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Sequence and structure of the mouse gene coding for the largest neurofilament subunit

(Intermediate filament; phosphorylation site; neuron-specific gene; CpG-rich island; intron positions; recombinant DNA)

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SUMMARY

We have determined the complete nucleotide sequence of the mouse gene encoding the neurofilament NF-H protein. The C-terminal domain of NF-H is very rich in charged amino acids (aa) and contains a 3-aa sequence, Lys-Ser-Pro, that is repeated 51 times within a stretch of 368 aa. The location of this serine-rich repeat in the phosphorylated domain of NF-H indicates that it represents the major protein kinase recognition site. The *nfh* gene shares two common intron positions with the *nfl* and *nfm* genes, but has an additional intron that occurs at a location equivalent to one of the introns in non-neuronal intermediate filament-coding genes. This additional *nfh* intron may have been acquired via duplication of a primordial intermediate filament gene.

INTRODUCTION

Neurofilaments are major cytoskeletal elements of nerve cells that play an important role in the control of axonal caliber. They are formed by the copolymerization of three neuron-specific proteins with apparent M_r's of 68 000 (NF-L) 145 000 (NF-M) and 200 000 (NF-H), as determined by SDS–polyacrylamide-gel electrophoresis (Hoffman and Lasek, 1975; Liem et al., 1978; Julien and Mushynski, 1982). In common with other IF proteins, the neurofilament subunits contain a highly conserved α-helical domain of approx. 40 kDa capable of forming coiled-coil structures (Geisler and Weber, 1982; Geisler et al., 1984; Hanukoglu and Fuchs, 1982; Quax et al., 1983; 1985; Steinert et al., 1983; Julien et al., 1985; 1986). A striking feature of neurofilament proteins is their C-terminal domains, which

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Abbreviations: aa, amino acid(s); bp, base pair(s); IF, intermediate filament; kb, kilobase(s) or 1000 bp; NF-H, the largest neurofilament subunit; *nfh*, gene coding for NF-H; *nfl* and *nfm*, genes coding for the small- and the mid-size neurofilament proteins, respectively; nt, nucleotide(s); SDS, sodium dodecyl sulfate.

retain a highly charged character despite their different lengths (Geisler et al., 1984; 1985; 1987; Julien et al., 1986; 1987; Lewis and Cowan, 1986; Robinson et al., 1987; Levy et al., 1987; Myers et al., 1987). In the case of NF-H, the tail domain is highly phosphorylated in axons (Julien and Mushynski, 1982; 1983; Geisler et al., 1985; Carden et al., 1985) and forms cross-links between neurofilaments and their surrounding structures (Julien et al., 1983; Hirokawa et al., 1984).

The *nfl* and *nfm* genes have been sequenced recently (Julien et al., 1986; 1987; Lewis and Cowan, 1986; Levy et al., 1987; Myers et al., 1987; Zopf et al., 1987) and found to be linked in the murine genome (Julien et al., 1986). The exon-intron organizations of the *nfl* and *nfm* genes are similar but the total lack of similarities with those of other IF-coding genes has led to the proposal that the ancestral neurofilament gene originated via an mRNA-mediated transposition event (Lewis and Cowan, 1986; Levy et al., 1987).

We report here the sequence and the exon-intron organization of the mouse *nfh* gene. The deduced amino acid sequence of NF-H contains in its C-terminal region an unusual serine-rich repeat that probably corresponds to the major phosphorylation site in neurofilament proteins. In addition, the structure of the *nfh* gene is consistent with the notion that an early duplication event may have separated neurofilament genes from the rest of the IF gene family.

MATERIALS AND METHODS

(a) Cloning

The screening at low stringency of a mouse genomic library in the cosmid vector pLTC with a *nfl* cDNA probe led to the isolation of a cross-hybridizing clone, designated cos3A1, that contained *nfh* exon sequences (Julien et al., 1986).

(b) Sequencing

For sequence analysis, DNA fragments from the *nfh* gene were subcloned into the M13mp18 vector or into the Bluescript plasmid (Stratagene, Inc.) to generate, with exonuclease III and mung-bean nuclease, overlapping deletion mutants. Sequencing was carried out by the dideoxy chain-termination method (Sanger et al., 1980). To confirm the transcription start point, mouse brain RNA (20 μg) was annealed to a 1.9-kb *Not*I-*Bam*HI probe labeled at the 5′-end and subjected to S1 nuclease analysis (Maniatis et al., 1982).

RESULTS AND DISCUSSION

(a) Sequencing of the *nfh* gene

Screening of a mouse genomic library at reduced stringency with a *nfl* cDNA probe yielded a cross-hybridizing cosmid clone, designated cos3A1. The restriction map of the genomic 40-kb insert is shown in Fig. 1. A 1.2-kb *Xho*I-*Bgl*II fragment that cross-hybridized with the *nfl* probe was found previously to include a small exon sequence corresponding to a highly conserved region of IF proteins and was used to detect on Northern blots a brain-specific mRNA of approx. 4 kb (Julien et al., 1986). This exon sequence is now identified as being in exon 3 of the *nfh* gene.

Mapping of the cos3A1 clone revealed the presence of a *Not*I site, 4 kb upstream from exon 3 (Fig. 1). The site recognized by *Not*I occurs infrequently and can indicate the position of a CpG-rich island surrounding the transcription start site of a vertebrate gene (Lindsay and Bird, 1987). Fragments flanking the *Not*I site were subcloned and sequenced by the dideoxy method of Sanger et al. (1980). In agreement with the prediction this region was found to contain a TATAAA box and to encode amino acid sequences corresponding to the N-terminal portion of NF-H (Geisler et al., 1985). The 5′ region of the gene was subsequently confirmed by S1 nuclease protection experiments that positioned the cap site at 20 nt downstream from the TATAAA sequence (data not shown).

The 3.9-kb *Xho*I-*Xho*I fragment, which contains exon 3, as well as the adjoining 3.0-kb *Xho*I-*Eco*RV fragment and the 5-kb *Eco*RV-*Sal*I fragment (Fig. 1) were subcloned into the Bluescript plasmid vector to produce overlapping deletion mutants that were used for dideoxynucleotide sequencing. The exon-intron

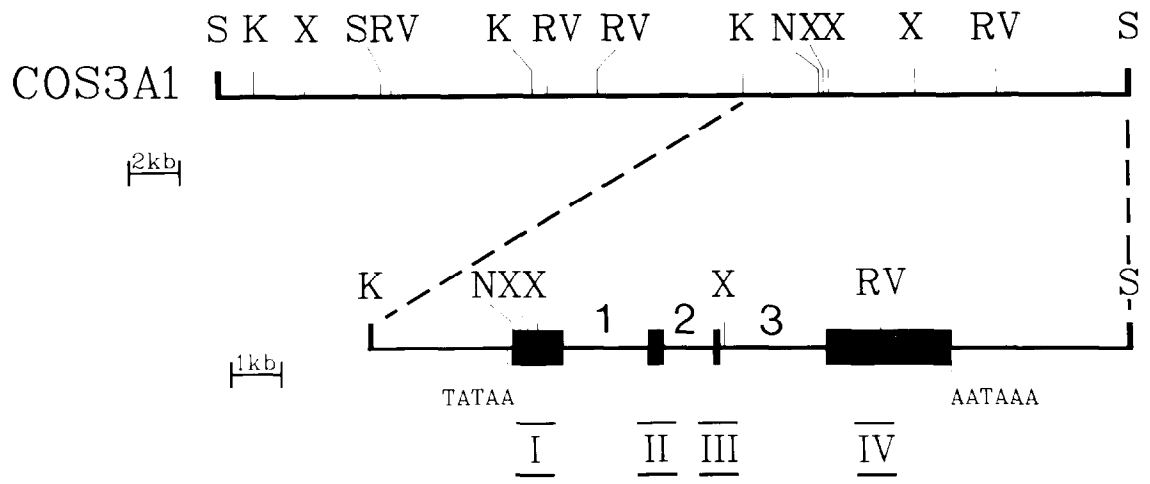

Fig. 1. Restriction cleavage map of the clone cos3A1 and exon-intron organization of the mouse neurofilament *nfh* gene. The clone cos3A1 was found to contain the *nfh* gene (Julien et al., 1986). Abbreviations of restriction enzyme names are: K, *Kpn*I; N, *Not*I; RV, *Eco*RV; S, *Sal*I; X, *Xho*I. The lowest line is a schematic representation of the *nfh* gene with exons as blackened boxes (marked I–IV). Introns are designated as 1–3 (see also Fig. 2).

organization of the *nfh* gene was elucidated by comparing the deduced amino acid sequences of open reading frames with partial NF-H sequences reported previously (Geisler et al., 1985; Robinson et al., 1986). The *nfh* gene has only three introns of 1.7, 1.1 and 2.0 kb, which are illustrated in the schematic presentation in Fig. 1. Approximately 50% of *nfh* intron sequences have been determined, and the exon-intron boundaries are shown in Fig. 2. All of the junctions follow the typical 5′GT-AG3′ rule (Breathnach et al., 1978). The intron positions in the *nfh* nucleotide sequence are also indicated by arrows in the sequence given in Fig. 3 and their significance will be discussed below.

(b) The multiple phosphorylation sites of NF-H: a serine-rich sequence repeated in tandem

The *nfh* gene encodes a protein of 1087 aa with a calculated M_r of 116000. This is much smaller than the M_r 200000 estimated on SDS gels. The high content of charged amino acids and of phosphate moieties in the C-terminal domain appears to be responsible for the anomalous gel-electrophoretic mobility of NF-H (Julien and Mushynski, 1982; Kaufmann et al., 1984; Georges and Mushynski, 1987). Indeed the central portion of the C-terminal domain has a very unusual protein structure. The amino acid sequence Lys-Ser-Pro is repeated 51 times. The repeat units, which are underlined in Fig. 3, are generally flanked by three amino acids (Ala, Gly, Ile)-Glu-(Ala, Val) or Glu-Lys-Ala. The sequences Val-Lys-Glu-Gly-Ala, Val-Lys-Glu-Asp-Ile and Val-Lys-Glu-Glu-Ala separate Lys-Ser-Pro units at the border of the repeated domain. The Lys-Ser-Pro repeats have also been found in the NF-H proteins of other species, including rat (Robinson et al., 1986), pig (Geisler et al., 1987), rabbit (Mack et al., 1988) and human (Lees, J.F., Shneidman, P.S., Skuntz, S.F., Carden, M.J. and Lazzarini, R.A., unpublished). A smaller number of copies of the repeats occur also in the NF-M protein (Myers et al., 1987; Levy et al., 1987). Interestingly, there is a correlation between the number of repeat

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                                    INTRON 1
TGGTTCCGAGgtgcgcgggacgcgggtggg...(1.7 kb)...ctgattccttccctacttagTGAGGTTGGA

                                    INTRON 2
CTCCTACCAGgtcgagcagagggagggtga...(1.1 kb)...tctgatctgtcttcccccagGACGCTATTC

                                    INTRON 3
CCGCTTACAGgtaagatgacccaggagctc...(2.0 kb)...cccattatccactccacagAAAGCTCCTGG
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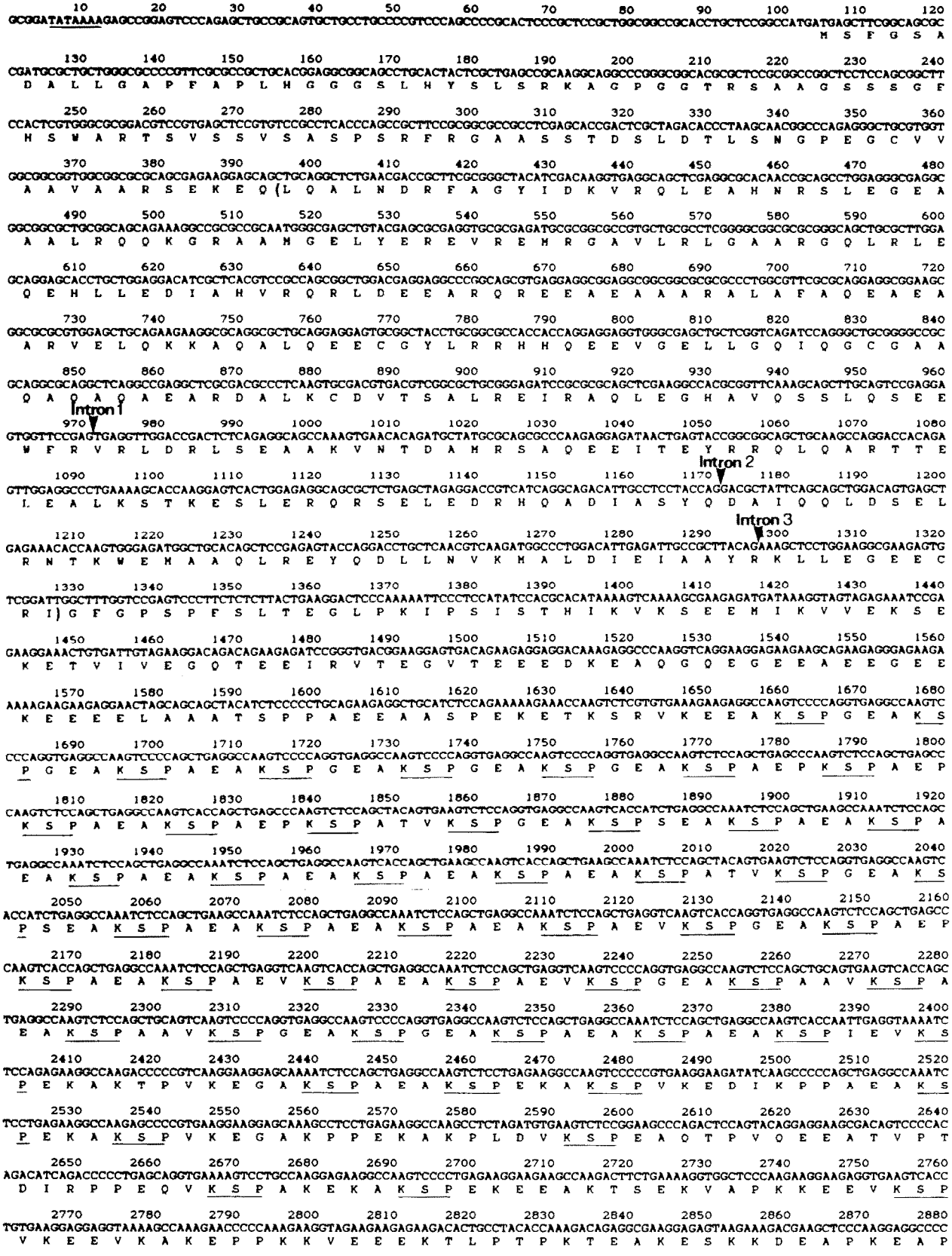
Fig. 2. Exon-intron junctions of the mouse *nfh* gene. Upper-case letters are exon sequences. For positions of introns 1–3 see Fig. 1.



(Fig. 3)

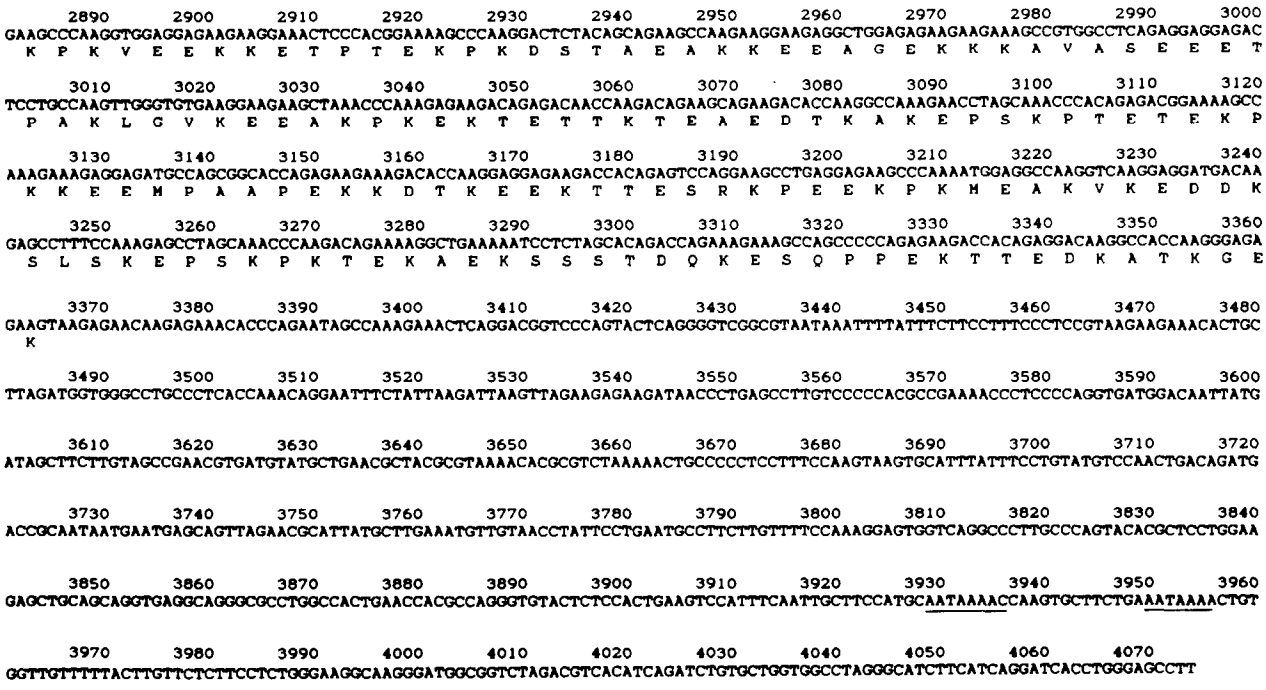


Fig. 3. Nucleotide sequence of the mouse *nfh* gene. The deduced amino acid sequence is shown under the nucleotide sequence. The arrowheads indicate the intron positions (1–3). The rod domain (see blackened boxes in Fig. 4) is delineated by parentheses (nt 395–1327). The TATAA box, the putative polyadenylation sites, and the Lys-Ser-Pro repeats of NF-H are underlined.

units and the extent of in vivo phosphorylation in NF-H and NF-M. The position of Lys-Ser-Pro repeats in the middle of the C-terminal domain corresponds to the location of multiple phosphorylation sites as defined previously by peptide mapping studies (Julien and Mushynski, 1983). Based on the phosphate content of NF-H (Julien et al., 1982; Georges et al., 1986) it can be estimated that about 25% of these sites are in a phosphorylated state in the axon in vivo.

(c) Structure and evolutionary origin of the *nfh* gene

The three introns in the *nfh* gene occur in the last α-helical segment of the protein and they do not delineate obvious functional subdomains (Fig. 4). Two *nfh* introns are found at the same locations in *nfl* and *nfm* genes indicating that the three genes were derived from a common ancestral gene. All introns in the *nfl* and *nfm* genes occur at locations non-homologous to intron locations in other members of the intermediate filament gene family. This led to the proposal that the primordial neurofilament gene evolved via an RNA-mediated transposition event (Lewis and Cowan, 1986; Levy et al., 1987).

The *nfh* gene contains an additional intron (intron 1) that occurs at an equivalent position in other members of the IF gene family (Fig. 4). Intron 1 of *nfh* is located only 5 nt upstream from the corresponding intron in the vimentin gene. It may have been integrated following the putative RNA-mediated transposition event. Alternatively, intron 1 of *nfh* may have been acquired via duplication of an ancestral IF gene with subsequent sliding of the exon-intron junction. The latter phenomenon has also occurred in keratin genes (Steinert et al., 1985). Thus, it is possible that an early gene duplication event separated neurofilament genes from the rest of the IF gene family. Accordingly, members of each IF branch would have gained and/or lost introns at different positions before divergence to give rise to all the different IF genes. Divergence of the ancestral neurofilament gene may have taken place in the earliest metazoa more than 700 million years ago as IF proteins are present in neuronal and non-neuronal cells of several invertebrates (Lasek et al., 1985; Bartnik et al., 1985; 1986). Following the latter evolutionary scheme, the neurofilament branch would have evolved with a first duplication of the ancestral *nfh* gene to give rise to the precursor of *nfm*

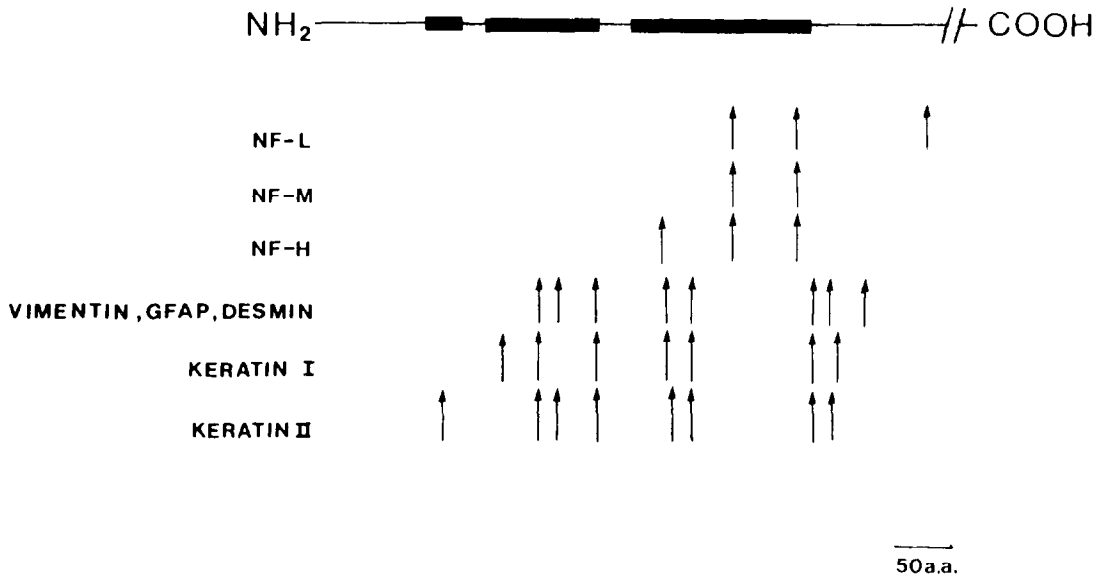


Fig. 4. Intron positions of intermediate filament genes in the structural regions of the proteins. The conserved α-helical regions are represented by blackened boxes. Arrows indicate the intron locations in each gene within their corresponding protein sequences. The *nfh* gene shares two intron positions with the *nfl* and *nfm* genes, but in addition its first intron occurs at a position equivalent in other IF genes, i.e., only 1 aa upstream from the corresponding vimentin intron. A more important intron shift of 4 aa occurred also at this position in the human type II keratin (Steinert et al., 1985).

and *nfl* genes. However, intron 1 of this precursor was lost prior to a subsequent gene duplication to yield the *nfm* and *nfl* genes. While the α-helical regions have been stringently conserved in the three neurofilament proteins, the presence of tandem repeat sequences in the C-terminal domains suggests that this region evolved with several recombination and amplification events. Hence the longer existence of NF-H would account for the more extensive tail region and the larger number of repeat sequences.

(d) Conclusions

The deduced amino acid sequence of the NF-H protein is remarkable. As expected, the protein shares with other IF proteins a homologous α-helical domain but the long C-terminal region in NF-H is very rich in charged amino acids and contains a repeated sequence, Lys-Ser-Pro, that represents the major protein kinase recognition site in neurofilament proteins. This concentration of charged amino acids in a domain of neurofilament subunits that forms a lateral projection may induce charge repulsions that increase the spacing between neurofilaments and thus determine axonal volume (Hoffman et al., 1987).

The actual structure of the *nfh* gene supports the common origin of neurofilament genes. However, in contrast to the *nfl* and *nfm* genes, the *nfh* gene contains an additional intron at a location homologous to one of the introns in non-neuronal IF genes. This additional *nfh* intron may reflect an early divergence of the neurofilament gene family via a duplication event.

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