Molecular genetics of neurofibromatosis type 1 (NF1)

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Abstract

Neurofibromatosis type 1 (NF1), also called von Recklinghausen disease or peripheral neurofibromatosis, is a common autosomal dominant disorder characterised by multiple neurofibromas, café au lait spots, and Lisch nodules of the iris, with a variable clinical expression. The gene responsible for this condition, NF1, has been isolated by positional cloning. It spans over 350 kb of genomic DNA in chromosomal region 17q11.2 and encodes an mRNA of 11–13 kb containing at least 59 exons. NF1 is widely expressed in a variety of human and rat tissues. Four alternatively spliced NF1 transcripts have been identified. Three of these transcript isoforms (each with an extra exon: 9br, 23a, and 48a, respectively) show differential expression to some extent in various tissues, while the fourth isoform (2.9 kb in length) remains to be examined. The protein encoded by NF1, neurofibromin, has a domain homologous to the GTPase activating protein (GAP) family, and downregulates ras activity. The identification of somatic mutations in NF1 from tumour tissues strongly supports the speculation that NF1 is a member of the tumour suppressor gene family. Although the search for mutations in the gene has proved difficult, germline mutation analysis has shown that around 82% of all the fully characterised NF1 specific mutations so far predict severe truncation of neurofibromin. Further extensive studies are required to elucidate the gene function and the mutation spectrum. This should then facilitate the molecular diagnosis and the development of new therapy for the disease.

(J Med Genet 1996;33:2-17)

Key words: neurofibromatosis 1; molecular genetics.

The disease

The neurofibromatoses (NF) are a group of neurocutaneous syndromes primarily affecting tissues derived from the neural crest. The extreme clinical heterogeneity of NF has evoked several attempts to classify the disease into

several discrete entities.¹⁻³ At least two distinctive forms, neurofibromatosis type 1 (NF1) and neurofibromatosis type 2 (NF2), were recognised and later confirmed by the cloning of two separate genes, the NF1 gene on chromosome 17⁴⁻⁶ and the NF2 gene on chromosome 22.^{7,8} Other NF related syndromes were also reported, such as segmental NF,⁹ Watson syndrome,¹⁰ Noonan syndrome,¹¹ spinal NF,¹² familial café au lait spots (CLS),¹³ and Schwannomatosis.¹⁴ It remains to be determined whether such disorders are genetically discrete.

NF1, also called von Recklinghausen disease or peripheral neurofibromatosis, is a common autosomal dominant disorder affecting about 1 in 3000 to 5000 people. It exhibits full penetrance and a high mutation rate with 30 to 50% of NF1 patients representing a new mutation.¹⁵⁻¹⁷ The NF1 mutation events show bias towards paternal origin.^{18,19} The condition is characterised by multiple CLS, neurofibromas, and Lisch nodules of the iris.¹⁵⁻¹⁷ Its clinical expression in a wide spectrum involving multiple body systems varies greatly from one patient to another, between families, and even within a given family carrying the same mutation. Although the three characteristic features (CLS, neurofibromas, and Lisch nodules) each occur in over 90% of all NF1 patients by puberty, the number of lesions is extremely variable. Other features are only present in a minority of NF1 cases, such as learning disabilities, seizures, macrocephaly, short stature, scoliosis, pseudoarthrosis, and malignancies.²⁰⁻²²

The cloning of the NF1 gene

The NF1 gene was isolated by positional cloning. Initial exclusion mapping suggested that the NF1 gene was most likely to be on either chromosome 5 or 17.²³ Seizinger *et al*²⁴ first reported linkage of the NF1 gene to the nerve growth factor receptor (NGFR) gene located at 17q22 with the marker pE51. Barker *et al*²⁵ established significant linkage with the markers derived from chromosome 17, especially the centromeric markers p3.6 (D17Z1) and pA10.41 (D17S71). White *et al*²⁶ observed that the centromeric marker p3.6, and a new marker pHHH202 (D17S33), which maps to 17q11.2,²⁷ were very close to the NF1 gene.

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The *NF1* gene structure. (A) The size of the *NF1* gene and the embedded genes. (B) The size of the *NF1* transcript. (C) The location of *NF1*-GRD and the alternatively spliced *NF1* transcripts.

These results coupled with other reports summarised by Skolnick *et al.*²⁸ indicated that the *NF1* gene was located on the long arm of chromosome 17. Further genetic analyses by eight teams with 31 markers clearly located the *NF1* gene to the proximal long arm of chromosome 17. The combination of these data resulted in an estimation of the most likely order of 13 loci on chromosome 17 as: pter-pA10.41-EW301-p17H8(centromere)-pHHH202-NF1-EW206-EW207-EW204-EW203-CRI.L581-CRI.L946-HOX2-NGFR-qter.²⁹ In this multipoint linkage map, the two markers pHHH202 and EW206 narrowed the location of the *NF1* locus to about 3 cM of 17q11.2.

Cytogenetic abnormalities from two unrelated *NF1* patients provided the first physical evidence that the *NF1* gene was on the proximal long arm of chromosome 17. One of the patients had a balanced translocation between chromosomes 1 and 17, t(1;17)(p34.3;q11.2).³⁰ The other carried a balanced translocation between chromosomes 17 and 22, t(17;22)(q11.2;q11.2).³¹ Somatic hybrid mapping analysis showed that the derived order of DNA markers and the translocation breakpoints agreed with the genetic map.^{31,32} Two of the DNA markers derived from chromosome 17 detected the breakpoints on PFGE gels. One (c11.1F10) identified abnormal PFGE fragments in both translocation patients and placed the breakpoints within a 600 kb region expected to contain the *NF1* gene.³² The other (17L1) detected abnormal PFGE fragments in the t(1;17) patient and her affected offspring.³³ These results led to the construction of a long range restriction map of the *NF1* region encompassing the two breakpoints which were separated by a minimum of 60 kb.³⁴ Both PFGE analysis with a jump clone pEH1³⁵ and chromosomal walking with cosmids cEVI20 and cEVI36³⁶ further indicated that the breakpoints were approximately 60 kb apart.

Subsequent search for coding sequences in the region between the translocation breakpoints identified three candidate genes

EVI2A,³⁷ EVI2B,³⁸ and OMGP,³⁹ and a pseudogene AK3.⁴⁰ However, none of these genes spanned either of the two translocation breakpoints and no *NF1* specific mutations could be found in these genes from *NF1* patients tested.³⁷⁻³⁹

The *NF1* transcript was finally identified by two American groups led by Drs Collins and White. Using PFGE and Southern blotting analyses, Viskochil *et al.*⁶ identified three large deletions (190 kb, 40 kb, and 11 kb) in the *NF1* candidate region. The 11 kb deletion did not contain any of the previously characterised genes located between the translocation breakpoints. However, it partially deleted the 3.8 kb *EcoRI* fragment (probe EE3.8) which was shown to span the *NF1* t(17;22) breakpoint and strongly hybridised to a 3.35 kb *EcoRI* mouse fragment on Southern blots from somatic cell hybrids.^{36,37} By screening several cDNA libraries with the conserved sequence EE3.8, a number of cDNA clones were identified from the translocation breakpoint region (TBR), generating an overlapping cDNA sequence of approximately 5 kb. One of these cDNA clones hybridised to an mRNA of about 11 kb. These cDNA clones represented part of a new gene, TBR. By sequencing these cDNA clones, a 4 kb sequence was obtained which contained a single open reading frame of about 3.3 kb.⁴ The TBR gene was found to be interrupted by the translocation t(17;22), the three large deletions, and six small *NF1* specific mutations in the coding sequence.⁴⁵

Simultaneously, Wallace *et al.*⁶ screened cDNA libraries with the jump clone pEH1 and the YAC clone A113D7,⁴¹ spanning the two translocation breakpoints. Two cDNA clones (p5 and B3A) were isolated covering a sequence of 2012 bp with a single open reading frame containing six exons, and spanning at least 33 kb of genomic DNA. Northern blotting analysis with the clone p5 led to the identification of a transcript of about 13 kb. This gene, initially termed NF1LT (*NF1* large transcript) gene, was found to be disrupted by the translocation t(17;22). An *NF1* specific de novo insertion of

0.5 kb was also detected in the gene. NF1LT and TBR represent the same gene, NF1, but were isolated from different cDNA clones.

The NF1 gene structure

NF1 spans over 350 kb of genomic DNA in chromosomal region 17q11.2 and encodes an mRNA of 11–13 kb containing at least 59 exons (figure). An 8457 bp open reading frame of the NF1 transcript predicts a protein of 2818 amino acids with an estimated molecular mass of 327 kDa.^{5 6 42–44}

There is a 360 amino acid region of the predicted gene product which shows homology to the catalytic domain of the mammalian GTPase activating protein (GAP) and the products of the yeast IRA1 and IRA2 genes.^{44 45} This region is referred to as NF1 GAP related domain (NF1-GRD) and is located in the central portion of the NF1 gene, encompassing exons 21–27 (figure).

The three genes, EVI2A, EVI2B, and OMGP, are embedded within intron 27 (figure).^{37–39} These genes are transcribed in the opposite orientation to NF1. The OMGP gene maps within 4 kb of the t(1;17) breakpoint. The EVI2A and EVI2B genes lie approximately 20 and 5 kb telomeric to the OMGP gene, respectively. The intronless AK3 pseudogene lies in intron 37 and is transcribed in the same orientation as NF1 (figure).⁴⁰

Four alternatively spliced NF1 transcripts have been identified. The first is called GRD II, with a 63 bp insertion (exon 23a) in the GAP related domain, and the second 3'ALT, with a 54 bp insertion (exon 48a) at the 3' end (figure).^{4 43 46 47} The third isoform of the NF1 transcript (termed here 5'ALT1), about 2.9 kb in length, has an open reading frame predicting a protein of 551 amino acid residues and sharing the same 547 amino-terminal residues with the original NF1 product (figure).⁴⁸ A recently identified isoform of the NF1 transcript (termed here 5'ALT2) contains a 30 bp insertion (exon 9br) between exons 9b and 10 (figure).⁴⁹

NF1 gene expression

THE NF1 mRNA

NF1 has been shown to be widely expressed in many tissues. The 13 kb transcript was detected in human brain, neuroblastoma, kidney, and melanoma.⁶ An 11–13 kb transcript was detected in murine kidney, brain, B16 melanoma cells, but not in mouse skin, spleen, thymus, or liver.⁴⁵ Expression of the NF1 gene was also found by RT-PCR analysis in many human tissues, including WBC, skin fibroblast, brain, spleen, lung, muscle, neuroblastoma, thymoma, neurofibroma, an NF1 neurofibrosarcoma cell line, a colon carcinoma cell line, and breast cancer.^{6 50 51} Ribonuclease protection assays and in situ hybridisation analysis showed that the NF1 transcript was also expressed in a variety of tissues in the chick embryo.⁵²

The alternatively spliced isoforms of the NF1 transcript have been investigated to determine

their expression pattern in different tissues. GRD I and GRD II are equally represented in the RT-PCR products amplified from EBV transformed lymphocytes either from NF1 patients or from normal controls.⁴³ These two isoforms have been found to have equal ratios in human kidney, placenta, and lung.⁵¹ Using RT-PCR, Andersen *et al*⁴⁶ were able to detect GRD I and GRD II transcripts in all tissues tested, but with a variation in the relative amounts of the two NF1 transcripts. These tissues included kidney, spleen, stomach, lung, colon, muscle, brain, peripheral nerve, skin fibroblasts, an EBV lymphoblastoid cell line, HeLa cells, HepG2, HEL, HT29, and NF88-2 cell lines, neuroblastoma, thymoma, breast carcinoma, and neurofibroma. Interestingly, the expression of these two isoforms has been observed to be associated with the differentiation status of a particular tissue.⁵⁰ GRD I predominates in fetal brain and undifferentiated primitive neuroectodermal tumours, whereas GRD II was predominantly expressed in adult brain and differentiated cell lines. GRD II expression was higher in 22 week fetal brain cells than in 20 week fetal brain cells. Treatment of the SH-SY5Y neuroblastoma cells with retinoic acid resulted in an immediate switch from GRD I to GRD II expression during the course of the induced differentiation. In contrast, Suzuki *et al*⁵¹ found a higher GRD I/GRD II ratio in human adult brain while GRD II was preferentially expressed in most (13/16) primary brain tumours analysed. Gutmann *et al*⁵³ used high doses of forskolin or 8-bromo-cAMP to induce both myelin PO protein and neurofibromin. They observed that differentiation induced by either cAMP stimulation or high density culture conditions was associated with predominant expression of GRD II and that GRD I expression was observed in untreated Schwann cells or those stimulated with mitogenic doses of forskolin or 8-bromo-cAMP. Using RNase protection and RT-PCR, Baizer *et al*⁵⁴ found that most chicken tissues including brain expressed predominantly GRD II throughout embryonic development. However, GRD I increased dramatically in brain during later development.

Gutmann *et al*⁴⁷ analysed 22 different human tissue samples to investigate the expression of the transcript isoform 3'ALT by RT-PCR. The highest expression of 3'ALT was found in adult cardiac muscle, skeletal muscle, and bladder. This isoform was also expressed in fetal cardiac muscle, adult left ventricle, and adult cardiac Purkinje cells. Trace levels of the 3'ALT expression was detected in brain and nerve.

Northern blot analysis enabled the simultaneous identification of at least three transcripts of about 2.9 kb, 11 kb, and 13 kb in both kidney and placenta.⁴⁸ The 11–13 kb transcripts are consistent with three transcripts reported previously.^{5 6} S1 mapping confirmed that the 2.9 kb mRNA was expressed as the NF1 transcript isoform 5'ALT1 lacking the GAP related domain.⁴⁸

Recently, the NF1 transcript isoform 5'ALT2, containing exon 9br, has been analysed by RT-PCR in several normal tissues and

brain tumours.⁴⁹ A high level expression of this isoform was observed in the central nervous system but no expression was detected in all the other normal tissues tested. Furthermore, the expression of this transcript decreased in medulloblastomas and oligodendrogliomas.

THE NF1 GENE PRODUCT: NEUROFIBROMIN

Using antisera raised against synthetic peptides or fusion proteins containing the GRD region of neurofibromin, a 220–320 kDa protein was identified in a variety of human and murine tissues such as HeLa cells, NIH3T3 cells, spinal cord, brain, neuroblastoma, and PC12 pheochromocytoma cell lines.^{55–60} Immunoprecipitation followed by western blotting allowed the detection of neurofibromin at highest levels in adult rat brain and spinal cord. Neurofibromin was also identified in the sciatic nerve and adrenal gland and, to a lesser extent, in liver, spleen, and pancreas. Only minute amounts of neurofibromin was detected in cardiac tissue. No neurofibromin was detectable in rat skeletal muscle, lung, kidney, or skin. Similarly, less neurofibromin was detected in human peripheral nerve than in human spinal cord. Immunostaining of tissue sections in the same study detected neurofibromin in neurones, oligodendrocytes, dorsal root ganglia, and non-myelinating Schwann cells but not in astrocytes or myelinating Schwann cells.⁵⁷

The observed size of neurofibromin (220–320 kDa) is smaller than that (327 kDa) predicted based on the NF1 open reading frame. Recently, the complete coding sequence has been subcloned into a baculovirus transfer vector and expressed in the infected Sf9 insect cells. The purified full length neurofibromin proved to be approximately 220 kDa.⁶¹ This discrepancy is most likely to be caused by protein folding during electrophoresis. There is no evidence of glycosylation or processing of the full length neurofibromin.⁶²

In a recent study, neurofibromin was identified in all parts of the brain, especially in large projection neurones such as pyramidal and Purkinjee cells.⁶³ Neurofibromin was also localised to keratinocytes and melanocytes in developing rat and human skin.⁶⁴ Gutmann *et al*⁶⁵ examined the expression of neurofibromin during in vitro myoblast differentiation. Differentiating C2C12 cells were shown to up-regulate the expression of NF1 mRNA and this resulted in an increase in neurofibromin levels. This was paralleled by a decreased level of activated p21^{ras}. Nakamura *et al*⁶⁶ observed strong expression of neurofibromin specific to the Schwann cells of normal peripheral nerves in an N-nitroso-N-ethylurea induced Syrian hamster NF1 model. Although neoplastic Schwann cells exhibited neurofibromin specific signals, the proportion of positive cells was diminished.

The availability of antibodies raised against portions of the NF1 product has also enabled the subcellular localisation of neurofibromin. DeClue *et al*⁵⁵ lysed NIH 3T3 cells in the absence of detergent and separated them into particulate and non-particulate fractions. By

immunoprecipitation analysis, neurofibromin was found solely in the particulate fraction while GAP was primarily in the soluble fraction. Hattori *et al*^{59,67} also located neurofibromin to the particulate fraction using similar strategies. In another study with 1% NP-40 in various cell lysates, NF1-GAP activity was found distributed between post 100 000 × *g* spin supernatant and the particulate fraction.⁶⁸ Immunohistochemical staining showed the location of neurofibromin in the cytoplasm of cells from various tissues.⁵⁷ Further experiments showed the co-localisation of neurofibromin with cytoplasmic microtubules.⁶⁹

NF1 gene function

TUMOUR SUPPRESSION

The identification of oncogenes and tumour suppressor genes has directly confirmed the involvement of genetic components in tumorigenesis.⁷⁰ Several genetic models have been proposed for tumorigenesis. Examples include the “two hit” model for retinoblastoma (RB),⁷¹ the dominant negative model for the mutant p53,⁷² and the multistep model for the familial adenomatous polyposis (FAP)/colorectal cancer syndrome (CRC).⁷³ In all these models, the inactivation of one or both alleles of a tumour suppressor gene is supposed to be involved in the development of a particular type of malignancy.

NF1 patients have multiple benign tumours which predispose to malignancies.^{15,74} Sequence analysis of NF1 showed a marked homology of a 360 residue region of predicted neurofibromin to the catalytic domain of mammalian GAP and yeast IRA1 and IRA2 gene proteins which can downregulate p21^{ras} activity.^{44,75} These observations suggest that NF1 is a tumour suppressor gene.

A second hit or a somatic mutation in a tumour suppressor gene may occur in tumour cells such as LOH or other types of genetic alterations which inactivate the gene. The first somatic mutations were detected in the NF1-GRD region by SSCP analysis and DNA sequencing.⁷⁶ These mutations involve an A to G transition leading to a Lys to Glu substitution at codon 1423 in exon 24 in colon adenocarcinoma, myelodysplastic syndrome, and anaplastic astrocytoma, and an A to C transversion resulting in a Lys to Gln substitution at the same codon in an anaplastic astrocytoma. This Lys is one of the 14 amino acids in the catalytic domain that are absolutely conserved across all members of the GAP family.^{44,75,77} In vitro site directed mutagenesis analysis of the A to G transition showed that the GAP activity of the mutant NF1-GRD is 200 to 400-fold lower than that of the wild type. Sawada *et al*⁷⁸ reported a mutational SSCP band in an NF1 exon in DNA extracted from a malignant Schwannoma of an NF1 patient. Ludwig *et al*⁷⁹ screened 57 patients with myelodysplastic syndrome (MDS) and 27 patients with acute myelocytic leukaemia (AML) for mutations in exon 24 and its flanking sequences using restriction enzyme digestion and PCR-SSCP analysis. None of these patients showed a codon

1423 mutation. However, a patient with chronic myelomonocytic leukaemia (CMML) exhibited a 3 bp deletion within the splice acceptor region in front of exon 24.

Some large somatic mutations identified in NF1 by Southern blotting include a loss of the wild type allele in three pheochromocytomas from NF1 patients,⁸⁰ a large homozygous deletion extending from the 5' end of the gene to intron 28 in a sporadic malignant melanoma,⁸¹ a 200 kb deletion in a neurofibrosarcoma from a patient with NF1,⁸² and variants in four out of 10 human neuroblastoma lines expressing little or no neurofibromin, one of which is a large homozygous deletion.⁶⁰

However, the actual mechanisms of tumorigenesis, in which NF1 is involved, remain unknown. No evidence of LOH has been observed in neurofibromas.⁸³ There may be several explanations for this. Firstly, neurofibromas may be multicellular in origin, as proposed by Fialkow *et al.*⁸⁴ However, Skuse *et al.*⁸³ showed that neurofibromas from all eight NF1 patients tested were of monoclonal origin by performing X chromosome specific RFLP analysis and methylation sensitive digestion with *HpaII*. The second explanation is that a second mutation in another gene may be required for the genesis of neurofibromas, or they may arise because of the loss of function of one allele. The latter is true in FAP, in which only one mutation is required for tumorigenesis.⁷³ The NF1 mutant allele may function in a dominant negative fashion causing partial functional inactivation of the wild type allele as does the mutant p53.⁷² The third explanation is that a second mutation in the NF1 gene may exist in neurofibromas, which is not identifiable by LOH. Such mutations may represent a point mutation or a small deletion or insertion.

In some malignant tumours from NF1 patients, however, LOH is confined to chromosomal region 17p only and one of the two p53 alleles has been deleted.^{85,86} Point mutations in the retained p53 allele have also been observed in neurofibrosarcomas.⁸⁵ Several other tumour suppressor genes may be located on chromosome 17.⁸⁷⁻⁹⁰ These observations suggest that the NF1 mutations may represent small alterations and may be involved in the multistep process of tumorigenesis in certain malignant tumours.

It should be noted that although no mutations in the NF1 gene have so far been detected in some types of tumours such as MDS, AML,⁷⁹ and familial gliomas,⁹¹ further mutation analysis in these tumours is required because only a small portion of the NF1 gene has been screened in such tumour studies.

NEUROFIBROMIN AND GAP ACTIVITY

Neurofibromin shows homology to various members of the GAP superfamily.^{44,45,92} These proteins include the mammalian GAP, the IRA1 and IRA2 gene products of *Saccharomyces cerevisiae*, the sar1 gene product of *Schizosaccharomyces pombe*, and *Drosophila* GAP1. The homology of neurofibromin to the mammalian GAP and the *Drosophila* GAP1 is re-

stricted to the catalytic domain of 360 amino acids. There is more extensive homology of neurofibromin to the yeast IRA1, IRA2, and sar1 gene proteins. The first mammalian GAP, a cytosolic protein with a molecular mass of 120 kDa (p120-GAP), was identified by its property of stimulating the conversion of the active GTP bound form of the ras proteins to the inactive GDP bound form.⁹³ The IRA1 and IRA2 genes encode large proteins of 2938 amino acids and 3079 amino acids respectively, which negatively regulate the yeast ras-cyclic AMP pathway.^{94,95} The sar1 gene product (80 kDa) regulates ras1 but does not appear to participate in the modulation of adenylate cyclase.⁷⁷ The *Drosophila* homologue GAP1 was also shown to be a negative regulator of ras1.⁹⁶ All these GAP-like proteins can down-regulate the activity of normal ras proteins. They may also function as effectors for ras proteins.⁹³

The ras genes were first discovered to be oncogenes in the acutely transforming Harvey and Kirsten rat sarcoma viruses.⁹⁷ These viral oncogenes were later found to be derived from cellular proto-oncogenes in the host's genome. Among more than 30 ras genes, three closely related ones are known as H-ras, K-ras, and N-ras, encoding a 21 kDa protein.^{87,98,99} The p21 ras protein (p21^{ras}) binds guanine nucleotides with high affinity and possesses an intrinsic GTPase activity, functioning as a single switch molecule: in the active GTP bound state, it is switched on; in the GDP bound state, it is switched off. This molecular switch plays a critical role in the control of cellular growth and differentiation. Activating mutations in ras lead to aberrant signalling for cell proliferation and are involved in the genesis of many human malignant tumours.¹⁰⁰

Genetic experiments suggest that the NF1-GRD, as other GAP-like proteins, can interact with yeast and mammalian ras proteins. Xu *et al.*⁷⁵ cloned DNA fragments covering the NF1 GAP related domain (NF1-GRD) into the yeast expression plasmid pKT10 and transformed the yeast IRA1⁻ and IRA2⁻ mutants with the constructs. They found that the IRA⁻ mutations were complemented by expressing the NF1-GRD peptide. Similar results were obtained in other studies by cloning and expressing cDNA fragments containing the NF1-GRD in a yeast expression system.^{101,102} Previous work has shown that either the complete protein or the catalytic domain of the mammalian GAP expressed in yeast can suppress the IRA1⁻ phenotype.^{94,103} Ballester *et al.*¹⁰¹ also showed that the expressed NF1-GRD peptide could inhibit both wild type and mutant activated human H-ras genes that were expressed in yeast. However, the human GAP can inhibit the wild type human H-ras protein but not the mutant H-ras protein.¹⁰³ Nur-E-Kamal *et al.*¹⁰⁴ found that both NF1-GRD and a fragment of 91 amino acids derived from NF1-GRD were able to reverse v-Ha-ras induced malignant phenotype. Nakafuku *et al.*¹⁵ examined a pool of mutagenised NF1 expression plasmids and obtained two mutant NF1 cDNA clones that could suppress oncogenic ras including RAS-

2Val19 cells. When expressed in mammalian cells, these mutant NF1-GRDs had the ability to reverse the morphological phenotype of v-ras transformed NIH3T3 cells. Wood *et al*¹⁰⁶ analysed two ras mutants in yeast. One of these mutants in the $\alpha 3$ region of ras was found to be less sensitive to neurofibromin activity and to bind less well to neurofibromin.

Biochemical analysis has shown that the GAP related domain of neurofibromin has the ability to accelerate the conversion of the active GTP bound p21^{ras} to the inactive form of GDP bound p21^{ras} and thus downregulates the ras activity. Xu *et al*⁷⁵ used the purified glutathion S-transferase/NF1-GRD fusion protein expressed in *E coli* and observed that this fusion protein strongly stimulated the GTPase activity of the yeast ras2 and human H-ras proteins. Ballester *et al*¹⁰¹ found that a peptide of 412 amino acids containing the NF1-GRD, when expressed in yeast, increased the H-ras GTPase activity. Martin *et al*¹⁰² used an epitope tagged peptide of 474 amino acids, which flanks the NF1-GRD and is expressed in the Sf9 insect cells, and showed that the GTPase activity of wild type ras protein was stimulated by the NF1-GRD. All these studies showed that the peptides containing the NF1-GRD did not stimulate the oncogenic ras mutants H-ras^{Val-12}, ras2^{Val-19}, ras^{Ala-42}, N-ras^{Asp12}, or N-ras^{Val-12}, although the GTPase activity of the N-ras^{Ala-38} effector mutant was slightly increased.^{75 101 102} Bollag and McCormick⁶⁸ reported that although both human GAP and NF1-GRD could bind oncogenic mutants of p21^{ras}, neither could stimulate their GTPase activity. Andersen *et al*⁶⁶ showed that both forms of the NF1-GRD, GRD I and GRD II, could not only complement the loss of IRA function but also increase the conversion of GTP bound ras to its GDP bound form, although the GRD II had a weaker effect. In addition, lower levels of neurofibromin were detected in malignant tumours from NF1 patients while normal levels of both p120-GAP and p21^{ras} were obtained in these tumours.^{58 107} In these studies, the total GTPase activating activity was found to be reduced. Furthermore, site directed mutagenesis analysis confirmed the decreased GTPase activating activity of the mutation at residue 1423 in the NF1-GRD region.^{76 108} Recently, Pouillet *et al*¹⁰⁹ extensively examined the role of lysine at codon 1423 by mutating it to all possible different amino acids and found that lysine was the only amino acid that produced a functional NF1 product having normal GAP activity. They also found that the mutation at codon 1434 from phenylalanine to serine could partially restore GAP activity in the lysine mutant at codon 1423.

Additionally or alternatively, neurofibromin may serve as a downstream effector for p21^{ras}. This model has also been proposed for the interaction between p120-GAP and p21^{ras}.⁹³ Mutations in the p21^{ras} effector domain inactivated the transforming ability of ras and blocked GTPase activation by p120-GAP.^{110 111} Similarly, the NF1-GRD peptides did not stimulate the GTPase activity of mutant p21^{ras} in the effector domain.^{68 75 102} Both p120 GAP

and NF1-GRD could bind well to constitutively signalling oncogenic p21^{ras} but interacted poorly with the effector mutant in which signalling is impaired.⁶⁸

The NF1-GRD and p120-GAP showed different affinities for p21^{ras}. The affinity of the NF1-GRD was estimated to be 20-fold higher than that of p120-GAP, while the specific activity of NF1-GRD was approximately 30-fold lower than that of p120-GAP.¹⁰² Further studies indicated that the NF1-GRD binds to p21^{ras} proteins up to 300 times more efficiently than p120-GAP.⁶⁸ However, at low N-ras concentrations, the p120-GAP and NF1-GRD activities were comparable. This suggests that neurofibromin may be a significant regulator of p21^{ras} activity, particularly at low p21^{ras} concentrations.¹⁰²

The NF1-GRD and human GAP also showed differences in sensitivity to inhibition by various lipids. Mitogenesis induced by lipid related mitogenic agents was found to depend on cellular ras activity.¹¹² It was later observed that several lipids inhibited the human GAP activity.¹¹³ Golubic *et al*¹¹⁴ reported that arachidonic acid inhibited the stimulation activity of the catalytic fragments of the GAP, NF1-GRD, and the yeast IRA2 protein while phosphatidic acid (containing arachidonic and stearic acid) inhibited NF1-GRD but not the catalytic fragments of the GAP or IRA2 protein. Han *et al*¹¹⁵ obtained similar inhibition of the GAP activity by arachidonic acid and also found that GAP stimulation of H-ras was increased by prostaglandins PGF2 α or PGA2 and decreased by PGI2, whereas NF1-GRD activity was unaffected by these prostaglandins. In another study, micromolar concentrations of arachidonate, phosphatidate, and phosphatidylinositol-4,5-bisphosphate affected only NF1-GRD. The p120-GAP activity was only weakly affected by these lipids.⁶⁸ Furthermore, the detergent dodecyl β -D-maltoside was found to be a more stable selective inhibitor specifically of the NF1-GRD activity. The selective inhibition of these lipids can be used to differentiate the two types of GTPase activating activity in human tissues and also indicates possible subtle distinctions in the biological functions of the NF1-GRD and p120-GAP.

Nearly all of the above experiments were performed with the NF1-GRD, not the full length neurofibromin. Bollag *et al*⁶¹ have purified full length neurofibromin obtained from a baculovirus/Sf9 expression system containing the complete coding sequence. To examine the GAP activity of full length neurofibromin, activation of the ras GTPase activity was assayed by monitoring phosphate release. The stimulation activity of full length neurofibromin was found to be similar to that of the truncated protein, NF1-GRD. Full length neurofibromin also showed similar sensitivity to the detergent dodecyl maltoside. Such observations allow a cautious extrapolation from results of experiments with the NF1-GRD peptide to interpretations of the full length neurofibromin activity.

Recently, Johnson *et al*¹¹⁶ introduced a full length NF1 cDNA into melanoma cell lines

deficient in neurofibromin expression. This introduction resulted in growth inhibition and induced differentiation in these cell lines. Overexpression of neurofibromin in NIH3T3 cells was growth inhibitory, but did not alter the level of GTP bound Ras in the cells. In addition, transformation by v-ras resistant to neurofibromin GAP activity was also inhibited by overexpressed neurofibromin. These results suggest that neurofibromin can inhibit Ras dependent growth by a mechanism independent of its GTPase activating function.

NEUROFIBROMIN AND TUBULIN

Tubulin has been found to be another inhibitor of the GTPase activating activity of both the NF1-GRD peptide and the full length neurofibromin.⁶¹ The interaction between neurofibromin, either truncated (NF1-GRD) or full length, and tubulin was shown by their copurification. This interaction was sensitive to the microtubule depolymerising agent colchicine. Bovine brain tubulin could cause significant inhibition of neurofibromin activity while the activity of p120-GAP was not affected by tubulin. Some tubulin binding determinants were localised to an 80 residue segment immediately N-terminal to the GAP related domain of neurofibromin by the identification of the reduced sensitivity of a truncated mutant neurofibromin to tubulin. In the same study, full length neurofibromin was found to be considerably less sensitive to phosphatidic acid than the truncated fragments (NF1-GRD and N-NF1-GRD) in the presence or absence of tubulin. This suggests that tubulin and phospholipids indeed bind to distinct sites and that other domains of full length neurofibromin impede the interaction of phospholipids with the GAP related domain.⁶¹ The relationship observed between neurofibromin and tubulin is consistent with the colocalisation of neurofibromin and microtubules.⁶⁹ Tubulin is the primary component of microtubules. Microtubules play a role in many aspects of cellular organisation.^{117,118} The interaction between neurofibromin and tubulin may be implicated in any cellular functions and it has been speculated that neurofibromin is a link between tubulin and the growth regulator ras.⁶¹

OTHER FUNCTIONS AND MODIFYING GENES

Neurofibromin is involved in the control of cellular growth and differentiation by at least three possible mechanisms: as an upstream downregulator of p21^{ras}, a downstream effector of p21^{ras}, and a link between tubulin and p21^{ras}. These mechanisms are not mutually exclusive and they may function in concert in various tissues. However, the NF1-GRD is the only domain that has been extensively examined and accounts for only about 13% of neurofibromin. To date, the vast majority of mutations identified in the NF1 gene do not disrupt the NF1-GRD. Furthermore, an isoform of neurofibromin lacking the NF1-GRD has been identified.⁴⁸ A recent study showed that when a mutation at codon 1434 from phenylalanine

to serine was introduced into wild type neurofibromin, the resulting product acquired the ability to suppress the activated phenotypes of RAS2Val19 cells. However, this suppression did not involve ras interaction since the mutant did not stimulate the intrinsic GTPase activity of RAS2Val19 protein and did not have an increased affinity for ras proteins.¹⁰⁹ All these data suggest that neurofibromin may participate in other biological activities.

A family based analysis of the variable expression of NF1 suggested a strong genetic component in the variation of NF1 expression but a minor role that the NF1 mutation type may play.¹¹⁹ Modifying genes may be involved in the variability of NF1 expression. This is supported by the observation that identical NF1 specific mutations do not cause the same phenotypes in unrelated NF1 patients.^{4,120-122} Five NF1 patients with large deletions removing the whole NF1 gene and its flanking sequences showed large numbers of neurofibromas.¹²³ This suggests that deletion of an unknown gene in the NF1 region may affect tumour initiation or development. Neurofibromin has several potential sites for phosphorylation, suggesting that neurofibromin could be regulated by kinases.⁴² The activity of p120-GAP was found to be modified by phosphorylation of tyrosine residues when activated by various growth factors.^{124,125} However, neurofibromin lacks similar domains to SH2 and SH3 of GAP which are thought to direct interactions with phosphotyrosine proteins involved in signal transduction.¹²⁶

The function of neurofibromin may be modified by different mechanisms. Neurofibromas are known to contain a large number of mast cells, the development of which can be induced by stem cell factor (SCF). The receptor of SCF is encoded by the c-kit gene. Hirota *et al*¹²⁷ observed that the c-kit mRNA was strongly expressed in mast cells in the neurofibromas and that the amount of SCF cDNA was greater in neurofibroma tissues than in normal skin tissues. Ryan *et al*¹²⁸ found that SCF was secreted by both normal Schwann cells and a malignant Schwannoma. The c-kit gene product was not expressed in normal Schwann cells but expressed in the malignant Schwannomas. Another observation suggested that certain growth factors such as platelet derived growth factor and transforming growth factor β 1 may be implicated in the development of neurofibromas in NF1.¹²⁹ These factors may thus function by direct or indirect interaction with neurofibromin. In addition, the functions of the three embedded genes (EVI2A, EVI2B, and OMGP) at the NF1 locus and their relationship with NF1 remain to be examined.³⁷⁻³⁹ These genes may participate in the modification of NF1 function. The search for homologous loci has shown NF1 related loci on chromosomes 2, 12, 14, 15, 20, 21, and 22.¹³⁰⁻¹³² The locus on chromosome 12 contains open reading frames homologous to NF1 in at least two exons and is expressed in a number of tissues.¹³¹ Such expressed sequences may also play a role in the regulation of NF1.

Germline mutation analysis

MUTATION SPECTRUM

Mutation analysis in the NF1 gene has proved difficult.¹³³ The techniques commonly used for mutation detection in the NF1 gene include two relatively simple ones: heteroduplex analysis (HA) and single strand conformation polymorphism analysis (SSCP). Reportedly, neither HA nor SSCP can identify 100% of point mutations.¹³⁴ Recently, several more sensitive techniques have been adapted to mutation analysis at the NF1 locus. Using denaturing gradient gel electrophoresis (DGGE), Valero *et al*¹³⁵ identified NF1 specific mutations in four of 70 unrelated NF1 patients by screening exons 29 and 31. Hoffmeyer *et al*¹³⁶ used a polymorphism in exon 5 of the NF1 gene to evaluate allele specific dosage of the NF1 mRNA in fibroblast-like cells and observed reduced amounts of mRNA from one of the NF1 alleles in six out of eight NF1 patients. Using chemical cleavage of mismatch (CCM), Purandare *et al*¹³⁷ screened 70% of the NF1 coding sequence amplified by RT-PCR from mRNA or by PCR from genomic DNA. They identified seven new NF1 specific mutations from 25 unrelated NF1 patients. In another mutation study, the protein truncation test (PTT) based on the *in vitro* transcription-translation of the whole NF1 coding sequence allowed the identification of truncated neurofibromin in 14 of 21 NF1 patients.¹³⁸

To date, over 100 NF1 specific germline mutations have been reported to the International NF1 Genetic Analysis Consortium.¹³⁹ Seventy-eight NF1 specific mutations have been described (table 1). The mutations include three cytogenetic rearrangements, 34 deletions, 12 insertions, two duplications, and 27 substitutions.

Interestingly, 82% (49/60) of all the fully characterised NF1 specific mutations are either nonsense or frameshift mutations. These mutations presumably lead to severe truncation of neurofibromin. The large deletions are also expected to cause severe truncation or complete removal of the gene. Nonsense and frameshift mutations have been reported to be associated with decreased or absent mRNA levels in a variety of both human^{159–162} and rodent genes.^{163–165} Mutations introducing an inappropriate stop codon have also been reported to result in undetectable protein.¹⁶⁶ Reduced amounts of mRNA from one of the NF1 alleles were observed in six of eight NF1 patients.¹³⁶ However, no mRNA reduction was observed from an NF1 patient with an inappropriate stop codon in the centre of NF1 in the same study. In contrast, a decrease in mutant mRNA level has been observed in a 10 bp duplication in exon 38¹⁵⁵ and a splice junction mutation in intron 31,¹⁵⁷ both of which cause frameshift.

Seven germline substitutions identified in the NF1 gene do not generate premature stop codons (table 1). These mutations replace amino acids that may be important for maintaining the tertiary structure of neurofibromin as found in the case of rhodopsin.¹⁶⁷ Analysis by site directed mutagenesis of the substitution A4267G in exon 24 showed a decreased GAP-

like activity of the mutant fusion protein.^{76 108 109}

The tandem duplication 5095dup42bp in exon 28 does not cause a frameshift and the predicted neurofibromin has an additional sequence of 14 amino acids, 13 of which contain a motif normally found in neurofibromin from codon 1701 to 1713.¹⁵⁴ It is not clear how this mutation affects gene function. Two exons of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene were observed to be duplicated in a patient with Lesch-Nyhan syndrome.¹⁶⁸ This duplication generated a transcript of increased length consistent with the size of the duplicated exons and the resulting enzyme had no detectable activity.¹⁶⁹ Another duplication in the collagen type 2A gene has been identified in spondyloepiphyseal dysplasia, resulting in excessive post-translational modification of the gene product.¹⁷⁰ It was suggested that the tandem duplication in NF1 might result in changes in protein folding, glycosylation, or excessive post-translational modification.¹⁵⁴ Alternatively, the duplication may alter the tertiary structure of mRNA and thus affect its stability or translation efficiency or both.¹⁵¹

The 3 bp deletion 2970delAAT results in loss of the codon ATG (methionine) with a silent change of codon 990 from ACA (threonine) to ACG (threonine).¹²² RT-PCR heteroduplex analysis has confirmed the expression of this mutation at the RNA level in this study. The neurofibromin has been found to be structurally abnormal (J Downward, personal communication).

The 320 bp insertion in intron 32 of NF1 results in a splicing error leading to loss of exon 33.^{6 146} Other examples of splicing errors in the NF1 gene resulted from four intronic substitutions in the splice consensus region.^{137 157 158} All these splicing abnormalities are expected to result in truncated neurofibromin.

The majority of the NF1 specific mutations are unique and have been found in only one family. However, six unrelated NF1 patients from different countries carry an identical transition (C5839T) in exon 31 (table 1). Three other examples of recurrent causative mutations include the mutations 2027insC in exon 13, 2970delAAT in exon 17, and 5449insC in exon 29, each of which was found in two unrelated NF1 patients (table 1).

Neurofibromin has been shown to function as a GAP-like protein.^{75 101 102} However, only six germline mutations have been described in the NF1-GRD region and all the other fully characterised small or point mutations do not interrupt this domain (table 1).

MUTATION MECHANISMS

Mutagenesis in mammalian genomes is shown to be influenced by genomic position.^{171 172} Mutations have been found to occur non-randomly with respect to the surrounding DNA sequence in human genes.^{173–175} NF1 has a mutation rate about 100-fold higher than the average mutation rate per generation per locus. The mutations observed so far in the NF1 gene are not randomly distributed. This suggests

Table 1 Germline mutations identified in the NF1 gene

Mutation	Location	Neurofibromin change predicted	No of patients	Reference
Chromosomal aberrations				
t(1;17) (p34.3;q11.2)		?	1	30
t(17;22) (q11.2;q11.2)		?	1	31
del(17) (pter→cen::q11.2 or 12→qter), +?r(17) (cen→q11.2 or q12)		?	1	140
Large deletions				
del(731–835)	Exon 6	Truncated	1	138
del(1528–1845)	Exons 10c–12a	Truncated	1	138
517bpd	Introns 32–33	Truncated	1	141
Large deletion	Introns 2–3	Loss of 28 amino acids	1	142
11kdel	3' end	?	1	5
40kdel	Undefined	?	1	143
40kdel	Containing EVI2	?	1	5
80kdel	Undefined	?	1	143
90kdel	5' end	?	1	144
190kdel	Undefined	?	1	5
Hemizygosity	Intron 27-exon 37	Truncated	2	145
>700kdel	NF1 and adjacent loci	No neurofibromin	5	123
Large insertions				
320bpins	Intron 32	Truncated	1	146
(splicing error: exon skipping)				
10kbins	Undefined	?	1	143
10kbins	Undefined	?	1	143
Small deletions				
2970delAAT	Exon 17	Loss of Met	2	122
3050delAATT	Exon 18	Truncated	1	138
del(4088–4110)	Exon 23	Truncated	1	138
4190delT	Exon 24	Truncated	1	147
4868delAC	Exon 28	Truncated	1	141
5010delG	Exon 28	Truncated	1	148
5077del(13bp)	Exon 28	Truncated	1	149
5108delAG	Exon 28	Truncated	1	150
5123delCCACC	Exon 28	Truncated	1	151
5679delACTG	Exon 30	Truncated	1	152
5843delAA	Exon 31	Truncated	1	135
5949delA	Exon 32	Truncated	1	152
?delGAGA	Exon 34	Truncated	1	153
?delT	Exon 34	Truncated	1	153
7260delT	Exon 41	Truncated	1	138
7745del(10bp)	Exon 44	Truncated	1	122
Small insertions				
2027insC	Exon 13	Truncated	2	138
5449insC	Exon 29	Truncated	2	143
5466insT	Exon 29	Truncated	1	143
5816insG	Exon 31	Truncated	1	150
5852insTT	Exon 31	Truncated	1	120
6519insG	Exon 34	Truncated	1	137
7485insGG	Exon 42	Truncated	1	137
Duplications				
5095dup(42bp)	Exon 28	14 additional amino acids	1	154
6922dup(10bp)	Exon 38	Truncated	1	155
Substitutions in coding sequences				
C1318T	Exon 10a	Truncated	1	138
C3049T	Exon 18	Truncated	1	138
G3497A	Exon 21	Gly1166Asp	1	137
C3826T	Exon 22	Truncated	1	138
A4256G	Exon 24	Lys1419Arg	1	137
A4267G	Exon 24	Lys1423Glu	1	76
C5242T	Exon 29	Truncated	1	135
C5260T	Exon 29	Truncated	1	135
C5380T	Exon 29	Truncated	1	138
T5795C	Exon 31	Leu1932Pro	1	4
C5839T	Exon 31	Truncated	6	4, 120, 121, 135, 156
T6339A	Exon 33	Truncated	1	138
T?A	Exon 33	Asn?Lys	1	141
C6427A	Exon 34	Leu2143Met	1	143
T6511G	Exon 34	Tyr2171Asp	1	143
G6624A	Exon 35	Truncated	1	138
C7486T	Exon 42	Truncated	1	137
G7522T	Exon 42	Truncated	1	138
Substitutions in introns and splicing errors				
A1721 + 3G (exon skipping)	Intron 11	Truncated	1	137
T5749 + 2G (exon skipping)	Intron 30	Truncated	1	137
A47768G (4 bp insertion in transcript)	Intron 31	Truncated	1	157
A6364 + 4G (exon skipping)	Intron 33	Truncated	1	158
Total			78	

that the sequence composition of NF1 also influences the mutation formation.

CpG dinucleotides show a high mutation rate in the human genome owing to spontaneous deamination of 5-methylcytosine¹⁷⁶ and about 32% of point mutations involve this dinucleotide.¹⁷³ Nine substitutions detected in NF1 occur within CpG dinucleotides. Six of

these point mutations involve a single CpG site in exon 31 representing an identical mutation C5839T (table 1). Other CpG involved substitutions include three C to T transitions in exons 10a,¹³⁸ 29,¹³⁵ and 42,¹³⁷ respectively. The NF1 coding sequence contains 107 CpG dinucleotides. Several positions are methylated both within and around the NF1 locus.¹⁷⁷ Fur-

ther mutational analysis may allow the identification of additional hot spots at such CpG sites in NF1.

Particular sequence patterns in the human genome are associated with the preferential formation of insertions and deletions.^{174 175} These patterns include direct repeats, palindromes, quasi-palindromes, symmetrical elements, and runs of identical bases. Preliminary examination of the local sequence environment flanking some deletions and insertions detected in NF1 has provided similar examples for such sequences.^{122 138 151 152 164}

Several NF1 related loci have been identified on chromosomes 2, 12, 14, 15, 20, 21, and 22.^{130–132} These sequences have homology greater than 90% with some NF1 exons and intronic sequences.¹³¹ The majority of these loci appear to represent pseudogenes, which may be involved in mutagenesis of NF1 by gene conversion.¹³¹ This mechanism might possibly explain the high mutation rate of NF1.

NF1 MUTATIONS AND PHENOTYPIC EXPRESSION

Whether particular NF1 mutations are correlated with the variable clinical expression clearly needs to be analysed. However, the current paucity of the disease causing mutations so far documented in NF1 causes difficulties in correlating the positions and types of mutations with the variable clinical features. The variability of phenotypic expression of NF1 even within families makes this more complicated.

No clear relationship between mutation size and clinical features has been found. However, large deletions of more than 700 kb have been detected in five NF1 patients. These mutations delete the whole NF1 gene and the three embedded genes (EVI2A, EVI2B, and OMGP) as well as the sequences flanking NF1. These patients have mild facial dysmorphism, mental retardation, numerous cutaneous neurofibromas, and plexiform neurofibromas.¹²³ They show a variable number of physical anomalies that were not apparently correlated with the extent of their deletion but a large number of neurofibromas for their age were observed in all these patients. This suggests that the variable NF1 phenotypes may be related to other genes at or near the NF1 locus, and that deletion of such unknown genes may lead to tumour initiation or development.¹²³

Two identical mutations (2970delAAT) in exon 17 (table 1) did not cause similar clinical features in two unrelated NF1 patients. One of them had developmental abnormalities, mild pulmonary stenosis, multiple CLS, axillary freckling, and freckles over the bridge of the nose, but had no neurofibromas, while the other had developmental delay, multiple CLS, and several neurofibromas. The C5839T transition within exon 31 has been identified in six unrelated NF1 patients (table 1). Clinical data were available from four of these patients. Two of them show scoliosis^{131 156} and the two others show “classical” NF1.¹³² In addition, affected members in the same family may have different manifestations.¹⁷⁸ These observations suggest

that disruption of the NF1 gene itself may not be enough to account for the clinical variability. They also appear to cast doubts on the validity of genotype-phenotype correlation in NF1.

It is not clear whether the genes (EVI2A, EVI2B, and OMGP) embedded in intron 27 of the NF1 gene play a role in the variable NF1 expression. EVI2A and EVI2B are the human homologues of murine *Evi-2A* and *Evi-2B* respectively.^{37 38} *Evi-2A* is a putative oncogene and is involved in retrovirus induced murine myeloid leukaemogenesis.¹⁷⁹ *Evi-2B* lies in the midst of a cluster of viral integration sites identified in retrovirus induced myeloid tumours and may thus also function as an oncogene in these tumours.³⁸ EVI2A is highly expressed in brain, bone marrow, and peripheral blood while EVI2B is expressed in bone marrow and peripheral blood but not in brain. Juvenile chronic myelogenous leukaemias occur more often in NF1 patients than in the general population.^{74 180 181} Recently, LOH was observed in bone marrow samples from five out of 11 children with NF1 and malignant myeloid disorders.¹⁸² In these cases, the retained NF1 allele was the one inherited from the affected parent. It is possible that mutations involving both NF1 and EVI2A (or EVI2B) may cause NF1/leukaemia syndromes. A 40 kb deletion has been reported to remove a number of NF1 exons, all of EVI2A, and the 5' exon of EVI2B.⁵ Several other large deletions have been reported to remove the whole NF1 gene and the three embedded genes.¹²³ However, none of the patients with these deletions shows features of leukaemia.

OMGP encodes the oligodendrocyte-myelin glycoprotein (OMGP),³⁹ which is expressed only in oligodendrocytes of the central nervous system. OMGP may function as a cell adhesion molecule in the myelin of the central nervous system.¹⁸³ No mutations involving only OMGP have been reported. As proposed by Viskochil *et al.*,³⁹ transcriptional regulation of these embedded genes might play a role in the NF1 phenotype. In the past, there have been isolated reports of patients with NF1 and multiple sclerosis (MS). MS is a disorder in which multiple plaques of demyelination occur in the central nervous system.¹⁸⁴ Perhaps the patients with NF1 and MS have a mutation disrupting both NF1 and OMGP, which may result in this particular phenotype.

Animal models for NF1

Animal models of human genetic disease have been very helpful in tackling a range of scientific and clinical problems.¹⁸⁵ Several NF1 models have been reported in different species. A *Drosophila* model has been generated by a lethal interaction of the eye colour mutant *prune* (pn) with *Killer-of-prune* (k-pn) allele of the abnormal wing disc (awd) locus.¹⁸⁶ The awd gene is the *Drosophila* homologue of the mammalian tumour metastasis gene nm23 and the pn gene is postulated to encode a GAP-like protein. In this model, hypertrophy of the neuroglia and the perineurium of the larval brain was observed. The lymph glands of the larvae

were found to be highly abnormal and melanotic pseudotumours were formed upon aging of the larvae. These manifestations involve cell types in *Drosophila* equivalent to those affected by human NF1. Spontaneous neurofibromas and hyperpigmented spots have been observed in the bicolour damselfish.^{187,188} The disease in the damselfish appears to be transmissible and the tumours tend to be more malignant than human neurofibromas. The Syrian hamster NF1 model has been achieved by N-nitroso-N-ethylurea treatment of pregnant Syrian golden hamsters.^{189,190} The hamster offspring develop similar neurofibromas and pigmented lesions to those observed in NF1 patients, and also show Wilms' tumours and other malignancies not typically seen in NF1 patients. Point mutations in the neo proto-oncogene have been identified in these hamster tumours but no mutations have been found in NF1.¹⁹⁰ A transgenic mouse model for neurofibromatosis has been described expressing the HTLV-I tat gene.^{191,192} Neurofibromas developed in the transgenic mice but no other features of human NF1 were observed.

The mouse NF1 gene was identified and characterised.¹⁹³ It encodes an approximately 12 kb mRNA with an open reading frame predicting a protein of 2841 amino acids. Sequence comparison of NF1 showed more than 98% amino acid sequence identity between mouse and man. Both the 5' and 3' end untranslated regions of the mouse NF1 transcript are highly conserved. Hajra *et al*¹⁹⁴ compared the promoter regions of human and mouse NF1 genes and found that the transcription sites were the same. Both contain a cAMP response element, AP2 consensus binding sites, and a serum response element. Recently, gene targeting has been used to disrupt the mouse NF1 gene by the insertion of a neomycin cassette into the murine DNA sequence equivalent to exon 31 of the human NF1.¹⁹⁵ Mice heterozygous for the targeted germline mutation show none of the classical symptoms observed in human NF1, but are highly predisposed to the formation of various tumour types, especially pheochromocytoma and myeloid leukaemia. Homozygosity for the mutation causes cardiac developmental abnormality and mid-gestational embryonic lethality. Branna *et al*¹⁹⁶ used a similar strategy to disrupt the mouse NF1 by generating a null mutation. The heterozygous mutant mice, aged up to 10 months, do not show any obvious abnormalities but the homozygous mutant embryos die in utero, exhibiting developmental abnormalities in the heart and various neural crest derived tissues.

Another animal homologue of the NF1 gene, the chicken NF1 gene, has been cloned^{22,54} and again the predicted amino acid sequence was highly conserved between chick and man. The chicken NF1 cDNA hybridised to a 12.5 kb transcript of RNA blots. The potential existence of spontaneous mutations in the chicken NF1 gene might provide another form of NF1 model. Alternatively, similar models might be generated by genetically mutating the chicken NF1 gene.

Molecular diagnosis of NF1

DNA testing provides a powerful tool in prenatal diagnosis and in presymptomatic or carrier screening for genetic diseases. At present, NF1 allele specific diagnosis with the mutations detected in the NF1 gene are only available to a few families. The molecular diagnosis of NF1 has been mainly based on linkage studies with available DNA markers.¹⁹⁷⁻¹⁹⁹ Although a battery of closely linked markers has been developed for prenatal and predictive testing, marker informativeness can not be achieved in all NF1 families. A number of documented intragenic polymorphisms appear to be in linkage disequilibrium with each other but no disequilibrium was found between the disease allele and any of these polymorphisms.^{200,201}

Several PCR based polymorphisms have been identified.²⁰²⁻²⁰⁶ The highly informative microsatellite in intron 38 has a heterozygosity of up to 0.82.²⁰³ Some polymorphisms have recently been found in the NF1 coding sequence including a polymorphic *RsaI* restriction site in exon 5²⁰⁷ and three single base substitutions in exons 23, 24, and 29 respectively.¹³⁷ These PCR based polymorphisms, in conjunction with the previously described markers identified by Southern blotting, as well as an increasing number of documented NF1-specific mutations, should make molecular diagnosis possible in more NF1 families (table 2).

The current demand for molecular diagnosis of NF1 is low. Many couples would probably request a prenatal diagnosis if it could predict disease severity. This is not possible at present. Presymptomatic DNA diagnosis is probably not going to be in huge demand because the clinical diagnosis of NF1 is usually straightforward, even in early childhood. Both the severity and prognosis of the disease are extremely variable. The observed variation may not only be related to different mutations disrupting the NF1 gene but also to the involvement of unknown modifying genes within or outside the NF1 locus.¹¹⁹ This implies that molecular prediction of disease severity and prognosis may either be very complicated or even impossible.

Therapy for NF1

No medical treatment is available to prevent or reverse the characteristic lesions of NF1. Instead, medical management is focused on genetic counselling and the early detection of treatable complications. Drug therapies directed at upregulating neurofibromin GAP activity or downregulating p21^{ras} activity may be useful for controlling the growth of neurofibromas. Several lipids have been found to inhibit the neurofibromin GAP activity.^{68,114} Tubulin has also been reported to inhibit the neurofibromin GAP activity.⁶¹ These findings may be helpful to the development of a drug capable of upregulating or replacing neurofibromin. Alternatively, pharmaceutical agents that block farnesylation and thus interfere with p21^{ras} activity may prove to have therapeutic potential in NF1.²¹³⁻²¹⁵ These agents have been

Table 2 Polymorphisms in the NF1 gene

Probe sequence	Restriction enzyme	Heterozygosity	Reference
Southern blotting			
AE25	<i>Bgl</i> II, <i>Pvu</i> II, <i>Taq</i> I	0.5	208
NF1C2	<i>Eco</i> RI	0.5	209
pRC9.4	<i>Bam</i> HI	0.45	210
fHB5E3	<i>Eco</i> RI	0.37	200
pHa39.3	<i>Eco</i> RI	0.42	200
pEV136.5	<i>Taq</i> I	0.43	200
fPL37.B2	<i>Bam</i> HI	0.42	200
pDV1.9	<i>Hind</i> III	0.41	200
fT315.1	<i>Eco</i> RI	0.49	200
f7G4.GL.2	<i>Bgl</i> II	0.41	200
GE2	<i>Eco</i> RI	?	211
PCR based			
<i>Rsa</i> I polymorphism in exon 5		0.40	207
T to C transition in intron 16		0.09	205
C to T transition in exon 23		?	137
G to A transition in exon 24		?	137
Compound nucleotide repeat in intron 26		0.72	212
<i>Alu</i> repeats in intron 27		High	202
CA/GT repeats in intron 27		0.46	204
CA/GT repeats in intron 27		0.72	204
C to T transition in exon 29		?	137
CA/GT repeats in intron 38		0.82	203
<i>Rsa</i> I polymorphism in intron 39		0.14	206
G to A transition in intron 41		0.43	205

found to inhibit the mitogenic effects of growth factors and the tumorigenic properties of neurofibromas. More useful therapies might involve drugs capable of inhibiting farnesyl transferase that adds farnesyl groups to the p21^{ras} protein.²¹⁶

The ultimate cure of an inherited disease may be gene therapy. Gene therapy for NF1 may not be easy for several reasons. First, because NF1 is an autosomal dominant disease, successful gene therapy may require correction of the mutant NF1 allele. Mutation analysis in NF1, however, showed that the majority of the causative mutations identified are expected to cause severely truncated neurofibromin. This suggests that NF1 may result from dosage insufficiency of normal neurofibromin. If this is true, introduction of a normal NF1 allele may be able to achieve therapy without correction of the mutant allele. Second, NF1 often involves multiple tissues with a high variation. It would be very difficult to deliver the normal NF1 copy to target tissues. This might be achieved only by germline gene therapy, about which there is still much ethical debate. Third, it has been difficult to generate a satisfactory NF1 animal model although several models in different species have been reported. The recently generated mouse models by gene targeting did not mimic human NF1.^{195,196} It may be possible to target the mouse NF1 gene using different mutations identified from NF1 patients and so to create a suitable NF1 mouse model. However, the involvement of modifying genes in the NF1 expression may make it more difficult to achieve such a model for gene therapy.

Possibly, a normal copy of NF1 may be transferred into target tumour tissues to achieve treatment. These tumours include NF1 associated malignant tumours and some sporadic malignancies not related to NF1. Nur-E-Kamal *et al*¹⁰⁴ observed that when the NF1-GRD was overexpressed in v-Ha-Ras transformed NIH3T3 cells, it greatly reduced their ability to form colonies in a soft agar. Further examination showed that a fragment of 91 amino acids (NF91) derived from the NF1-GRD was

able to reverse the v-Ha-Ras induced malignant transformation as the NF1-GRD. These results suggest that the NF1-GRD or even the smaller fragment NF91 may be used as a potential cure for human cancers caused by mutations in the ras gene.

Conclusions and speculations

The isolation of the NF1 gene has advanced the understanding of the molecular basis of the disease and the gene function. Mutation analysis has allowed the identification of over 100 disease causing germline mutations in the NF1 gene. However, these mutations are only responsible for a small proportion of all NF1 patients. Recent applications of some techniques with high sensitivity, such as CCM, DGGE, and PTT, have proved to be highly efficient in mutation detection in the NF1 gene. It is essential to use such techniques for further mutation search in the gene, especially the regions not yet examined which include the coding sequence, splice site consensus sequences, and other essential elements for gene expression. The functions of the three embedded genes (EVI2A, EVI2B, and OMGP) and their relationship to NF1 remain unknown, and thus mutation analysis in these genes may also be interesting. On the other hand, extensive analysis of somatic mutations in a variety of human tumours should help us understand the possible mechanisms by which the NF1 gene is involved in tumorigenesis. Further mutation analysis should lead to molecular diagnosis for more NF1 families and may also provide clues for tumour diagnosis in the future.

Evidence is accumulating that NF1 as a tumour suppressor gene plays an important role in the control of cell growth and differentiation. Neurofibromin genetically and biochemically shows GTPase activating activity, down-regulating the activity of normal ras proteins. However, this may not be the only way neurofibromin functions. Many questions need to be answered. For example, what would be the effect of the interaction between neuro-

fibromin and tubulin? How does the differential expression of the alternatively spliced NF1 transcripts contribute to the gene function in different tissues? Do these transcript isoforms play different roles? Are there any modifying genes to NF1? If so, where do these genes lie in the genome and are they associated with the variable clinical expression of NF1? To address such questions more precisely, further studies are required by using a variety of strategies. Examples include in vitro site directed mutagenesis in different domains of the NF1 gene and in vivo interruption of the mouse NF1 gene by generating different types of mutations through gene targeting. Hopefully, this would lead to the generation of a proper mouse model for NF1. Breeding heterozygous NF1 mice against different strains should help to identify modifying genes. Further detailed studies on the interaction of neurofibromin with p21^{ras}, tubulin, growth factors, or other proteins are also important for the elucidation of the gene function. Further knowledge of the gene function may also lead to the development of new therapy for NF1.

We thank Drs N Thomas and A Clarke for their invaluable comments on the manuscript. M H Shen is also grateful to Dr W Brown for his encouragement during the preparation of this manuscript. This work was supported by Action Research.

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