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Deletions Spanning the Neurofibromatosis I Gene: Identification and Phenotype of Five Patients

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Summary

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder characterized by marked variation in clinical severity. To investigate the contribution to variability by genes either contiguous to or contained within the NF1 gene, we screened six NF1 patients with mild facial dysmorphology, mental retardation, and/or learning disabilities, for DNA rearrangement of the NF1 region. Five of the six patients had NF1 gene deletions on the basis of quantitative densitometry, locus hemizygosity, and analysis of somatic cell hybrid lines. Analyses of hybrid lines carrying each of the patient's chromosomes 17, with 15 regional DNA markers, demonstrated that each of the five patients carried a deletion >700 kb in size. Minimally, each of the deletions involved the entire 350-kb NF1 gene; the three genes—EVI2A, EVI2B, and OMG—that are contained within an NF1 intron; and considerable flanking DNA. For four of the patients, the deletions mapped to the same interval; the deletion in the fifth patient was larger, extending farther in both directions. The remaining NF1 allele presumably produced functional neurofibromin; no gene rearrangements were detected, and RNA-PCR demonstrated that it was transcribed. These data provide compelling evidence that the NF1 disorder results from haploid insufficiency of neurofibromin. Of the three documented de novo deletion cases, two involved the paternal NF1 allele and one the maternal allele. The parental origin of the single remaining expressed NF1 allele had no dramatic effect on patient phenotype. The deletion patients exhibited a variable number of physical anomalies that were not correlated with the extent of their deletion. All five patients with deletions were remarkable for exhibiting a large number of neurofibromas for their age, suggesting that deletion of an unknown gene in the NF1 region may affect tumor initiation or development.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder characterized by marked variation in expression. The diagnosis is confirmed by the presence of two or more specific findings, primarily multiple neurofibromas, café-au-lait spots, axillary or inguinal freckling, Lisch nodules of the iris, and occasionally optic glioma and certain bone abnormalities (Stumpf et al. 1988). A wide range of other manifestations are found in NF1 patients, from mental retardation and learning disabilities to CNS tumors and cancer (Huson et al. 1989*b;* Riccardi 1992). Investigating the molecular bases of variable expressivity became feasible with the cloning of the NF1 gene (reviewed in Viskochil et al. 1993). The NF1 gene was mapped by genetic linkage analysis to the pericentromeric region of chromosome 17 (Barker et al. 1987; Seizinger et al. 1987). The fortuitous identification of two NF1 patients with intragenic balanced translocations proved instrumental in cloning the gene (Cawthon et al. 1990; Wallace et al. 1990). At an estimated 350 kb in length, NF1 is a large gene with a transcript of 11-13 kb (Viskochil et al. 1990; Wallace et al. 1990). The 2,818-amino-acid gene product (Marchuk et al. 1991), designated "neurofibromin," con-

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tains a small central domain that shares sequence and functional homology with mammalian GTPase activating protein (GAP); both neurofibromin and GAP regulate ras activity (Martin et al. 1990; Xu et al. 1990). While the predominant tissues affected in the disorder are of neural crest origin, the ubiquitous expression of the NF1 gene (Wallace et al. 1990) suggests that some defective NF1 alleles could have functional consequences in other tissues, thereby contributing to phenotypic variation.

Current evidence indicates that the existence of more than one NF1 locus is highly unlikely. Genetic linkage analyses in over 170 families and molecular identification of NF1 mutations in affected individuals provide compelling evidence in support of a single NF1 gene at 17q11.2, responsible for both familial and sporadic cases of the disorder (Stephens et al. 1987; Goldgar et al. 1989 and references therein; Cawthon et al. 1990; Viskochil et al. 1990; Clementi et al. 1991; Wallace et al. 1991; Kayes et al. 1992*a*). NF1 allelic heterogeneity appears to be the norm, with many different NF1 mutations described (Cawthon et al. 1990; Viskochil et al. 1990; Kayes et al. 1992*a;* Stark et al. 1992; Upadhyaya et al. 1992; Weiming et al. 1992). This is not unexpected in light of the high mutation rate of the disorder, estimated at 1×10^{-4} per gamete per generation (Huson et al. 1989*a*). A 1%–3% recurrence rate has been reported for one nonsense mutation (Estivill et al. 1991). Further mutational analyses will be important in identifying functional domains of neurofibromin other than the GAP-related domain and, perhaps, in associating them with specific manifestations of the disorder. Other loci and nongenetic factors may also influence expression of the disorder (Riccardi 1993). Recent statistical evidence suggested that the genotype of modifying loci may be a critical determinant (Easton et al. 1993).

Linked genes that may modify the phenotype include genes either contiguous to or contained within the NF1 gene. While genes contiguous to NF1 have yet to be identified, there are three known genes located within an intron of the NF1 gene and transcribed from the alternate strand. Two of the contained genes, EVI2A and EVI2B, are the human homologues of genes where retroviral insertion results in murine myeloid tumors (O'Connell et al. 1990; Cawthon et al. 1991). The third is the oligodendrocyte myelin glycoprotein (OMG) that may play a role in CNS myelination (Buchberg et al. 1990; Mikol et al. 1990; Viskochil et al. 1991). What contribution, if any, defects in these genes would make to the NF1 phenotype is unclear.

We described previously a large de novo NF1 deletion that included and extended well beyond the NF1 gene in a patient with NF1, mental retardation, and mildly dysmorphic facial features (Kayes et al. 1992*a*). To determine if other NF1 patients with intellectual impairment and mild dysmorphism also carried rearrangements in the NF1 gene region, we screened additional patients and extended the characterization of our original deletion patient (Kayes et al. 1992*a*). In the present report, we describe extensive deletions found in five of six patients and compare their NF1 manifestations, intellectual impairment, and physical features.

Subjects, Material, and Methods

Subjects

Patient UWA106-3 has been described, in part, in a previous report (Kayes et al. 1992*a*). The other five patients were referred by neurofibromatosis clinics on our specific inquiry for NF1 patients with mild dysmorphism and/or intellectual impairment. Patients UWA128-3 and UWA110-3 were referred by W.B., UWA69-3 and UWA123-3 by V.M.R., and UWA119-1 by A.R. Genetic information and clinical manifestations are detailed in table 1. Patients designated as hyperteloric had inner-canthal or interpupillary distances >95th percentile; individuals with short stature had height <5th percentile (Jones 1988). Immortalized cell lines were established for each patient and for their parents, when available, by transformation of peripheral leukocytes with Epstein-Barr virus (Neitzel 1986). Blood samples were obtained from the clinically unaffected parents of UWA106-3 (Kayes et al. 1992*a*), UWA119-1, and UWA123-3. Paternity and pedigree structure were verified by analysis of the segregation of alleles at several informative and highly polymorphic loci, including D13S115, D13S71, ACTC, D18S46, and APOC2. Some of the additional 58 unrelated NF1 patients analyzed in the polymorphism studies have been described elsewhere (Stephens et al. 1987, 1989, 1992).

Somatic Cell Hybrids

To isolate the chromosome 17 homologues of each candidate deletion patient, human/hamster somatic cell hybrids were constructed by a modification of the method of Puck et al. (1989). Thirty to 50 million human immortalized lymphoblasts were added to hamster hypoxanthine ribosyl transferase–deficient RJK cells (Fuscoe et al. 1983) at 50%–75% confluency in serum-free medium. After a 30-min incubation for cell adherence, the medium was removed and the cells overlaid with polyethylene glycol 1500 (Boehringer-Mannheim)

Table 1

Phenotype of Patients with NF1 Gene Deletions

	Patient Number				
	UWA106-3	UWA119-1	UWA123-3	UWA128-3	UWA69-3
Age at exam (years)	20	25	5	29	16
Sex	M	M	M	F	M
Family history of NF1	no	no	no	u	u
More than six café-au-lait spots	+	+	+	+	+
More than two neurofibromas	+	+	+	+	+
Plexiform neurofibroma(s)	+	+	+	+	+
Optic glioma	−	−	u	−	u
Intellectual impairment[a]	IQ 46	MR	LD	LD	IQ 59
Macrocephaly	−	+	+	−	+
Hypertelorism	+	+	+	−	+
Down-slanting palpebral fissures	−	−	+	+	+/−
Ptosis	+	−	+	+	+/−
Micrognathia	+	+/−	+	+	+
Broad neck	+	−	+	+	+
Low posterior hairline	−	u	−	+	−
Short stature	−	−	−	+	+
Scoliosis/kyphoscoliosis	−	−	−	+	+
Shield chest	−	−	−	+	−
Cubitus valgus	−	−	−	+	+
Short fingers	−	−	−	+	−

Note.—Features were present (+), absent (−), suggested (+/−), or not determined (u). Additional features displayed by individuals include UWA106-3—hypotonia at birth and absent coccyx; UWA123-3—fetal hydronephrosis, edema at birth, unilateral cryptorchidism, hyperelastic skin, small atrial septal defect, and intermittant cyanosis, in infancy, due to small jaw and thick tongue; and UWA128-3—nasal obstruction and respiratory problems at birth.

[a] Intelligence quotient (IQ); unknown IQ value (MR); and learning disability (LD).

for 30 s and washed with serum-free medium. After overnight incubation in nonselective media (RPMI and 5% fetal bovine serum), the fused cells were trypsinized and distributed among 10 plates in RPMI selective medium containing 1 × hypoxanthine, aminopterin, and thymidine (Gibco BRL). Ouabain (Sigma Chemical) was added to a final concentration of 0.5 μg/ml as needed to control overgrowth by human lymphoblasts. After 2–3 wk, visible colonies were transferred and grown for 2 wk in 24-well plates. Clones were assayed for the presence of one or both chromosomes 17 by PCR amplification using primers specific for any one of three chromosome 17 loci for which the patient was heterozygous. The loci included D17S33, which lies centromeric to the NF1 gene (White et al. 1987; Ainsworth and Rodenhiser 1991), and two loci telomeric to NF1, including D17S250 (Weber et al. 1990) and THRA1 (thyroid hormone receptor, alpha 1) (NIH/CEPH Collaborative Mapping Group 1992 and references therein). To ensure that we chose a hybrid cell line with a significant proportion of cells carrying a single human chromosome 17, lines identified initially by PCR were rescreened using the less sensitive method of Southern blot hybridization. DNA from candidate hybrid cell lines was hybridized to D17S36 (Stephens et al. 1987), a locus not deleted in any of the patients, as was determined by densitometry; cell lines that generated a strong hybridization signal were selected for analysis.

DNA Markers

To screen genomic DNA of patients for NF1 gene rearrangements and RFLPs, the overlapping cDNAs GE2, FF1, FF13, FB5D, AE25, and P5 were used (Marchuk et al. 1991 and references therein). DNA from whole blood or lymphoblasts individually digested with *Bam*HI, *Bgl*II, *Hin*dIII, *Hin*fI, *Msp*I, and *Pvu*II was transferred to membrane and hybridized to radiolabeled probes as described elsewhere (Kayes et al. 1992*b*). Membranes were hybridized sequentially to each of the overlapping NF1 cDNAs and to the distal locus D17S36 that was used as a control for differences in DNA concentration per lane.

Southern blot analysis to detect rearrangements in the hybrid cell lines used three cDNAs—GE2, AE25, and B3A—which define the 5′, central, and 3′ regions of the gene, respectively (Marchuk et al. 1991). End clones from an NF1 YAC contig were AN2 from YAC A113D7 and AH1 from YAC A43A9 (Marchuk et al. 1992). Probes for loci genetically linked to NF1 were obtained from the American Type Culture Collection (Rockville, MD), including VAW212 (D17S117), VAW215R3 (D17S120), VAW210M1 (D17S115) (Fain et al. 1989), EW206 (D17S57), EW207 (D17S73) (Fain et al. 1987), and HHH202 (D17S33) (White et al. 1987). L946 (D17S36) (Stephens et al. 1987) was a gift of Collaborative Research (Waltham, MA). A highly informative Alu polymorphism intragenic to the NF1 gene was amplified by PCR (Weber and May 1989; Xu et al. 1991). Products were electrophoresed through Long Ranger gel matrix (AT Biochem, Malvern, PA), and dried gels were exposed to Kodak X-Omat AR film overnight.

NF1 Transcriptional Analysis

Total RNA was isolated from 1×10^7 lymphoblasts of each patient by using the RNAid Plus kit (BIO101; LaJolla, CA). One-thirtieth of the total RNA yield was reverse transcribed using random hexamers and the Geneamp RNA-PCR kit (Perkin Elmer, Norwalk, CT). NF1 was amplified from the total cDNA yield by a PCR of 100 μl containing 1.4 mM $MgCl_2$, 1 × PCR buffer, 2.5 U *Taq* polymerase, and 15 pmol each of primers NF-6423 and NF-6790. After an initial incubation for 2 min at 94°C, the reaction was cycled 35 times at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. Primers, designated by their base-pair location in the sequence by Marchuk et al. (1991), were NF-6423 (5′-CAATTCCCTTGATGTGGCA) in exon 33 and NF-6790 (5′-TATCTCTCATGCATGCCTC), which crosses the junction between exons 34 and 35. Exons 33–35 were numbered 6–8 elsewhere (Cawthon et al. 1990; R. M. Cawthon, personal communication). Direct sequencing of amplified product was by the method of Tracy and Mulcahy (1992). Automated sequence analysis and oligonucleotide synthesis was performed by the University of Washington Molecular Pharmacology Facility.

Results

Screening for NF1 Gene Rearrangements

To facilitate detection of NF1 gene rearrangements by Southern blot analysis, we identified restriction endonucleases that did not reveal common RFLPs. With these enzymes, novel fragments or decreased band densities would be less likely to result from variation at a polymorphic site. Southern blots of DNA from 25 unrelated individuals were digested with six different restriction endonucleases and were probed with each of the overlapping NF1 cDNAs. The following cDNA-endonuclease combinations revealed no RFLPs among the 50 NF1 alleles tested: GE2-*Pvu*II or *Hin*fI; FB5D-*Hin*dIII; FF1-*Pvu*II; FF13-*Bam*HI or *Bgl*II; P5-*Hin*dIII, *Bgl*II, or *Pvu*II; AE25-*Hin*dIII or *Msp*I; and B3A-*Hin*dIII (data not shown). Using these cDNA-endonuclease pairs, we performed Southern blot analysis on the six NF1 patients with intellectual impairment and mild dysmorphism. While novel fragments were not observed, five of six patients had bands of reduced density on probing with each of the cDNAs, suggesting reduced NF1 copy number (data not shown). The exception was patient UWA110-3.

NF1 copy-number studies were consistent with analysis of an NF1 intragenic polymorphism. Patient UWA110-3 was heterozygous, demonstrating the presence of two NF1 alleles (fig. 1*A*, lane 4). The remaining five patients—UWA69-3, UWA106-3, UWA123-3, UWA128-3, and UWA119-1—had only a single band, indicating that they were either hemizygous or homozygous at this site (fig. 1*A*, lanes 5–8, 10). Analysis of alleles for the clinically unaffected parents of the sporadic patient UWA119-1 demonstrated that he did not inherit a maternal NF1 allele (fig. 1*A*, lanes 10–12), providing compelling evidence for a deletion of the maternal allele.

Analyses of Somatic Cell Hybrids

To determine unambiguously that the NF1 gene was deleted and to map its extent, somatic cell hybrids were constructed for the five candidate deletion patients. Hamster cell lines carrying each human chromosome 17 of a patient were identified by PCR and Southern blot analyses. An illustrative example for patient UWA106-3 is shown in figure 1*B*. Analysis of genomic DNA demonstrated that the patient was heterozygous at the D17S33 locus (lane 5). The presence of D17S33 allele 1 and allele 2 in hybrids 36 and 41, respectively (lanes 6 and 7), indicates that these cell lines each carry one of the chromosomes 17 of this patient. The hybridization of NF1 cDNA GE2 to the cell hybrid 41, but not to 36, demonstrated that the chromosome 17 in hybrid 36 was deleted for this region of the NF1 gene (lanes 6 and 7). As expected, the hamster-specific GE2 fragment (lane 2) was also observed in both hybrid lines (lanes 6 and 7). Similar analyses identified a pair of hy-

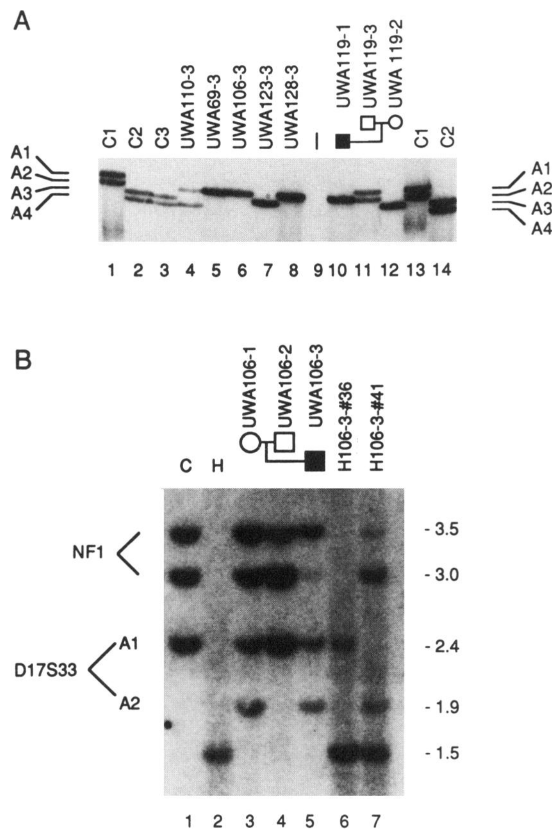

Figure 1 Analysis of genomic DNA and somatic cell hybrids. *A*, Analysis of an NF1 intragenic polymorphism. Genomic DNA was amplified by PCR using primers flanking an intragenic Alu polymorphism (Xu et al. 1991). Control individuals C1, C2, and C3 display the four alleles (A1–A4) at this locus. Amplified products from patients and clinically unaffected parents are indicated. Lane 9 was empty. *B*, Analysis of somatic hybrid cell lines derived from patient UWA106-3. The NF1 cDNA, GE2, and the probe HHH202 for the polymorphic locus D17S33 were hybridized simultaneously to a Southern blot membrane of *Rsa*I-digested DNA. Human fragments homologous to GE2 and the two alleles of D17S33 (A1, A2) are indicated. C = DNA of unaffected control individual; H = hamster cell line RJK; UWA106-1 and UWA106-2 = genomic DNA of the clinically unaffected parents; UWA106-3 = genomic DNA of patient; and H106-3-#36 and H106-3-#41 = hamster/human hybrids, each carrying one of the chromosomes 17 of patient UWA106-3. Fragment size (in kb) was determined by size standards (not shown). It is not known why the relative intensity of the human-specific GE2 fragments vary among the patient, his parents, and hybrid 41. This variation was sometimes observed among different unaffected individuals and between other patients and their derivative hybrid lines. We propose that a GE2-homologous polymorphic fragment revealed by *Rsa*I comigrates with the NF1-GE2 fragment in some individuals. Multiple NF1-homologous loci, presumably pseudogenes, have been identified on different chromosomes (Legius et al. 1992). Since our hybrid lines carry other undetermined human chromosomes, the variations between genomic DNA and hybrid lines could reflect the presence or absence of the human chromosome carrying the polymorphic pseudogene in the hybrid line.

brid lines that carried each of the chromosomes 17 of the other four candidate deletion patients. The GE2 region of the NF1 gene was deleted from one allele of each patient.

The deletion in each patient was delineated by mapping 15 different NF1 regional markers on the somatic cell hybrid carrying the deleted chromosome 17 (fig. 2). Patient UWA106-3 had the largest deletion. The NF1 cDNAs, the YAC contig end clones AH1 and AN2, and the flanking loci D17S120, D17S117, D17S115, D17S57, or D17S73 failed to hybridize to the somatic cell hybrid line carrying the deleted chromosome. Sequences homologous to each of the NF1 cDNAs were also deleted from the chromosomes of patients UWA69-3, UWA119-1, UWA123-3, and UWA128-3. In these four chromosomes, the centromeric deletion breakpoints mapped between the YAC end clone AH1 and either D17S117 or D17S120, and the telomeric breakpoint mapped between AN2 and either D17S115 or D17S57 (fig. 2). Parallel analyses of the hybrid cell lines carrying the alternative chromosome 17 for each patient gave positive hybridization signals for all loci tested and no evidence for rearrangement of the other NF1 allele (data not shown).

NF1 Transcriptional Analysis

To determine if the single remaining NF1 gene was transcribed, total RNA from the lymphoblasts of each patient was reverse transcribed and amplified by PCR. Product from each of the five patients comigrated with that of an unaffected control individual (fig. 3). Direct sequencing of the product from patient UWA119-1 (data not shown) demonstrated that it was identical to the published sequence of exons 33 and 34.

Frequency of NF1 Deletions

To determine if deletions spanning the NF1 gene were frequent, we analyzed unrelated NF1 patients for heterozygosity at an NF1 intragenic polymorphic site (Xu et al. 1991). Fifty-eight patients, who fulfilled the criteria for a diagnosis of NF1 but were unselected with regard to NF1 family history, intellectual impairment, and distinctive features, were analyzed. We found that 40 of the 58 patients were heterozygous, implying that deletion of this site and, therefore, of the NF1 gene are not common among the general NF1 patient population.

Patient Phenotype

Since patients were referred on the general criteria of NF1 with either dysmorphism and/or mental retarda-

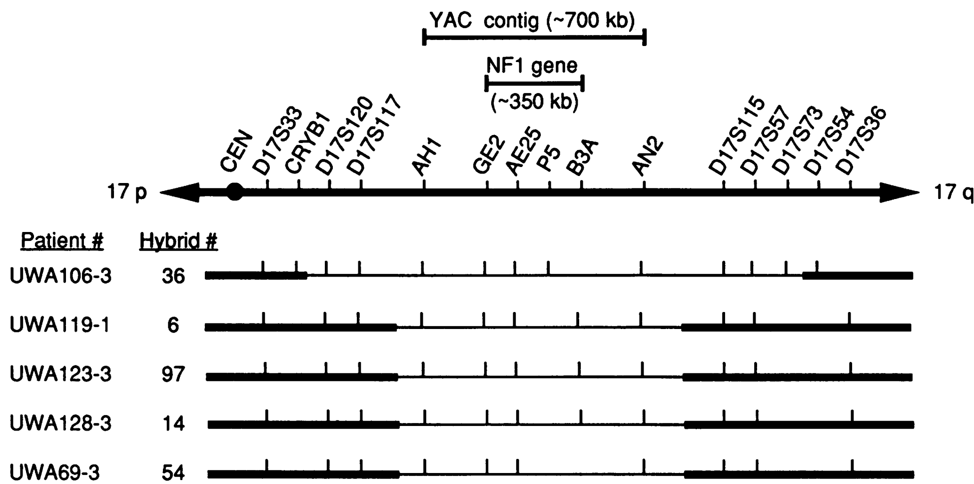

Figure 2 Mapping the extent of NF1 gene deletions. The top line represents the map of the chromosome 17 region surrounding the NF1 gene; the relative position of markers is indicated, including the centromere (CEN); drawing is not to scale. GE2, AE25, and B3A are NF1 cDNAs from the 5′, middle, and 3′ ends of the gene, respectively (Marchuk et al. 1991), and AH1 and AN2 are end clones from a YAC contig (Marchuk et al. 1992). Flanking this region, the order of genetically linked markers is indicated. Two ambiguities in locus order include the relative order of D17S120 and D17S117 and the location of D17S115 relative to D17S57 and D17S73 (Kayes et al. 1992*b* and references therein). Depicted below the map are the hybrid cell lines carrying the deleted chromosome for each patient. Vertical marks on thin lines denote loci absent from the hybrid line; vertical marks on thick lines denote loci present in the hybrid line; and absence of a vertical mark denotes that the locus was not tested.

tion, each patient was reevaluated to assess similarities and differences in their clinical features. Genetic and clinical findings are summarized in table 1. Each patient fulfilled the criteria developed by an NIH Consensus Committee for a diagnosis of NF1 (Stumpf et al. 1988; table 1). Three patients were sporadic cases; no manifestations of NF1 were observed in either parent, and paternity was consistent with genetic studies employing highly polymorphic markers (data not shown; Kayes et al. 1992*a*). For patients UWA128-3 and UWA69-3, the putative sporadic presentation could not be confirmed, as both parents were not available for examination.

The five patients carrying NF1 regional deletions were remarkable for their large number of cutaneous neurofibromas. In the three postpubertal patients—UWA106-3, UWA119-1, and UWA128-3—the total number of cutaneous neurofibromas was estimated as 101–500. These were densely distributed on the trunk and were less numerous on the extremities (fig. 4*A*). In the prepubertal and pubertal patients, UWA123-3 and UWA69-3, there were multiple neurofibromas on the trunk, in addition to patches of pebbly textured skin suggestive of large numbers of subcutaneous neurofibromas (fig. 4*B*). These individuals had an estimated 51–100 total cutaneous neurofibromas. Optic gliomas were not detected in the three patients—UWA106-3, UWA119-1, and UWA128-3—who underwent magnetic resonance imaging of the head (table 1). None of the patients had a slit-lamp examination for Lisch nodules.

Three patients—UWA106-3, UWA119-1, and UWA69-3—were mentally retarded, as determined by formal testing during childhood. The other two patients were not formally tested but had a history of learning difficulties. UWA128-3 repeated grades in elementary school, completed her education through high school, and is employed. UWA123-3, at 5 years of age, displays fine-motor and speech articulation difficulties but is not globally delayed.

The deletion patients exhibited a variable number of dysmorphic features (table 1 and fig. 5). Patient UWA128-3 had the most striking phenotype, with down-slanting palpebral fissures, ptosis, micrognathia, short and broad neck, low posterior hairline, short stature, shield chest, cubitus valgus, and short fingers. Cytogenetic analysis of 50 cells demonstrated a 46,XX karyotype. Patient UWA123-3 had hypertelorism, down-slanting palpebral fissures, ptosis, micrognathia, and broad neck. The facial features of patient UWA69-3 were less striking, with hypertelorism, micrognathia, and broad neck, but his body habitus was remarkable for short stature and cubitus valgus. Patients UWA106-

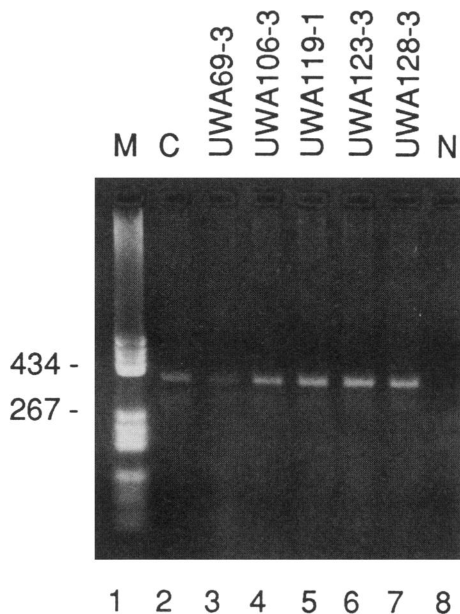

Figure 3 Transcription of the remaining NF1 allele. Purified total RNA from lymphoblasts of each patient and a control individual was reverse transcribed, and the cDNA for exons 33 and 34 was amplified by PCR. Products are as follows: lane 1 (M), size markers from *Hae*III digest of pBR322; lane 2 (C), unaffected, unrelated individual as a positive control; lanes 3–7, NF1 deletion patients as indicated; and lane 8 (N), negative control without RNA.

3 and UWA119-1 had fewer physical anomalies (table 1). With the exception of UWA123-3, who had a small atrial septal defect at birth, there was no evidence of congenital heart defects among these patients.

The one patient not deleted for the NF1 region, UWA110-3, inherited NF1 from his mother, a high school graduate who manages a small business. She was the first individual with documented NF1 in the family, with findings limited to café-au-lait spots and cutaneous neurofibromas. UWA110-3 has an IQ of 50 and was raised in institutional settings and foster homes. Clinical features related to NF1 included multiple café-au-lait spots, axillary freckling, and 10 truncal neurofibromas of ~1 cm in diameter. He had a normal karyotype of 46, XY, and fragile X studies were negative. He was without hypertelorism, micrognathia, or broad neck. He was thin with long thin fingers and height at the 45th percentile.

Discussion

NF1 Gene Deletions

We identified deletions in NF1 patients by screening initially for apparent differences in gene copy number on Southern blots probed with NF1 cDNAs. To reduce the probability that decreased band density reflected RFLPs rather than copy-number differences, we identified a restriction endonuclease(s) for each NF1 cDNA probe that did not reveal variant-sized fragments on Southern blots of unrelated individuals. Given that analyses with these cDNA-endonuclease combinations revealed no RFLPs in 50 sampled chromosomes, the upper 95% confidence bound for the frequency of an RFLP would be .058. The 25 unrelated individuals included 17 patients with NF1 and 8 patients with features of NF1 that were insufficient to fulfill the criteria for a confirmed diagnosis of the disorder. Using these patients, rather than unaffected individuals, offered two advantages. First, the polymorphism frequency may be more accurate, since the population of interest was being screened, and second, Southern blot–detectable rearrangements causal for NF1 may be detected. One variant fragment was revealed by *Pvu*II and the cDNA FF13, in 1 of the 25 patients tested, but studies of other family members demonstrated that this variant did not cosegregate with the affected phenotype. This observation demonstrates the value of collecting blood from the patient's family members whenever possible, i.e., for differentiation of infrequent neutral polymorphisms from mutations. One disadvantage of screening patients, rather than unaffected individuals, is that an NF1 mutational hot spot at a restriction endonuclease site could be misinterpreted as a polymorphism. However, putative mutations can be tested easily by assaying whether the variant band(s) cosegregate with the NF1 phenotype in family studies. No variant-sized fragments were detected among the 17 unrelated NF1 patients by using the cDNA-endonuclease pairs that were informative for analysis of NF1 gene copy number or rearrangements. These data suggest that the majority of NF1 mutations will not be detectable by conventional Southern blot analysis with cDNA probes.

In our initial screen for reduced NF1 copy number, five of six NF1 patients showed Southern blot fragments of reduced density. Two unambiguous methods were used to confirm these results. First, a highly informative NF1 intragenic polymorphism was amplified in each patient. Consistent with the screening results, patient UWA110-3 was heterozygous, while the other five patients were either hemi- or homozygous (fig. 1*A*). Second, deletions were confirmed and mapped in each of these five patients by the construction and analyses of somatic cell hybrid lines carrying each of the chromosomes 17 (figs. 1*B* and 2). The five patients each carried a deletion that encompassed the 350-kb NF1 gene and

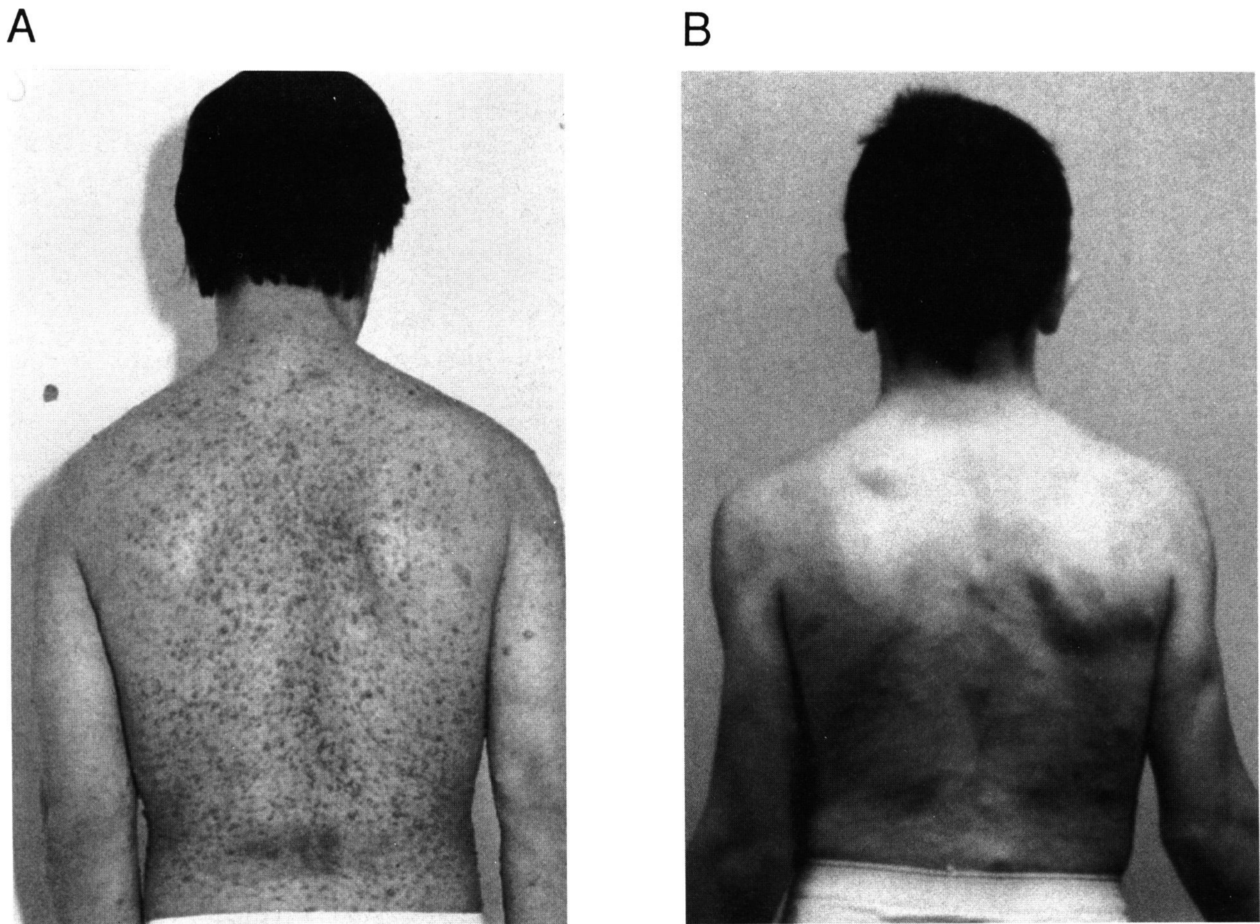

Figure 4 Involvement of truncal skin. Back views of postpubertal patient UWA119-1 (*A*) and pubertal patient UWA69-3 (*B*) document the multiple cutaneous neurofibromas of both patients, and for UWA69-3 (*B*) the patches of pebbly textured skin are suggestive of multiple subcutaneous neurofibromas.

the complete NF1 YAC contig estimated at 700 kb in length (fig. 2). The largest deletion, carried by patient UWA106-3, extended farther, to include several genetically linked flanking loci (fig. 2). These data for UWA106-3, determined by analysis of somatic cell hybrids, are consistent with our previous quantitative densitometric analysis of the patient's genomic DNA (Kayes et al. 1992*a*). Genetically, this deletion has a length of ~10.4 cM, the sex-averaged distance between D17S33 and D17S54 (Goldgar et al. 1989; Kayes et al. 1992*b*). The deletion breakpoints of patients UWA69-3, UWA119-1, UWA123-3, and UWA128-3 all map to the same two intervals (fig. 2). The proximal breakpoints lie between the YAC end clone AH1 and either D17S117 or D17S120, two loci whose relative order is not known. The distal breakpoints lie between the YAC end clone AN2 and D17S115 or D17S57. These data suggest the possibility that recombinogenic sequences may flank the NF1 gene. Mapping and sequencing the breakpoints will not only address this possibility but will be important for genotype/phenotype correlation. The breakpoints may involve different genes and/or create novel fusion peptides that influence the phenotype.

Frequency of Large NF1 Gene Deletions

An important question arising from our data is whether gene deletions are frequent among individuals with NF1. Previous studies employing conventional and pulse field gel electrophoresis suggest that large gene rearrangements are uncommon. Eight presumably intragenic rearrangements were identified among 200

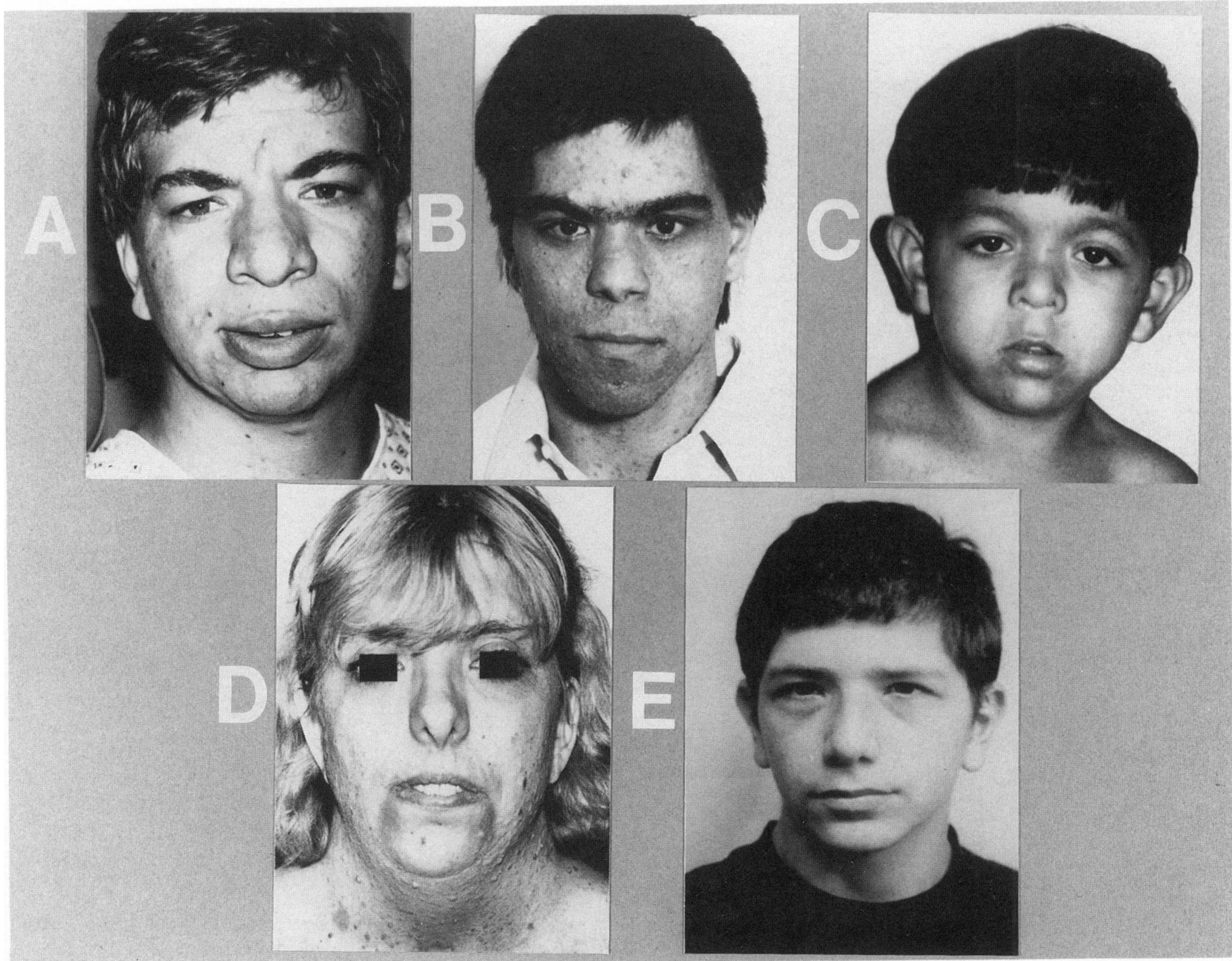

Figure 5 Facial features of the five NF1 deletion patients. Patient photographs are UWA106-3 (*A*), UWA119-1 (*B*), UWA123-3 (*C*), UWA128-3 (*D*), and UWA69-3 (*E*). Eyes were masked on patient request.

unrelated NF1 patients screened (Upadhyaya et al. 1990, 1992; Viskochil et al. 1990). However, since the probes employed were intragenic (with one exception) and the results dependent on detecting a break point, it is unlikely that these studies would have detected deletions of the magnitude reported here. To address the question of deletion frequency, we determined that 68% of unrelated NF1 patients (n = 58), unselected for specific physical features or intellectual abilities, were heterozygous for an NF1 intragenic polymorphism. This is comparable to the number of heterozygotes predicted on the basis of allele frequencies in an unaffected population (Xu et al. 1991). These results suggest that large deletions encompassing the NF1 gene will not be the rule among the general NF1 patient population.

Parental Origin of NF1 Gene Deletions

A preference for de novo mutation of the paternally derived NF1 allele has been demonstrated in 22 of 24 families by haplotype analysis (Jadayel et al. 1990; Stephens et al. 1992). The molecular nature of the NF1 mutations in these families is unknown. It was unexpected that we found one maternally derived de novo deletion among only three total cases. Identification of the parental origin of additional deletions is needed to determine if the mechanism(s) responsible for NF1 gene deletion is subject to a parent-of-origin effect. A role for genomic imprinting, either in enhancing mutation of the paternally derived NF1 allele or in protecting the maternally derived allele from mutation, has been hypothesized (Stephens et al. 1992). If the NF1

gene is imprinted sometime during development, it has no dramatic effect on the phenotype, as demonstrated by patients UWA106-3 and UWA123-3 with an expressed maternal NF1 allele and patient UWA119-1 with an expressed paternal allele (figs. 1*A* and 3 and table 1; Kayes et al. 1992*a*).

Genotype/Phenotype Correlations

Minimally, each deletion encompassed four genes: the NF1 gene and the embedded genes EVI2A, EVI2B, and OMG. The functional consequences of a deletion in any one of these four genes are unclear. Screening NF1 patients for mutation of embedded genes has been unproductive, with one exception (Cawthon et al. 1991; Viskochil et al. 1991). A 40-kb deletion involving (*a*) exonic regions of NF1 and EVI2B and (*b*) the entire EVI2A gene was identified in a patient whose phenotype was not distinctive (Viskochil et al. 1990, 1991). The deletion of OMG in our five patients with intellectual impairment is consistent with speculation that OMG alterations may play a role in learning disabilities of NF1 patients (Cawthon et al. 1990). However, intellectual disability could also result from either (1) deletion of other genes critical to CNS development that are located in this region or (2) simply the loss of >700 kb of DNA.

We presume that the single remaining NF1 allele in our five patients produces functional neurofibromin. No rearrangements were detected with overlapping cDNA probes, and RNA-PCR demonstrated transcription of this allele in lymphoblasts (fig. 3). The development of NF1 manifestations in what appears to be the absence of any defective neurofibromin provides strong evidence that the disorder results from haploid insufficiency of neurofibromin.

In comparison with the values from the population-based study of Huson et al. (1989*b*), our deletion patients had a large number of neurofibromas for their age. It is intriguing to speculate that this phenotype is related to the large DNA deletion. An interesting and testable hypothesis is that deletion of an unknown nearby gene predisposes to development of neurofibromas. Located within the minimal deleted region currently defined by D17S120/D17S117 and D17S115/D17S57 (fig. 2), this gene could have tumor-suppressor activity or could modify the NF1 phenotype by another mechanism. Analysis of deletions carried by additional patients will be necessary to test this hypothesis and to narrow the location of the putative modifying gene. An increased number of neurofibromas is unlikely to result directly from an NF1 null mutation, since at least one such patient did not have this phenotype (Schmidt et al. 1987). Whether an NF1 null allele predisposes to increased numbers of neurofibromas is unknown; the number of tumors observed in most patients is either not determined or not reported (Ledbetter et al. 1989; Upadhyaya et al. 1990; Estivill et al. 1991; Stark et al. 1992). However, the recent report of a role for the 3′ UTR in growth and differentiation (Rastinejad and Blau 1993) suggests that a deletion involving an entire gene may not necessarily result in the same phenotype as would null alleles that are transcribed.

The NF1 deletion patients described here may represent the critical resources for determining whether the NF1 gene behaves as a tumor-suppressor gene. Although the gene is often considered a tumor suppressor on the basis of the focal nature of NF1 manifestations and the development of the disorder in individuals with one inactive allele, an NF1 somatic mutation in an involved nonmalignant tissue of an NF1 patient has yet to be documented. Examination of neurofibromas from our patients should facilitate detection of any second-hit mutation, since there is only one NF1 allele to screen. While a tumor-suppressor role for neurofibromin has yet to be demonstrated in benign neurofibromas, recent evidence supports such a role in neoplasia. A homozygous NF1 deletion in a neurofibrosarcoma from an NF1 patient has been identified (Legius et al. 1993). NF1 somatic mutations in malignancies of NF1-unaffected individuals (Li et al. 1992) and in melanoma and neuroblastoma cell lines (Andersen et al. 1993; The et al. 1993) also implicate neurofibromin in tumorigenesis. A corollary of this hypothesis would be that individuals hemizygous for the NF1 gene, such as those reported here, may be at higher risk for CNS and visceral neoplasms, as well.

Although our five patients carry deletions of generally similar structure, they exhibit a wide range of physical anomalies (table 1 and fig. 5). Some anomalies fall within the spectrum of features described in patients with Noonan syndrome, a genetic disorder of highly variable presentation that can include short stature, broad or webbed neck, thorax abnormalities, congenital heart defects, mental retardation, and distinctive facies marked by hypertelorism, down-slanting palpebral fissures, low-set posteriorly rotated ears, deeply grooved philtrum, micrognathia, and low posterior hairline (Allanson 1987 and references therein). Among the five deletion patients, we found no correlation between the number of physical anomalies and the extent of the deletion. The two patients displaying the greatest

number of anomalies normally associated with Noonan syndrome—patients UWA123-3 and UWA128-3—have deletions that are smaller than, and internal to, that of patient UWA106-3, who exhibits few such features (table 1 and figs. 2 and 5). These data suggest that the physical anomalies of these patients are part of the variable expression of the NF1 disorder. Supporting this conclusion is the recent description of a family with features of both Noonan and Watson syndromes, with a 42-bp in-frame tandem duplication in the NF1 gene (Tassabehji et al. 1993). In contrast to the NF1 deletions, this defective allele is presumably transcribed and translated into abnormal neurofibromin. Together, these data suggest that some "Noonan" features can develop in NF1 patients with either reduced or abnormal neurofibromin. Our findings emphasize the need for molecular evaluation of the NF1 gene in patients reported previously in the literature as presenting with "neurofibromatosis-Noonan syndrome" (Allanson et al. 1985; Mendez 1985; Opitz and Weaver 1985; Meinecke 1987; Quattrin et al. 1987; Abuelo and Meryash 1988; Stern et al. 1992). Similarity in phenotype between certain NF1 patients and Noonan syndrome patients may be the result of similar developmental or physiological events. It has been proposed that in utero edema is responsible for some of the features of Noonan syndrome (Allanson et al. 1985; Witt et al. 1987). It also occurs in Turner syndrome, in which affected individuals have a facial appearance, neck changes, and chest alterations similar to those in Noonan syndrome (Shepard and Fantel 1986). One of our patients with NF1 also had in utero edema (table 1).

It is interesting to note that the unusual phenotypic features of some NF1 individuals, including two of our patients, have been commented on elsewhere. A photograph of patient UWA69-3 as a young child has been published (fig. 2-1 in Riccardi and Eichner 1986; Riccardi 1992). The dysmorphologist David Smith examined patient UWA128-3 in 1970 and noted in his clinic documentation that she represented an entity not previously described in the literature at that time. Kaplan and Rosenblatt (1985) described three children from two families with NF1, dull intellect, and a distinctive facies strikingly similar to those of our patients.

Our data suggest that individuals with sporadic NF1, dysmorphism, and intellectual impairment may be more likely to carry large deletions of the NF1 gene. Further studies are needed to determine precisely the contribution of an NF1 null allele or of an extensive deletion involving contained or contiguous genes to the phenotypic manifestations of large numbers of neurofibromas, dysmorphic features, and/or intellectual impairment.

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