Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2

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Neurofibromatosis type 2 (NF2) is a monogenic dominantly inherited disease predisposing carriers to develop nervous system tumours. To identify the genetic defect, the region between two flanking polymorphic markers on chromosome 22 was cloned and several genes identified. One is the site of germ-line mutations in NF2 patients and of somatic mutations in NF2-related tumours. Its deduced product has homology with proteins at the plasma membrane and cytoskeleton interface, a previously unknown site of action of tumour suppressor genes in humans.

THE neurofibromatoses are autosomal dominant diseases which primarily affect the nervous system. Neurofibromatosis type 1 (NF1), which occurs with an incidence of 1/3,000, predisposes mainly to the development of peripheral neurofibromas, cafe au lait macules, optic nerve gliomas and bony abnormalities. Neurofibromatosis type 2 (NF2), which occurs with an incidence of 1/37,000 (ref. 1), is mainly associated with the development of schwannomas, and also, to a lesser extent, to meningiomas and ependymomas[2]. Juvenile cataracts are also frequently seen in NF2. Though clinical studies first suggested the existence of at least two different forms of neurofibromatosis, definite proof of the existence of two distinct syndromes was provided only recently when it was determined that the genetic defects causing NF1 and NF2 map to separate chromosomes. Linkage studies showed that, in contrast to NF1 which maps to chromosome 17 (refs 3,4). NF2 maps to chromosome 22 (ref. 5). Further genetic studies of NF2 have shown it to be a genetically homogeneous disease[6]. Deletion studies in tumours suggests that loss of the NF2 gene is also important in the development of sporadic schwannomas[7,8] and meningiomas[9–11], which together represent 30% of all primary brain tumours. This implies that NF2 might be caused by mutations in a gene with tumour suppressor activity.

Segregation studies in affected families, and characterization of a germ-line deletion in one NF2 pedigree have determined that NF2 maps to the interval between D22S212 and D22S32 (ref. 12 and M. S. *et al.*, unpublished results), a region small enough to attempt the identification of the NF2 gene using a positional cloning strategy.

TABLE 1 Rearrangements in NF2 patients detected by N1.1 cDNA

Patient	Restriction enzyme	Size (kb) Normal	Abnormal
4774	*Sfi*I	50	70
	*Bss*HII	130	210
	*Eag*I	115	195
R2642	*Bss*HII	130	145
	*Eag*I	115	130
4702	*Eco*RI	20	19
	*Hin*dIII	7	6
	*Hin*cII	13	12
R267	*Eco*RI	20	19

None of the abnormal bands were present in 40 control individuals tested by PFGE (individuals R2642 and 4774) and 90 control individuals by the Southern blots.

Cloning of the D22S212/D22S32 region

Using an extended panel of somatic cell hybrids, D22S212, D22S32 and four additional loci (Cos5/6, Kil764, Kil045, LIF) were sublocalized to two adjacent subregions (groups 7 and 8) that flank a translocation breakpoint recurrently observed in Ewing's sarcoma (refs. 13,14 and unpublished results). These loci were progressively expanded by the isolation of overlapping cosmids using chromosome 22-specific libraries[15]. In this process several contiguous sequences (contigs) were connected so that a total of four cosmid contigs was finally obtained. Isolation of yeast artificial chromosomes and/or Southern analysis of total human DNA provided an estimate of the sizes of the gaps between these contigs indicating that the distance between D22S212 and D22S32 was about one million base pairs (Fig. 1).

Search for submicroscopic chromosome alteration

Forty-two unrelated NF2 patients were screened for rearrangements in the D22S212/D22S32 region using pulsed-field gel

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TABLE 2 Oligonucleotides used to screen for mutation in the SCH gene

	Region screened (codon number)	Oligonucleotide sequence
Set 1	39 to 80	5′ GC clamp 1- TTGCTCACAGTGTCCTTCCC 3′
		5′ M13 reverse- TCAGCCCCACCAGTTTCATC 3′
Set 2	150 to 172	5′ M13 direct- ATCTTTAGAATCTCAATCGC 3′
		5′ GC clamp 2- AGCTTTCTTTTAGACCACAT 3′
Set 3	226 to 270	5′ M13 direct- CCACAGAATAAAAAGGGCAC 3′
		5′ GC clamp 2- GATCTGCTGGACCCATCTGC 3′
Set 4	334 to 374	5′ M13 direct- TCGAGCCCTGTGATTCAATG 3′
		5′ GC clamp 2- AAGTCCCCAAGTAGCCTCCT 3′
Set 5	376 to 446	5′ GC clamp 1- CCCACTTCAGCTAAGAGCAC 3′
		5′ M13 reverse- CTCCTCGCCAGTCTGGTG 3′
Set 6	527 to 579	5′ M13 direct- TCTCACTGTCTGCCCAAG 3′
		5′ GC clamp 2- GATCAGCAAAATACAAGAAA 3′

5′ GC clamp 1 = 5′ CCCCGCCCGGCCCGCCCGCCCCCCGCCCCCCCTCCCGGCCCGCCCCCCTGGCGCCCCGC 3′
5′ GC clamp 2 = 5′ CCCCACGCCACCCGACGCCCCAGCCCGACCCCCCCGCGCCCGGCGCCCCCGC 3′
5′ M13 direct = 5′ TGTAAAACGACGGCCAGT 3′
5′ M13 reverse = 5′ CAGGAAACAGCTATGACC 3′

Six genomic sequences containing in each case a whole exon were analysed using the computer programs Meltmap and 5QHTX[35] to determine the optimal set of two oligonucleotides. One member of each pair of oligonucleotides contains a GC-rich sequence (CG clamp 1 or 2) [28]. The other member contains a sequence (M13 direct or M13 reverse) used to sequence the amplified product with commercially available fluorescent primers. All PCR reactions were done in 10 μl with AmpTAQ Kit (Cetus) using a Perkin Elmer 9600 DNA cycler (35 cycles with denaturing step 95 °C for 30 s, annealing temperature as specifically indicated in each case for 30 s and elongation at 72 °C for 90 s). Denaturing gradient gel electrophoresis (DGGE) was done as published[44]. Specific conditions for PCR amplification and DGGE analysis were as follows: PCR annealing temperature, set 1 and 4, 62 °C; set 2, 3, 5 and 6, 60 °C. DGGE analysis: denaturation gradients (100% denaturant is 7M urea and 40% formamide), set 1, 2, 3 and 6, 30% to 80%; set 4 and 5, 40% to 90%. Electrophoresis was done for set 1, 3, 4 and 6 for 3 h; set 2 for 2 h and set 5 for 4 h. Amplified products were purified on Centricon 100 ultra-filtration devices (Amicon, Epernon, France)[22] and direct sequencing was done using PRISM *Taq* fluorescent primer Idt (Applied Biosystems).

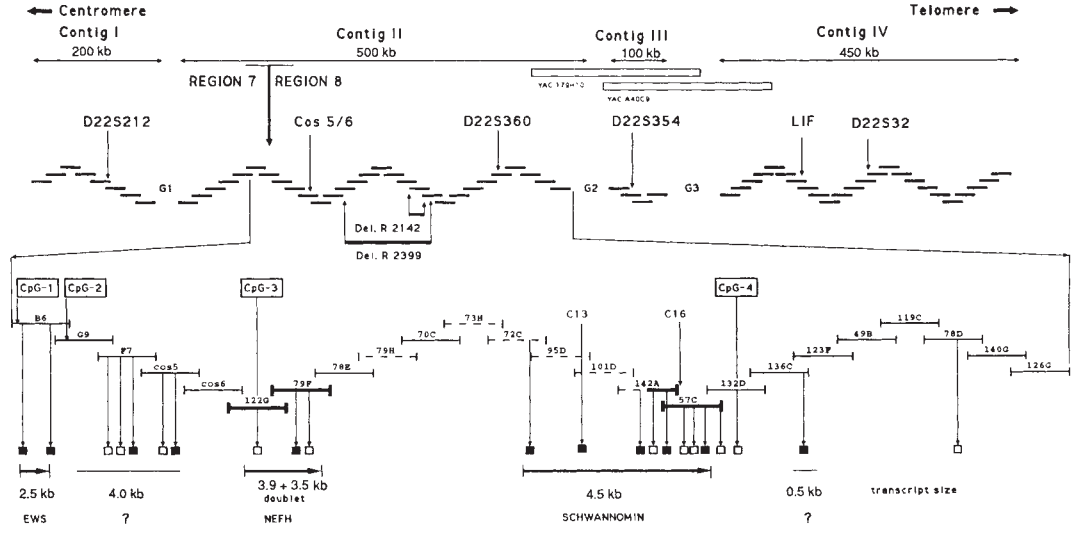

FIG. 1 Regional map of the NF2 locus. The relative position of the four contigs of cosmids, their size and their orientation with respect to the centromere and telomere of the long arm of chromosome 22 are shown. The minimum number of cosmids (tiling path, each bar corresponds to one cosmid) and the loci that were initially expanded are indicated below. The boundary between region 7 and 8 defined by a chromosome 22 mapping panel of somatic cell hybrids is indicated [13]. G1 to G3 refer to the three gaps between the four contigs. G1 has been estimated to be about 0.5 kb by Southern blotting experiments. G2 and G3 are about 100 kb and 20 kb, respectively, and have each been spanned with a YAC clone (M. Giovannini & L. Selleri, unpublished results). The positions of the deletion detected in individuals R2142 and R2399 are shown. In the lower part of the figure a portion of the tiling path of contig II is shown in greater detail. Cosmids that have been shown by FISH to be preserved or deleted in the NF2 patient R2399 are shown in thick or dotted lines, respectively (cosmid 142A is split by the deletion). The position of four clusters of GC nucleotides are indicated as follows: CpG-1 contains site(s) for *Mlu*I, *Bss*HII and *Sac*II; CpG-2, CpG-3 and CpG-4 each contain site(s) for *Sac*II, *Bss*HII and *Not*I. *Eco*RI fragments demonstrating phylogenetic conservation with rodent DNA (rat and mouse) are indicated by squares. They are black when the fragment is able to detect a transcript in RNAs from at least one of the tested human cell lines. The size of the detected transcript is indicated. When known, the direction of transcription is indicated by an arrow. The positions of the C13 and C16 probes are indicated.

METHODS. Expansion of six loci from the vicinity of the NF2 gene (D22S212, Cos5/6, Kil764, Kil045, LIF and D22S32) was obtained as previously described[15]. FISH was done as described in ref. 41. Each cosmid between B6 and 126G was digested with *Eco*RI. Aliquots were double digested with the following enzymes containing two CG dinucleotides in their target sequence (*Bss*HII, *Sac*II, *Not*I, *Nru*I and *Mlu*I) and alteration in the migration pattern was analysed. Individual *Eco*RI fragments were also gel purified and used as probes on Southern blots made from *Eco*RI digested human rat and mouse DNAs[42]. To reveal phylogenetic conservation two stringency conditions were used for washing (2 × SSC, 50 °C and 0.1 × SSC, 65 °C in both cases for 5 min). Northern blots were done using standard conditions [42] with RNAs extracted from the following cell lines: HL60 (promyelocytic leukaemia), HeLa (cervix carcinoma), SKNBZ (neuroblastoma), 2102 EP and Tera 2 (teratocarcinomas), CCL225 (colon carcinoma), OZ (glioma), EW24 (Ewing's sarcoma), Bewo (choriocarcinoma).

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electrophoresis (PFGE) and single-copy probes derived from the previously identified cosmids. Two individuals had abnormal fragments when probed with C16 isolated from the cosmid 57C (Fig. 2*a*): patient R2399 and R2142 displayed the expected 130 kilobase (kb) *Bss*HII fragment plus new fragments of 85 kb and 90 kb, respectively. With the same probe, both patients also had abnormal band size with *Sac*II and *Eag*I. In contrast, C13, a probe 40 kb centromeric to C16 and which detects the same *Bss*HII fragment in normal individuals, failed to detect the rearrangements in R2142 and R2399, suggesting that C13 maps to an area deleted in both patients.

The presence and extent of the deletions were tested using fluorescent *in situ* hybridization (FISH) on interphase nuclei or metaphase chromosomes using nine cosmids (Fig. 1). Both copies of chromosome 22 of patient R2399 were detected with the two most centromeric (122G, 79F) and the most telomeric (57C) cosmids. In contrast, five cosmids located between these two borders (79H, 73H, 72C, 95D and 101D) hybridized to a single copy of chromosome 22, confirming the presence of a deletion. For cosmid 142A, the second most telomeric cosmid tested, the recurrent observation of a strong signal on one copy of chromosome 22 and a weak signal on the other suggested that it straddled the distal end of the deletion. We concluded that patient R2399 has a germ-line 130 kb interstitial deletion flanked by cosmids 79F and 142A. For patient R2142, similar FISH experiments demonstrated that only cosmid 101D was deleted (Fig 2*b*). Cosmid 95D, which partially overlaps with cosmid 101D, gave variable signal on one copy of chromosome 22, compatible with a partial deletion. Cosmids 57C, 142A and 72C were not deleted, suggesting that the deletion in R2142 is about 40 kb. To identify candidate genes for NF2 the segment of contig II into which these two deletions map was further studied.

Search for genes in the D22S212/D22S32 region

We made a systematic analysis of over 100 *Eco*RI fragments isolated from the 24 cosmids shown on the lower part of Fig. 1. To identify clusters of CG dinucleotides, which frequently mark the 5′ region of genes[16], each fragment was examined for the presence of restriction sites which contain two CGs in their recognition sequence. Four clusters of at least three different restriction sites were found. Each *Eco*RI fragment was also tested for its ability to hybridize rodent DNA and, when phylogenetic conservation was found, to detect transcripts on northern blots made with RNAs from various human cell lines. When the different data generated by this analysis were put together on the map, the position of four genes became evident. The most centromeric gene, associated with the CpG-1 cluster, marks the limit between subregions 7 and 8 (ref. 13). It has been called EWS because of its constant rearrangement in Ewing's sarcoma[17]. The closest identified gene on the telomeric side of

TABLE 3 germ-line and somatic SCH mutations in NF2-patients and NF2 related tumours

Sample	Mutation	Altered codon	Consequence	Family analysis
SP 10	CT deletion	54	Frame-shift	new mutation
SP 16	C to T	57	Non-sense	ND
IC 2	C to T	57	Non-sense	ND
GL 9	AGgt to AGtt	junction 80/81	Splice donor	segregates with NF2
ST 2	AGgt to AGtt	junction 80/81	Splice donor	ND
SP 4	agTA to atTA	junction 149/150	Splice acceptor	ND
EB D	GGgt to GGat	junction 172/173	Splice donor	segregates with NF2
EB N	C insertion	253	Frame-shift	new mutation
GL 18	C deletion	262	Frame-shift	ND
EB G	C to T	262	Non-sense	ND
GL 2C to T	341	Non-sense	ND	
SP 20	TGAACGC del	349 to 351	Frame-shift	new-mutation
RE 1	GAACGCAAGAGG replaced by CGAGAGAAGCA	350 to 353	Frame-shift	segregates with NF2
RF 10	T to C	360	Leu to Pro	segregates with NF2
GL 5	AGgt to AGat	junction 447/448	Splice donor	segregates with NF2
DNA from tumour				Tumour type Chr.22 allele
IC-BE	C TO T*	57	Non-sense	schwannoma preserved
28A	C to T*	262	Non-sense	schwannoma lost
IC-J	C to T†	262	Non-sense	schwannoma lost
IC-F	C to T†	341	Non-sense	schwannoma lost
IC-AB	GGAA del†	43 and 44	Frame-shift	meningioma preserved
IC-PO	TC del†	61	Frame-sshift	meningioma lost

A group of 90 independent NF2 patients was screened for mutations on 6 different exons. Fifteen patients had an abnormally migrating band which was sequenced and revealed a mutation in each case. The predicted consequence of the mutation on the schwannomin structure is given. 'Segregates with NF2' means that the mutation was present in all studied affected members (at least two affected individuals in two different generations per family) and absent from all studied unaffected members. 'New mutation' indicates that the affected patients has two unaffected parents. In each of these three families, the SCH mutation of the patient was not present in either of his/her parents. The diagnostic criteria for NF2 were those defined by the 1991 NIH consensus conference statement[36]. Non-paternity was excluded by typing the following highly polymorphic loci D5S346 and CAMBC (refs 37, 38). No familial relationship were found for patients GL 9 and ST 2 and for patients SP 16 and IC 2s ND, Other family members were not studied. Thirty schwannomas and 30 meningiomas from either NF2 or sporadic patients were also screened. Six tumours revealed the presence of an SCH mutation. DNAs from the blood of the six corresponding patients were also investigated. Schwannomas 28A and IC-BE developed in NF2 patients EB-G and IC 2, respectively. IC-J and IC-F correspond to the schwannomas of the sporadic patients J and F described in ref. 39. IC-AB and IC-PO are two meningiomas removed from sporadic patients. 'Lost', Somatic losses of heterozygosity in the tumour DNAs observed for the following loci: 28A: IGLV, BCR, CRYB2A, D22S32, D22S15, D22S29 and ARSA (ref. 40); IC-F: D22S170, IGLV, D22S32, PDGFB, D22S80 (ref. 25 and unpublished result); IC-J: D22S24, D22S9, D22S170 and D22S20 (ref. 39 and unpublished result); IC-PO: D22S20, PDGFB, D22S171 (unpublished result). 'Preserved', All observed chromosome 22 heterozygosities in the constitutional DNA were preserved in the tumour DNA.

*Mutation observed in the tumour is identical to that found in the patient blood DNA.

†Mutation is not observed in the patient blood DNA and is therefore somatic.

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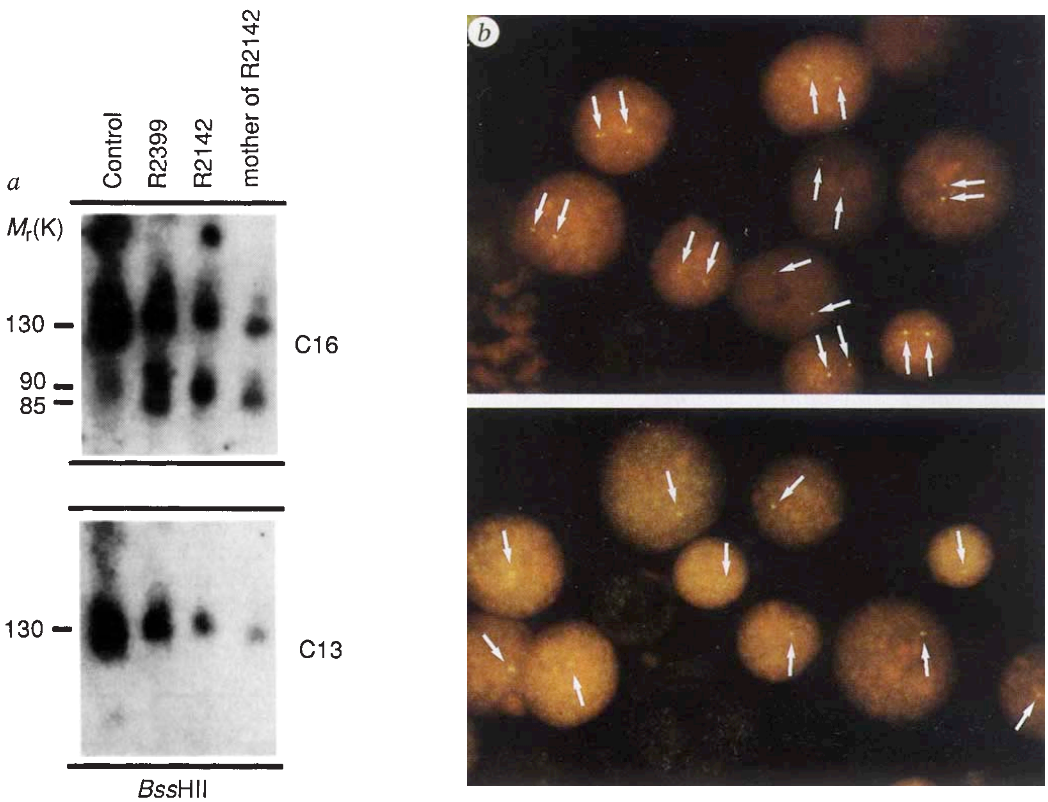

c

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GGGTCCTCGCGGCCCATGCTGGCCGCTGGGGACCCGCGCAGCCCAGACCGTTCCCGGGCCGGCCAGCCGCCACCATGGTGGCCCTGAGGCCTGTGCAGCAACTCCAGGGGGGCTAAAGGGCTCAGAGTGCAGCCCGTGGGGCGCGA

                15              30              45              60              75              90             105             120             135
ATG GCC GGG GCC ATC GCT TCC CGC ATG AGC TTC AGC TCT CTC AAG AGG AAG CAA CCC AAG ACG TTC ACC GTG AGG ATC GTC ACC ATG GAC GCC GAG ATG GAG TTC AAT TGC GAG ATG AAG TGG AAA GGG AAG GAC
Met Ala Gly Ala Ile Ala Ser Arg Met Ser Phe Ser Ser Leu Lys Arg Lys Gln Pro Lys Thr Phe Thr Val Arg Ile Val Thr Met Asp Ala Glu Met Glu Phe Asn Cys Glu Met Lys Trp Lys Gly Lys Asp

               150             165             180             195             210             225             240             255             270
CTC TTT GAT TTG GTG TGC CGG ACT CTG GGG CTC CGA GAA ACC TGG TTC TTT GGA CTG CAG TAC ACA ATC AAG GAC ACA GTG GCC TGG CTC AAA ATG GAC AAG AAG GTA CTG GAT CAT GAT GTT TCA AAG GAA GAA
Leu Phe Asp Leu Val Cys Arg Thr Leu Gly Leu Arg Glu Thr Trp Phe Phe Gly Leu Gln Tyr Thr Ile Lys Asp Thr Val Ala Trp Leu Lys Met Asp Lys Lys Val Leu Asp His Asp Val Ser Lys Glu Glu

               285             300             315             330             345             360             375             390             405
CCA GTC ACC TTT CAC TTC TTG GCC AAA TTT TAT CCT GAG AAT GCT GAA GAG GAG CTG GTT CAG GAG ATC ACA CAA CAT TTA TTC TTC TTA CAG GTA AAG AAG CAG ATT TTA GAT GAA AAG ATC TAC TGC CCT CCT
Pro Val Thr Phe His Phe Leu Ala Lys Phe Tyr Pro Glu Asn Ala Glu Glu Glu Leu Val Gln Glu Ile Thr Gln His Leu Phe Phe Leu Gln Val Lys Lys Gln Ile Leu Asp Glu Lys Ile Tyr Cys Pro Pro

               420             435             450             465             480             495             510             525             540
GAG GCT TCT GTG CTC CTG GCT TCT TAC GCC GTC CAG GCC AAG TAT GGT GAC TAC GAC CCC AGT GTT CAC AAG CGG GGA TTT TTG GCC CAA GAG GAA TTG CTT CCA AAA AGG GTA ATA AAT CTG TAT CAG ATG ACT
Glu Ala Ser Val Leu Leu Ala Ser Tyr Ala Val Gln Ala Lys Tyr Gly Asp Tyr Asp Pro Ser Val His Lys Arg Gly Phe Leu Ala Gln Glu Glu Leu Leu Pro Lys Arg Val Ile Asn Leu Tyr Gln Met Thr

               555             570             585             600             615             630             645             660             675
CCG GAA ATG TGG GAG GAG AGA ATT ACT GCT TGG TAC GCA GAG CAC CGA GGC CGA GCC AGG GAT GAA GCT GAA ATG GAA TAT CTG AAG ATA GCT CAG GAC CTG GAG ATG TAC GGT GTG AAC TAC TTT GCA ATC CGG
Pro Glu Met Trp Glu Glu Arg Ile Thr Ala Trp Tyr Ala Glu His Arg Gly Arg Ala Arg Asp Glu Ala Glu Met Glu Tyr Leu Lys Ile Ala Gln Asp Leu Glu Met Tyr Gly Val Asn Tyr Phe Ala Ile Arg

               690             705             720             735             750             765             780             795             810
AAT AAA AAG GGC ACA GAG CTG CTG CTT GGA GTG GAT GCC CTG GGG CTT CAC ATT TAT GAC CCT GAG AAC AGA CTG ACC CCC AAG ATC TCC TTC CCG TGG AAT GAA ATC CGA AAC ATC TCG TAC AGT GAC AAG GAG
Asn Lys Lys Gly Thr Glu Leu Leu Leu Gly Val Asp Ala Leu Gly Leu His Ile Tyr Asp Pro Glu Asn Arg Leu Thr Pro Lys Ile Ser Phe Pro Trp Asn Glu Ile Arg Asn Ile Ser Tyr Ser Asp Lys Glu

               825             840             855             870             885             900             915             930             945
TTT ACT ATT AAA CCA CTG GAT AAG AAA ATT GAT GTC TTC AAG TTT AAC TCC TCA AAG CTT CGT GTT AAT AAG CTG ATT CTC CAG CTA TGT ATC GGG AAC CAT GAT CTA TTT ATG AGG AGA AGG AAA GCC GAT TCT
Phe Thr Ile Lys Pro Leu Asp Lys Lys Ile Asp Val Phe Lys Phe Asn Ser Ser Lys Leu Arg Val Asn Lys Leu Ile Leu Gln Leu Cys Ile Gly Asn His Asp Leu Phe Met Arg Arg Arg Lys Ala Asp Ser

               960             975             990            1005            1020            1035            1050            1065            1080
TTG GAA GTT CAG CAG ATG AAA GCC CAG GCC AGG GAG GAG AAG GCT AGA AAG CAG ATG GAG CGG CAG CGC CTC GCT CGA GAG AAG CAG ATG AGG GAG GAG GCT GAA CGC ACG AGG GAT GAG TTG GAG AGG AGG CTG
Leu Glu Val Gln Gln Met Lys Ala Gln Ala Arg Glu Glu Lys Ala Arg Lys Gln Met Glu Arg Gln Arg Leu Ala Arg Glu Lys Gln Met Arg Glu Glu Ala Glu Arg Thr Arg Asp Glu Leu Glu Arg Arg Leu

              1095            1110            1125            1140            1155            1170            1185            1200            1215
CTG CAG ATG AAA GAA GAA GCA ACA ATG GCC AAC GAA GCA CTG ATG CGG TCT GAG GAG ACA GCT GAC CTG TTG GCT GAA AAG GCC CAG ATC ACC GAG GAG GAG GCA AAA CTT CTG GCC CAG AAG GCC GCA GAG GCT
Leu Gln Met Lys Glu Glu Ala Thr Met Ala Asn Glu Ala Leu Met Arg Ser Glu Glu Thr Ala Asp Leu Leu Ala Glu Lys Ala Gln Ile Thr Glu Glu Glu Ala Lys Leu Leu Ala Gln Lys Ala Ala Glu Ala

              1230            1245            1260            1275            1290            1305            1320            1335            1350
GAG CAG GAA ATG CAG CGC ATC AAG GCC ACA GCG ATT CGC ACG GAG GAG GAG AAG CGC CTG ATG GAG CAG AAG GTG CTG GAA GCC GAG GTG CTG GCA CTG AAG ATG GCT GAG GAG TCA GAG AGG AGG GCC AAA GAG
Glu Gln Glu Met Gln Arg Ile Lys Ala Thr Ala Ile Arg Thr Glu Glu Glu Lys Arg Leu Met Glu Gln Lys Val Leu Glu Ala Glu Val Leu Ala Leu Lys Met Ala Glu Glu Ser Glu Arg Arg Ala Lys Glu

              1365            1380            1395            1410            1425            1440            1455            1470            1485
GCA GAT CAG CTG AAG CAG GAC CTG CAG GAA GCA CGC GAG GCG GAG CGA AGA GCC AAG CAG AAG CTC CTG GAG ATT GCC ACC AAG CCC ACG TAC CCG CCC ATG AAC CCA ATT CCA GCA CCG TTG CCT CCT GAC ATA
Ala Asp Gln Leu Lys Gln Asp Leu Gln Glu Ala Arg Glu Ala Glu Arg Arg Ala Lys Gln Lys Leu Leu Glu Ile Ala Thr Lys Pro Thr Tyr Pro Pro Met Asn Pro Ile Pro Ala Pro Leu Pro Pro Asp Ile

              1500            1515            1530            1545            1560            1575            1590            1605            1620
CCA AGC TTC AAC CTC ATT GGT GAC AGC CTG TCT TTC GAC TTC AAA GAT ACT GAC ATG AAG CGG CTT TCC ATG GAG ATA GAG AAA GAA AAA GTG GAA TAC ATG GAA AAG AGC AAG CAT CTG CAG GAG CAG CTC AAT
Pro Ser Phe Asn Leu Ile Gly Asp Ser Leu Ser Phe Asp Phe Lys Asp Thr Asp Met Lys Arg Leu Ser Met Glu Ile Glu Lys Glu Lys Val Glu Tyr Met Glu Lys Ser Lys His Leu Gln Glu Gln Leu Asn

              1635            1650            1665            1680            1695            1710            1725            1740            1755
GAA CTC AAG ACA GAA ATC GAG GCC TTG AAA CTG AAA GAG AGG GAG ACA GCT CTG GAT ATT CTG CAC AAT GAG AAC TCC GAC AGG GGT GGC AGC AGC AAG CAC AAT ACC ATT AAA AAG CTC ACC TTG CAG AGC GCC
Glu Leu Lys Thr Glu Ile Glu Ala Leu Lys Leu Lys Glu Arg Glu Thr Ala Leu Asp Ile Leu His Asn Glu Asn Ser Asp Arg Gly Gly Ser Ser Lys His Asn Thr Ile Lys Lys Leu Thr Leu Gln Ser Ala

               1770            1785
 AAG TCC CGA GTG GCC TTC TTT GAA GAG CTC TAG CAGGTGACCCAGCCACCCCAGGACCTGCCACTTCTCCTGCTACCGGGACCGCGGGATGGACCAGATATCAAGAGAGCCATCCATAGGGAGCTGGCTGGGGGTTTCCGTGGGAGCTCCAGAACTTTCCCCAGCT
Lys Ser Arg Val Ala Phe Phe Glu Glu Leu End
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FIG. 2 Analysis of deletions in NF2 individuals. *a*, Pulsed-field electrophoresis blots showing rearrangements. From left to right, *Bss*HII digests of a normal control, R2399, R2142 and the affected mother of R2142 hybridized with the probe C16 (upper panel) and the probe C13 (lower panel). C16 detects the expected 130 kb fragment in all individuals; a new 85 kb fragment is seen in individual R2399 and a new 90 kb fragment is seen in individual R2142 and her mother. C13 does not detect the rearranged fragments. *b*, Fluorescent *in situ* hybridization (FISH) of cosmids to the cell line R2142. Top panel, interphase nuclei hybridized with cosmid 101D. Only one chromosome 22 is seen indicating that the cosmid 101D is entirely deleted from the second copy of chromosome 22. Bottom panel, interphase nuclei hybridized with the cosmid 142A. Both copies of chromosome 22 are seen confirming the presence of two intact copies of chromosome 22 at this locus. *c-e*, Nucleotide and predicted amino-acid sequence of N1.1 cDNA, and amino-acid homologies. *c*, Entire nucleotide sequence of the N1.1 cDNA. The predicted start site is at postion 1–3 and the in-frame stop codon is at position 1,786–1,788. The predicted amino-acid sequence of 595 residues is shown beneath the respective codons. An in-frame stop codon in the 5′ untranslated sequence is 89 ▶

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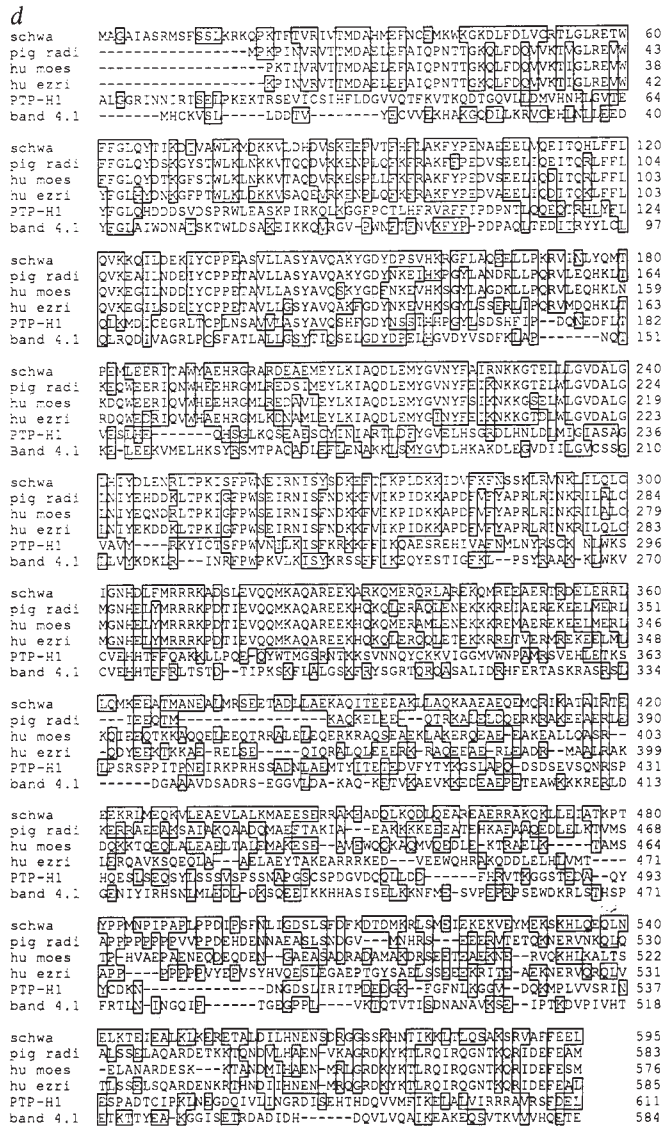

bp upstream of the first ATG. Potential phosphorylation sites are double underlined, potential glycosylation sites are single underlined (EMBL data base accession number Z22664SCHWANNOM). *d*, Homologies to the following proteins: radixin, moesin, ezrin, protein tyrosine phosphatase 1 and erythrocyte protein band 4.1 for the entire protein. *e*, Homology of schwannomin for the rat repetitive element L1 transposon for a small section of the C-terminus domain.

METHODS. *a*, High M_r DNA of the lymphoblastoid cell lines was prepared in agarose plugs by standard methods. Restriction digests were done in agarose according to the manufacturer's protocol. The PFGE Pharmacia apparatus at pulse-time settings optimized for separating the desired restriction fragments was used. Southern transfer and hybridizations were as described elsewhere[42]. Lambda ladder M_r markers were purchased from BRL. *b*, Whole cosmid DNA was labelled by nick translation using biotin-14-dATP and hybridized to metaphase chromosome spreads and interphase nuclei of the respective lymphoblastoid cell lines. The signal was detected with Avidin FITC and amplified with Avidin-conjugated antibody[41]. *c–e*, The entire N1.1 clone was sequenced in both directions using the dideoxynucleotide method and nested deletion produced using the Henikoff method [43]. The sequence was further verified by sequencing of genomic clones. The homologies were determined as described in the text.

EWS is expressed as a 4 kb transcript. CpG-2 may provisionally be associated with this unknown gene and awaits characterization. The third gene has a neuronal pattern of expression and a transcript size identical to that of the neurofilament heavy-chain gene, NEFH, a gene previously located on chromosome 22 (refs 18,19). Use of NEFH complementary DNAs confirmed this identification and showed that CpG-3 is located in the 5′ region of this gene. The fourth gene was detected by 5 different *Eco*RI fragments which hybrized to the same 4.5 kb RNA. The corresponding gene, called SCH, is transcribed in the centromeric to telomeric orientation. No CpG clusters in its 5′ region had been identified in the systematic analysis of *Eco*RI fragments. The CpG-4 cluster located in its 3′ region is probably related to a more distally located gene which may correspond to the 0.5 kb transcript detected by one *Eco*RI fragment or to another gene which escaped our detection procedure.

Isolation of a candidate gene

The deletion in patient R2399, although removing the 5′ end of SCH, did not alter the NEFH gene. Furthermore, the deletion of patient R2142 appeared to lie entirely within the SCH gene. Therefore we isolated the SCH gene as the most likely candidate for involvement in NF2. The phylogenetically conserved sequence C13 which cross-hybridized to pig, monkey, rat, mouse, chicken and hamster DNA (data not shown), was used to isolate a single 2.0 kb cDNA clone, N1.1, from a human fetal brain cDNA library (Stratagene). Southern analysis using DNA from cosmids in the region and chromosome 22 hybrid cell lines showed that N1.1 mapped back to the correct region.

Southern analysis using the N1.1 cDNA detected seven *Eco*RI fragments. The positions of these *Eco*RI fragments on the cosmid contig show a genomic span of about 100 kb suggesting the presence of many large introns (Fig. 3*a*).

To identify additional germ-line rearrangements, N1.1 was used to probe PFGE blots for 42 unrelated NF2 and 40 control individuals and Southern blots for 60 unrelated NF2 patients and 90 control individuals. In contrast to control DNAs which all demonstrated a consistent pattern of hybridizing bands, four NF2 patients revealed multiple additional fragments compatible with the occurrence of an altered SCH allele (Table 1). One of them, individual 4707, is a member of a large pedigree segregating NF2. Seven of seven affected individuals segregated the altered *Eco*RI fragments; the new fragment was not seen in any of the unaffected family members. The rearranged fragment maps to a 20 kb *Eco*RI fragment which cross-hybridizes to the cDNA. Because these rearrangements were likely to be causally related to NF2, we investigated the SCH gene further.

Tissue specificity and homology

Northern analysis revealed a 4.5 kb message in mouse fetal brain, human kidney, lung, breast, ovary, placenta and neuroblastoma but not in mouse adult spinal cord and brain, and not in human adult brain, liver and pancreas (Clontech multiple tissue northern blot). The highest expression was seen in the fetal brain, though the presence of signals in many other tissues suggests widespread expression.

Sequence analysis revealed an open reading frame of 1,785 bases for a predicted protein of 595 amino acids which was named schwannomin, a word derived from schwannoma, the most prevalent tumour seen in NF2 (Fig. 2*c*). The predicted molecular mass is 66K. The probable start site at position 1–3 is the most likely because it is the first ATG after an in-frame stop codon 89 base pairs (bp) upstream and because it contains the Kozak consensus sequence[20]. No polyadenylation signal or poly (A) tail was detected. Our 5′ untranslated sequence is 144 bp and the 3′ untranslated is 135 bp. Based on results obtained with the northern blots, where a 4.5 kb message is detected, we predict that we are missing about 2.3 kb of untranslated sequence of the SCH messenger RNA.

Sequence homology searches using GENBANK and the

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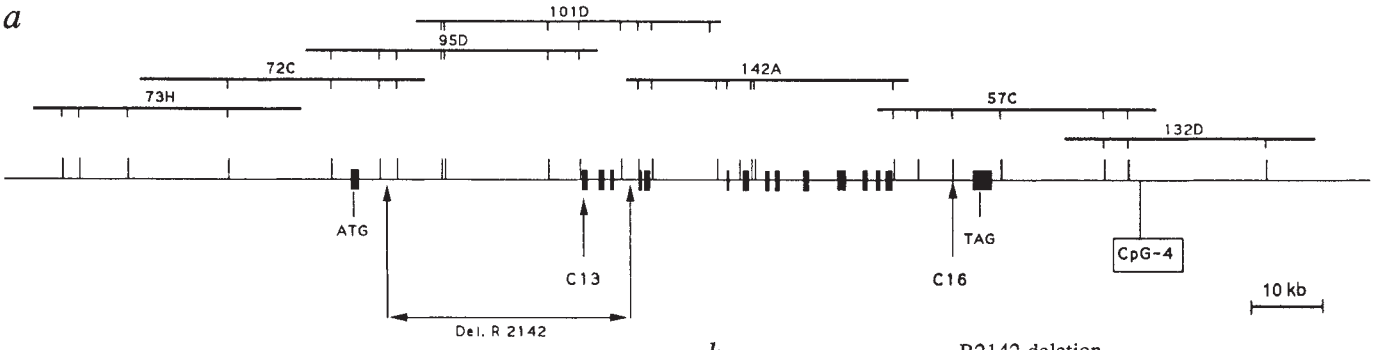
BLAST e-mail server indicated that this is a novel sequence[21]. But significant amino-acid homology was found with moesin (48% for entire protein; 62% for the N terminal), ezrin (48% for entire protein; 62% for the N terminal), radixin (47% for entire protein; 62% for the N terminal), protein tyrosine phosphatase 1 (25% for the entire protein; 44% for the N terminal), erythrocyte protein 4.1 (25% for the entire protein; 46% for the N terminal) and rat repetitive element L1 transposon (15 of 22 amino acids in the C terminus; refs 24–27 and W. T. Lankes, H. Furthmayr and M. R. Amieva, sequence submitted to EMBL/Genbank/DDBJ Databanks) (Fig. 2*d*,*e*). The predicted secondary structure of schwannomin is similar to the structures of moesin, ezrin and radixin: a large N-terminal domain followed by a large α-helix domain and a small C terminus.

Search for point mutations in NF2 patients

To obtain further evidence of SCH alteration in NF2 patients, exons and intron–exons boundaries within the coding sequence of SCH were determined (Fig. 3*a*). Specific exons were amplified by polymerase chain reaction (PCR) and the resulting products were analysed using denaturing gradient gel electrophoresis, an efficient screening method for detecting point mutation[28] (Table 2). Survey of a series of 90 unrelated NF2 patients revealed 15 abnormal migration patterns. Direct sequencing of the corresponding bands identified 15 genetic variants. With one exception, which causes a non-conservative leucine to proline substitution, all these variants can be predicted to lead to the synthesis of a truncated SCH protein (Table 3). Whenever it was possible to investigate several family members in two generations, the SCH mutations were shown to segregate with the disease. On three occasions, the DNA variants were only present in the patient constitutional DNA and not in either of their unaffected parents providing the strongest evidence of a causal relationship between the occurrence of a new mutation and the development of the disease.

Germ-line mutation in patient R2142

To identify the deletion breakpoints of the cell line R2142 we probed *Eco*RI digests with all the *Eco*RI fragments of the SCH region. Variant *Eco*RI bands were only identified with the two *Eco*RI fragments shown in Fig. 3*a*, providing evidence that the borders of the deletion were lying within these two fragments and predicting a 36 kb deletion compatible with the conclusion derived from the previous PFGE and FISH studies. This deletion, entirely contained within the SCH gene, is predicted to remove the second, third and fourth coding exon. Reverse-transcriptase PCR of RNA isolated from the lymphoblastoid cell line R2142, using primers internal to the cDNA yielded in addition to the expected 764 bp fragment, a smaller 431 bp fragment. Sequencing revealed that the latter fragment originated from a SCH mRNA in which the first and the fifth coding exon were adjacent thus confirming the initial prediction. The



b

R2142 deletion

N1.1 TCTCAAGAGGAAGCAACCCAAGACGTTCACCGTGAGGATCGTCACCATGGACGCCGAGAT
R2142 TCTCAAGAGGAAGCAACCCAAGACGTTCACCGTGAGGATCGTCACCATGGACGCCGAGAT

N1.1 GGAGTTCAATTGCGAGATGAAGTGGAAAGGGAAGGACCTCTTTGATTTGGTGTGCCGGACT
R2142 GGAGTTCAATTGCGAG

N1.1 CTGGGGCTCCGAGAAACCTGGTTCTTTGGACTGCAGTACACAATCAAGGACACAGTGGCCT
R2142

N1.1 GGCTCAAAATGGACAAGAAGGTACTGGATCATGATGTTTCAAAGGAAGAACCAGTCACC
R2142

N1.1 TTTCACTTCTTGGCCAAATTTTATCCTGAGAATGCTGAAGAGGAGCTGGTTCAGGAGATCA
R2142

N1.1 CACAACATTTATTCTTCTTACAGGTAAAGAAGCAGATTTTAGATGAAAAGATCTACTGC
R2142

N1.1 CCTCCTGAGGCTTCTGTGCTCCTGGCTTCTTACGCCGTCCAGGCCAAGTATGGTGACTACGAC
R2142 TATGGTGACTACGAC

N1.1 CCCAGTGTTCACAAGCGGGGATTTTTGGCCCAAGAGGAATTGCTTCCAAAAAGGGTAATA
R2142 CCCAGTGTTCACAAGCGGGGATTTTTGGCCCAAGAGGAATTGCTTCCAAAAAGGGTAATA

N1.1 AATCTGTATCAGATGACTCCGGAAATGTGGGAGGAGAGAATTACTGCTTGGTACGCAGAG
R2142 AATCTGTATCAGATGACTCCGGAAATGTGGGAGGAGAGAATTACTGCTTGGTACGCAGAG

N1.1 CACCGAGGCCGAGCCAGGGATGAAGCTGAAATGGAATATCTGAAGATAGCTCAGGACCTG
R2142 CACCGAGGCCGAGCCAGGGATGAAGCGTAAATGGAATATCTGAAGATAGCTCAGGACCTG

N1.1 GAGATGTACGGTGTGAACTACTTTGCAATCCGGAATAAAAAGGGCACAGAGCTGCTGCTT
R2142 GAGATGTACGGTGTGAACTACTTTGCAATCCGGAATAAAAAGGGCACAGAGCTGCTGCTT

N1.1 GGAGTGGATGCCCTGGGGCTTCACATTTATGACCCTGAGAACAGACTGACCCCCAAGATCT
R2142 GGAGTGGATGCCCTGGGGCTTCACATTTATGACCCTGAGAACAGACTGACCCCCAAGATCT

N1.1 CCTTCCCGTGGAATGAAATCCGAAACATCTCGTACAGTG
R2142 CCTTCCCGTGGAATGAAATCCGAAACATCTCGTACAGTG

FIG. 3 *EcoRI* restriction map of the SCH gene, position of coding exons and characterization of the aberrant transcript in patient R2142. *a*, Vertical bars and black boxes indicate the position of *Eco*RI sites and of the coding exons, respectively. The first and last coding exons are indicated by ATG and TAG, respectively. The position of each exon within its *Eco*RI fragment is only indicative. The positions of the C13 and C16 probes are also provided. The position of the R2142 deletion is shown. The positions of the two *Eco*RI fragments detecting rearranged bands in patient R1242 DNA are marked by arrows. *b*, The sequence of the reverse transcriptase PCR (RT-PCR) products from R2142 RNA. Comparison to the N1.1 sequence shows an in-frame deletion of 333 bp of the expressed SCH gene in R2142.
METHODS. The *Eco*RI restriction map of the 7 cosmids was generated using standard procedures[42]. *Sau*3A-digested cosmid DNAs were also subcloned in M13 mp18 and the resulting mini-libraries were screened with the N1.1 cDNA. The positive clones were directly sequenced using dideoxy chain termination methods and an Applied Biosystems automatic sequencer. Missing exons were searched among restriction fragments generated by appropriate digestion of the cosmid DNAs using N1.1 cDNA subfragments as probes. The sequence of the intron–exon junctions are deposited in the EMBL data base (accession number X7265 HSSCH01 to X72670 HSSCH16). Total RNA was prepared from the R2142 lymphoblastoid cell line by standard methods. RT-PCR was done using the primers TCTCAAGAGGAAGCAACCCA and CGCTGTACGAGATGTTTCGG (double underlined in Fig. 2*c*), the Perkin-Elmer GeneAmp kit and the manufacturer's protocol. The annealing temperature was 55 °C for 1 min, elongation at 72 °C for 1.5 min and denaturation at 94 °C for 1 min for 30 cycles. The expected 764bp RT-PCR product plus a new 431 bp product were visualized on agarose gels. The 431 bp fragment was purified and sequenced after asymmetric PCR amplification.

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reading frame is preserved in this aberrant mRNA and its predicted translation product is a schwannomin protein in which amino acids 39 to 149 are deleted (Fig. 3*b*). Because patient R2142 is affected, it is expected that this abnormal SCH protein is non-functional.

Search for SCH mutations in NF2-related tumours

DNA from 30 schwannomas and 30 meningiomas were also investigated for mutations in the same 6 coding exons. Somatic SCH mutations were observed in two schwannomas (tumours IC-J and IC-F) and two meningiomas (tumours IC-AB and IC-PO) which developed in patients not affected by NF2. In addition, in two schwannomas (tumours 28A and IC-BE) from NF2 patients, the SCH germ-line mutations were detected (Table 3). Loss of heterozygosity for chromosome 22 alleles in tumours IC-J, IC-F and IC-PO indicate that both copies of SCH are inactivated in three tumours. The absence of a normal SCH allele in tumour 28A and detection of the germ-line SCH mutation in the remaining chromosome leads to the same conclusion for a fourth tumour. Thus functional inactivation of SCH by a two-hit mechanism may operate in the tumorigenic process of both schwannomas and meningiomas.

Discussion

The homology of schwannomin to erythrocyte protein 4.1 and ezrin/moesin/talin family of genes suggests that schwannomin sublocalizes to the cell membrane and acts as a membrane-organizing protein. From these homologies we predict that the N-terminal domain binds to integrins and the α- helix domain binds to components of the cytoskeleton[23,25,29]. The protein may normally act like protein 4.1 which links transmembrane glycoproteins to the spectrin–actin complex of the cytoskeleton or, like talin, which interacts with vinculin and integrins, thereby regulating organization of cell shape and perhaps cytoplasmic extensions. For example, schwannomin might normally lead to stable cell–cell and cell–matrix interactions. Its absence may lead to cell migration, changes in cell shape or loss of contact inhibition. Ezrin and moesin are found preferentially in retraction fibres, blebs, microspikes, filopodia and lamellipodia, structures involved in cell exploration, attachment, movement, and events in epithelial–mesenchymal transformations in development.

Clearly, the elucidation of the role of schwannomin in the development of the NF2 phenotype awaits further analysis of its biochemistry and cellular biology. However, identification in NF2 patients of SCH mutations which almost always lead to a truncated protein, or of deletion involving a part of or a complete copy of the SCH gene, clearly indicates that loss of one functional copy of SCH causes the disease. The identification of potentially inactivating somatic SCH mutations in tumours known to occur in NF2 patients further substantiates the hypothesis that SCH has tumour-suppressor activity. Deletion studies have implicated a tumour-suppressor gene on chromosome 22 in the genesis of a group of tumours not seen with a high frequency in NF2: gliomas[30], pheochromocytomas[31], colon carcinoma[32] and breast cancer[33]. Genotypic analysis of such tumours known to lose chromosome 22 frequently will determine more precisely the tumour types in which functional inactivation of SCH may occur and thus document further the range of its potential tumour-suppressor activity.

Finally the demonstration that SCH mutations are associated with the NF2 phenotype has immediate implications. First, members of NF2 families can now be directly tested for mutations in this gene. Those individuals that have not inherited the gene will be spared repeated medical evaluation. Second, the phenotypic manifestations of NF2, which vary widely among patients and families[34], can now be analysed on the basis of fully characterized SCH mutation.

Note added in proof: Since submission of this article a candidate gene for NF2 has been reported by Trofatter *et al.* (*Cell* **72**, 791–800, 1993). Its cDNA and deduced translation product, termed Merlin, are almost identical to the SCH cDNA and to the schwannomin protein. □

Received 2 March; accepted 15 April 1993.

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ACKNOWLEDGEMENTS. We thank M. Giovannini and L. Selleri for their contribution to YAC isolation and characterization, and T. Melot, M. Peter, A. Krizus, L. Lebuis, M. Rousseau and J. Sarrazin for technical assistance; K. Morgan and M. Fujiwara of the NCE medical genetics computer unit for their assistance with the database searches; F. Resche, J. M. Sterkers, J. Philippon, P. Vialat and J. F. Gusella for providing cell lines or samples; and the NF2 patients and their families for their cooperation. This work was supported by grants from the European Community Commissions, the Ministere de la Recherche et de la Technologie, the Ligue Nationale contre le Cancer, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Association Française contre les Myopathies, the Council for Coordinating Cancer Research, the Association Neurofibromatose France, Medical Research Council of Canada, the National Neurofibromatosis Foundation Inc. and the Fonds de Recherche en Santé du Québec, the A. & M. Axson Johnson's Fond, the Swedish Medical Research Council, S. and L. Walker Foundation and the National Institutes of Health. K.H.X., C.D. and E.B. are recipient of fellowships from Comité Départemental de l'Yonne de la Ligue Contre le Cancer and from Association pour la Recherche sur le Cancer and from the Dutch Praeventiefonds respectively.

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