Functional analysis of the human neurofilament light chain gene promoter

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Received November 5, 1992; Revised and Accepted December 21, 1992

ABSTRACT
We have carried out a structural and functional analysis on the human NF-L (H-NF-L) gene. It contains a methylatlon-free island, spanning the 5' flanking sequences and the first exon and a number of neuronal-specific DNase I hypersensitive sites have been identified in the upstream region as well as within the body of the gene. Analysis in cell lines and transgenic mice using a combination of these sites has revealed the presence of a conserved element(s) between -300bp and -190bp which is required for neuronal-specific expression.

INTRODUCTION
Neurofilaments (NFs) are neuron-specific intermediate filaments that are composed of three proteins with apparent molecular weights of 68000 (NF-L), 145000 (NF-M), and 200000 (NF-H) as determined by SDS-gel electrophoresis (for review, see 1). Expression of NFs occurs as early as 9-10 days of gestation in the mouse central and peripheral nervous system and coincides with the appearance of postmitotic neurons (2). The genes are differentially regulated during development; for example, NF-L and NF-M genes are expressed early in the embryo, with a progressive increase during development, while the NF-H mRNA accumulates to significant levels only post-natally (3). Analysis of their expression in pheochromocytoma PC 12 cells which mimicks neuronal development has shown that the NF-L and NF-M genes are at least in part controlled at the level of transcription, whereas the induction of NF-H by nerve growth factor is controlled post-transcriptionally (4-5).

Sequence analysis of a genomic clone of the human NF-L gene has shown that a 6.5kb EcoRI fragment contains all of the coding sequence and one of the polyadenylation sites (6). This fragment also contains 2.3kb 5' flanking sequence. The human NF-L promoter has a typical TATA box 30bp upstream of the cap site and a potential Sp1 binding site at -110bp, relative to the cap site. The promoter and the first exon are very GC-rich especially in the region from -300bp and +200bp from the cap site (64%). This is a typical feature of methylation-free islands, which are present at the 5' end of a large number of genes (7,8).

We report here the characterization of the promoter of the human NF-L gene. We show that the gene has a number of neuronal-specific DNase I hypersensitive sites in the 5' flanking sequence and within the body of the gene. To assess the role of some of these hypersensitive sites, the expression of a number of deletion constructs have been analysed both in cells and transgenic mice. These experiments demonstrate the presence of a brain-specific element(s) between -300bp and -190bp relative to the cap site which is conserved between the human and mouse genes.

MATERIALS AND METHODS
Constructs
The human NF-L constructs are cloned into pUC19 unless otherwise stated. The promoter deletions are as follows: The -2300bp construct contains 2300bp 5' upstream sequence and is identical to the sequenced 6.5kb EcoRI fragment (6). The -300bp construct is deleted to a BamHI site which is -300bp from the cap site and extends downstream as far as the RI site (7).

The -300 Sal contains the same -300bp promoter deletion, but extends to a SalI site which is 3.1kb downstream of the RI site in exon IV. The -300 Sal 5'ESV and -300 Sal 3'ESV constructs contain a 220bp NcoI-PvuII fragment from the SV40 enhancer which was modified by addition of linkers to either a BamHI fragment or a SalI fragment and placed at the 5' and 3' end of the -300bp construct, respectively. The -190bp, -90bp and -70bp deletion mutants contain 190bp, 90bp and 70bp promoter fragments, respectively linked to the rest of the gene and were generated using restriction enzyme sites Apal, BssHII and Nael, respectively. These were also modified by linker addition so as to contain a BamHI recognition sequence. The -55bp deletion mutant contains 55bp of promoter fragment and was generated by Bal31 digestion of the -300bp construct. The construct lacking the first intron hypersensitive sites was prepared by deleting a 1kb BalI-StuI fragment from the first intron of the -300bp construct.

The human Thy-1 gene used for C1300 transfections was a kind gift of Dr. E. Spanopoulou and the human β-globin construct used as cotransfection control in the HeLa transfections was as described (9).

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DNase I hypersensitive site mapping

DNase I sensitivity assays were carried out as described by Enver et al. (10) with the modification described by Grosveld et al. (11). DNA was prepared from DNase I-treated nuclei using the procedure of Luster and Ravetch (12). The probes used for Southern analysis of this DNA are described in the corresponding figure legends.

Cell culture and transfections and RNA analysis

HeLa and C1300 cells (13) were obtained from the American Type Culture Collection and were grown in α-MEM supplemented with 10% foetal calf serum. Gene transfer was performed by calcium phosphate co-precipitation (14,15). 30μg of supercoiled DNA were used per 90mm plate (10^6 cells). The calcium phosphate-DNA was left on the cells for 12–16 hours then the medium was changed and the cells harvested 48 hours after transfection for RNA preparation.

RNA extraction of the transfected cells was performed in 3M lithium chloride, 6M urea including a 5 minute sonication step (41). DNA S1 probes were end labelled at one end with T4 polynucleotide kinase (Boehringer Mannhein) and γ32p-ATP (3000 Ci/m mole, New England Nuclear) or fill-in with reverse transcriptase (Super RT, Anglia-Biotec) and the appropriate α32p-labellled deoxynucleotide triphosphate (6000 Ci/m mole, New England Nuclear). Restriction fragments used as probes are described in the corresponding figure legends. Hybridisation and S1 protection reactions were carried out as described previously (16,17, also see below).

RESULTS

Chromatin structure of the human NF-L gene

The presence of a methylation-free island in the human NF-L gene expected on the basis of high GC content was confirmed by Southern blot analysis. The DNA was digested with the methylation-sensitive enzyme Hpa-II to show that the presence of unmethylated CpG dinucleotides from -1.7kb to +1.5kb relative to the cap site in all tissues examined including germ-line (data not shown).

We next localised all of the DNase I-hypersensitive sites within a 6.5kb EcoR I fragment containing the human NF-L gene and 2.3kb of 5' flanking region. Since there are large populations of non-neuronal cells in brain tissue, we used a neuronal cell line to determine the position of these sites. Nuclei were prepared from an NF-L expressing (SKNSH) and non-expressing (HeLa) cell line to determine the position of these sites. Nuclei were prepared from an NF-L expressing (SKNSH) and non-expressing (HeLa and K562) human cell lines and subjected to limited DNase I digestion. DNA was isolated, digested with various restriction enzymes, subjected to Southern blotting and hybridised to several probes spanning the entire NF-L gene. When EcoRI-digested DNA was hybridised with the 3' 4.5kb EcoR I-BamHI probe A (figure 1, left panels), several hypersensitive sites (HS3–6) were observed in DNA isolated from the SKNSH cells, but none with the non-neuronal HeLa (data not shown) or K562 DNA. As a control the K562 blot was also probed with a β-globin probe to show the presence of the locus activating region hypersensitive sites (data not shown, 42). When hybridised with a probe from the 5' end of the EcoRI fragment (probe B), the positions of the three promoter hypersensitive sites were mapped to -1.1kb (HS1), -900bp (HS2) and -250bp (HS3) (figure 1, middle).
Hybridisation of SstI digested DNA with the 450bp SstI-XhoI probe C (figure 1, right) further resolved the strongest hypersensitive site (HS5) into two subsites, HS5A and HS5B, which are 100bp apart and are situated in the first intron around position +3600bp (relative to the cap site). The two weaker sites (HS4 and HS6) were mapped to be at +3400bp, in the first exon and at +5000bp, in the second intron. The positions of the six nerve-specific hypersensitive sites are summarised in figure 1.

Transient transfections in HeLa cells

Based on the location of DNase I hypersensitivity sites and sequence homology to the promoter of the mouse NF-L gene (20) a number of deletions of the human NF-L gene were cloned in pUC and tested by transfection in HeLa cells (figure 2). As a control the gene with the −300bp deletion was also cloned upstream and downstream of SV40 enhancer to drive expression in HeLa cells (−300Sal in pBSV, −300 Sal 5’ESV and 3’ESV, respectively). All NF-L plasmids were cotransfected with a human β-globin gene (9) to control for transfection efficiency. The results show that the NF-L gene is expressed at low levels in this transient assay system unless driven by the SV40 enhancer.

Figure 2. Expression analysis of Human NF-L promoter deletions in transiently transfected HeLa cells. Upper panel: The various human NF-L promoter deletions were co-transfected with a human BF-globin gene in a pBSV-based vector (9) by the calcium phosphate precipitation method. The cells were harvested after 48 hours and total RNA was analysed by an S1 nuclease protection assay as follows: 50μg of RNA was hybridized to a mixture of the following probes; i) a 980bp BstEII-Apal endlabelled 3' human BF-globin (h-Bf-glob) probe spanning exon III and IV and ii) a 350bp RsaI end-labelled 5' human NF-L probe. Hybridisation was carried out at 54°C in 80% formamide after which the products were digested at 25°C for 2 hours using 100u of SI nuclease and analysed by fractionation on a denaturing polyacrylamide gel. Protected fragments of 121nt for the human NF-L gene and 320nt for the human BF-glob gene are indicated by arrows. The uppermost arrow indicates the position of rehybridised 350bp human NF-L input probe. Transfer RNA (tRNA), C1300 and HeLa RNA were used as negative controls. A construct lacking a promoter (−20bp) was used as negative control. GRK is a kidney tumour derived cell line expressing human BF-glob RNA (40) and RNA prepared from this line was used as a positive control for the human BF-glob mRNA protected fragment. The marker (M) is end-labelled HinfI digested pBR322.

Figure 3. Expression analysis of human NF-L promoter deletions in transiently transfected C1300 cells. Human NF-L deletion constructs (see figure 2) were co-transfected with a human BF-globin construct into mouse C1300 neuroblastoma cells by the calcium phosphate precipitation method. The cells were harvested after 48 hours and total RNA was analysed by an S1 nuclease protection assay as follows: 50μg of RNA was hybridised to a mixture of the following probes: i) a 980bp BstEII-Apal endlabelled 3' human BF-glob (h-Bf-glob) probe spanning exon III and IV and ii) a 350bp RsaI end-labelled 5' human NF-L probe. Hybridisation was carried out at 54°C in 80% formamide after which the products were digested at 25°C for 2 hours using 100u of SI nuclease and analysed by fractionation on a denaturing polyacrylamide gel. Protected fragments of 121nt for the human NF-L gene and 320nt for the human BF-glob gene are indicated by arrows. The uppermost arrow indicates the position of rehybridised 350bp human NF-L input probe. Transfer RNA (tRNA), C1300 and HeLa RNA were used as negative controls. A construct lacking a promoter (−20bp) was used as negative control. GRK is a kidney tumour derived cell line expressing human BF-glob RNA (40) and RNA prepared from this line was used as a positive control for the human BF-glob mRNA protected fragment. The marker (M) is end-labelled HinfI digested pBR322.

Figure 4. Analysis of a −2300bp human NF-L promoter deletion in adult transgenic mouse lines. Various lines of transgenic mice bearing the −2300bp human NF-L promoter deletion construct were established as described in Materials and Methods. Total RNA was prepared from various tissues of two month old offspring from transgenic mice lines 6, 13, 1 and 14, as well as an aged-matched non-transgenic (N-Tg) mouse. 40 μg of RNA was hybridized with a mixture of two probes; i) a 350bp RsaI end-labelled 5' human NF-L (HNF-L) to detect the transgene and ii) a 270bp XmaI end-labelled 5' mouse NF-H (mNF-H) probe to correct for possible differences in the mRNA levels. The specific activity of the mNF-H was twice that of the HNF-L probe. Hybridisation was performed at 60°C in 80% formamide and the samples were digested at 37°C for 2 hours after which the protected fragments were fractionated by denaturing polyacrylamide gel electrophoresis. Protected bands of 121nt for the endogenous mNF-H and 121 nucleotides for the transgene. SKNSH RNA was used as the positive control for the transgene signal. Abbreviations: brain (B), kidney (K), liver (L), spleen (S), ovaries (O), testes (Te), and thymus (T).
Nevertheless, the activity of the promoter in the absence of a SV40 enhancer is sufficiently high to observe that activity is only increased two to three fold when the promoter is deleted from -300 to -190bp. Further deletions which leave less than 90bp of the promoter result in a very low level of expression, suggesting the presence of a basic promoter element between -90 and -70 which is required for expression in this heterologous cell type. Although we have not rigorously confirmed this, preliminary footprinting experiments (data not shown) indicate that this region may contain a protein binding site present in HeLa cells, but not SKNSH cells. More importantly, we conclude from this experiment that deletions of the promoter regions (-2.3kb to -300bp) which contain neuronal-specific hypersensitive sites either have no effect or appear to increase rather than decrease expression in HeLa cells.

**Transient expression in C1300 cells**

To be able to analyse the promoter deletion constructs in neuronal cells, we tested a number of cell lines for their transfection efficiency. Six NF positive cell lines were used: PC12 (21), SKNSH (22), B104 (23), C1300 (13) SMS-KCN and SMS-KAN (24) of which C1300 gave the highest transfection efficiency and this cell line was therefore used for the analysis of the NF-L mutant constructs. The human Thy-1 gene which is expressed in neuronal cells in vivo (25) was used as a control. Fig. 3 shows that the deletion of the upstream promoter (-2300bp to -300bp) enhances the expression of NF-L when compared to the Thy-1 control. A similar result has been reported by Shneidman et al. (26). A further deletion to -190bp leads to a strong decrease in NF-L expression, indicating the presence of a neuron-specific positive element. Further deletion to -90 and/or -55 leads to a further small decrease in activity. Interestingly even the -55bp promoter construct retains a low level of transcriptional activity.

**Expression analysis in transgenic mice**

Because the effects observed in the C1300 cells were small we decided to analyse the effect of the promoter deletions which had the most pronounced effects in cell culture in transgenic mice. The first construct that we microinjected contained 2.3kb of 5’ flanking sequence linked to the rest of the HNF-L gene. It
which contains HS3, but not HS1 and HS2 (figure 5). This tested four transgenic mice containing the -300bp promoter copies (mouse # 1 carries 5 copies, data not shown). We then examined the expression of the transgene in the thymus indicating that the gene is sensitive to position effects (figure 4). A similar result has been shown to be expressed in a developmentally specific manner (figure 5). The lower intensity signal of the control mouse NF-H gene relative to that of the NF-L transgene when compared to the two month old mice is consistent with the known early expression of NF-L during development. This result therefore argues against a developmental role for hypersensitive sites HS1 and HS2.

To further determine the location of the neuronal tissue specific element in the -300 promoter HNF-L promoter, we examined (on the basis of the C1300 cell transfection experiments) the expression of a 5' deletion containing 190bp of upstream sequence linked to the rest of the gene. We obtained three transgenic mice. Only two of these mice expressed the transgene in the brain, but at very reduced levels relative to those observed for the -300bp mice (figure 6). Ectopic HNF-L expression was also detected in both mice. We therefore conclude that an element important for neuronal-specific expression has been lost by this deletion and as a consequence that the transgene is more sensitive to position-effects. However, the gene is still expressed at a low level further emphasizing that the region between -190bp and -55bp is important for basal expression.

It is clear from the results with the -300bp promoter this residual promoter fragment is sufficient to allow the HNF-L gene to be expressed in a developmentally specific manner (figure 5). However, we had previously shown that a 2.3kb HNF-L promoter fragment linked to the Thy-1 gene was not expressed early in development, but rather mimicked the expression pattern of the Thy-1 gene (28). This suggested that either the NF-L gene carries a developmental specific element in the gene or that a known brain-specific element (in the first intron) of the Thy-1 gene is sensitive to position effects (figure 4). A similar result has been shown to be expressed in a developmentally specific manner (figure 5). The lower intensity signal of the control mouse NF-H gene relative to that of the NF-L transgene when compared to the two month old mice is consistent with the known early expression of NF-L during development. This result therefore argues against a developmental role for hypersensitive sites HS1 and HS2.

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Figure 7. Analysis of -300bp human NF-L promoter deletion lacking the first intron hypersensitive sites (HS5A and HS5B) in adult and embryonic stage transgenic mice. A transgenic mouse line bearing a construct consisting of the -300 NF-L promoter deletion as well as an internal Ball-Stul first intron deletion was established. A total of 8 nine day (E9) embryo offspring from a mating between a transgenic heterozygote and wild type mouse were dissected and RNA was prepared from the head regions. Transgenic embryos (1 and 3) and were identified by Southern blot analysis of placental DNA (not shown) and the transgene expression in transgenic (1,3) as well as non-transgenic (2, 4–8) E9 brain was analysed by an SI nuclease protection assay. Brain RNA from a two-month old transgenic adult carrying the same construct (adult Tg) was also analysed in order to compare the ratio of the NF-L transgene expression to that of the endogenous NF-H. As a positive control for endogenous mNF-H expression as well as a negative control for transgene expression RNA from the -55 deletion line 16 (see figure 5) designated -55bp Tg was analysed in parallel. The transfer RNA (tRNA) track served as a control for nonspecific probe digestion products.

Figure 8. Comparison of human and mouse NF-L promoter regions. The sequence of the human NF-L promoter in the region of -300 to -180 was compared to the homologous region in the mouse gene. Dashes indicate identity of bases at that position.
gene has a dominant effect. The obvious candidate for such an intragenic NF-L element would be the strong hypersensitive sites HSS A and B. We therefore analysed the expression pattern of a human NF-L gene construct containing 300bp upstream promoter sequence linked to the rest of the gene but lacking the first intron strong hypersensitive sites (HSS A and HSSB) (see figure 2). Two transgenic mice were obtained of which only the one carrying two copies of the gene could be established as a line. This line was bred and total brain RNA was analysed from a two month old transgenic mouse and 9 day embryos by S1 nuclease protection (figure 7). The developmental role for the first intron was assayed by the ratio of HNF-L transgene RNA to endogenous NF-H RNA. The two 9 day embryos which carried the transgene both expressed the HNF-L transgene, but not the NF-H endogenous gene whereas the two month old animal expresses both genes (figure 7). We therefore conclude that hypersensitive sites HSS A and HSSB are not required for the correct developmental expression of the gene.

**DISCUSSION**

It has been shown for the murine NF-L gene, that 1.5kb of the sequence upstream of the transcriptional start site is sufficient to yield brain-specific expression of the homologous gene (29) as well as heterologous genes (30) in transgenic mice. Further promoter deletions have not been analysed for the murine NF-L gene to permit a functional comparison to be made of the minimal regulatory sequences that are needed for neuronal-specific expression in human and mouse genes. Using large fragments of flanking sequences, brain-specific expression has been observed for a number of neuronal-specific genes in transgenic mice (31,32,33,34,28), including the HF-H gene (35). However in only one case has a functional element been defined to a very small region. Analysis of neural-specific SCG10 promoter sequences fused to a marker gene in transgenic mice (36) as well as cell lines (37) has identified a silencer element in the 5'-flanking sequences. It has been proposed that neuronal specificity of the SCG10 gene is achieved through selective repression in non-neuronal tissues (36), making this an alternative mechanism for gene activation in the nervous system.

We have studied the possible in vivo role of the hypersensitive sites of the human NF-L gene by analysing the expression pattern of a series of deletion constructs in transgenic mice and cultured cells. Murine NF-L, NF-M and NF-H genes as well as the chicken NF-M gene have recently been shown to be expressed after transfection in non-neuronal cells (38,26,39). Using transient transfections, negative regulatory regions were found between -2300bp and -285bp in our transient assays in C1300 cells. In agreement with these results, negative regulatory elements were found between -300bp and -925bp in the mouse NF-L gene as well as upstream regions of the murine NF-M and NF-H genes that suppressed transcription in both neuronal and non-neuronal cells (26). Since these elements behave similarly in all cell types, their importance in regulating NF expression remains unclear. We have also observed an increase in expression upon deleting from -2300bp to -300bp in our transient assays in C1300 cells. In agreement with the above mentioned data this effect is also observed in HeLa cells. Although the expression level in the transgenic mice cannot be strictly quantitated as a function of copy number, the average expression per copy of the transgene is higher with the -2300bp promoter than with the -2300bp promoter. This result agrees with that obtained in the transfections and we conclude that the upstream region plays a negative regulatory role. It is not clear however whether it has a neuronal-specific regulatory role, because the data obtained in non neuronal cells were obtained by transient transfections in cells that do not show any hypersensitive sites in this region of the promoter of the endogenous NF-L gene.

When the human NF-L promoter is deleted from -300bp to -190bp there is a clear negative effect on neuronal expression of the gene both in transgenic mice and the cell cultures. Sequence comparison reveals a high degree of homology between the mouse and the human NF-L gene in the region between -300bp and -190bp (over 65%, figure 8). We have carried out preliminary experiments on the biochemical characterization of the promoter region and footprint data show that potential neuron cell specific factor binding sites are centered around -230bp and -285bp (data not shown).

A further deletion which leaves only a 55bp promoter including a TATA box was still expressed, albeit at low levels in C1300 cells. However, the -55bp promoter is not expressed in transgenic mice indicating that there are no downstream elements capable of driving minimal expression. It is clear that HSS A and HSSB are not required for the tissue-specific or temporal regulation of the gene contrary to what has been suggested previously (28) because the -300bp promoter when linked to a gene which lacks HSS A and HSSB is still expressed correctly. It is therefore not clear what role if any is played by the downstream sequences which contain HS4, 5 and 6. Of course the possibility remains that the sites HSS A and 5B do fulfill an as yet unclear function and that the other hypersensitive sites still present in this construct may functionally substitute for the deleted sites, reflecting a level of redundancy in the NF-L regulatory sequences.

**ACKNOWLEDGEMENTS**

We are grateful to Eugenia Spanopoulou for the Thy1 constructs and helpful discussion. ML was supported by the FRSQ Quebec. PF was supported by Cooley's Anemia Foundation. The work was supported by the MRC UK.

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