

Ancient Missense Mutations in a New Member of the *RoRet* Gene Family Are Likely to Cause Familial Mediterranean Fever

The International FMF Consortium*

Summary

Familial Mediterranean fever (FMF) is a recessively inherited disorder characterized by dramatic episodes of fever and serosal inflammation. This report describes the cloning of the gene likely to cause FMF from a 115-kb candidate interval on chromosome 16p. Three different missense mutations were identified in affected individuals, but not in normals. Haplotype and mutational analyses disclosed ancestral relationships among carrier chromosomes in populations that have been separated for centuries. The novel gene encodes a 3.7-kb transcript that is almost exclusively expressed in granulocytes. The predicted protein, pyrin, is a member of a family of nuclear factors homologous to the Ro52 autoantigen. The cloning of the FMF gene promises to shed light on the regulation of acute inflammatory responses.

Introduction

The hereditary periodic fever syndromes are a relatively recently recognized group of inherited inflammatory disorders that are characterized by episodic, self-limited bouts of fever accompanied by unexplained arthritis, sterile peritonitis, pleurisy, and/or skin rash (reviewed in Kastner, 1996). To date, three clinical diseases have been identified in this class: the recessively inherited familial Mediterranean fever (FMF, MIM249100) and Dutch type periodic fever (MIM260920, also known as the hyperimmunoglobulinemia D syndrome), and the dominantly inherited familial Hibernian fever (MIM142680). In addition, there are a number of other related but clinically distinguishable conditions (Gertz et al., 1987; Karenko et al., 1992) that have been described in case reports but have not yet been organized into specific nosologic entities.

Of the three well-defined hereditary periodic fever syndromes, FMF is by far the most common and has attracted the most investigative interest. FMF is seen

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primarily in individuals of non-Ashkenazi Jewish, Armenian, Arab, and Turkish background. In these populations, the carrier frequency has been estimated to be as high as 1:5 (Rogers et al., 1989; Daniels et al., 1995; Yuval et al., 1995), raising speculation that heterozygotes may have some selective advantage, perhaps manifested by increased resistance to an as yet unidentified infectious agent. Depending on poorly defined hereditary and environmental factors (Pras et al., 1982), homozygotes often develop progressive systemic amyloidosis from the deposition of the acute phase reactant serum amyloid A (SAA). Prior to prophylactic treatment with colchicine, FMF amyloidosis was a significant cause of renal failure and death in the Middle East. In the absence of an accurate diagnostic test, FMF patients in countries where the disease is less prevalent often experience years of attacks and several exploratory surgeries before the correct diagnosis is made and adequate treatment with colchicine is begun.

FMF attacks are characterized by a massive influx of polymorphonuclear leukocytes (PMNs) into the affected anatomic compartment. Inciting factors are unclear, and, even off treatment, patients can sometimes inexplicably go for long periods without symptoms, only to relapse into a frequent pattern of attacks at some later time. At the biochemical level, patients have variously been reported to have abnormal levels of a C5a inhibitor (Matzner and Brzezinski, 1984), neutrophil-stimulatory dihydroxy fatty acids (Aisen et al., 1985), and dopamine β -hydroxylase (Barakat et al., 1988), but none of these findings has led to the identification of the genetic basis of FMF.

In the absence of other functional clues, we undertook a positional cloning approach to identify the FMF gene (designated *MEFV*). Initial linkage studies placed *MEFV* on chromosome 16p (Pras et al., 1992) in a panel of non-Ashkenazi Jewish families. Linkage to this region has subsequently been confirmed and extended to all four major affected ethnic groups (Shohat et al., 1992; Pras et al., 1994; French FMF Consortium, 1996), although differences in severity among subpopulations has suggested the possibility of allelic heterogeneity (Pras et al., 1997). Early studies demonstrated a substantial founder effect in the Moroccan Jewish population (Aksentijevich et al., 1993), and as markers closer to *MEFV* have been identified, it has become apparent that a high percentage of all North African Jewish FMF carrier chromosomes (93% in our hands) are descended from a common ancestor (French FMF Consortium, 1996; Levy et al., 1996; Balow et al., 1997). This same ancestral haplotype was observed in one third of the Armenian carrier chromosomes we have studied and in about 15% of Iraqi Jewish FMF chromosomes.

Using publicly available polymorphic markers, we narrowed the candidate region to an approximately 1 Mb interval lying between the polycystic kidney disease (*PKD1*) and tuberous sclerosis (*TSC2*) genes on the telomeric end, and the CREB-binding protein (*CREBBP*) gene on the centromeric end (Sood et al., 1996; see Figure 1). The extensive physical maps constructed around these genes (Dackowski et al., 1996; Giles et al., 1997) did not, however, extend into the *MEFV* region, and large insert YACs mapping to this interval (Doggett

et al., 1995) were found to be unstable. We therefore established a highly redundant contig comprised of small-insert YACs, P1s, PACs, BACs, and cosmids that completely spans the interval (Sood et al., 1997). Using an independent panel of non-Ashkenazi Jewish families and newly developed microsatellites from Genethon, the French FMF Consortium (1996) identified markers \sim 600 kb apart defining the *MEFV* interval by intrafamilial recombinants. By comparing FMF carrier chromosomes in their panel with the ancestral haplotype noted above, the French Consortium found "historical recombinants" that further narrowed the candidate interval to a \sim 250 kb YAC. Our own data defined a 285 kb candidate interval by observed intrafamilial recombinants and an \sim 200 kb minimal region by historical recombinants (Balow et al., 1997). Both of these sets of data are consistent in their placement of the FMF gene.

We now report the cloning of the FMF gene from this 200 kb interval. Our strategy involved developing a detailed transcript map from this region by a combination of exon amplification, direct cDNA selection, single-pass sequencing of a minimal tiling path of cosmids, and, for selected areas, finished sequencing. During the construction of the transcript map, we identified new microsatellites that permitted further refinement of the candidate interval to the centromeric-most 115 kb. Using two trapped exons as probes, we isolated a 3.7 kb clone from a leukocyte cDNA library. The protein product is predicted to be 781 amino acids in length and is a member of a family of proteins that includes the Ro52 autoantigen (Itoh et al., 1991) and RFP (ret finger protein, the gene encoded by the 5' half of the hybrid *ret* transforming gene; Takahashi et al., 1988). We have identified three missense mutations in the cloned cDNA, all of which encode changes in the same highly conserved C-terminal domain. None of the three mutations is present in any of a large panel of normal control chromosomes. Two of these mutations are found on FMF carrier chromosomes in populations that, in some cases, have been geographically separated for over 2000 years, indicating that most cases of FMF are descended from a very ancient pool of founders, and suggesting common origins for several Middle Eastern populations.

Results

Positional Cloning Strategy

Figure 1 depicts schematically the recent steps in our positional cloning strategy. Based on early genetic localization data, we established a contig spanning the 1 Mb interval between *D16S94* and *D16S2622* (Sood et al., 1997). Microsatellite markers from this interval defined a critical region of 285 kb (*D16S468*–*D16S3376*) based on observed recombinations in a panel of 61 families, and a smaller candidate interval of \sim 200 kb (*D16S3082*–*D16S3373*) based on historical recombinants. Transcripts were then identified by a combined strategy of exon amplification, direct cDNA selection, and single-pass sequencing. This led to the isolation of 9 full-length cDNA clones (8 of which were previously uncharacterized), including 3 classical zinc finger genes, 2 members of the olfactory receptor family, and a novel caspase.

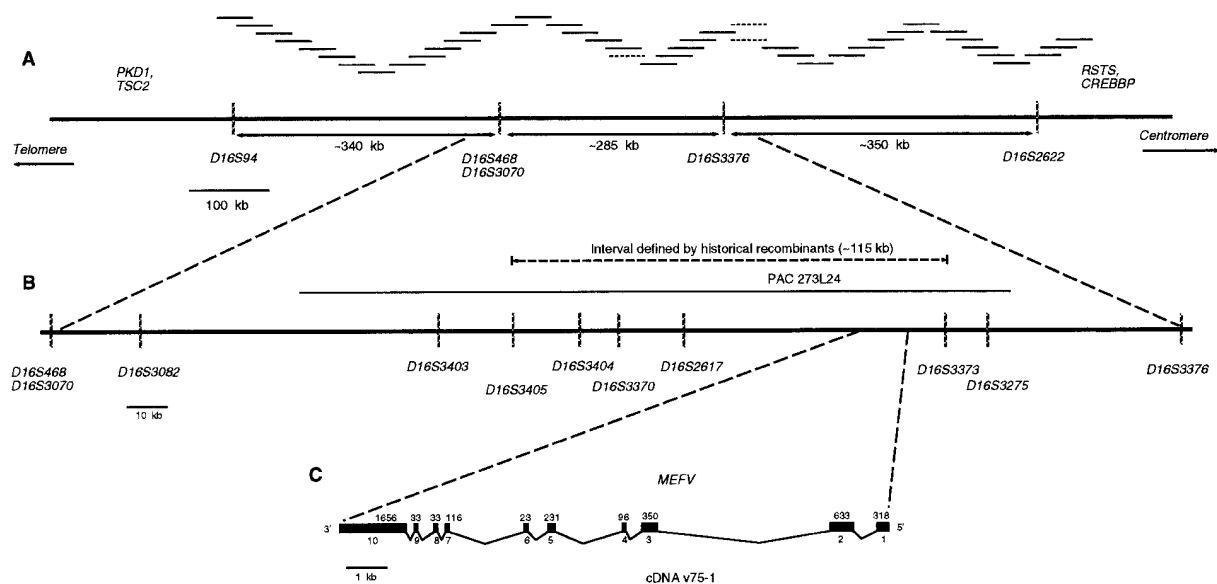


Figure 1. Schematic Representation of the *MEFV* Region on Chromosome 16p13.3

(A) ~1 Mb region over which a cosmid contig was constructed (Sood et al., 1997). *PKD1*, polycystic kidney disease 1; *TSC2*, tuberous sclerosis 2; *RSTS*, Rubinstein-Taybi syndrome; *CREBBP*, CREB binding protein gene. (B) ~285 kb candidate interval defined by intrafamilial recombination events in our panel of families (Balow et al., 1997). *D16S3405*–*D16S3373* is the minimal candidate interval defined by historical recombinants in the present study. (C) Representation of the genomic structure of the v75-1 gene. Shaded boxes represent exons; introns are drawn to scale. Numbers above the boxes represent the size of the exons, in bp. Numbers below the boxes reflect the order of the exons, with (1) being the most 5'.

The furthest centromeric of these 9 cDNAs, clone v75-1, was isolated by solution hybridization of a leukocyte cDNA library with biotinylated oligonucleotide probes derived from two exons trapped from PAC 273L24.

In parallel, analyzing finished sequence from ~90 kb in the center of the *D16S3082*–*D16S3373* interval, we found two new microsatellites, *D16S3404* and *D16S3405* (Figure 1B). In one non-Ashkenazi Jewish family, evidence for a historical recombination event that occurred between *D16S3404* and *D16S3405* in the highly conserved non-Ashkenazi Jewish haplotype (designated haplotype A) was observed, thereby excluding the region telomeric of *D16S3405* (and four candidate genes encoded therein) from further consideration.

Characterization of cDNA v75-1

The translated v75-1 cDNA sequence is shown in Figure 2, and the exon-intron structure deduced from the genomic sequence of two cosmids is depicted in Figure 1C. Although there is an excellent Kozak consensus (Kozak, 1996) at the initial methionine, the reading frame remains open in the cDNA upstream of this. Nevertheless, there are no splice-acceptor consensus sequences or in-frame methionines with good Kozak sequences before the first stop upstream in genomic DNA, and the transcript size by Northern blot (see below) is 3.7 kb, thus suggesting that the sequence presented is full-length.

Consistent with the initial analysis of single-pass genomic sequence from this region, BLASTX (Altschul et al., 1990) demonstrated homologies with a number of molecules implicated in inflammation. These include the 52 kDa Ro/SS-A ribonucleoprotein, against which patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome frequently make autoantibodies, the

interferon-inducible transcriptional regulator Staf-50 (Tissot and Mechti, 1995), and rpt-1, a mouse down-regulator of IL-2 (Patarca et al., 1988). Homology to the latter protein is in a domain extending from residues 385 to 550, while homology to the former two proteins is particularly high through a domain at the C-terminal end of v75-1 (double-boxed in Figure 2). This domain is also found in the *ret* finger protein (RFP) and has been termed an rfp (Takahashi et al., 1988) or B30.2 domain (Vernet et al., 1993; Henry et al., 1997). Analysis with the SEG algorithm (Wootton, 1994) indicated a high likelihood that this domain assumes a globular conformation.

Additional analyses of the putative protein product, termed pyrin to connote its relationship to fever, indicate that it is very positively charged, with a predicted pI > 8 and with lysine and arginine making up 13% of the amino acid composition. Moreover, the PSORT algorithm (Nakai and Kanehisa, 1992) detected two overlapping nuclear targeting signals. The first is a four-residue pattern composed of a histidine and three lysines (starting at residue 419), and the second is a Robbins/Dingwall consensus (boxed in Figure 2, residues 420–437; Robbins et al., 1991). In addition, a PROSITE search (Bairoch et al., 1997) identified a bZIP transcription factor basic domain (Shuman et al., 1990) at residues 266–280. The region between residues 375 and 407 (denoted by plus signs in Figure 2) contains an arrangement of cystine and histidine residues with spacing that conforms closely to a B box-type zinc finger domain (Reddy et al., 1992).

Northern analysis using a probe with unique sequence from exon 2 of v75-1 showed an abundant ~3.7 kb transcript in peripheral blood, but there was no significant expression in 14 other normal tissues, including

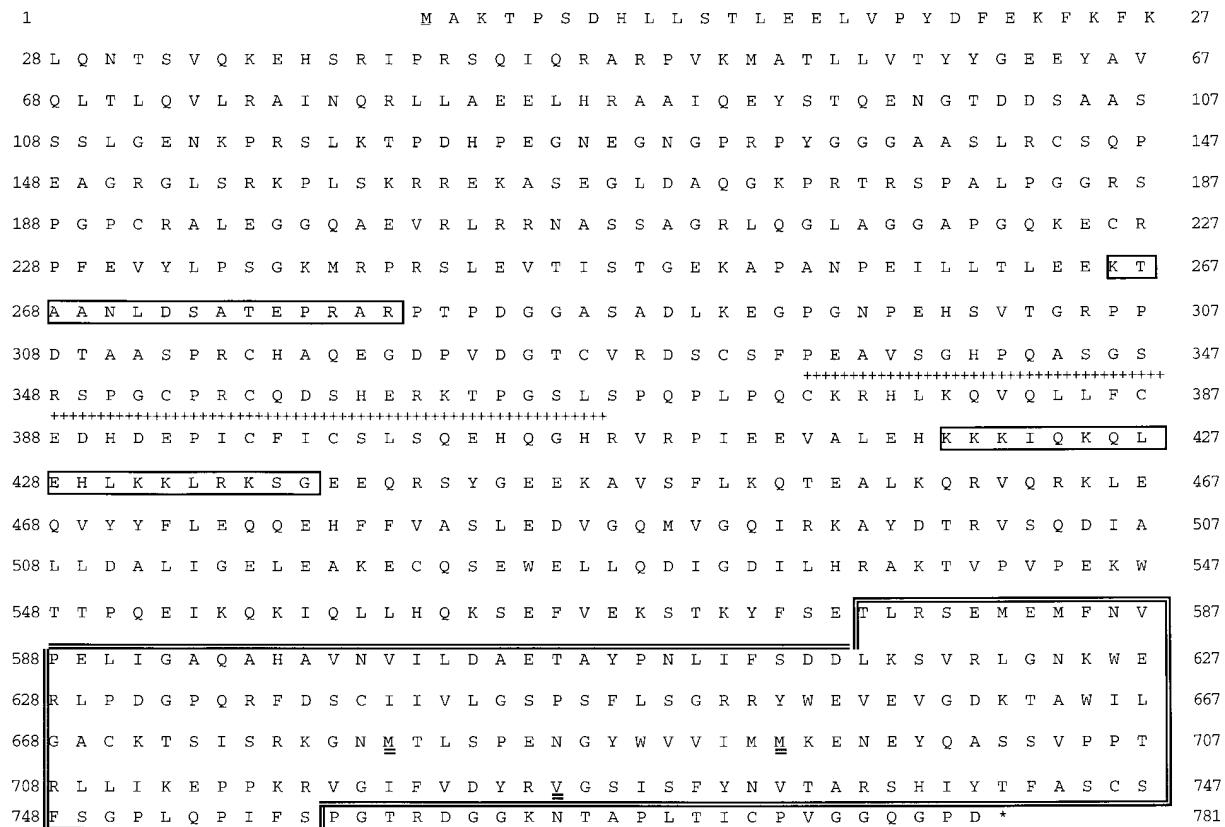


Figure 2. The Predicted Protein Sequence Encoded by cDNA v75-1

The boxed segment from aa 266 to 280 is a bZIP transcription factor basic domain; the boxed segment from aa 420 to 437 is a Robbins/Dingwall consensus nuclear targeting signal. The segment indicated by plus signs between residues 375 and 407 is a potential B-box zinc finger domain. The region double-boxed from residue 577 to 757 is a rfp, or B30.2, domain. Within this box, the three mutated amino acids are double-underlined.

spleen and thymus (Figure 3A). Bone marrow and lymph nodes were also negative for v75-1 expression (data not shown). Among a panel of tumor cells, only the SW480 colorectal adenocarcinoma cell line showed significant expression. Expression of v75-1 was absent in HL-60 promyelocytic leukemia, K-562 erythroleukemia, and Burkitt's lymphoma. Even by RT-PCR, message was not detectable in a peritoneal fibroblast cell line, or in fetal or adult brain (data not shown). Figure 3B demonstrates pyrin expression in PMNs, but not in lymphocytes. We also found that pyrin is not expressed in monocytes purified from peripheral blood (data not shown).

Mutational Analyses

Direct sequencing of RT-PCR products or amplified exons from the eight cDNAs telomeric to v75-1 failed to identify disease-associated mutations. In contrast, three different missense mutations were identified in exon 10 of v75-1 (Figure 4) after screening a total of 165 individuals from 65 families. The first mutation, a G→C transversion at nt 2040 that results in the substitution of isoleucine for methionine (M680I), was observed in the homozygous state in the affected offspring of a single Armenian family. The second, an A→G transition at nt

2080 causing a substitution of valine for methionine (M694V), was observed in a large number of affected individuals bearing four apparently distinct disease-associated haplotypes (see below). The third, a T→C transition at nt 2177, results in the substitution of alanine for valine (V726A) and was observed in affected individuals bearing the C haplotype in a Druze family and in other FMF patients and carriers bearing this haplotype. When the remaining nine exons of v75-1 were sequenced in these haplotypes, no additional mutations were identified.

Ancestral Relationships Among FMF Carrier Haplotypes

Table 1 explores the relationship of v75-1 mutations with specific disease-associated haplotypes in our study population. Haplotype A is the disease-associated haplotype observed in a very high percentage of North African Jewish carrier chromosomes, but that is seen less frequently in the Armenian and Iraqi Jewish populations. Haplotypes F and G are additional North African Jewish disease-associated haplotypes, each observed in a single family, that appear by microsatellite analysis to be different from the A haplotype (Table 1A). Haplotype B is yet another distinct FMF haplotype observed in the

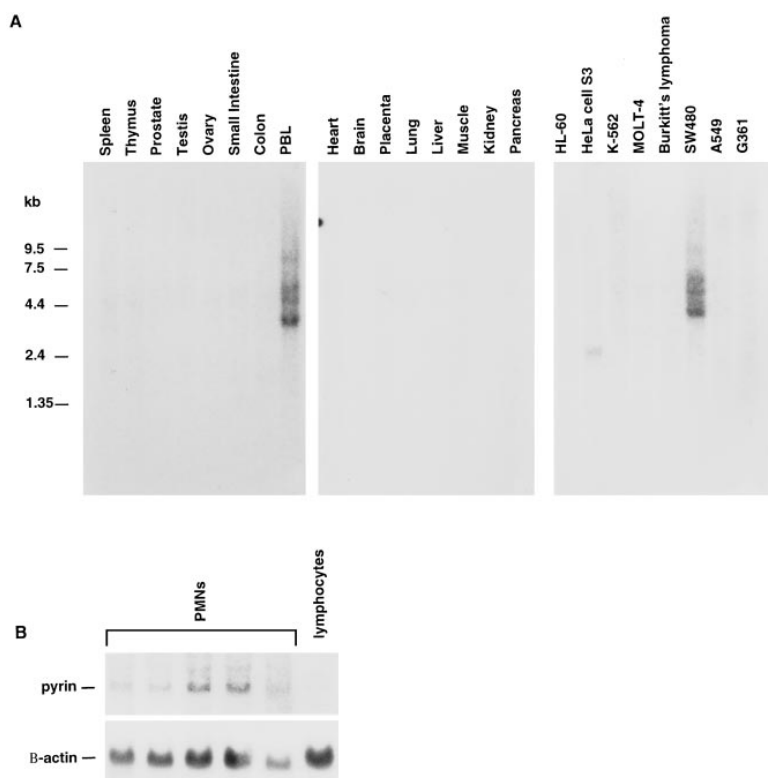


Figure 3. Expression Profile of the v75-1 Gene

(A) Results of hybridization of a probe derived from exon 2 on multiple-tissue Northern blots. A transcript of 3.7 kb is present in peripheral blood leukocytes and colorectal adenocarcinoma SW480. HL-60, promyelocytic leukemia; K-562, erythroleukemia; MOLT4, lymphoblastic leukemia; A549, lung carcinoma; G361, melanoma.

(B) Hybridization of the same exon 2 probe on Northern blot with mRNA from purified PMNs and lymphocytes. PMN lanes represent preparations from different individuals. A β-actin control is shown below.

Iraqi Jewish population, while the C haplotype is seen in patients from several different ethnic groups. The J and K haplotypes have only been observed in Armenian affected individuals. Table 1A indicates no easily discernible relationship among these haplotypes at the level of microsatellites, except that haplotypes J and K are identical in the region from *D16S3403* to *D16S2617*.

Table 1B summarizes data on 11 single nucleotide polymorphisms (SNPs) that we have identified from within the v75-1 gene for each of the haplotypes defined in Table 1A. Nine of these polymorphisms represent synonymous substitutions within exons of this gene, while the remaining two are seen in introns 4 and 6. The A, F, and G haplotypes are identical at all 11 polymorphisms, and, although the B haplotype differs from A, F, and G at the 5' end of the gene, SNPs for all 4 haplotypes converge beginning at exon 3. Similarly, SNPs for the C, J, and K haplotypes also converge by exon 3. The "AFGB" and "CJK" core SNP haplotypes from exon 3 to 10 were also observed on significant numbers of ethnically matched normal chromosomes (not shown).

Table 1C, which correlates haplotypes with v75-1 mutations, suggests that ancestral relationships among these subsets of chromosomes are likely. Thus, the convergent A, F, G, and B haplotypes all bear the M694V mutation, while we have not observed M694V on haplotypes that do not share the AFGB core SNPs. Similarly, Armenian haplotypes J and K converge within v75-1 and both bear the same M680I mutation, while this mutation has not been observed on haplotypes that do not have the CJK core SNPs. The C haplotype bears the same convergent SNP haplotype as J and K, suggesting an independent mutational event on an ancestral CJK-type chromosome.

For any given haplotype, only one mutation was present. Altogether, these three mutations accounted for 78 carrier chromosomes in our panel. There were several other carrier chromosomes with different microsatellite haplotypes for which we did not observe any of these three mutations (these latter carrier chromosomes have thus far only been screened for exon 10 mutations). In four separate families we have observed M694V/V726A compound heterozygotes. In one such family, the parents are of Ashkenazi and Iraqi Jewish ancestry, with the Ashkenazi father carrying the V726A mutation on the C haplotype FMF chromosome. This haplotype establishes an ancestral link between Ashkenazi Jews, who have lived in Eastern Europe for the last 1500 years, and the Druze, who have lived in isolated communities in what is now Israel and Lebanon for the last ~1000 years. The only other Ashkenazi Jewish carrier whom we have genotyped also has the V726A mutation.

The M680I, M694V, and V726A mutations were not present on any of 105 normal chromosomes in our study population (defined by haplotype analysis in well-characterized families), nor in almost 200 North American control chromosomes. It is also noteworthy that although no A haplotype-bearing noncarrier chromosomes could be identified, two noncarrier chromosomes with the B haplotype (both by microsatellites and SNPs) did not have the M694V mutation, further strengthening the connection between the presence of the mutation and disease. Moreover, 42% of a panel of 24 Middle Eastern normal chromosomes bear the AFGB SNP core haplotype but do not carry M694V, which is further evidence that M694V is disease-causing, and not a private polymorphism.

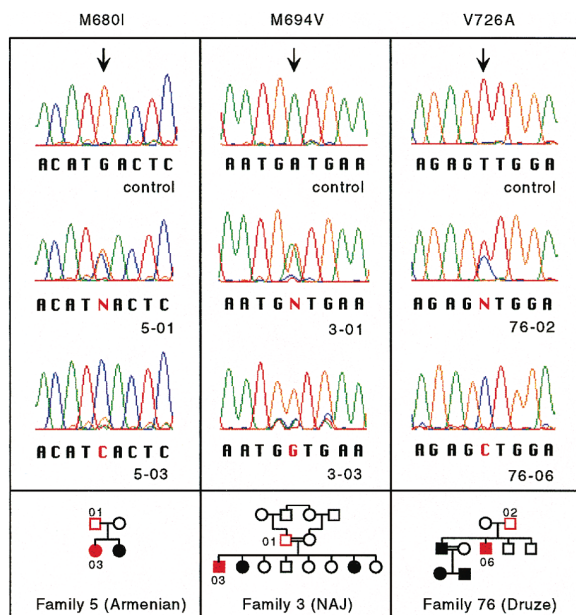


Figure 4. DNA Sequence Electropherograms Demonstrating the M680I, M694V, and V726A Substitutions

For each mutation, individuals who are homozygous for the normal allele are shown at the top, heterozygotes between the normal and mutant allele are shown in the middle, and homozygotes for the mutation are shown at the bottom. On the pedigree diagram, the heterozygote is shown as an open red symbol, and the homozygote for the mutation is shown as a closed red symbol. The control for the M680I mutation is individual 76-06, the control for M694V is 76-02, and the control for V726A is 3-01.

Discussion

In this paper, we describe the molecular cloning of a new cDNA, v75-1, from the familial Mediterranean fever candidate region on chromosome 16p13.3 and present compelling evidence that this gene is *MEFV*, the gene causing FMF. The cornerstone of the argument equating v75-1 with *MEFV* is the identification of three different v75-1 mutations on FMF carrier chromosomes in multiple ethnic groups, mutations that are not seen in a panel of almost 300 normal control chromosomes.

It is extremely unlikely that the substitutions we have identified in v75-1 are actually polymorphisms in tight linkage disequilibrium with "real" mutations on a nearby gene. This hypothesis would require that there be three such v75-1 polymorphisms on three different haplotypes, each in perfect linkage disequilibrium with the mutations on the real FMF gene. While not impossible, such a scenario is at the least unnecessarily complex. It is also unclear where such a closely linked gene would be located. The historical recombinants at the 5' (centromeric) end of v75-1 exclude the interval between *D16S3373* and v75-1. On the telomeric side, the 5' end of a novel zinc finger gene is located within 10 kb of the 3' end of v75-1, but thorough screening has revealed no mutations in this latter gene (data not shown). Moreover, there are no trapped exons, direct-selected cDNAs, or expressed sequence tag (EST) hits that map to the interval between them. Finally, and most importantly, the

observation of normal chromosomes that bear disease-associated microsatellite and SNP haplotypes but do not have the M680I, M694V, or V726A mutations is strong evidence that these are not just haplotype-specific polymorphisms.

While these genetic arguments firmly establish the identity of v75-1 as the FMF gene, data on v75-1 tissue distribution are highly consistent with the clinical phenotype. Based on the nature of the inflammatory infiltrate and the anatomic localization of inflammation in FMF, *MEFV* gene expression might be predicted to be observed in granulocytes and/or serosal cells. Multiple-tissue Northern blots demonstrated high levels of expression in peripheral blood leukocytes, but not in lymph node, spleen, or thymus, which are comprised largely of lymphocytes. From this we deduced that pyrin is most likely expressed primarily in mature granulocytes, an inference that was subsequently confirmed by direct observation.

Computational analyses of wild-type and mutant pyrin molecules suggest a mechanism by which the mutations identified may cause disease. All three mutations are clustered within 46 amino acids of one another in the highly conserved B30.2 (rfp) globular domain at the C-terminal end of the predicted protein, and they probably affect the secondary structure of this domain. Based on BLASTX homologies and its restriction to granulocytes, pyrin is likely to be a nuclear factor that controls the inflammatory response in differentiated PMNs. Given that FMF is a disease of excessive inflammation, and that pyrin is homologous to rpt-1, a known down-regulator of inflammation, pyrin may be a negative auto-regulatory molecule in PMNs. The FMF-associated mutations and resultant pyrin structural changes would then, after some undefined event that triggers inflammation, prevent the normal pyrin-mediated negative feedback loop.

None of these three mutations results in a truncated protein, and the periodic nature of inflammatory attacks in FMF is consistent with a protein that functions adequately at steady state but decompensates under stress. Several other diseases with periodic manifestations follow this paradigm, including sickle cell anemia (β -globin; Weatherall et al., 1995) and hyperkalemic periodic paralysis (adult skeletal muscle sodium channel; Ptáček et al., 1991). Both are characterized by missense mutations encoding an abnormal protein with reduced function. Patients experience symptoms when environmental factors (hypoxia or acidosis in the case of sickle cell, excess consumption of potassium in periodic paralysis) induce additional conformational changes in the protein (sickle cell) or destabilize a delicate equilibrium (periodic paralysis). Similarly, environmental triggers may play a role in FMF, and the identification of pyrin should provide clues regarding the proximal steps in the cascade that leads to an attack.

Just as protein-truncating mutations in globin produce thalassemia and not sickle cell disease, more profound loss-of-function mutations in pyrin may produce a phenotype much different from FMF. It is even conceivable that the total lack of pyrin may lead to generalized, uncontrolled inflammation and may therefore be embryonically lethal. Mouse models may shed light on these questions.

Table 1. FMF Carrier Haplotypes and cDNA v75-1 Mutations

A. Extended Microsatellite Haplotypes

Haplotype	Ethnic Grp ^b	Microsatellite Markers ^a									
		S468	S3082	S3403	S3405	S3404	S3370	S2617 ↓	S3373	S3275	S3376
A	NAFJ, IrJ, Arm	212	148	1100	165	118, 122	153	104	185	184	211
F	NAFJ	204	152	1000	165	118	149	104	187	188	215
G	NAFJ	202	150	1000	185	94	141	107	187	184	211
B	IrJ	206	138	1000	187	94	141	110	185	186	217
C	IrJ, AshJ, Dr, Arm	202	140	1100	165	110	149	107n ^c	187	186	201
J	Arm	202	136	1100	165	118	149	107n	183	188	215
K	Arm	202	132	1100	165	118	149	107n	185	196	211

B. cDNA v75-1 Intragenic Haplotypes

Haplotype	Ethnic Grp	Single Nucleotide Polymorphisms ^d										
		×2.1 306	×2.2 414	×2.3 495	×2.4 605	×3.1 901	i4.1 ^e	×5.1 1422	×5.2 1428	×5.3 1530	i6.1 ^f	×9.1 1764
A	NAJ, IrJ, Arm	C	G	A	A	C	A	G	A	T	C	A
F	NAFJ	C	G	A	A	C	A	G	A	T	C	A
G	NAFJ	C	G	A	A	C	A	G	A	T	C	A
B	IrJ	T	A	C	G	C	A	G	A	T	C	A
C	IrJ, AshJ, Dr, Arm	T	A	C	G	T	G	A	G	C	T	G
J	Arm	T	A	C	G	T	G	A	G	C	T	G
K	Arm	C	G	A	G	T	G	A	G	C	T	G

C. cDNA v75-1 Mutations

Mutation	aa	Haplotypes	Normal Chromosomes
2040 G→C	Met680Ile	J (1/1) K (1/1)	0/293
2080 A→G	Met694Val	A (62/62) B (5/5) F (1/1) G (1/1)	0/295
2177 T→C	Val726Ala	C (8/8)	0/283

^aIn each case the "D16" has been omitted at the beginning of the locus designation. The position of *MEFV* relative to these markers is indicated by the arrow. Microsatellites are listed telomeric to centromeric, from left to right.

^bNAFJ = North African Jewish, AshJ = Ashkenazi Jewish, IrJ = Iraqi Jewish, Arm = Armenian, Dr = Druze.

^cDenotes the presence of a null allele with standard *D16S2617* primers, but 107 bp amplicon when an alternative primer pair is used (Balow et al., in press).

^dDesignated by exon or intron; ×2.1 indicates the first polymorphism in exon 2. Below each designation is the nucleotide position in the sequence, with 1 being the adenosine in the ATG initiation codon. Note that the polymorphisms are listed 5' to 3', from left to right, which is the opposite orientation on the chromosome from that shown in (A).

^eIntron 4, nucleotide position 1356 + 43.

^fIntron 6, nucleotide position 1610 + 95.

It would appear that at least some of the phenotypic variation in FMF may be attributable to the difference between the M694V and V726A mutations. The M694V mutation is very common in the populations with the highest incidence of systemic amyloidosis (especially North African Jews), while V726A is seen in populations in which amyloid is less common (Iraqi and Ashkenazi Jews, Druze, and Armenians). Nevertheless, M694V-associated haplotypes can comprise a significant minority of carrier chromosomes in populations where severe amyloidosis is exceedingly rare. One possible explanation could be that the "milder" V726A mutation is "protective" against amyloid, in which case M694V/M694V homozygotes would be amyloid-prone but V726A homozygotes and compound heterozygotes would not.

Although founder effects clearly have played a role in the population genetics of FMF, selection for heterozygotes remains an attractive hypothesis. The probable

role of pyrin in the homeostatic control of inflammation is consistent with heterozygotes exhibiting heightened responses to a specific pathogen or class of pathogens. The strikingly high gene frequencies for FMF in more than one Middle Eastern population could then be explained by increased heterozygote resistance to such an organism. Since the gene frequency for FMF is much lower in the East European Ashkenazi Jewish population (Yuval et al., 1995), it is possible that such a selective factor would have been endemic to the Mediterranean basin. The identification of pyrin should lead to testable hypotheses regarding the host inflammatory response in FMF heterozygotes.

Our data indicate that the M694V and V726A mutations are very old. Based on the convergence of several carrier haplotypes, it is likely that all modern M694V chromosomes are descended from a common founder. This mutation is seen on the B FMF haplotype commonly observed in Iraqi Jews, a group that remained relatively

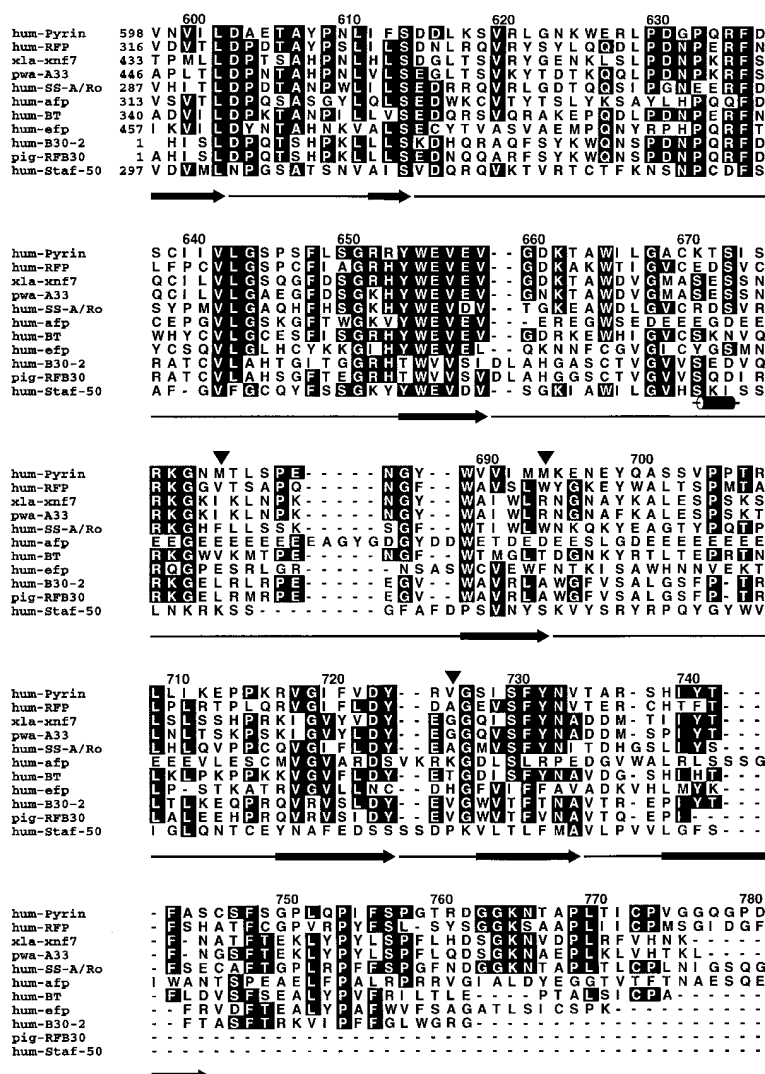


Figure 5. Multiple Sequence Alignment of the C-Terminal End of Human Pyrin

Sequences shown have statistical similarity as assessed by BLAST (Altschul et al., 1990). Search cutoffs used to identify homologs were a Karlin-Altschul score of two aligned sequences ≥ 70 with a probability $\leq 10^{-3}$. At each position, residues occurring in a majority of the sequences are shown in inverse type. The numbering scheme at the top of the figure is based on the sequence of pyrin. The positions of secondary structural elements in the wild-type pyrin (here, all β sheets) as predicted by the profile neural network method PHDsec (Rost and Sander, 1993, 1994) are shown as bold, horizontal arrows below the alignment. The residues altered in the three mutants are indicated by downward-pointed arrowheads above the alignment. hum-RFP, RET finger protein (SWISS-PROT P14373); xla-xnf7, nuclear phosphoprotein xnf7, *Xenopus laevis* (PIR A43906); pwa-A33, zinc-binding protein A33, *Pleurodeles waltl* (SWISS-PROT Q02084); hum-SS-A/Ro, 52 kDa RO protein (SWISS-PROT P19474); hum-afp, acid finger protein (GenBank U09825); hum-BT, butyrophilin (GenBank U90552); hum-eef, estrogen-responsive finger protein (PIR A49656); hum-B30-2, B30-2 gene, (PRF 2002339); pig-RFB30, ring finger protein RFB30, *Sus scrofa* (EMBL Z97403); hum-Staf-50, transcription regulator Staf-50 (PIR A57041).

isolated from the rest of the Jewish population from the time of the Babylonian captivity (~2500 years ago) until the formation of the state of Israel. Similarly, the observation of the V726A mutation and C microsatellite haplotype in Armenians, Ashkenazi Jews, and Druze FMF patients establishes a historical lower boundary on the age of this mutation of perhaps 2000 years.

It is also possible, of course, that the M694V mutation, and its surrounding SNPs, has been transferred to four different haplotypes by interchromosomal gene conversion rather than by meiotic recombination events. In that case, the estimation of the age of the original founder mutation would not be possible. Finally, the possibility of multiple independent origins of M694V and M680I cannot be formally excluded, but the concordance of the SNP haplotypes close to the mutation (Table 1B and 1C) renders this possibility rather unlikely.

The identification of pyrin as the molecule responsible for FMF also focuses interest on the *RoRet* family of proteins, all of which share a B-box zinc finger as well as a 170 amino acid rfp (B30.2) domain. The spacing

between these two structural elements is also highly conserved, suggesting that precise orientation of the two domains with respect to one another is required for function. The conservation of the rfp domain (Figure 5) in molecules as diverse as butyrophilin (a milk protein with probable receptor function; Jack and Mather, 1990), Ro52 (a ribonucleoprotein that is autoantigenic in patients with SLE and Sjögren's syndrome), A33 (a factor that binds polytene chromosomes in the newt; Bellini et al., 1993), and xnf7 (a factor that binds mitotic chromosomes in the frog; Reddy et al., 1991) is remarkable; the sequence identity among these proteins ranges from 40% to 60%. Scrutiny of B30.2 mutations in pyrin may provide insights into this evolutionarily conserved but poorly understood domain.

The identification of pyrin mutations as the cause of FMF will substantially advance our understanding of this disorder and will provide a tool to address questions ranging from population genetics to structural biology. It represents the first step in the delineation of an important new pathway in the control of inflammation.

Experimental Procedures

Patients and DNA Samples

Forty-four families of non-Ashkenazi Jewish descent (18 Moroccan, 14 Libyan, 5 Tunisian, 2 Egyptian, and 5 Iraqi) and 5 Arab/Druze families were identified and sampled at the Chaim Sheba Medical Center in Tel-Hashomer, Israel. Twelve Armenian families were recruited from Cedars-Sinai Medical Center in Los Angeles. Details of this panel have been previously described (Balow et al., 1997). One additional Ashkenazi/Iraqi Jewish family was also studied. The diagnosis of FMF in all families was according to established clinical criteria (Sohar et al., 1967). Patient consent was obtained from participants in the study after approval by the human experimentation committees at each institution. DNA was extracted from whole blood or from Epstein-Barr virus-transformed lymphocytes by standard techniques.

Genotyping

Patients were genotyped as previously described (Balow et al., 1997). Additionally, genotyping by PCR was carried out with three new markers that were developed in this study and are described here (*D16S3403*, *D16S3404*, and *D16S3405*). Sequences of oligonucleotides and PCR conditions used to amplify these markers are available from the Genome Database.

Direct cDNA Selection

Cosmids, BAC, and P1 clones in the FMF candidate region were biotinylated using BioPrime (Life Technologies, Gaithersburg, MD). cDNAs were prepared from combined mRNA from fetal brain, fetal liver, and human lymph node by reverse transcription and ligation of an EcoRI/NotI adaptor, which also served as a PCR primer, to second-strand cDNAs. cDNAs were directly hybridized to biotinylated templates that were recovered using streptavidin-labeled magnetic beads. Conditions for blocking, hybridization, binding, and elution of cDNAs from magnetic beads (Dyna) were as described (Parimoo et al., 1991). After two rounds of selection, eluted cDNAs were amplified with CUA-tailed EcoRI/NotI adaptor primers and subcloned into the pAMP10 vector (Life Technologies, Gaithersburg, MD) to yield libraries of selected cDNAs. Recombinant clones were arrayed on blots. Clones that hybridized to either repetitive or ribosomal sequences were excluded from further analysis. To confirm their origin, unique clones were individually hybridized to EcoRI digests of cosmid/BAC/P1 DNAs and DNAs from chromosome 16-specific human-hamster hybrid lines. Clones were then hybridized to each other and were binned into groups. Representative clones of each group were hybridized to multiple-tissue Northern blots and sequenced.

Exon Trapping

Exon trapping was performed on PAC clone 273L24 (Sood et al., 1997) as previously described (Buckler et al., 1991). PAC clone 273L24 was partially digested with Sau 3AI. The reaction products were size-fractionated by agarose gel electrophoresis, and DNA fragments 2 kb and larger were isolated from the gel. Partially digested DNA (50 ng) was ligated with 10 ng of exon-trapping vector pSPL3 that had been previously cleaved with BamHI and dephosphorylated with calf intestinal alkaline phosphatase (Promega, Madison, WI). Ligation products were electroporated into *E. coli* DH12B, and the electroporated cells cultured en masse in LB broth with 200 mg/ml ampicillin for 16 hours at 37°C with shaking. DNA prepared from the culture was used to transfect COS-7 cells (ATCC 30-2002) using lipofectACE reagent (Life Technologies, Gaithersburg, MD). Total RNA was isolated from transfected COS-7 cells with Trizol reagent (Life Technologies) followed by ethanol precipitation. First-strand cDNAs of transcription products from pSPL3 were primed with the oligonucleotide SA2. Specific amplification of trapped exons was as follows: PCR primed with oligonucleotides SA2 and SD6 was performed, followed by digestion of the PCR products with BstXI. A second PCR reaction using the digestion products was primed with oligonucleotides dUSD2 and dUSA4. The resulting DNA fragments were cloned into pAMP10 vector and sequenced. Two hundred clones were sequenced and 20 independent exons were

identified by visual inspection and hybridization to DNA fragments from the FMF critical region, with several exons identified more than one time.

cDNA Identification by Solution Hybridization

Solution hybridizations were carried out using the GeneTrapper cDNA Positive Selection System (Life Technologies, Gaithersburg, MD). Two trapped exons, v66 and v75, were used as starting material. PCR screening of Superscript cDNA libraries (Life Technologies, Gaithersburg, MD) derived from human brain, liver, leukocytes, spleen, and testis were used to determine the tissue-specific expression of these exons. The leukocyte cDNA library was used in further experiments since it showed significant expression of v66 and v75 exons. GeneTrapper experiments were performed with sense and antisense primers from both exons, assuming both orientations of these exons in the putative transcript. The following oligonucleotides were synthesized and PAGE-purified: v66GT1: AAG CTC ACT GCC TTC TCC TC; v66GT2: GAG GAG AAG GCA GTG AGC TT; v75GT1: GAC TTG GAA ACA AGT GGG AG; v75GT2: CTC CCA CTT GTT TCC AAG TC. Oligos were biotinylated and hybridized to single-stranded DNA from the leukocyte cDNA library (one primer per reaction), followed by cDNA capture using paramagnetic streptavidin beads and repair using the corresponding nonbiotinylated oligos. Colony hybridization of lifts using ³²P-dCTP end-labeled oligos was used to identify positive clones. Gel-purified inserts from these clones were hybridized to cosmid contig blots to distinguish cDNA clones mapping to the FMF region from false positive clones due to homologous domains. All positive clones were identified by the primers v66GT2 and v75GT2, and no clones were identified by the other set of primers.

Oligonucleotides for Exon Amplification

Oligonucleotides used to amplify pyrin exons were as follows (all oligo sequences are given 5' to 3'): exon 1 forward, AACCTGCCTTTT CTGCTCA; exon 1 reverse, CACTCAGCACTGGATGAGGA; exon 3 forward, GAACTCGCACATCTCAGGC; exon 3 reverse, AAGGCCCA GTGTGTCCAAGTGC; exon 4 forward, TTGGCACCAGCTAAAGAT GGC; exon 4 reverse, TCTCCCTCTACAGGGATGAGC; exon 5 forward, TATCGCTCTGCTCTGGAATC; exon 5 reverse, CACTGTGG GTCACCAAGACCAAG; exon 6 forward, TCCAGGAGCCAGAAAGTA GAG; exon 6 reverse, TTCTCCCTATCAAATCCAGAG; exon 7 forward, AGAATGTAGTTCATTTCAGC; exon 7 reverse, CATTCTGT AACGCAGGGTTT; exon 8/9 forward, ACCTAATCCAGCTTCTCTC TGC; exon 8/9 reverse, AGTTCTTCTGGAACGTGGTAG; exon 10a forward, CCAGAAGAACTACCTGTCCC; exon 10a reverse, AGAGC AGCTGGCGAATGTAT; exon 10b forward, GAGTGGAGGTTGGAG ACA; exon 10b reverse, TCCTCCTGAAATCCATGG.

Northern Blot Analysis

To determine transcript size and level of expression in various tissues, multiple-tissue Northern blots (Clontech) were hybridized with probes derived from various exons of the gene. These exons were amplified and purified as part of the sequencing protocol for mutation analysis. Larger exons (2, 5, and 10) were labeled by random-priming using Stratagene Prime-It Kit and ³²P-dCTP (ICN). Hybridization and washing of blots were essentially as described in Sambrook et al. (1989), except using Hybridisol I (Oncor) prepared hybridization buffer. Hybridization was detected by autoradiography, with 4 hour exposures. Northern blots with mRNA from highly purified peripheral blood lymphocytes, PMNs, and monocytes were the kind gift of Drs. H. Lee Tiffany and Harry Malech.

Mutation Detection by Fluorescent Sequencing

Approximately 100 ng of genomic DNA template was used in PCR reactions to amplify exons and flanking intronic sequences according to the supplier's recommendations for AmpliTaq Gold (Perkin Elmer, Branchburg, NJ) and Advantage-GC Genomic PCR Kit (Clontech, Palo Alto, CA). The PCR primers were tailed with one of the following sequences: -21M13 forward: GTAAACGACGCGC CAGT; -28 M13 reverse: CAGGAAACAGCTATGACCAT; -40 M13 forward: GTTTTCCAGTCACGACG. After amplification, reactions were run on 1% agarose gels and gel-purified using either QIAquick

gel extraction kit (QIAGEN, Santa Clarita, CA) or Microcon/Micro-pure/Gel Nebulizer system (Amicon, Beverly, MA). Alternatively, PCR products were column-purified with Microcon-100 (Amicon). Purified amplicons were sequenced with dye primer chemistry (PE Applied Biosystems, or Amersham, Cleveland, OH). Sequencing reactions were ethanol-precipitated and run on an ABI 377 automated sequencer. Sequence data were analyzed with either Autoassembler 1.4 (PE Applied Biosystems, Branchburg, NJ) or Sequencher 3.0 (Gene Codes Inc., Ann Arbor, MI).

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