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Identification of the Functional Profilin Gene, Its Localization to Chromosome Subband 17p13.3, and Demonstration of Its Deletion in Some Patients with Miller-Dieker Syndrome

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Summary

Profilin is a conserved actin-monomer-binding protein which is found in all eukaryotes, including yeast. Although amino acid sequence analysis and RNase protection analysis suggest a single profilin isoform in mammalian cells, Southern blot analysis of human and somatic cell hybrid DNA indicates several loci in the human genome which hybridize with the profilin cDNA. We therefore isolated human genomic clones to analyze these genetic loci in detail. Only one of the cloned loci has typical features of a functional gene, including upstream transcriptional elements and typical exon-intron structure. Four other isolated loci are all diverged, intronless pseudogenes and are likely to be nonfunctional. The functional gene was localized to human chromosome band 17p13 by analysis of somatic cell hybrids and by in situ chromosomal localization. The Miller-Dieker syndrome (MDS), a rare congenital disorder manifested by characteristic facial abnormalities and lissencephaly (smooth brain), is associated with microdeletions of the distal 17p region. RFLP analysis of a patient with MDS, and analysis of somatic cell hybrids containing partially deleted chromosomes 17 from patients with MDS, using the profilin gene probe, indicate that profilin is localized to chromosome subband 17p13.3. These results also indicate that profilin is the first identified cloned gene which is part of the genetic material deleted in some patients with MDS but that other patients have smaller deletions not affecting the profilin locus. Thus, single allelic deletion of the profilin locus may contribute to the clinical phenotype of the MDS in some patients but does not play a major role in the essential phenotype.

Introduction

Profilin is a 15-kD actin-monomer-binding protein expressed at relatively high levels in mammalian cells (Carlsson et al. 1976; Blikstad et al. 1980; DiNubile and Southwick 1985). It is thought to serve an important storage-pool function for actin monomers, and its affinity for actin is reduced by binding of polyphosphoinositides in vitro (Lassing and Lindberg 1985, 1988). Reversible interactions between profilin and actin and between profilin and phosphoinositides are thought to occur in vivo to permit regulated actin filament assembly in response to cell surface–mediated signaling events (Markey et al. 1981; Lind et al. 1987). Profilin has recently been shown to be localized ultrastructurally to the membranes of human platelets and polymorphonuclear leukocytes (Hartwig et al. 1989), supporting an in vivo interaction with phosphoinositides.

Profilin-like proteins and/or their genes have been identified and cloned in multiple eukaryotic species including Acanthamoeba (Reichstein and Korn 1979; Ampe et al 1988*b*) and yeast (Oechsner et al. 1987). In yeast, the single profilin gene is reportedly essential for viability (Magdolen et al. 1988). Clear sequence conservation among the various profilins is limited to

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the N-terminal and C-terminal regions of the protein, the latter of which is thought to be the actin-binding domain, on the basis of both cross-linking to actin and preliminary structural information derived from analysis of crystals of the protein (Magnus et al. 1986). In Acanthamoeba, three different isoforms of the protein have been identified (Ampe et al. 1985, 1988*b*; Kaiser et al. 1985), which vary predominantly in the central region of the protein and have identical sequence in the N-terminal 21 residues and in 31 of 34 residues at the C-terminus.

In mammals, evidence for profilin isoforms has been much more limited. Profilin has been isolated and sequenced from bovine spleen and human platelets (Ampe et al. 1988*a*), with the finding of a single isoform in those tissues. In addition, RNase protection analysis of RNA from several tissues by using the profilin cDNA also suggested the presence of a single profilin mRNA (Kwiatkowski and Bruns 1988). A recent report (Fujiki and Pollock 1988), however, describes the isolation from a murine sarcoma ("sarcoma 180" cells), of a profilin with novel amino acid composition. Moreover, Southern blot analysis of human and somatic cell hybrid DNA has indicated the presence of at least four loci in the human genome which hybridize strongly to the human profilin cDNA probe, including 5′ and 3′ untranslated regions (Kwiatkowski and Bruns 1988).

We therefore examined these profilin-related genetic loci in detail, to determine which of them was the functional profilin gene and whether the others were functional or nonfunctional related genes.

Material and Methods

A human genomic library in EMBL3 was the gift of Drs. David Bonthron and Stuart Orkin and was used as described elsewhere (Kwiatkowski et al. 1988). DNA sequencing was performed on M13mp18 and M13mp19 single-stranded vectors and double-stranded plasmid vectors by using Sequenase (U.S. Biochemical, Cleveland). Southern blot analysis was performed on DNA prepared from human leukocyte or lymphoblastoid or somatic cell hybrid cell lines by standard methods, using random hexanucleotide labeled probes and GeneScreen Plus paper (du Pont, Wilmington, DE), as described elsewhere (Kwiatkowski and Bruns 1988). Oligonucleotides were synthesized by the phosphotriester method. Plasmid pPFN was used as the full-length profilin cDNA probe, with insert length 0.8 kb (Kwiatkowski and Bruns 1988).

S1 nuclease analysis was performed as described elsewhere (Kwiatkowski et al. 1988). In brief, restriction enzyme–digested plasmid DNA was phosphatased with calf intestinal phosphatase (Boehringer-Mannheim Diagnostics, Federal Republic of Germany), end-labeled with γ^{32}P ATP and polynucleotide kinase, digested with a second restriction enzyme, and size fractionated by PAGE. Isolated probe (10,000 cpm) probe was hybridized to 10 μg cellular RNA in a solution of 80% formamide, 0.05% SDS, 1 mM EDTA, 10 mM Pipes pH 6.4, 0.4 M NaCl by heating at 90°C for 5 min and then at 65°C for 30 min and then was incubated at 42°C for 8–12 h. S1 nuclease (3–100 U; New England Nuclear) was then added in 0.3 M NaCl, 30 mM sodium acetate pH 4.5, 3 mM $ZnSO_4$, and digestion proceeded for 30 min at 22°C. Digestion products were analyzed on 7 M urea–6% polyacrylamide sequencing gels. Probes were also sequenced by the Maxam and Gilbert (1980) procedure and were run on the same gel.

In situ hybridization to metaphase spreads of human chromosomes was performed according to a method described elsewhere (Morton et al. 1984). Plasmid PFNHP1.0 was radiolabeled to a specific activity of 1.39×10^7 cpm/μg, by nick-translation with all four ^{3}H-labeled deoxynucleotide triphosphates. Metaphase chromosomes were obtained from peripheral blood lymphocyte cultures established from karyotypically normal human males.

Karyotypic and Southern blot analyses were performed on a phenotypically normal mother and on her child affected with Miller-Dieker syndrome (MDS). The child had typical MDS facies, including micrognathia and microcephaly, and lissencephaly and died at 6 mo of age. Chromosome preparations from the mother indicated a derivative chromosome 17, presumed to be the reciprocal product of a balanced translocation involving 17p13 and an unidentified chromosome (Krauss et al. 1988). The child inherited the der(17) from the balanced translocation and was thus monosomic for 17p13-pter sequences and potentially trisomic for the sequences transferred to the der(17).

Somatic cell hybrids, containing abnormal chromosomes 17 from patients with MDS and variable-sized microscopic or submicroscopic deletions of 17p, were used in Southern blot analysis for regional localization on chromosome 17. The method of generation of the hybrids, and their use in ordering of cloned genes and anonymous DNA fragments in the 17p13 region, are described in detail elsewhere (vanTuinen et al., 1987, 1988; Ledbetter et al. 1989). Results from this mapping panel are consistent with the hypothesis that all deletions of chromosome 17 which occur in MDS pa-

tients are simple in the sense that a single contiguous region of chromosome 17 is always lost. This panel of hybrids is therefore useful in mapping genes on chromosome 17 relative to other cloned markers. Since human chromosome 17 is homologous to mouse chromosome 11, the mouse-rat hybrid F(11)J, containing mouse chromosome 11 as its only mouse DNA (Killary and Fournier 1984), was used to map the profilin gene in the mouse.

Results

Isolation and Classification of Human Profilin Genomic Clones

Some 500,000 phage clones of a human genomic library in EMBL3 (average insert 15–20 kb, roughly three genome equivalents) were screened with the full-length human profilin cDNA, pPFN. Fourteen positive clones were identified and isolated for analysis. Restriction mapping and Southern blot analysis of the clones permitted the determination of five distinct groups of clones (see below). Four oligonucleotides (see fig. 1) were used to assess the sequence relationship of the clones to the profilin cDNA (data not shown). The oligonucleotides were chosen from the 5′ and 3′ regions of the cDNA to reduce the possibility that introns might occur in the middle of the oligo sequences. Only three clones hybridized to all four oligonucleotides; the other 11 hybridized to one (1) or two (10). These three clones had the same detailed restriction map and were subjected to sequence analysis. This locus is denoted the PFN1 gene, on the basis of its localization to chromosome 17 (see below). The remaining 11 genomic clones were grouped into four distinct loci by restriction mapping and Southern blot analysis of *Sau*3A digests of the clones by using pPFN as probe (data not shown). These other genomic loci are denoted PFNA, PFNB, PFNC, and PFND.

The Functional Profilin Gene, PFN1

The nucleotide sequence and restriction map of one of the genomic clones derived from the PFN1 locus are shown in figure 1. PFN1 had typical features of a functional gene. To confirm that it was a functional locus and to determine the site of transcriptional initiation, S1 nuclease protection analysis was performed using the *Nco*I-digested, ^{32}P-labeled 5′ fragment of the PFN1 gene. Results are shown in figure 2, in comparison with

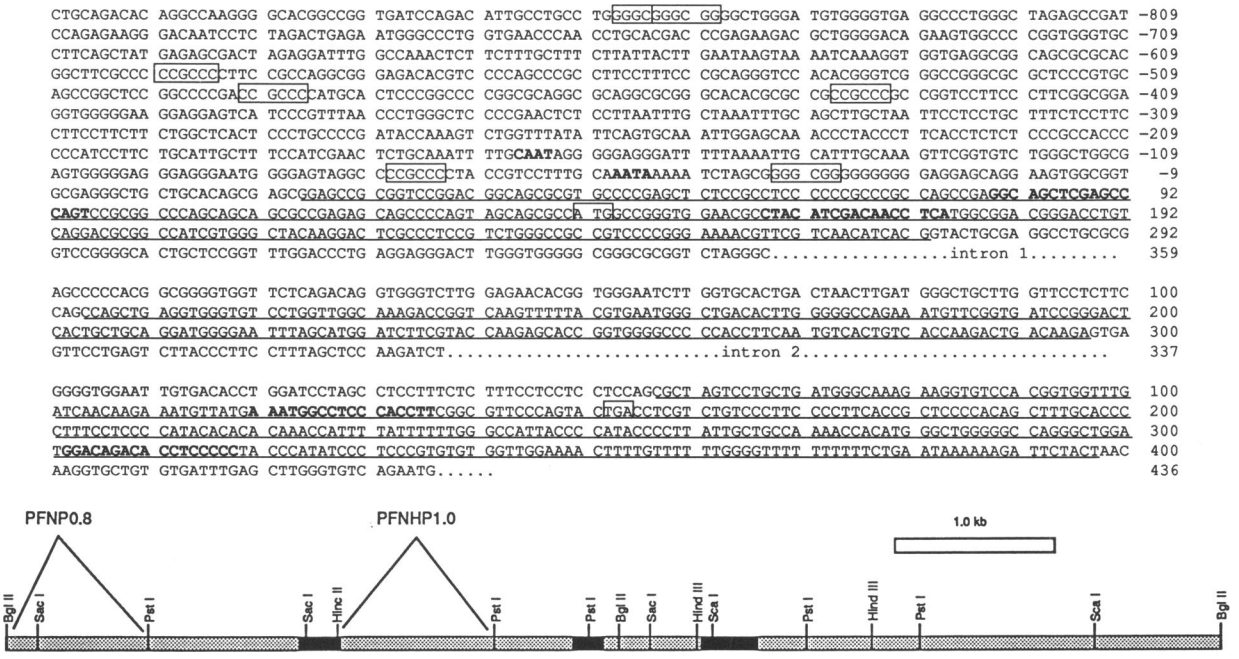


Figure 1 Nucleotide sequence and restriction map of the PFN1 locus. *a,* Nucleotide sequence of PFN1. Copies of the Sp1 promoter element are boxed; CAAT and TATA elements are in boldface. The sequence of the full-length profilin cDNA is underlined; four oligonucleotides within the cDNA sequence are indicated in boldface; the initiator ATG and terminator TGA are also boxed. *b,* Restriction map of PFN1. A size standard is indicated; exon sequences are indicated by filled boxes. PFNP0.8 and PFNHP1.0 are, respectively, the 0.8-kb and 1.0-kb fragments indicated.

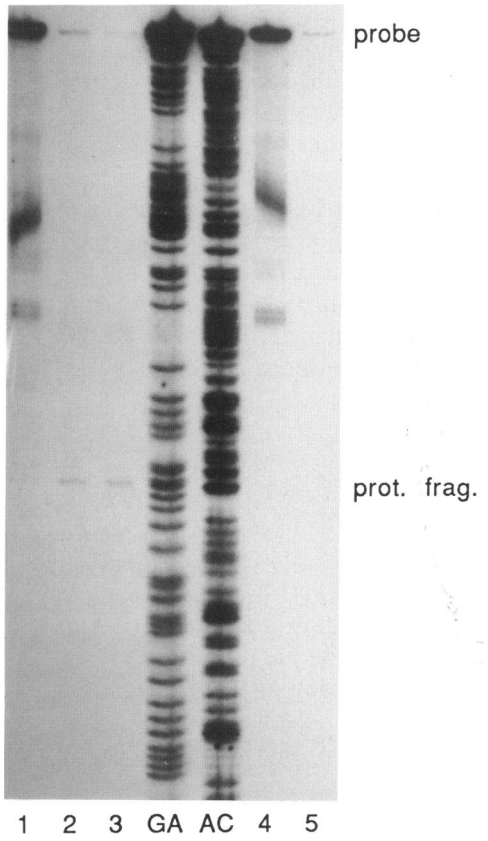


Figure 2 Definition of the transcription-initiation region of PFN1 by S1 nuclease protection. The *Nco*I-digested (base 143, fig. 1*a*), end-labeled 5′ fragment of the PFN1 locus was used. Lanes 1–3, S1 nuclease analysis of 10 μg human uterus RNA; after hybridization, 30, 100, and 300 U S1 nuclease were added to the samples loaded in lanes 1–3, respectively. Lanes GA and AC, Maxam-Gilbert sequencing reactions of the same probe. Lanes 4 and 5, S1 nuclease analysis of 10 μg yeast tRNA; after hybridization, 30 and 100 U S1 nuclease were added to the samples loaded in lanes 4 and 5, respectively. A protected fragment of size 143 bases (to base 0; fig. 1*a*) is seen.

adjacent Maxam-Gilbert sequencing reactions. The labeled fragment is protected 15 bases past the last base of the longest cDNA and defines the site of transcriptional initiation (denoted as base 1 in fig. 1*A*, top). Other features of the PFN1 locus that are consistent with its identification as the functional profilin gene included (1) several upstream GC-rich regions, the longest of which was bases −631 to −401 with 81% GC, and seven copies of the Sp1 promoter element GGGCGG (Dynan and Tjian 1985) (boxed); (2) CAAT and TATA boxes (Breathnach and Chambon 1981) (shown in bold) at positions −165 and −56; (3) two introns, of length 1.5 and 0.55 kb, with perfect matches for the mammalian splice consensus sequence; (4) absence of a poly A tail at the 3′ end, in comparison with the cDNA; and (5) a perfect sequence match in the exons with the cDNA.

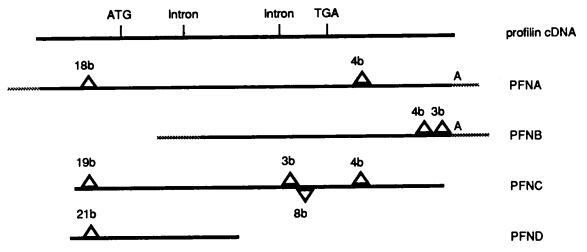
Two *Bgl*II-*Bgl*II fragments, as shown in figure 1*B*, were derived from the same genomic clone as well as two smaller PFN1 locus-specific probes (see below). These probes are PFNP0.8, a 0.8-kb *Bgl*II-*Pst*I fragment located upstream of the transcriptional initiation site, and PFNHP1.0, a 1.0-kb *Hin*cII-*Pst*I fragment derived from the first intron of the gene.

Loci PFNA–PFND

Complete restriction mapping and partial sequence analysis were performed on representative genomic clones derived from loci PFNA–PFND. Comparison of double-stranded sequence data with those of the profilin cDNA is shown schematically in figure 3 (full sequence information is available on request). In contrast to PFN1, sequence regions similar to the profilin cDNA did not contain intervening sequence. Moreover, sequence comparison indicated that all were significantly diverged from the profilin cDNA, with only 86.0%–88.7% identical nucleotide residues. There were also significant nucleotide deletions in all of PFNA–PFND (fig. 3). Although larger deletions occurred primarily in the 5′ and 3′ untranslated regions, single base deletions/additions were found in PFNA, PFNC, and PFND within the coding region, causing frameshifts. For these three loci, the initiator methionine sequence had also diverged. PFNB was truncated at its 5′ end relative to the coding region of the profilin cDNA (fig. 3), without evident splice-site sequence. The remainder of its sequence was in-frame without stop codons but with multiple nucleotide and amino acid substitutions. For PFNA and PFNB, the sequence determined extended beyond the 3′ untranslated region of the cDNA, demonstrating a poly A tail, strongly suggesting that they were derived through an RNA intermediate.

Sequence comparisons of PFNA–PFND both with the cDNA sequence and with each other indicated that PFNA, PFNB, and PFND were less diverged from each other than from the cDNA. In addition, sequence entirely 3′ to the cDNA from PFNA and PFNB had high similarity, suggesting that after a single retrotranscription event multiple copies of the gene were generated. This possibility is also suggested by the observation that all of the loci which extended into the 5′ region of the cDNA contained an 18–21-base deletion at exactly the same site.

The similarity between PFN1 and PFNA at the 5′ end of PFNA extended to base −18 of the PFN1 se-

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PFNA        TTCTTTTTTTAAGTGGCGGAGCGAGGGCTACT
                      ||||||||| ||||||||| ||
PFN1  -28   GAGGAGCAGGAAGTGGCGGTGCGAGGGCTGCT   4
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Figure 3 Comparison between the profilin cDNA, pPFN, and loci PFNA, PFNB, PFNC, and PFND. *a*, Representation of sequenced portions of the profilin loci. Regions similar to the profilin cDNA are indicated by a dark line; those distinct from the cDNA are indicated by a cross-hatched line. Triangles indicate sites of deletion (△) and insertion (▽) of length ≥3 bases relative to the profilin cDNA sequence; the sites of introns present in PFN1 are also indicated; poly A tails are indicated by A. *b*, Sequence comparison between the 5′ regions of PFNA and PFN1.

quence (fig. 3*B*), and at that point PFNA abruptly diverged with an AT-rich sequence. This observation is also consistent with pseudogene formation following retrotranscription (Weimer et al. 1986), but it suggests that transcription of mRNA in PFN1 initiates at base −18 upstream of the transcription initiation site indicated by S1 nuclease protection. It is possible that a more proximal site is used occasionally or was used in the past at the time of generation of the PFNA pseudogene.

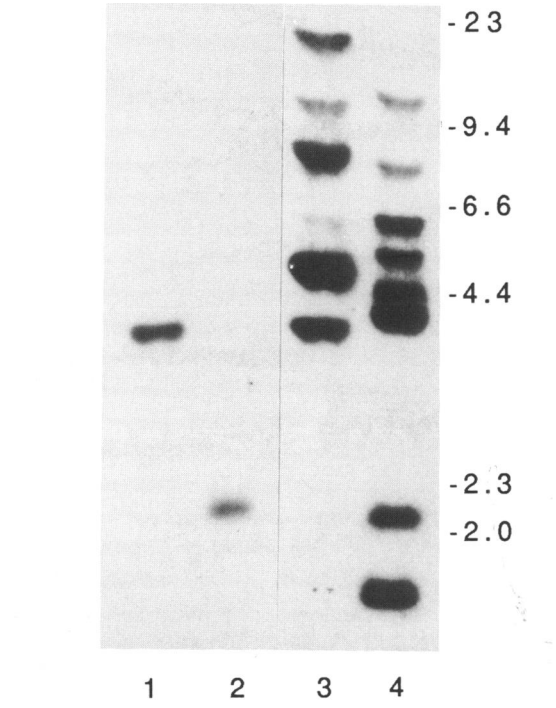
PFN1 Is Localized to Chromosome 17p13

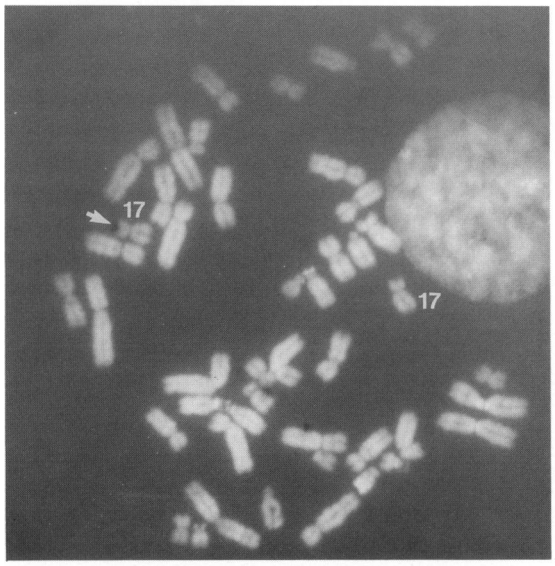
Previous somatic cell hybrid analysis (Kwiatkowski and Bruns 1988) using the profilin cDNA indicated that profilin loci were dispersed in the genome, with distinct Southern blot bands localized to chromosomes 17 (PFN1), 1 (PFN2), 6 and/or 13 (PFN3), and a fourth independent segregant (PFN4) whose precise location could not be determined (nomenclature per HGM9.5 [McAlpine et al. 1988]). A 4.0-kb *Bgl*II-*Bgl*II genomic fragment represented the PFN1 locus in this analysis. The restriction map of the isolated genomic clones containing the functional profilin gene indicated that the functional gene was contained in two *Bgl*II fragments, each of this size (fig. 1*B*). PFNHP1.0, the intron fragment of the functional profilin gene, was used in Southern blot analysis of *Bgl*II-digested human DNA, in comparison with a pPFN-probed Southern blot, to confirm the derivation of this band from the functional profilin gene (fig. 4*A*). Southern blot analysis of both *Bgl*II and *Sac*I genomic digests also demonstrates that PFNHP1.0 is a single-copy sequence in the human genome, with single reactive bands, in contrast to pPFN.

The PFNHP1.0 probe was then used in in situ hybridization to metaphase spreads, to determine the regional localization on chromosome 17. The in situ analysis was initially performed with the observer (C.C.M.) blinded to the results of the somatic cell hybrid analysis. Several peaks of hybridization were noted, including, in order of magnitude, 13q12-q14, 17p13, 1p36.1-p36.3, 18p11.3, and 1p31-p32. After these data were collected, the results of both the previous hybrid mapping and the assignment of PFN1 to chromosome 17 were considered, and the hybridization to 17p13 was concluded to be the regional assignment. Analysis of 50 metaphase preparations from a normal male that were hybridized with the tritium-labeled PFNHP1.0 probe indicated that 12% of spreads had silver-grain deposition at band p13 of at least one chromosome 17. Of the nine silver grains observed to be located on or beside chromosome 17, six (3.1% of the total number of grains observed overlying chromosomes) were localized in band p13. The reason(s) for the relatively low specificity and multiple peaks of hybridization when a single-copy intron-specific probe is used in this analysis is not evident. The correlation between (*a*) chromosomes hybridizing in situ with the intron-specific probe and (*b*) the localization of profilin by somatic cell hybrids (i.e., chromosomes 1 and 13) is also not understood. However, to confirm the regional localization on chromosome 17, additional chromosomes 17 were analyzed by in situ hybridization. In 122 metaphase preparations, 24 grains were found on or beside chromosome 17; 12 (50%) of these were localized at 17p13. The remaining grains were evenly distributed over the chromosome, without any secondary peaks. The aggregate grain-localization data and a representative metaphase spread are shown in figures 4*B* and 4*C*.

PFN1 Is Deleted in Some Patients with MDS

Since this localization suggested that profilin might be a part of the genetic material on chromosome 17 that is deleted in MDS, we sought to confirm it by RFLP analysis of both a phenotypically normal mother and her child affected with MDS. As shown in figure 5, digestion of human DNA with *Rsa*I permits the identification of a simple two-allele polymorphism by using PFNP0.8 (see fig. 1*B*) as probe. Among 21 unrelated Americans, the frequencies of bands A1 and A2 were .52 and .48. The two alleles demonstrated codominant segregation among seven informative families which in-





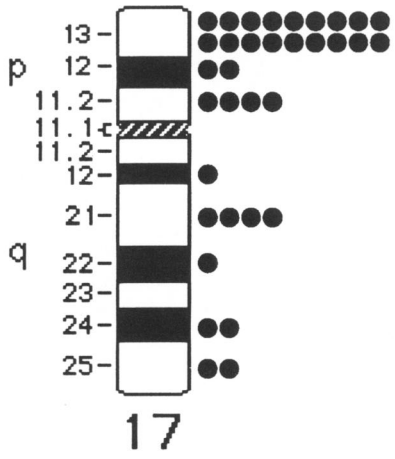


Figure 4 Chromosomal localization of PFN1. *a,* Southern blot analysis of human DNA, using PFNHP1.0 (lanes 1 and 2) and the profilin cDNA (lanes 3 and 4). Lanes 1 and 3, *Bgl*II digest; lanes 2 and 4, *Sac*I digest. Unique 4.0-kb *Bgl*II and 2.1-kb *Sac*I fragments react with the PFNHP1.0 probe. *b,* Chromosome 17 idiogram indicating the relative location of silver grains seen after in situ chromosomal hybridization. *c,* Representative metaphase spread showing hybridization with PFNHP1.0 at 17p13.3.

cluded 53 individuals. Application of the probe to *Rsa*I-digested DNA from the mother and MDS-affected child (fig. 5, lanes 6 and 7, respectively) demonstrated absence of the maternal allele in the child, a finding consistent with deletion of the maternal PFN1 allele. Additional polymorphic 17p probes, including H37, Z22, and 144 (see fig. 6), for which the mother was heterozygous, were also absent in the child.

We extended these observations by Southern blot analysis of a panel of somatic cell hybrids, containing partially deleted human chromosomes 17, derived from patients with MDS (Ledbetter et al. 1989). Profilin was deleted in three of the somatic cell hybrids containing cytogenetically visible deletions of 17p (fig. 6). The smallest of these deletions (hybrid MH74 in patient MDS-1) has a breakpoint in the most distal subband,

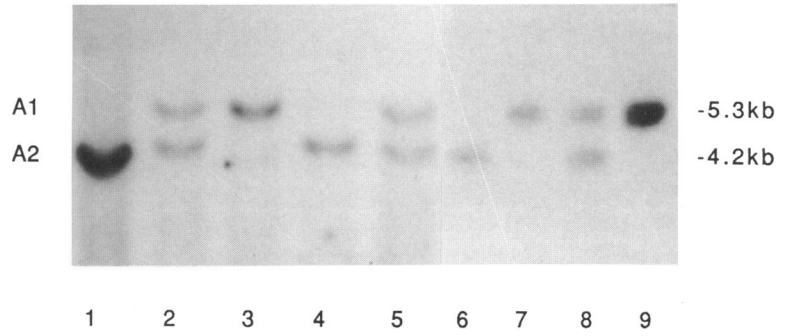


Figure 5 RFLP analysis using the PFNP0.8 probe. Autoradiographs of Southern blots are shown, of DNA derived from seven unrelated individuals, after digestion with *RsaI* by using the PFNP0.8 probe (lanes 1–5, 8 and 9), and of DNA derived from a phenotypically normal mother (lane 6) and her MDS-affected child (lane 7). No maternal allele is seen in the child.

p13.3, of chromosome 17, therefore localizing profilin to this subband. Profilin was not deleted in one hybrid containing a smaller, visible deletion (hybrid AY1 in patient MDS-12) or in two hybrids from patients with submicroscopic deletions (hybrid HD13 in patient MDS-6 and hybrid BR8 in patient MDS-9). This indicates that profilin is both outside of the smallest region of overlap of these deletions and proximal to the MDS critical region.

Hybridization to *BglII*-digested DNA from the mouse-rat hybrid containing mouse chromosome 11 showed both the mouse-specific (9.4-kb) and rat-specific (15-kb) bands to be present (data not shown), indicating that profilin maps to mouse chromosome 11.

Discussion

We have demonstrated both that there is a single functional profilin gene among those loci recognized by the profilin cDNA probe and that it is localized to human chromosome 17p13.3. The perfect sequence match and structural features of PFN1 strongly argue that it is the functional profilin gene. The other profilin loci identified are processed retropseudogenes, have significantly diverged from the profilin mRNA sequence, and are unlikely to be transcriptionally active. In addition, previous RNase protection analysis using RNA probes covering the entire profilin mRNA gave no evidence of partially protected fragments, as would be expected if any of PFNA–PFND were transcriptionally active.

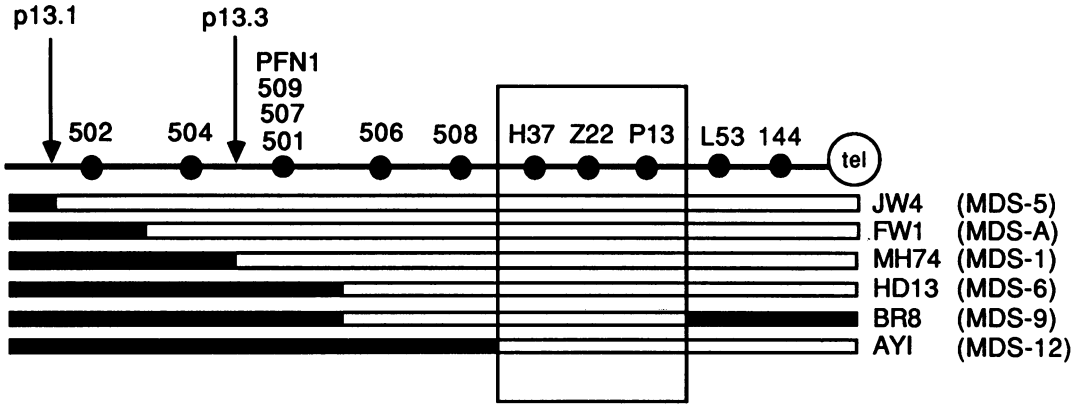


Figure 6 Localization of PFN1 within subband 17p13.3. Twelve anonymous DNA probes (502–144, shown at top) were previously ordered on 17p by their presence or absence in somatic cell hybrids containing deletion chromosomes 17 from MDS patients (Ledbetter et al. 1989). The PFN1 position, as based on its hybridization to the same hybrid panel, is shown. Probes are ordered from centromere (at left) to telomere (tel). Cytogenetically visible breakpoints at subbands p13.1 and p13.3 are designated by arrows. Below the map are bars representing the results of hybridization of the 12 anonymous probes and PFN1. Filled bars represent positive hybridization (not deleted), and open bars represent negative hybridization (deleted). The box indicates the smallest region of overlap for all deletions, which defines the MDS critical region.

These observations conflict with the report of the occurrence of a distinct profilin isoform in the murine "sarcoma 180" cell line (Fujiki and Pollock 1988). It is possible that this novel profilin isoform is derived from PFN1 by a mutational event, as has been reported for actin in the HuT-14 cell line (Lin et al. 1985). Alternatively, there could be two functional genes in mice, in contrast to the situation in humans. Finally, genes encoding widely diverged forms of profilin might not be detected by this method of analysis.

Analysis of a panel of somatic cell hybrids containing deletions of distal 17p refined the localization of PFN1 to subband p13.3. This is the most telomeric location of any cloned gene on 17p mapped to date. Previously, tumor antigen p53 and RNA polymerase II were the most distally located genes on 17p, in the interval p13.100-p13.105 (vanTuinen et al. 1988). Every human gene on human chromosome 17 maps to mouse chromosome 11 (Buchberg et al. 1989), thereby representing the largest conserved syntenic group among autosomes. The localization of profilin to mouse chromosome 11 further extends this homology to the most distally localized gene on human 17p.

MDS is a rare disorder manifested by characteristic facial abnormalities and classical, or type 1, lissencephaly (smooth brain) (Dobyns et al. 1984). Most cases of MDS have a subtle cytogenetically detectable deletion of chromosome 17p, involving subband 13.3 (Schwartz et al. 1988; Ledbetter et al. 1989). However, other patients with this syndrome have no abnormality visible by cytogenetic study. MDS is thought to be a "contiguous gene syndrome" (Schmickel 1986), i.e., a complex disease phenotype associated with chromosomal deletions affecting multiple, unrelated genetic loci physically contiguous on a chromosome (Ledbetter et al. 1989). PFN1 is the only currently recognized functional gene deleted in some MDS patients and therefore is potentially involved in the phenotypic expression of these patients. The fact that it is not deleted in patients with smaller visible or submicroscopic deletions indicates profilin does not play a major role in the primary phenotypic features in MDS, such as the neuronal migration defect producing lissencephaly. It will be of interest to examine the tissues of patients with MDS who have lost one copy of the profilin gene, to see whether there are any detectable effects on cellular profilin levels. If profilin is in fact reduced, then effects on cell structure, actin filament organization, and phosphoinositide metabolism will all be of interest, as profilin is postulated to have important roles in all of these processes.

Acknowledgments

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