to the MNTB neuron (for example, small downward blips following each main EPSC in Fig. 6a). They produce small EPSCs with delayed onset, fluctuating size and failures. For recording of EPSCs, the intracellular solution contained (in mM): potassium gluconate 97.5, CsCl 32.5, EGTA 5, HEPES 10, $MgCl_2$ 1, ATP 2, GTP 0.2 (pH 7.2). Because of the large size of AMPA EPSCs, an intracellular Na^+-channel blocker, QX314 (2 mM) was added to improve the quality of voltage clamp. The NMDA (*N*-methyl-D-aspartate) EPSC was largely blocked by Mg^{2+} (1 mM), with a residual contribution to the total EPSC at −60 mV of less than 5%. Bicuculline (10 μM) and strychnine (1 μM) were added to the artificial cerebral spinal fluid (ACSF) to block inhibitory inputs. The same intracellular solution was used for recording presynaptic action potentials and potassium currents, except that CsCl was replaced by KCl and no QX314 was added. For recordings of presynaptic calcium currents, the intracellular solution contained (in mM): CsCl 110, EGTA 0.5, HEPES 40, $MgCl_2$ 1, ATP 2, GTP 0.2, Na_2-phosphocreatinine 12, TEA 10 (pH 7.2) and extracellular ACSF solution was supplemented with 10 mM TEA, 0.3 mM 4-aminopyridine (4-AP) and 1 μM tetrodotoxin (TTX). The series resistance for postsynaptic recordings was 4–6 MΩ and 8–12 MΩ for presynaptic recordings. The series resistance compensation was at least 85%, with a lag of 10 μs. Data were filtered at 2–5 kHz, digitized and acquired on-line with pClamp6 software (Axon Instruments) running on a 486 computer. Current amplitude analysis and single exponential fitting were done using the same software. Averaged data are expressed as mean ± standard error (s.e.). For experiments illustrated in Fig. 6, slices were incubated in ASCF containing 0.2 mM EGTA-AM (in 0.1% dimethylsulphoxide (DMSO)) (Molecular Probes) for 30 min and then washed in normal ACSF for at least 30 min before recording. The same manipulation with DMSO alone in two control experiments produced no detectable effects. Cyclothiazide, strychnine and QX314 were purchased from Research Biochemical International; thapsgargin, 4-AP, TEA, TTX and bicuculline were from Sigma.

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An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy

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Fukuyama-type congenital muscular dystrophy (FCMD), one of the most common autosomal recessive disorders in Japan (incidence is 0.7–1.2 per 10,000 births), is characterized by congenital muscular dystrophy associated with brain malformation (micropolygria) due to a defect in the migration of neurons[1]. We previously mapped the FCMD gene to a region of less than 100 kilobases which included the marker locus *D9S2107* on chromosome 9q31 (refs 2–4). We have also described a haplotype that is shared by more than 80% of FCMD chromosomes, indicating that most chromosomes bearing the FCMD mutation could be derived from a single ancestor[5]. Here we report that there is a retrotransposal insertion of tandemly repeated sequences within this candidate-gene interval in all FCMD chromosomes carrying the founder haplotype (87%). The inserted sequence is about 3 kilobases long and is located in the 3′ untranslated region of a gene encoding a new 461-amino-acid protein. This gene is expressed in various tissues in normal individuals, but not in FCMD patients who carry the insertion. Two independent point mutations confirm that mutation of this gene is responsible for FCMD. The predicted protein, which we term fukutin, contains an amino-terminal signal sequence, which together with results from

transfection experiments suggests that fukutin is a secreted protein. To our knowledge, FCMD is the first human disease to be caused by an ancient retrotransposal integration.

After localizing the FCMD gene to the centromeric vicinity of the *D9S2170* locus by linkage-disequilibrium mapping[4] and founder haplotype mapping[5], we constructed a cosmid contig harbouring *D9S2105* and *D9S2107* (ref. 6). To screen DNA from FCMD patients for genomic rearrangement, we hybridized each cosmid clone of the contig (Fig. 1a) to Southern blots of genomic DNA from FCMD patients. When the whole insert of cosmid clone cE6, which included *D9S2170*, was used as a probe, we found that FCMD DNAs digested with *Pvu*II specifically lacked the normal 5-kilobase (kb) band and instead had a new 8-kb band. When the same DNAs were digested with *Pst*I or *Bgl* II, the normal band was missing and a new, larger band appeared, indicating that a 3-kb fragment had been inserted into the genomic DNA of most of our FCMD patients (Fig. 1b). When a fragment of this cosmid, E6f3, was used as a probe, most patients showed an abnormal 8-kb homozygous band; the remaining patients gave both a normal 5-kb and an abnormal 8-kb band (Fig. 1c). Detailed analysis confirmed the insertion of a 3-kb sequence within a 1.4-kb *Eco*RI fragment of cE6 in most FCMD patients (Fig. 1a). This insertion allele cosegregated with the FCMD founder haplotype (138-192-147-183 for *D9S2105-D9S2170-D9S2171-D9S2107*).

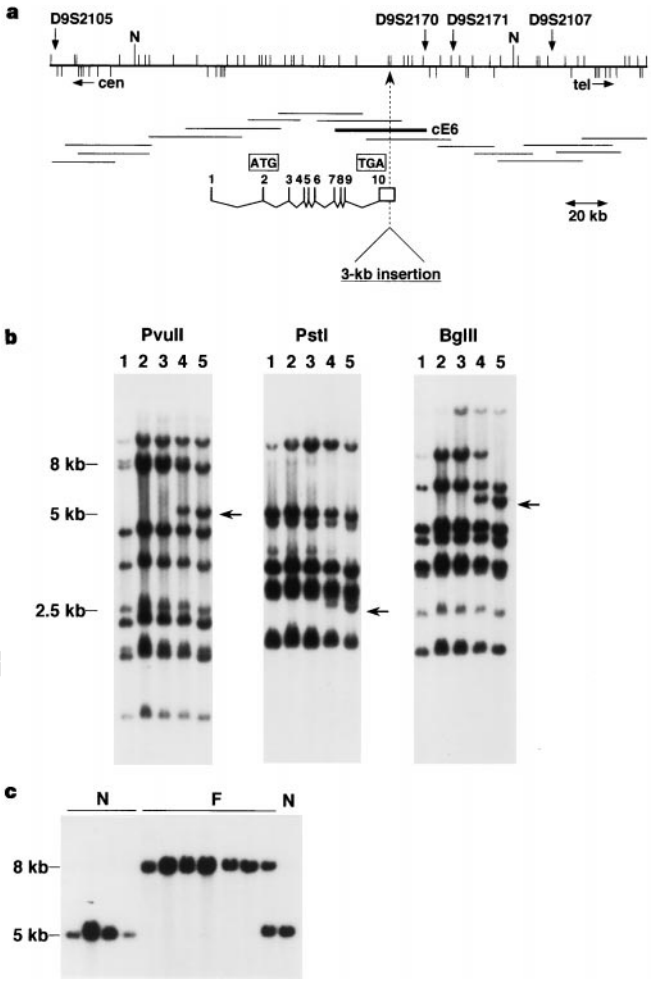

Figure 1 Genomic insertion in FCMD patients. **a**, The FCMD gene region on chromosome 9q31. Horizontal lines, individual cosmids of the cosmid contig; short vertical lines, *Eco*RI cutting sites; N, *Not*I cutting sites. The genomic structure of the FCMD gene is shown below the map. **b**, Southern blot analysis of FCMD patients. Genomic DNA was digested with *Pvu*II, *Pst*I or *Bgl*II. Lanes: 1–3, patients homozygous for the founder haplotype; 4, patient heterozygous for the founder haplotype; 5, a normal control. The probe used was the whole insert of cosmid clone cE6. Bands seen in the normal control were absent in FCMD patients (arrows) and new bands larger than the missing band were evident. **c**, *Pvu*II-digested blot hybridized with a fragment probe, E6f3, derived from cE6. F, FCMD patients; N, normal controls.

Using the 1.4-kb *Eco*RI fragment of cE6 as a probe to screen an adult brain complementary DNA library, we obtained 2.1-kb and 3.2-kb cDNAs with poly(A) tracts. Screening of additional cDNA libraries or RACE (rapid amplification of cDNA ends) experiments yielded 13 additional clones. Sequencing of all 15 cDNA clones revealed that the composite cDNA spans 7,349 base pairs (bp) and an open reading frame of 1,383 bp begins with an initiator ATG codon at base 112. The predicted protein has 461 amino acids, a calculated relative molecular mass of 53.6K and a calculated isoelectric point of 8.29 (Fig. 2). The original two cDNA clones resulted from polyadenylation at two consensus sites (bases 6,266 and 7,327), and corresponded to the two bands seen on northern blot (Fig. 3).

Northern blots revealed 6.5-kb and 7.5-kb transcripts in poly(A)$^+$ messenger RNA from a variety of human tissues, with the strongest signals being obtained from heart, brain, skeletal muscle and pancreas. This may explain why congenital muscular dystrophy in FCMD patients is associated with a brain anomaly and often with fibrosis of the heart muscle as well[1]. Transcripts were also detected in cultured lymphoblasts (Fig. 3).

The genomic structure of this gene was determined by Southern hybridization against cosmid clones using as probes DNA sequences corresponding to each exon, and by DNA sequencing. The gene spans more than ~100 kb of genomic DNA and is composed of 10 exons; exon 2 includes the translation-initiation codon (Fig. 1a).

Searches of the nucleotide databases revealed no significant similarities to genes of known function. The predicted protein sequence was compared against the SwissProt and PIR databases, and against the predicted translation products of GenBank, EMBL, DDBJ and PDB databases. No significant similarity was found to any known proteins, although weak similarities to the predicted translation products from *Caenorhabtitis elegans* cosmids W02B3 and T07A4 (*U22833* and *Z48055*) were detected. A search for protein motifs revealed one *N*-glycosylation site. The PSORT program[7] for prediction of protein localization sites indicated that the FCMD protein has an N-terminal signal sequence with a possible cleavage site at codon 21, but that it lacks a transmembrane region; another program, TMpred[8], however, predicted one (amino acids 288–306) transmembrane segment (Fig. 2).

To characterize the inserted DNA, we isolated a clone containing the insertion from a genomic library constructed from an FCMD patient who was homozygous for the insertion. Sequence analysis revealed that the inserted DNA fragment, which was 3,062 bp long, was composed of $(TCTCCC)_{41}$; 27 copies of a 49-bp sequence (5′-GGGAGGGAGGTGGGGGGGTCAGCCCCCCG-CCTGGCCAGCCGCCCCATCC-3′, tandemly repeated); a SINE (short interspersed sequence)-type human retroposon sequence;

```
  1 MSRINKNVVLALLTLTSSAFLLFQLYYYKHYLSTKNGAGLSKSKGSRIGF
                                                    #
 51 DSTQWRAVKKFIMLTSNQNVPVFLIDPLILELINKNFEQVKNTSHGSTSQ
                    #
101 CKFFCVPRDFTAFALQYHLWKNEEGWFRIAENMGFQCLKIESKDPRLDGI
151 DSLSGTEIPLHYICKLATHAIHLVVFHERSGNYLWHGHLRLKEHIDRKFV
201 PFQKLQFGRYPGAFDRPELQQVTVDGLEVLIPKDPMHFVEEVPHSRFIEC
251 RYKEARAFFQQYLDDNTVEAVAFRKSAKELLQLAAKTLNKLGVPFWLSSG
301 TCLGWYRQCNIIPYSKDVDLGIFIQDYKSDIILAFQDAGLPLKHKFGKVE
351 DSLELSFQGKDDVKLDVFFFYEETDHMWNGGTQAKTGKKFKYLFPKFTLC
401 WTEFVDMKVHVPCETLEYIEANYGKTWKIPVKTWDWKRSPPNVQPNGIWP
451 ISEWDEVIQLY
```

Figure 2 Amino-acid sequence of the protein (fukutin) encoded by of the FCMD gene. The predicted hydrophobic signal sequence is underlined. A potential *N*-linked glycosylation site is indicated by a broken line. Amino acids mutated in the families described here are indicated by a hash symbol.

a polyadenylation signal (AATAAA); and poly(A). Furthermore, a target-site duplication, consisting of a direct repeat of AAGAAAAAAAAAATTGT at both ends, indicated retrotransposal insertion of this 3-kb fragment. The tandem-repeat sequence was almost identical to one observed in the human gene encoding complement component 2 (*Z11739*), the genomic region near the Huntington's disease gene (*Z69654*).

By comparing cDNA and genomic sequences, we conclude that the 3-kb insertion was in the 3′ untranslated region of this gene (between bases 5,889–5,890). Most (125 of 144, 87%) FCMD chromosomes contained this insertion, whereas it was found in only one of 176 chromosomes in unrelated normal individuals; the frequency of one in 88 individuals corresponded well to that of FCMD carriers in the Japanese population[1].

To determine the effect of this genomic insertion on the FCMD transcript, we did northern blot analysis of poly(A)$^+$ mRNA isolated from cultured lymphoblasts of several patients. The transcript of this gene was nearly undetectable in FCMD patients who carried the insertion homozygously, and significantly lower than normal in patients heterozygous for the insertion and another mutation haplotype (Fig. 3b). The results indicated that the insertion or other mutations in the FCMD gene are likely to cause a decrease in transcription and/or an instability of mRNA, resulting in loss of function.

To search for inactivating mechanisms in FCMD chromosomes without the 3-kb insertion, we screened exons and flanking intronic sequences throughout the coding region by using single-stranded conformation polymorphism (SSCP) analysis in four patients. A product of the polymerase chain reaction (PCR) corresponding to exon 3 of the gene in patient H.M. (Fig. 4a, left), who carried the founder insertion from her father and the 130-201-157-183 haplotype from her mother (corresponding to lane 3 of the northern blot shown in Fig. 3b), showed a mobility shift. Sequence analysis showed a C-to-T transition at base 250 in the maternal allele, resulting in premature termination (CGA to TGA, R47X; Fig. 4b, left). Analysis of other members of H.M.'s family by PCR and restriction-fragment length polymorphism (RFLP) indicated that this mutation segregated with the disease and with the microsatellite haplotype. Furthermore, five other families (those of Y.S., R.M., A.G., A.T. and A.Y.) carrying the same haplotype had the same nonsense mutation (Fig. 4c, left). In addition, we identified in patient T.I. a 2-bp deletion at bases 298–299 (codon 63) causing a frameshift and a premature stop at codon 75 (Fig. 4a, b, right). The patient's Japanese mother carries an insertion-chromosome; her American father (of English and German extraction) does not. The 2-bp deletion was present in the chromosome inherited from her father (Fig. 4c, right). Taking together, our results indicate that our cDNA and its predicted protein, fukutin, are the FCMD gene and its product.

To investigate the localization of fukutin in skeletal muscle, we raised six polyclonal and three monoclonal antibodies; however, none of these detected specific signals on either control or FCMD skeletal muscle (data not shown). Computer analysis suggested that fukutin could be a membrane protein or an extracellular protein. Next, we transfected COS-7 cells with plasmids carrying fukutin–GFP (where GFP is green fluorescent protein) or fukutin–HA (HA is haemagglutinin). Transfected cells gave an intracellular signal that revealed a perinuclear ribbon-like Golgi apparatus (Fig. 5a); the labelling patterns of each transfectant were almost identical. Fukutin co-localized with a Golgi marker, Golgi 58K protein (Fig. 5b, c). Gradually, the signals assume a granular cytoplasmic distribution (Fig. 5d), suggesting that fukutin passes through the Golgi before being packaged into secretory vesicles. The signal was not seen at the plasma membrane, however, where most proteins responsible for the muscular dystrophies are located. Transfection experiments using mouse myoblast cell line C2C12 cells gave similar results.

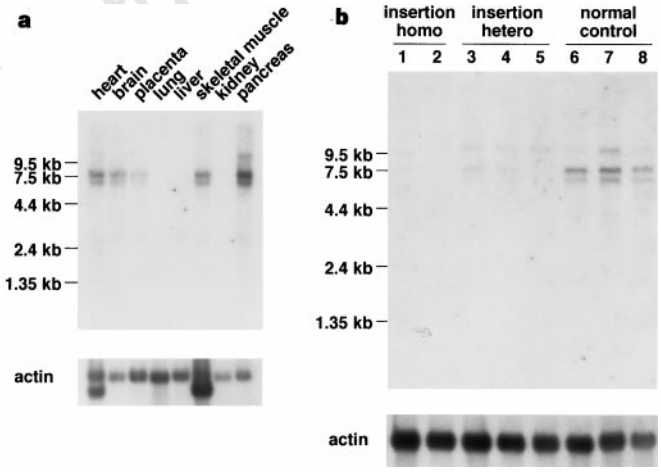

Figure 3 Northern blot analysis of the FCMD gene. **a**, Tissue-specific expression of FCMD mRNA in human tissues. An RNA blot containing 2 μg poly(A)$^+$ RNA from each of eight human tissues was hybridized with FCMD cDNA (clone II-5 containing the coding sequence) and β-actin (shown below) cDNA probes. **b**, RNA blot containing 2 μg poly(A)$^+$ RNA from lymphoblasts of FCMD patients and normal controls, hybridized with the same probes as in **a**. Note that the signals are almost absent or abnormally low in FCMD patients who carry the insertion homozygously or heterozygously, respectively.

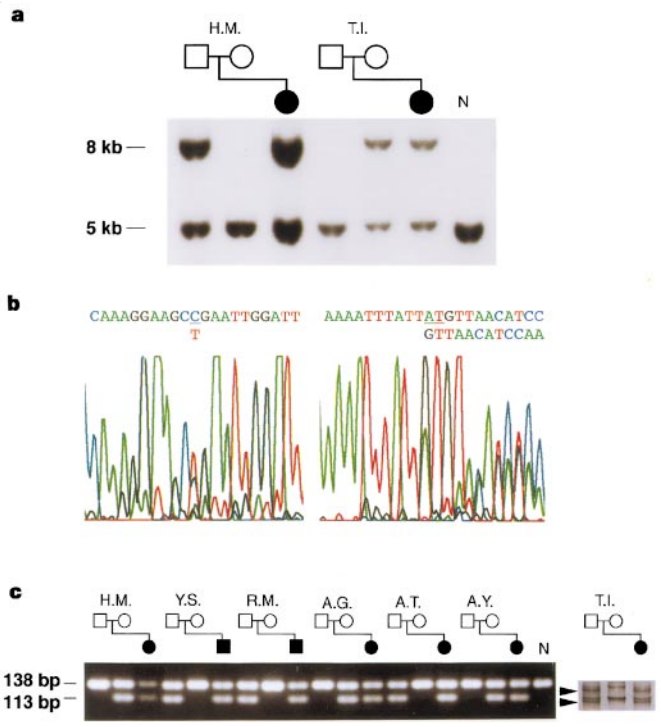

Figure 4 Segregation of point mutations in the families of H.M. and T.I. **a**, Patients H.M. and T.I. are heterozygous for the founder-insertion allele. Patient T.I. is the daughter of an American father and Japanese mother. **b**, Patients were shown to carry C250T (left) and Δ298–299 (right) mutations, both of which cause premature termination (R47X and frameshift). **c**, *Alu*I, which cleaves DNA at AGCT sequences, was used to confirm the mutation in the family of H.M. and in five other families (those of Y.S., R.M., A.G., A.T. and A.Y.) carrying the same haplotype (left); the mutant allele has an AGCT sequence. In this case, the *Alu*I digests of the normal PCR product are 138 bp and 4 bp; *Alu*I digestion of the mutant allele divided it into 113-bp, 25-bp and 4-bp fragments. In the family of H.M. the (non-insertion) mutant allele is derived from the mother. SSCP analysis showed that the 2-bp deletion in patient T.I. was derived from her caucasian father (right, arrowheads).

To confirm that fukutin is a secreted protein, we looked for it in the culture medium of COS-7 cells expressing fukutin–GFP by using metabolic labelling and immunoprecipitation with an anti-GFP polyclonal antibody. This antibody specifically precipitated fukutin from medium from COS-7 cells transfected with fukutin–GFP but not from control culture medium (Fig. 5e), indicating that fukutin–GFP was being secreted into the COS-7 cell culture medium. We conclude that fukutin is a secreted protein rather than a plasma-membrane protein.

Our results are evidence that the principal FCMD ancestor chromosome carries a 3-kb insertion within the 3′ untranslated region that results in dysfunction of the FCMD gene. How can insertion into the 3′ untranslated region cause a reduction in the corresponding mRNA? As the 3′ untranslated region affects the stability of mRNA[9], the inserted sequence may alter its secondary structure and render it unstable. Defects in the dystrophin–glycoprotein complex[10] lead to a loss of linkage between laminin α2 in the extracellular matrix and actin in the subsarcolemmal cytoskeleton, cause various muscular dystrophies[11,12]. Decreased immunostaining of a sarcolemmal glycoprotein, β-dystroglycan[13], and of an extracellular matrix protein linked with α-dystroglycan, the laminin α2 chain[14], has been observed in FCMD muscle. Abnormalities in basal lamina in FCMD muscle and brain have been seen by electron microscopy[15,16]. In light of these observations, we suggest that fukutin may be located in the extracellular matrix, where it interacts with and reinforces a large complex encompassing the outside and inside of muscle membranes; alternatively, as a secreted protein, fukutin may cause muscular dystrophy by an unknown mechanism. Another important manifestation of FCMD is a brain anomaly called micropolygyria (type II lissencephaly), in which neuronal lamination of normal six-layered cortex is lacking because of a defect in the migration of neurons[1]. At least four genes implicated in cortical dysgenesis disorders have been proposed to function in the migration and assembly of neurons during cortical histogenesis[17–21]. Of these, like fukutin, reelin is a secreted glycoprotein that has several structural features characteristic of extracellular matrix proteins. Discovery of the FCMD gene represents an important step towards greater understanding of the pathogenesis of muscular dystrophies and also of normal brain development. □

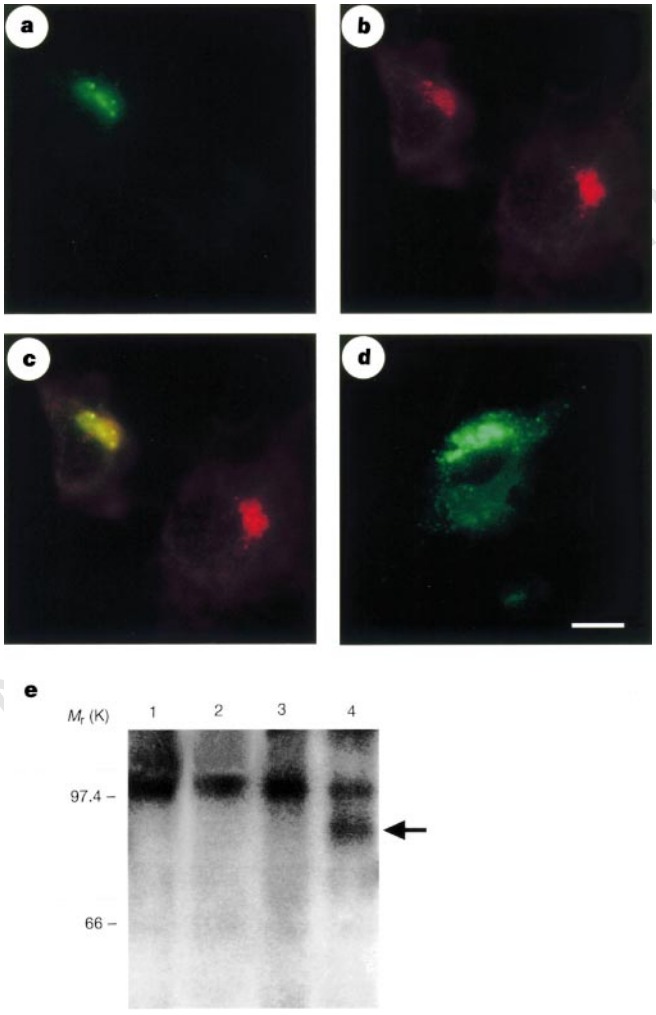

Figure 5 Cellular localization of fukutin. **a**, COS-7 cells transfected with a fukutin–GFP construct show a perinuclear ribbon-like signal (green). **b**, Immunolocalization of the same cell with anti-Golgi 58K protein monoclonal antibody (red) reveals an almost identical localization with fukutin–GFP. **c**, Superimposition of the two labelling methods. **d**, C2C12 cells transfected with a fukutin–GFP construct; the signal appears to have a granular cytoplasmic distribution. Scale bar, 10 μm. **e**, Secretion of fukutin. COS-7 cells were transfected with fukutin–GFP (lanes 3 and 4) or without it (lanes 1 and 2). The ^{35}S-labelled supernatant was immunoprecipitated with no antibody (lanes 1 and 3) or with anti-GFP antibody (lanes 2 and 4). A specific band of M_r ~86K (arrow) was detected only in the sample containing both fukutin–GFP and anti-GFP.

Methods

FCMD families and Southern/northern blot analysis. DNA samples from 63 families[2–4] and 23 additional FCMD families were analysed. Southern blots were analysed by competitive hybridization. Multiple-tissue northern blots were purchased from Clontech. Total RNA was isolated from lymphoblasts of patients and normal controls by extraction with acid–phenol (Trizol, Gibco-BRL). Poly(A)$^+$ RNA (2 μg) was selected by oligo(dT) chromatography (Oligo-dT Latex, Takara).

Screening of cDNA libraries, RACE and sequence analysis. A cDNA library from human adult brain (caudate nucleus) was supplied by Y. Misumi. Libraries from fetal brain were purchased from Clontech. RACE experiments were performed using the Marathon cDNA amplification kit and Marathon Ready cDNA from adult heart (Clontech) in parallel. Sequencing was done using the PRISM ReadyReaction DyeDeoxy Terminator cycle-sequencing kit with AmpliTaq-FS (Perkin–Elmer) on an ABI Model 377 DNA sequencer (Perkin–Elmer).

SSCP analysis. Primers ex3F (5′-GTTGCATGCTGGACTTTGAA-3′) and ex3R (5′-CATAAAGCACTTGGTAAAGGGC-3′) for exon 3, and primers ex4F (5′-GACTGTTGTGTTGGCTTACTGG-3′) and ex4R1 (5′-GATACTGCAGTG-CAAATGCAG-3′) for exon 4, were used to amplify patient DNA. SSCP analysis was performed as described[22].

Cell transfection and immunofluorescence. A cDNA fragment (bases 60–1,494) containing an open reading frame was subcloned by PCR into the vector pEGFP-N1 (Clontech) and the vector pcDNA3.1(+) (Invitrogen). On fukutin–HA, an HA epitope tag (YPYDVPDYA) was added to the C terminus of fukutin. COS-7 and C2C12 cells were transfected with plasmid DNA with Lipofect-AMINE (Gibco-BRL) in Opti-MEM (Gibco-BRL). The localization of fukutin–GFP, fukutin–HA and Golgi 58K protein was determined by fluorescence microscopy one day after transfection.

Metabolic labelling and immunoprecipitation. Fukutin-GFP expressed COS-7 cells were incubated in DMEM containing [^{35}S]methionine and [^{35}S]cysteine. Culture medium was collected and pre-cleared by centrifugation. Anti-GFP polyclonal antibody (Clontech) was added to the samples. Immunoprecipitates were captured with protein A–Sepharose-4b (Zymed) and washed with RIPA buffer (10 mM Tris-HCl, pH 7.4, 1% Nonidet-P40, 0.1% Na deoxycholate, 0.1% SDS and 150 mM NaCl). Samples were electrophoresed on 10% SDS–polyacrylamide gels.

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Developmental selection of *var* gene expression in *Plasmodium falciparum*

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The protozoan *Plasmodium falciparum* causes lethal malaria[1]. Adhesion of erythrocytes infected with *P. falciparum* to vascular endothelium and to uninfected red blood cells (rosetting) may be involved in the pathogenesis of severe malaria[2–4]. The binding is mediated by the antigenically variant erythrocyte-membrane-protein-1 (PfEMP-1)[5–8], which is encoded by members of the *P. falciparum var* gene family[9,10]. The control of expression and switching of *var* genes seems to lack resemblance to mechanisms operating in variant gene families of other microbial pathogens[11,12]. Here we show that multiple, distinct *var* gene transcripts (about 24 or more) can be detected by reverse transcription and polymerase chain reaction in bulk cultures of the rosetting parasite FCR3S1.2, despite the adhesive homogeneity of the cultures. We also detected several *var* transcripts in single erythrocytes infected with a ring-stage parasite of FCR3S1.2, and found that different *var* genes are transcribed simultaneously from several chromosomes in the same cell. In contrast, we detected only one *var* transcript, FCR3S1.2 *var*-1, which encodes the rosetting PfEMP-1 protein[13], in individual rosette-adhesive trophozoite-infected cells, and we found only one PfEMP-1 type at the erythrocyte surface by labelling with ^{125}iodine and immunoprecipitation. We conclude that a single *P. falciparum* parasite simultaneously transcribes multiple *var* genes but, through a developmentally regulated process, selects only one PfEMP-1 to reach the surface of the host cell.

We studied the transcription and expression of *var* genes in a recently cloned and phenotypically homogeneous parasite culture, FCR3S1.2, which has a rosetting rate of greater than 90%. We first used reverse transcription and polymerase chain reaction (RT-PCR) to amplify the *var* gene messenger RNAs from unsynchronized cultures at the eighteenth generation after cloning, when most of the parasites were at the ring stage. We used degenerate primers that mapped to conserved stretches of the Duffy-binding-like (DBL)-1 domain of PfEMP-1 (ref. 14) (Fig. 1). We cloned products of amplification by RT-PCR, and sequenced 15 of these recombinant plasmids containing inserts of 500–600 base pairs (bp). As the parasites were phenotypically homogeneous, we assumed that there would be a corresponding sequence homogeneity in the transcript population. Analysis of the inserts showed, however, that only ~30% of the sequences (5 of 15) corresponded to the FCR3S1.2

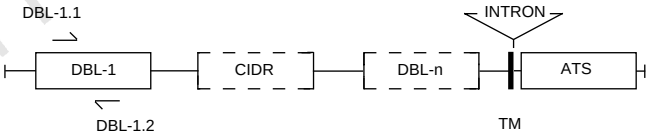

Figure 1 General structure of *var*/PfEMP-1. The semi-conserved Duffy-binding-like (DBL)-1 domain is present in all *var* genes sequenced to date. Downstream of the DBL-1 domain there is a cysteine-rich interdomain region (CIDR) and a variable number ($n = 0$–4) of less conserved DBL structures. A putative transmembrane domain (TM) is followed by the highly conserved acidic terminal sequence (ATS), which is presumably cytoplasmically located. We designed the degenerate oligonucleotide primers DBL-1.1 and DBL-1.2 from short, conserved amino-acid sequences flanking relatively variable stretches (400–700 bp) in the DBL-1 domain[14]. Boxes with dashed outlines indicate that these domains may or may not be present in a given PfEMP-1 molecule.

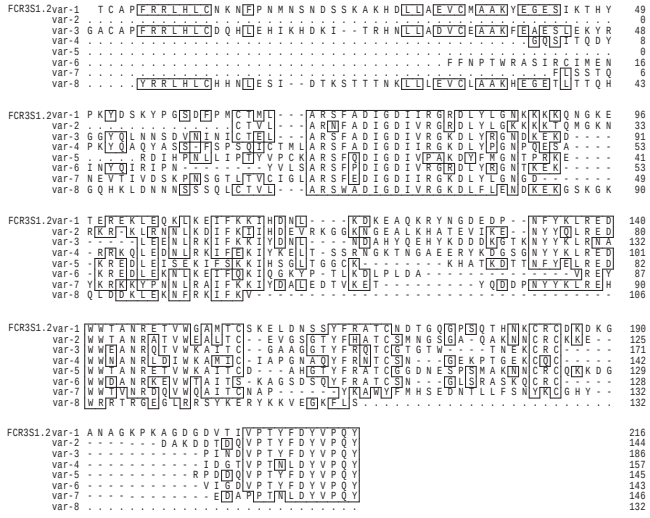

Figure 2 Predicted amino-acid sequences of *var* transcripts from bulk cultured FCR3S1.2. The figure shows the alignment of the unique sequences (*var 1–8*) that were amplified by RT-PCR with the DBL-1.1 and DBL-1.2 primers. Amino-acid similarity is indicated by boxes. Gaps and incomplete sequences are represented by dashed and dotted lines, respectively.