to the parameters recommended by the manufacturer and by using DyNAzyme EXT polymerase (Finnzymes, Finland). Annealing was performed through a temperature gradient in all steps. The nested PCR reactions were analysed by agarose gel electrophoresis and major bands were excised from the gel and purified (QIAquick gel extraction kit, Qiagen, Hilden, Germany). Purified products were ligated into pGEM-T easy vector and many recombinant clones were picked for subsequent sequencing analysis.

Northern blotting analysis

Human brain MTN Blot II, containing approximately 2 μg of poly(A) + RNA per lane from eight different human brain tissues, was purchased from Clontech. α-^{32}P-dCTP-labelled random primed 0.3 kb fragment of the human NPFF coding region was used as a probe in the hybridization done according to the manufacturer's instructions using ExpressHyb hydridization solution (Clontech). The hybridized and washed filter was exposed for 5 days to Biomax MS film (Kodak, Rochester, NY, USA). The filter was subsequently stripped according to the manufacturer's instructions and rehybridized with α-^{32}P-dCTP-labelled random primed 1 kb fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. After washing, the filter was exposed to Biomax MS film for 24 h. Quantification of the northern blot hybridization signal was done by digitizing the X-ray film images with a computer-based MCID image analysis system (Imaging Research, St Catharines, Ontario, Canada). The values are presented as the relative optic density (ROD) of the NPFF-specific band area for each lane divided by the GAPDH-specific band area of the corresponding lane.

Real-time quantitative RT–PCR

For the construction of standard curves to be used in real-time quantitative RT–PCR, plasmids containing full-length human NPFF cDNA (accession number AF005271) and rat NPFF cDNA (accession number AF148700) or part of the human porphobilinogen deaminase (PBGD) gene (accession number M95623.1) and rat GAPDH gene (accession number NM_017008.1), containing the amplicon to be studied, were linearized and the concentration of the linearized plasmids was estimated both by spectrophotometry and by agarose gel electrophoresis against a molecular mass standard. The linearized plasmids were adjusted to contain 10^6 copies/mL according to known concentration, size (bp) and molecular mass of the plasmids and subsequently sequence-specific standard curves were generated by doing 10-fold serial dilutions (10^1–10^6 copies/mL). Reverse transcription was performed on 5.0 μg DNase I (Promega) treated total RNA using first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). The presence of contaminating genomic DNA was controlled by doing cDNA synthesis reactions omitting the reverse transcriptase (minus RT controls). The LightCycler (Roche Biochemicals, Mannheim, Germany) instrumentation together with TaqMan chemistry was used to amplify the specific gene transcripts. The following primers and fluorogenic probes, using FAM (6-carboxy-fluorescein) as the reporter dye at the 5′-end and TAMRA (6-carboxy-tetramethyl-rhodamine) as a quencher dye at the 3′-end, were used (see Table 1): for human NPFF mRNA precursor forward primer hNPFF-TAQ-UP, TaqMan probe hNPFF-TAQ-PROBE (overlaps intron I junction), reverse primer hNPFF-TAQ-DO; and for human PBGD mRNA forward primer hPBGD-TAQ-UP, TaqMan probe hPBGD-TAQ-PROBE, reverse primer PBGD-TAQ-DO to check the integrity and amount of input total RNA in each sample. For rat NPFF mRNA precursor forward primer rNPFF-TAQ-UP, TaqMan probe rNPFF-TAQ-PROBE (overlaps intron I junction), reverse primer rNPFF-TAQ-DO; and for rat GAPDH mRNA forward primer rGAPDH-TAQ-UP, TaqMan probe rGAPDH-TAQ-PROBE, reverse primer rGAPDH-TAQ-DO. The PCR was performed in glass capillaries in a 20-μL final volume composing of 2 μL LightCycler–FastStart DNA Master Hybridization Probes reaction mix, $MgCl_2$ in an end concentration of 2 mM for human NPFF, 3 mM for rat GAPDH and 4 mM for human PBGD and rat NPFF, 0.5 μM primers and 0.2 μM TaqMan probe. Template (cDNA, plasmid standards, minus RT controls, water as negative control) was always added to the PCR reaction in a volume of 2 μL, thus corresponding to approximately 600 ng input total RNA for the cDNA samples. The relative copy number of the amplicon was determined at each run by using the sequence-specific standard curves. The amplification was performed for 45–55 cycles, starting with a 10-min initial denaturation at 95°C and then a 10-s denaturation at 95°C, followed by an annealing and elongation for 30 s at 60°C for each cycle. Following RT–PCR amplification, data were analysed using LightCycler 3 analysis software. Subsequently, the amplicon copy value for human NPFF or rat NPFF in each sample, run in duplicate, was normalized to the amplicon copy value for human PBGD or rat GAPDH, also run in duplicate, in the same sample. The final data are presented as number of NPFF precursor mRNA copies per 10 000 human PBGD mRNA or 10 million rat GAPDH mRNA copies per sample. The specificity of the PCR reactions was also verified by ethidium bromide staining on 2.5% agarose gels (data not shown).

Construction of chimeric human NPFF-luciferase reporter constructs

To prepare 5′-deletion constructs of the proximal human NPFF promoter a 214-, 551- and 830-bp promoter fragment was amplified from human genomic DNA by PCR. PCR reactions were performed with Pfu DNA polymerase (Promega) using an Eppendorf Mastercycler gradient machine with the following programme: (i) 95°C for 2 min, (ii) 95°C for 45 s, (iii) 57°C for 30 s, (iv) 72°C for 3 min with a 1-s addition after each cycle, (v) steps ii–iv for 35 cycles followed by a 72°C 5-min final extension. The PCR products were subsequently cut with *Nhe*I (cutting site inserted in H-DO primer) and inserted in *Nhe*I–*Sma*I cut promoterless pGL3-basic vector (Promega) upstream of the firefly luciferase cDNA in sense

orientation. The orientation and sequence of the inserts were verified by sequencing across the insert-vector junctions in both ends. The combination of primers that were used to generate the PCR products are as follows (see Table 1): H-DO/H-UP 0.2 (construct HNF5′0.2-LUC), H-DO/H-UP 0.5 (construct HNF5′0.5-LUC) and H-DO/H-UP 0.8 (construct HNF5′0.8-LUC). For a schematic representation of the yielded constructs see Fig. 7(a). For each original human construct, two truncated forms were produced by PCR with shorter 3′-ends (see Fig. 7a). For the first set of 3′-deletion constructs the following combination of primers were used: H-DO#1/H-UP 0.2 (construct HNF5′0.2#1-LUC), H-DO#1/H-UP 0.5 (construct HNF5′0.5#1-LUC) and H-DO#1/H-UP 0.8 (construct HNF5′0.8#1-LUC). For the second set of 3′-deletion constructs the following combination of primers were used in PCR: H-DO#2/H-UP 0.5 (construct HNF5′0.5#2-LUC) and H-DO#2/H-UP 0.8 (construct HNF5′0.8#2-LUC). The PCR reaction conditions and parameters were identical as for the original constructs except that annealing was performed at 50°C. The PCR products were processed and subcloned as previously described.

Transient transfections and reporter gene assays

Luciferase reporter constructs were transfected into cells growing in monolayers with Fugene 6 transfection reagent (Roche) according to the manufacturer's instructions. In brief, cells were plated at a density of 1×10^5 cells in 1 mL medium in 12-well dishes (Nunc, Naperville, IL, USA) 16–24 h before transfection. One microgram of each reporter construct was co-transfected with 0.5 μg pSV-β-galactosidase vector (pSV-β-gal, Promega) per well. The transfections were done in duplicates or triplicates and repeated at least three times. Cells were transiently transfected for a total time of 48 h after which the transfectants were collected and measured for luciferase activity according to the protocol in the Luciferase assay system kit (Promega) with 20 μL of cell extract using a Luminoscan luminometer (Labsystems, Finland). Luciferase activity was normalized to expression of pSV-β-galactosidase by using β-galactosidase enzyme assay system (Promega) with 50 μL of cell extract. For NGF stimulation of PC12 cells, NGF (50 ng/mL) was added to the culture medium when seeding the cells 16–24 h before transfections. 24 h post transfection fresh NGF (50 ng/mL) was added to the transfectants and the cells were collected 48 h post-transfection.

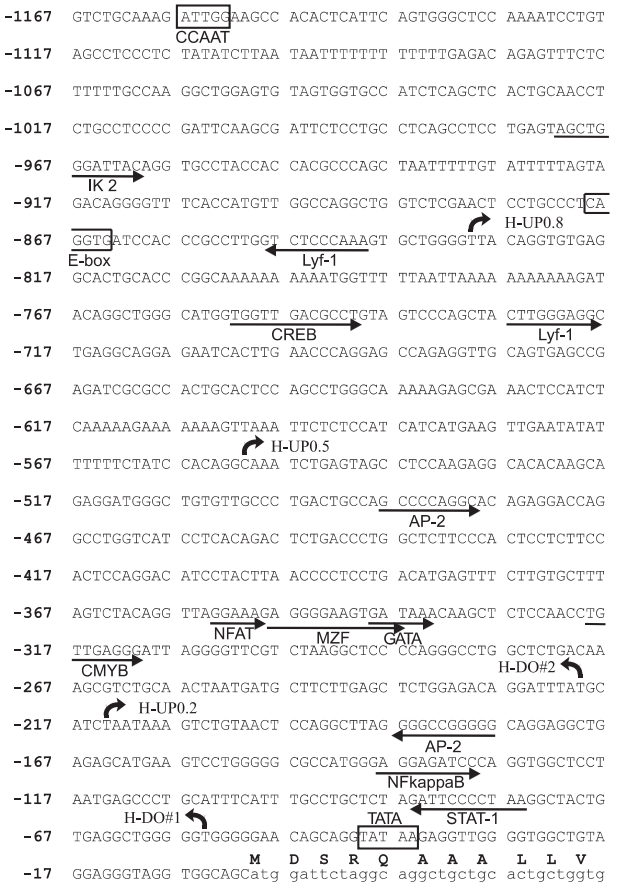

Fig. 1 Nucleotide sequence of the proximal human NPFF promoter region. Relative nucleotide numbering according to the translational start site as + 1. Protein encoding nucleotides with lowercase letters and amino acid shortenings marked by bold uppercase letters above the nucleotide sequence. Potential *cis*-acting elements are underlined with arrows, indicating the orientation, and the corresponding names are marked underneath. TATA elements, CCAAT elements and potential E-boxes are enboxed. The 5′- and 3′-ends of the primers used in constructing the chimeric reporter plasmids are indicated by arrows and named according to Table 1.

Results

Cloning, structure and sequence analysis of the human NPFF promoter

By using a genome walking strategy, a total of 4.7 kb of the human NPFF promoter region was cloned and sequenced[1] (Genebank entry AF503515). A sequence analysis of the first 1.2 kb of the promoter region (in respect to the translational start site) is presented in Fig. 1. The 5′-flanking region of the human NPFF gene is not especially GC-rich, having a GC content of about 50%. A putative TATA element was found 40 bp upstream from the translational start site. One potential CCAAT box was located at position − 1153 bp. Potential binding sites for the well-known tissue-specific transcription factors MZF and NF-AT were found and in addition to these the human promoter displayed consensus sequences for GATA, IK 2 and Lyf-1 (Fig. 1). Additionally, the promoter region displayed consensus binding sites for c-Myb, CREB and AP-2 and one potential E-box was found at position − 869 bp.

Determination of the transcription start site

A 5′-end for the human NPFF precursor mRNA has been previously presented in Perry *et al*. (1997), determined by sequencing one positive clone from a human testis cDNA library screening. The 5′-end has, however, not been confirmed or determined by other methods. By using 5′-RACE and primer extension, we confirmed the 5′-end presented previously, but also discovered a novel region for

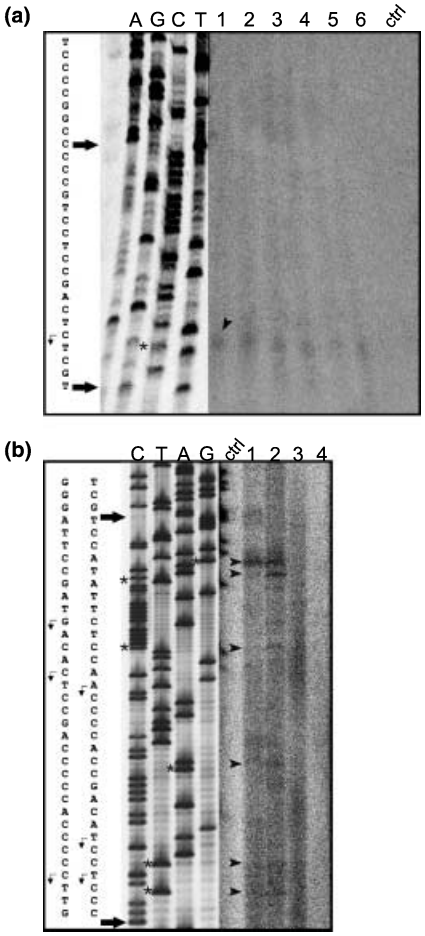

Fig. 2 Primer extension assays. (a) PE_1 experiment. The antisense primer hWALKnest-DO (Table 1) was used as a primer in a reverse transcription reaction using 30 μg total RNA isolated from U-251 MG (lanes 1,2), 250 ng poly(A) + RNA from A549 (lane 3) and U-251 MG (lane 4) and 30 μg total RNA from Jurkat (lanes 5,6). Annealing was performed at 45°C (lanes 1, 5) or at 68°C (lanes 2, 3, 4, 6 and ctrl). Control reaction was performed on 30 μg yeast tRNA. Primer extension analysis revealed one weakly extended product (arrowhead) at position − 166 relative to the translational start site. The size was determined by comparison of a sequencing ladder generated by using the same primers as for the primer extension with plasmids containing a genomic fragment corresponding to the 5′-region of the human NPFF gene and the corresponding nucleotide is marked by an asterix. The sequence was read from marked arrows and the presented sequence is antisense sequence. (b) PE_2 experiment. The antisense primer hNPFF-PE (Table 1) was used as a primer in a reverse transcription reaction using 250 ng (lane 1) and 500 ng (lane 2) poly(A) + RNA isolated from human spinal cord, 100 μg total RNA from human medulla (lane 3) and 100 μg total RNA from U-251 MG cells (lane 4). Control reaction was performed on 100-μg yeast tRNA. Primer extension analysis revealed several extended products (arrowheads) in lanes 1 and 2, corresponding to nucleotides − 15, − 18, − 30, − 51, − 66 and − 70 bp, marked by asterixes in the sequencing ladder. The sequence was read from marked arrows and the presented sequence is antisense sequence.

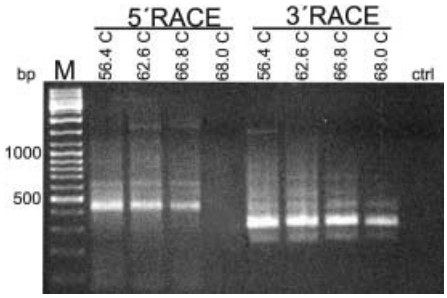

Fig. 3 5′- and 3′-RACE assays. Agarose gel electrophoresis of PCR products from 5′- and 3′-RACE assays, which were run and analysed as explained in detail in Experimental procedures. Control included omission of template. Annealing was performed through a temperature gradient with the temperatures indicated above the lanes.

transcription initiation. The primer hWALKnest-DO, suitable to confirm the published 5′-end, was used for primer extension (PE_1) and hybridized to total or poly(A) + RNA from different human cell lines. Although very weak, a single primer extension product was generated in all cell lines used (Fig. 2a). By comparison with a DNA ladder generated on a genomic plasmid by the same oligonucleotide primer, the transcriptional initiation site was located three nucleotides upstream of the previously reported 5′-end (Figs 2a and 4). Control reactions with yeast tRNA did not yield any visible extension products (Fig. 2a). Another primer, hNPFF-PE, more downstream from hWALKnest-DO, was used to study the possible use of transcriptional start sites (PE_2) in the region where the 5′-end for the rat and mouse NPFF gene has been mapped (Vilim *et al*. 1999). Several extended products were found in reactions with poly(A) + RNA from human spinal cord, but none was seen with total RNA from human medulla, human astrocytoma cell line U-251 MG and yeast tRNA (Fig. 2b). As presented in Figs 2(b) and 4, the extended products were located at the positions − 15, − 18, − 30, − 51, − 66 and − 70 bp in respect to the translational start site. The results indicate the use of two distinct regions for transcription initiation and we saw a need to reconfirm the results by carrying out a 5′-RACE assay.

By using human spinal cord poly(A) + RNA as a template for the construction of the RACE library, several extended RT–PCR products were seen after a round of nested PCR through a temperature gradient (Fig. 3). Transformed clones of the products were sequenced and the 5′-ends were located at the positions −9, − 13, − 15, − 39, − 164 and −204 bp (Fig. 4). Most of the sequenced clones exhibited the 5′-end found at position −13 and −15 bp. The 5′-RACE results support the primer extension results in showing the use of two distinct regions for transcription initiation. Although not all of the 5′-ends found by primer extension and 5′-RACE match, the 5′-ends at positions −15 and −164 bp were found with both methods (Fig. 4). By searching several established

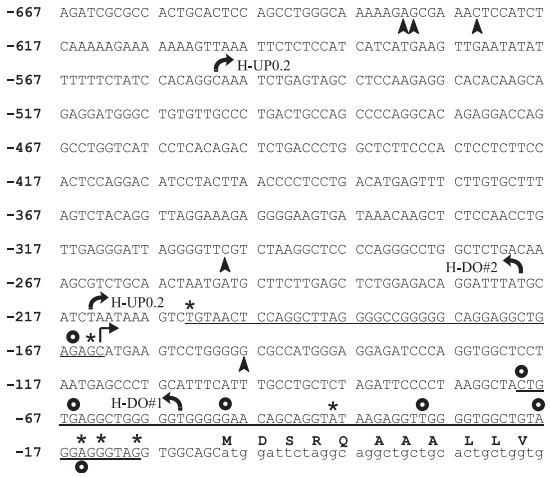

Fig. 4 Summary of identified human NPFF transcription start sites by primer extension, 5′-RACE assay and dbEST search. Relative nucleotide numbering according to the translational start site as + 1. Protein-encoding nucleotides with lowercase letters and amino acid shortenings marked by bold uppercase letters above the nucleotide sequence. The bent arrow indicates the position of mRNA 5′-end identified in Perry *et al.* (1997); the circles indicate 5′-ends identified by primer extension analysis; asterixes indicate 5′-ends identified by 5′-RACE assay; and arrowheads underneath the sequence indicate 5′-ends of EST cDNA clones found in the dbEST databank. The sequence region underlined twice corresponds to region 1 for transcription initiation and the sequence region underlined once to region 2. The 5′- and 3′-ends of the primers used in constructing the chimeric reporter plasmids are indicated by arrows and named according to Table 1.

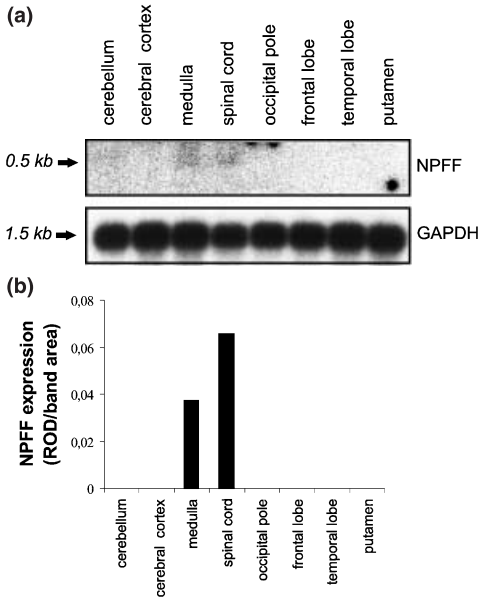

Fig. 5 Northern blot analysis. (a) A purchased human brain poly(A) + RNA northern blot containing about 2 μg poly(A) + RNA per lane was hybridized with α-^{32}P-dCTP-labelled random primed 0.3 kb fragment of the human NPFF coding region. After high-stringent washes, a weak 500 bp band was visible only in medulla and spinal cord. (b) Rehybridization with α-^{32}P-dCTP-labelled random primed 1 kb fragment of the human GAPDH gene. A strong 1.5 kb band demonstrates overall equal loading and quality of input poly(A) + RNA in all lanes. The data presented in the chart underneath the panels are the measured relative optic density (ROD) of the NPFF-specific band area adjusted with the ROD of the corresponding GAPDH-specific band.

databanks for human expressed sequence tag (EST) clones, we found six cDNA clones which displayed longer mRNA transcripts in respect to their 5′-UTR end than the one reported previously. The 5′-ends of these human NPFF EST clones are located at positions − 148, − 300, − 602, − 621, − 627 and − 628 bp (Fig. 4).

3′-RACE

To confirm that no alternative 3′-end variants for the human NPFF mRNA exist, we conducted a 3′-RACE assay. As shown in Fig. 3, a round of nested PCR produced a major product and some minor products, and sequencing of several transformed clones confirmed the published 3′-end as the only variant found with this method (data not shown). The length of the poly(A) tail was, however, variable (from 19 to 30 adenine residues).

Northern hybridization analysis

A northern filter containing approximately 2 μg poly(A) + RNA per lane from eight human brain regions was hybridized with a human NPFF cDNA probe. In the human brain regions studied, a small band of approximately 500 bp was found only in medulla and spinal cord (Fig. 5). Rehybridization with a human GAPDH probe demonstrated overall equal loading and quality of input poly(A) + RNA in all lanes (Fig. 5). As seen in the bar chart of Fig. 5, the NPFF hybridization signal was found after normalization to be higher in the spinal cord than in the medulla.

NPFF gene expression by quantitative RT–PCR

We examined the level of endogenous NPFF gene expression in a variety of cell lines to be used for transient transfections by real-time quantitative RT–PCR. The human lung carcinoma cell line A549 was chosen due to preliminary results of NPFF mRNA expression in the lung (unpublished result), the human astrocytoma cell line U-251 MG to represent a glial cell line and the human neuroblastoma cell lines SK-N-SH and SK-N-AS as representative neuronal cell lines. The rat pheochromocytoma cell line PC12 was chosen as a well-documented cell line in neuropeptide research and because it serves as an excellent model for sympathetic neurone-like cells. The highest level of human NPFF precursor mRNA was detected in the astrocytoma cell line U-251 MG followed by the neuroblastoma cell line SK-N-AS (Fig. 6). Although the levels in these cell lines were higher than for the lung carcinoma cell line A549 and neuroblastoma SK-N-SH, the

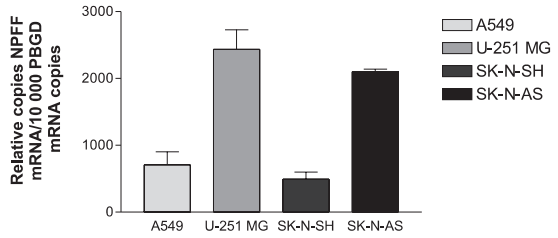

Fig. 6 Real-time quantitative human NPFF mRNA RT–PCR on human cell lines. Reverse-transcribed DNase I-treated total RNA from human lung carcinoma A549, astrocytoma U-251 MG and neuroblastoma SK-N-SH and SK-N-AS were subjected to RT–PCR using Lightcycler instrumentation against a sequence-specific standard for human NPFF and human PBGD. The final data are presented as the mean of the amount of NPFF precursor mRNA copies per 10 000 human PBGD mRNA copies from at least two independent experiments. Quantitatively, U-251 MG and SK-N-AS were found to express significantly higher levels of NPFF than A549 and SK-N-SH, although the expression level for all the cell lines must be considered low.

mRNA expression is to be considered very low (approximately 250–300 copies NPFF mRNA/300 ng total RNA) in all the human cell lines studied. The levels of NPFF mRNA were normalized to human porphobilinogen deaminase (PBGD) mRNA expression (Fink *et al*. 1998), as we found that the levels of β-actin were very variable between the cell lines used (data not shown). The PBGD gene, which is pseudogene-free and ubiquitously and consistently expressed in also complex tissues (Fink *et al*. 1998), was found to work extremely reliably as a quantitation reference between the cell lines used in this study. The level of endogenous NPFF mRNA can hereby function as a positive and quantitative reference for identifying true promoter regions for the NPFF gene.

The level of endogenous NPFF mRNA in PC12 cells was also examined by quantitative RT–PCR by using rat-specific oligonucleotides. PC12 cells were found to express measurable amounts of NPFF mRNA as seen in Fig. 8(a). PC12 cells were also stimulated by NGF for 72 h and the levels of NPFF mRNA was measured. As seen in Fig. 8(a) a significant increase ($p < 0.05$ by unpaired *t*-test), which was approximately twofold, in NPFF mRNA expression was seen. An inducible promoter activity during NGF stimulation can therefore be proposed.

Functional analysis of the 5′-flanking region of the NPFF gene

To examine whether the 5′-flanking region of the human NPFF gene contained a functional promoter, we generated a series of chimeric NPFF promoter/luciferase gene reporter constructs, as depicted schematically in Fig. 7(a). The vector pGL3 basic used in these constructs, which encodes the firefly luciferase gene, does not contain a functional promoter and is entirely dependent upon the

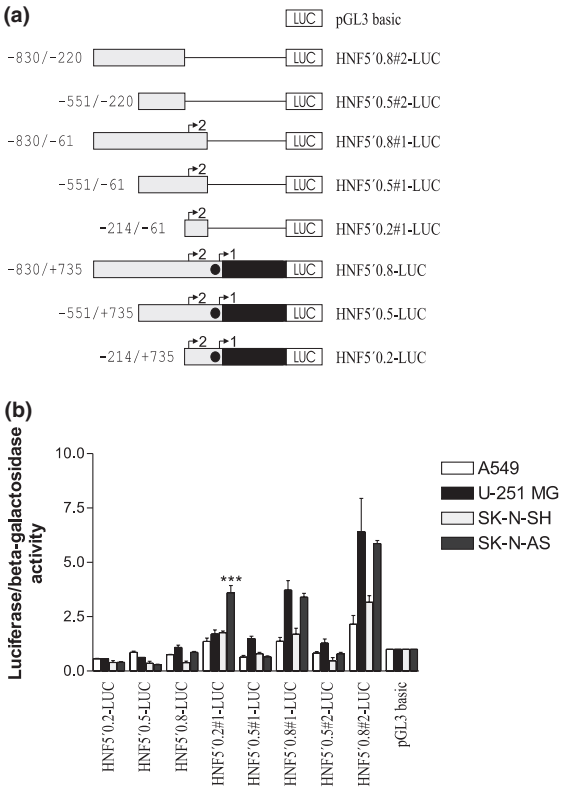

Fig. 7 Functional characterization of the 5′-flanking region of the human NPFF gene. (a) A schematic presentation of the chimeric constructs used. Nucleotide numbering is presented as the translational start site as + 1. The novel identified transcription start sites are marked by a bent arrow marked by number 1. The reported transcriptional start site is indicated by a bent arrow marked by number 2. ●, A potential TATA element. The black region indicates the coding region, the grey region the 5′-flanking region. (b) The constructs presented in (a) were transiently co-transfected with a β-galactosidase expression plasmid (pSV-β-gal) into human lung carcinoma A549, astrocytoma U-251 MG and neuroblastoma SK-N-SH and SK-N-AS cell lines. The background activity of the promoterless luciferase plasmid pGL3 basic is given an activity of 1. The data are the means ± SEM of all experiments. Statistical analysis was conducted with one-way ANOVA. The activity of the HNF5′0.2#1-LUC construct was significantly higher ($p < 0.001$ by one-way ANOVA) in SK-N-AS cells.

functional promoter activity of the ligated heterologous gene sequence. The yielded constructs were used to transiently transfect A549, U-251 MG, SK-N-SH, SK-N-AS and PC12 cells.

The basal constructs HNF5′0.2-LUC and HNF5′0.5-LUC containing 735 bp of the human NPFF coding region, including both introns, and 214 bp and 551 bp of the human NPFF promoter, respectively, were not able to drive the expression of the luciferase gene in any of the cell lines studied (Figs 7b and 8b). HNF5′0.8-LUC, with 830 bp of the promoter region exhibited some promoter activity above background in U-251 MG and PC12 cells (Figs 7b and 8b).

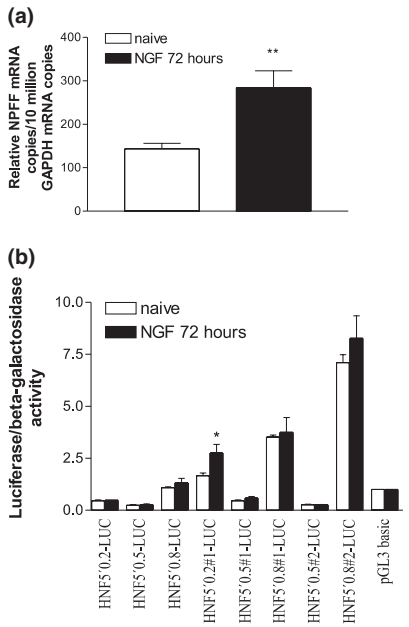

Fig. 8 (a) Reverse-transcribed DNase I-treated total RNA from naive and NGF differentiated (72 h) rat pheochromocytoma PC12 cells were subjected to real-time quantitative RT–PCR using Lightcycler instrumentation against a sequence-specific standard for rat NPFF and rat GAPDH. The final data are presented as the mean of the amount of NPFF precursor mRNA copies per 10 million GAPDH mRNA copies. A significant increase in NPFF precursor mRNA ($p < 0.01$ by unpaired *t*-test) was seen after 50 ng/mL NGF differentiation for 72 h. (b) Functional characterization of the human NPFF chimeric promoter constructs in naive and NGF-differentiated (72 h) PC12 cells. The general activity of the human constructs in naive PC12 cells were comparable to the human cell lines studied. After 72 h of NGF differentiation, HNF5′0.2#1-LUC exhibited a significant increase in promoter activity ($p < 0.05$ by unpaired *t*-test) as compared to the promoter activity seen in naive cells.

When deleting the region reaching from + 735 to − 60 bp from the basal constructs (version #1 of each construct, Fig. 7a), some promoter activity was to be seen for the HNF5′0.2#1-LUC construct in all cell lines, but significantly highest in SK-N-AS cells (Figs 7b and 8a, $p < 0.001$ by one-way ANOVA). The HNF5′0.5#1-LUC construct failed to drive the expression of the luciferase gene in all cell lines except U-251 MG. HNF5′0.8#1-LUC produced a significant promoter activity in all cell lines, being highest in U-251 MG, SK-N-AS and PC12 cells. The deleted region contained 735 bp of the coding region, the consensus TATA box and some of the novel transcription start sites found by primer extension and 5′-RACE (see Fig. 4). When deleting the region reaching from − 61 bp − 219 bp from the basal constructs (version #2, Fig. 7a), the HNF5′0.5#2-LUC construct only produced a barely measurable promoter activity in U-251 MG cells. In the HNF5′0.8#2-LUC construct, the same deletion resulted in an additional increase in luciferase activity from the HNF5′0.8#1-LUC construct in all cells, being highest in U-251 MG, SK-N-AS and PC12 (Figs 7a and 8a). The deleted area contained all the identified transcription start sites by primer extension and 5′-RACE. These results suggest that the area spanning from − 552 bp to − 830 bp contains crucial elements for human NPFF promoter activity, as the constructs containing this area exhibited the highest promoter activity. Some masking of this area was nonetheless seen when the coding region containing both introns was attached (HNF5′0.8-LUC), as no luciferase activity was produced. The ability of this human NPFF promoter region to produce promoter activity was not abolished by deletion of the consensus TATA-box, and the promoter activity was, in fact, increased indicating negative regulatory function in the promoter region including the TATA-element. By comparing Figs 6 and 7(b), it can be concluded that the region spanning from − 552 to − 830 bp can be characterized as a promoter region for the human NPFF gene, as the activity in the functional analysis correlates with the endogenous level of NPFF mRNA transcription in the cell lines studied. Interestingly, the 5′-ends of four of the human NPFF EST clones are located in this region (see Fig. 4). A potential silencer element was also indentified in the region reaching from − 220 bp to − 551 bp, as all constructs containing this region, but lacking the region from − 551 to − 830 bp, failed to produce any promoter activity.

Inducible transcriptional activity of the human NPFF promoter

Because we were able to demonstrate a significant increase in NPFF mRNA in NGF-stimulated PC12 cells (Fig. 8a), we wanted to characterize an element in the human promoter region responsible for inducible transcriptional activity produced by NGF. By transfecting the same constructs as used for the human cell lines in naive PC12 cells, we could clearly conclude that the function of the chimeric constructs was similar in naive PC12 cells as in the human cell lines (Figs 7b and 8b). This indicates a similar mechanism for NPFF gene transcription in rat and human and supports the use of a rat cell line in the analysis of the human NPFF promoter. Subsequently, PC12 cells stimulated with NGF were transiently transfected with the chimeric constructs. We were able to measure a significant increase in luciferase activity (Fig. 8b, $p < 0.05$ by unpaired *t*-test) after 72 h of NGF stimulation in cells transfected with the HNF5′0.2#1-LUC construct. The addition of 5′-end promoter sequence interfered with this mechanism, as the cells transfected with the HNF5′0.5#1-LUC and HNF5′0.8#1-LUC constructs exhibited unchanged promoter activity after NGF stimulation. The deletion of the promoter region present in HNF5′0.2#1-LUC (nt − 214 to nt − 61) also abolished the NGF induced luciferase activity as seen in constructs HNF5′0.5#2-LUC and HNF5′0.8#2-LUC (Fig. 8b). The

increase in luciferase activity in the HNF5′0.2#1-LUC transfected cells was approximately twofold, which correlates with the increase seen in NPFF mRNA after NGF stimulation as determined by real-time quantitative RT–PCR (Fig. 8a).

Discussion

To study the molecular mechanisms responsible for the basal transcriptional regulation of the human NPFF gene and possible mechanisms behind an inducible transcription, we cloned the promoter region of the human NPFF gene. In this study, 4.7 kb of the promoter region was cloned and sequenced. The human NPFF promoter region was found not to be especially GC-rich, the average for the first 1.5 kb being 50%, yet somewhat higher than the average of 40% given for the mammalian genome. The structural analysis disclosed that the proximal 5′-flanking region contains a canonical TATA box, a CCAAT box and an E-box motif. E-boxes are known to be important in the expression of several neuropeptides, including pro-opiomelanocortin (POMC; Therrien and Drouin 1993), CGRP (Ball *et al*. 1992), PPT (Paterson *et al*. 1995) and [arginine]vasopressin (AVP; Coulson *et al*. 1999). The canonical TATA box element found is conserved in the mouse and rat NPFF gene, residing approximately 30 bp upstream from the mRNA 5′-terminus (Vilim *et al*. 1999). The function of this TATA element is, however, not verified and a non-functional TATA box-like sequence has been found for example in the human prepro-orexin gene promoter (Sakurai *et al*. 1999). As seen in Fig. 4, primer extension and 5′-RACE analysis performed in this study indicate that multiple transcription initation sites might be used by the human NPFF gene, and these initiation sites are clustered in two distinct regions, one which correlates with the start sites for the rat and mouse NPFF gene, here called region 1 (Vilim *et al*. 1999), and one which correlates with the previously identified mRNA 5′-end, region 2 (Perry *et al*. 1997). Multiple transcription initiation sites are common for genes lacking a functional TATA box (for a review on TATAless promoters see Smale 1997) and the distinct use of initiation sites can be cell- or tissue-specific. There is evidence of CNS genes in which multiple promoters choreograph appropriate developmental or tissue-specific gene expression (Timmusk *et al*. 1993; Pathak *et al*. 1994; Myers *et al*. 1998) or increase the complexity of mRNA transcripts within the same tissue type (Liu and Fischer 1996). The primer extension assay failed to produce extended products with total RNA from human medulla and the astrocytoma cell line U-251 MG, although products were seen from poly(A) + RNA from human spinal cord (Fig. 2b). This might indicate the region to which these initiation sites were mapped (region 1) to be used for transcription initiation only in spinal cord cells, but it could also reflect the larger amount of undegraded target mRNA present in the human spinal cord sample or methodological differences when using poly(A) + RNA versus total RNA and tissues versus cell lines.

We can conclude that the expression pattern for the human NPFF gene generally seems to be similar to the NPFF gene expression in rat and mouse (Vilim *et al*. 1999; Brandt *et al*. 2000), as a detectable transcript of approximately 500 bp was visible only in medulla and spinal cord as revealed by northern blot analysis. Characterization of the human NPFF promoter revealed the region reaching from − 552 bp to − 830 bp to be producing the highest promoter activity observed with the chimeric constructs used. The activity levels in the cell lines are well correlated with the endogenous NPFF mRNA levels, thus confirming the region to contain strong positively acting elements for the NPFF gene. The region reaching from − 220 to − 550 bp was found to possess transcriptionally suppressing features, which is consistent with the view that negative regulation may be used as a general mechanism to restrict the expression of certain genes to subpopulations of neurones (Mandel and McKinnon 1993). It can be concluded that the sequence composition of this potential silencer element does not show any homology to other known neurone-specific silencer elements (Mori *et al*. 1992; Li *et al*. 1993; Bessis *et al*. 1997; Givogri *et al*. 2000; Seth and Majzoub 2001), but it is already well known that the combination of the basal promoter with repressor molecules and cell-type-specific enhancers are required to achieve tissue-specific expression of several other neuronal genes, including a variety of neuropeptides (Quinn 1996). The functional properties of this element remain to be studied further in more detail.

The PC12 cell line is a clone derived from a pheochromocytoma tumour of the rat adrenal medulla, which has become a well established model to study the action of NGF (Greene and Tischler 1976). These cells stop dividing and differentiate morphologically and biochemically into sympathetic neurone-like cells when treated with NGF. Because we could clearly see the same activity pattern for the human NPFF constructs between the human cell lines and naïve PC12 cells, we could conclude that the rat PC12 cell line reflects the human milieu for basal NPFF cell transcription. By stimulating PC12 cells for 72 h with biologically active NGF, we could quantitatively measure a significant increase in NPFF mRNA copy number. Subsequently, the chimeric constructs were transfected in NGF-induced PC12 cells for the equal period of time. As compared with the naïve cells, the NGF-stimulated transfectants exhibited a significant increase in luciferase activity only when transfected with the HNF5′0.2#1-LUC construct. Within this construct, consensus binding sites for AP-2, NFκB and STAT-1 reside (Fig. 1). The presence of binding sites for AP-2 and NFκB is highly interesting in the potentially NGF-responsive area in HNF5′0.2#1-LUC. It is already well established that neuropeptide Y (NPY) gene expression is up-regulated in

NGF-stimulated PC12 cells (Allen *et al*. 1987) and that a NGF response element (NGFRE) lies in the rat and human NPY gene promoter (Higuchi *et al*. 1992; Minth-Worby 1994). AP-2α has recently been shown to be the nuclear protein binding to the NGFRE in response to NGF (Li *et al*. 2000). NGF has also been shown to increase NFκB activity in various neuronal and non-neuronal populations (Wood 1995; Maggirwar *et al*. 1998; Yoon *et al*. 1998; Hamanoue *et al*. 1999). Nonetheless, the mechanism of NPFF gene induction following NGF stimulation might not necessarily be due to a direct interaction between NGF and the NPFF promoter elements, but due to secondary effects associated with differentiation of PC12 cells into sympathetic neurone-like cells. It may however, be pointed out that the same construct which exhibited the NGF responsive activity increase also exhibited significantly higher activity in SK-N-AS cells as compared to the other human cell lines (Fig. 7b), thereby supporting the concept that important elements for NPFF-specific gene transcription reside within this region. It therefore remains to be analysed if the regulatory elements causing the observed promoter activity in the NGF-induced PC12 cells and SK-N-AS cells are equal and thus represent a general concept of this promoter in response to growth factors, e.g. during inflammation, differentiation and brain development by NGF or other growth factors.

Although we have not yet identified the transcription factors and their exact cognate regulatory elements that are responsible for basal and inducible transcriptional activity, we have clearly identified regions of chromosomal DNA that play an important role in human NPFF gene expression. We have also been able to demonstrate an inducible transcriptional activation of the NPFF gene in PC12 cells by NGF, which will serve as a tool for future studies concerning the NPFF gene. NPFF gene expression is tissue- and cell-type-specific and the *in vivo* cell type specificity of NPFF gene expression makes it an attractive model for investigating the *cis* elements involved in restricting gene expression mainly to a subset of neurones, a phenomenon which still is poorly understood. Understanding the regulation of the NPFF gene is particularly important because the peptides play significant roles in inflammatory (Panula *et al*. 1996; Pertovaara *et al*. 1998) and neuropathic pain (Wei *et al*. 1998) and in opiate tolerance (Malin *et al*. 1990; Panula *et al*. 1996). Under experimentally induced conditions, NPFF mRNA expression is up-regulated in an inflammatory, but not in a neuropathic, state in the spinal cord (Vilim *et al*. 1999). Indeed, there is experimental evidence suggesting that NPFF may produce a selective attenuation of tactile allodynia in neuropathic rats, an effect partly independent of naloxone-sensitive opioid receptors, and that NPFF in the periaqueductal grey matter attenuates antinociception induced by intracerebrally administered morphine (Wei *et al*. 1998). During inflammation it has furthermore been shown that NPFF in the spinal cord produces a submodality-selective potentiation of the antinociceptive effect induced by brain stem–spinal pathways, also independent of naloxone-sensitive opioid receptors (Pertovaara *et al*. 1998). It is thus possible that regulation of endogenous NPFF peptide levels in selected sites may be beneficial in treatment of inflammatory and/or neuropathic pain.

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