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A Homoplasmic Mitochondrial Transfer Ribonucleic Acid Mutation as a Cause of Maternally Inherited Hypertrophic Cardiomyopathy

Robert W. Taylor, PHD,* Carla Giordano, MD,† Mercy M. Davidson, PHD,† Giulia d'Amati, MD, PHD,‡ Hugh Bain, MD,§ Christine M. Hayes, BSC,* Helen Leonard, MD,§ Martin J. Barron, PHD,* Carlo Casali, MD, PHD,‖ Filippo M. Santorelli, MD,¶ Michio Hirano, MD,† Robert N. Lightowlers, PHD,* Salvatore DiMauro, MD,† Douglass M. Turnbull, MD, PHD*

Newcastle upon Tyne, United Kingdom; New York, New York; and Rome, Italy

OBJECTIVES The purpose of this study was to understand the clinical and molecular features of familial hypertrophic cardiomyopathy (HCM) in which a mitochondrial abnormality was strongly suspected.

BACKGROUND Defects of the mitochondrial genome are responsible for a heterogeneous group of clinical disorders, including cardiomyopathy. The majority of pathogenic mutations are heteroplasmic, with mutated and wild-type mitochondrial deoxyribonucleic acid (mtDNA) coexisting within the same cell. Homoplasmic mutations (present in every copy of the genome within the cell) present a difficult challenge in terms of diagnosis and assigning pathogenicity, as human mtDNA is highly polymorphic.

METHODS A detailed clinical, histochemical, biochemical, and molecular genetic analysis was performed on two families with HCM to investigate the underlying mitochondrial defect.

RESULTS Cardiac tissue from an affected child in the presenting family exhibited severe deficiencies of mitochondrial respiratory chain enzymes, whereas histochemical and biochemical studies of the skeletal muscle were normal. Mitochondrial DNA sequencing revealed an A4300G transition in the mitochondrial transfer ribonucleic acid (tRNA)^{Ile} gene, which was shown to be homoplasmic by polymerase chain reaction/restriction fragment length polymorphism analysis in all samples from affected individuals and other maternal relatives. In a second family, previously reported as heteroplasmic for this base substitution, the mutation has subsequently been shown to be homoplasmic. The pathogenic role for this mutation was confirmed by high-resolution Northern blot analysis of heart tissue from both families, revealing very low steady-state levels of the mature mitochondrial tRNA^{Ile}.

CONCLUSIONS This report documents, for the first time, that a homoplasmic mitochondrial tRNA mutation may cause maternally inherited HCM. It highlights the significant contribution that homoplasmic mitochondrial tRNA substitutions may play in the development of cardiac disease. A restriction of the biochemical defect to the affected tissue has important implications for the screening of patients with cardiomyopathy for mitochondrial disease. (J Am Coll Cardiol 2003;41:1786–96) © 2003 by the American College of Cardiology Foundation

Over the last decade, mutations in the mitochondrial deoxyribonucleic acid (mtDNA) have become increasingly recognized as important causes of disease (1,2), with the number of different pathogenic defects now described exceeding 200 (3). Many take the form of mtDNA rearrangements, but an increasing number of maternally inherited mtDNA point mutations have been described, affecting protein coding, ribosomal ribonucleic acid (mt-rRNA), or more commonly, transfer ribonucleic acid (mt-tRNA) genes (4). Recent epidemiologic studies have estimated the incidence of mtDNA mutations to be at least 1 in 8,000 in a stable Caucasian population within the Northeast of England (5). The majority of reported pathogenic mtDNA mutations are heteroplasmic, a situation in which both the mutated and wild-type forms of mtDNA are present within the same cell. Homoplasmic mtDNA mutations (in which all copies of the genome are affected) have been reported in association with tissue-specific disease, such as Leber's hereditary optic neuropathy (LHON) and sensorineural hearing loss, affecting protein-encoding (6,7), 12S mt-rRNA (8), and the mt-tRNA$^{Ser(UCN)}$ (9–11) genes.

A number of mtDNA point mutations have previously been described in patients presenting with cardiomyopathy (12,13). In this report, we describe two families with hypertrophic cardiomyopathy (HCM) associated with a homoplasmic A4300G mutation in the mt-tRNA^{Ile} gene. In both families, the clinical features and associated histochemical and biochemical abnormalities were confined to the heart. We were able to study the cardiac tissue of

From the *Department of Neurology, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom; †Department of Neurology, Columbia University College of Physicians and Surgeons, New York, New York; §Department of Paediatric Cardiology, Freeman Hospital, Newcastle upon Tyne, United Kingdom; and ‡Dipartimento di Medicina Sperimentale e Patologia, ‖Istituto di Clinica delle Malattie Nervose e Mentali, and ¶Molecular Medicine, Children's Hospital "Bambino Gesù," Università di Roma-La Sapienza, Rome, Italy. This work was supported by grants provided to Drs. Turnbull and Lightowlers from the Wellcome Trust, U.K., to Dr. Davidson from the American Heart Association, and to Dr. Giordano from Telethon-Italia.

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Abbreviations and Acronyms

COX	= cytochrome *c* oxidase
HCM	= hypertrophic cardiomyopathy
LHON	= Leber's hereditary optic neuropathy
LV	= left ventricle or ventricular
mtDNA	= mitochondrial deoxyribonucleic acid
mt-tRNA^{Ile}	= mitochondrial transfer ribonucleic acid gene for isoleucine
PCR	= polymerase chain reaction
RFLP	= restriction fragment length polymorphism
SDH	= succinate dehydrogenase

affected individuals from both families, which revealed not only severely decreased respiratory chain activity but also very low steady-state levels of mt-tRNA^{Ile}.

METHODS

Clinical profile. FAMILY 1. The family tree is reported in Figure 1A. Patient no. IV-02 was admitted at age 14 months following a five-day history of a sore throat and swollen glands and a one-day history of lethargy. His development up to this stage was normal. He was in cardiac failure with poor peripheral perfusion. An echocardiogram showed gross dilation of the left ventricle (LV), with grossly diminished global function (Table 1). The LV was hypertrophied. He continued to deteriorate and died of a cardiac arrest two days after admission. Pathologic examination revealed marked concentric hypertrophy of the LV, with no evidence of myocarditis but with hypertrophied myocytes.

The proband (Patient no. IV-03) was carefully monitored from birth. Left ventricular hypertrophy was noted at 11 months, but LV function was good. He was followed until the age of five years at regular intervals; clinical examination, electrocardiography, and echocardiography confirmed biventricular hypertrophy, but with preserved function. The child himself was not restricted in his activities. At five years, he was admitted as an emergency with a four-day history of lethargy. He was in cardiac failure with very poor ventricular function (Table 1). His condition rapidly worsened. He died eight days after admission, despite full supportive therapy. Both surviving boys (Patient nos. IV-01 and IV-04) are asymptomatic. They have evidence of mild LV hypertrophy (both IV-01 and IV-04) and dilation (IV-01) based on both clinical and echocardiographic measures, although neither boy has experienced episodes of cardiac failure. Their mother and maternal grandmother are asymptomatic, and the mother has a normal electrocardiogram (ECG) and echocardiogram. There is no other history of cardiomyopathy in the family.

FAMILY 2. The detailed clinical features of this large family have been reported previously (14,15). Briefly, a diagnosis of nonobstructive idiopathic HCM was made at 20 years in the proband (IV-01), who underwent heart transplantation 14 years later for congestive heart failure. A maternal cousin (IV-09) with LV hypertrophy progressed to congestive heart failure in six months (Table 1) and is now waiting for heart transplantation with ventricular mechanical assistance. Family history was highly suggestive of a maternal pattern of transmission of the disease (Fig. 1B). All affected members, both symptomatic and asymptomatic, had echocardiographic and ECG features of nonobstructive HCM (Tables 1 and 2). Neurological examination was normal.

Cell culture. Fibroblast cultures were obtained by explant culture of skin biopsy samples from the probands of both families. Myoblast cultures from the proband of family 1 (IV-03) were obtained as described elsewhere (16). In the absence of primary skeletal muscle cultures, fibroblasts from the proband of family 2 (IV-01) and a cousin (IV-03) were converted to myoblasts using the muscle differentiation gene, MyoD, as described by Sancho et al. (17). Fibroblasts were grown in minimal essential medium (MEM) (Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS), 1% MEM nonessential amino acids, and 1% MEM vitamins. Myoblasts were grown in Hams F10 (Life Technologies) supplemented with 20% FCS and 1% chick-embryo extract (ICN Flow Biomedicals, Irvine, California). In addition, both growth media contained 100 μg/ml streptomycin and 100 U/ml benzylpenicillin and were further supplemented with sodium pyruvate (110 μg/ml) and uridine (50 μg/ml) to maintain respiratory-deficient cells.

Histochemical and biochemical analyses of respiratory chain enzymes. Heart tissue from the left and right ventricles of the probands from families 1 and 2 was obtained at autopsy (within 2 h of death) and heart transplantation, respectively. The samples were frozen in isopentane previously cooled to −190°C in liquid nitrogen. Cryostat sections (8 μm) were assayed for succinate dehydrogenase (SDH) and cytochrome *c* oxidase (COX) activities (18). In some cases, sections were dual-stained for both activities.

Respiratory chain enzyme activities were evaluated in mitochondrial fractions (19) prepared from fresh skeletal muscle, cardiac samples, and cultured skin fibroblasts from the proband of family 1 and whole-cell lysates of cultured skin fibroblasts from the proband of family 2. The activities of the individual respiratory chain complexes were determined spectrophotometrically, as previously described (19), using the matrix marker citrate synthase as a standard.

Molecular genetic analyses. FAMILY 1. Total DNA was isolated from skeletal muscle, heart (LV), and cultured myoblasts from the proband and from circulating blood lymphocytes obtained from his mother, maternal grandmother, and a maternal cousin, according to standard procedures.

FAMILY 2. Total DNA was isolated from skeletal muscle, heart, circulating lymphocytes, and cultured skin fibroblasts from the proband and from circulating blood lymphocytes from 23 family members spanning four generations of this large pedigree and included affected and nonaffected individuals.

The sequence of the entire mitochondrial genome in the

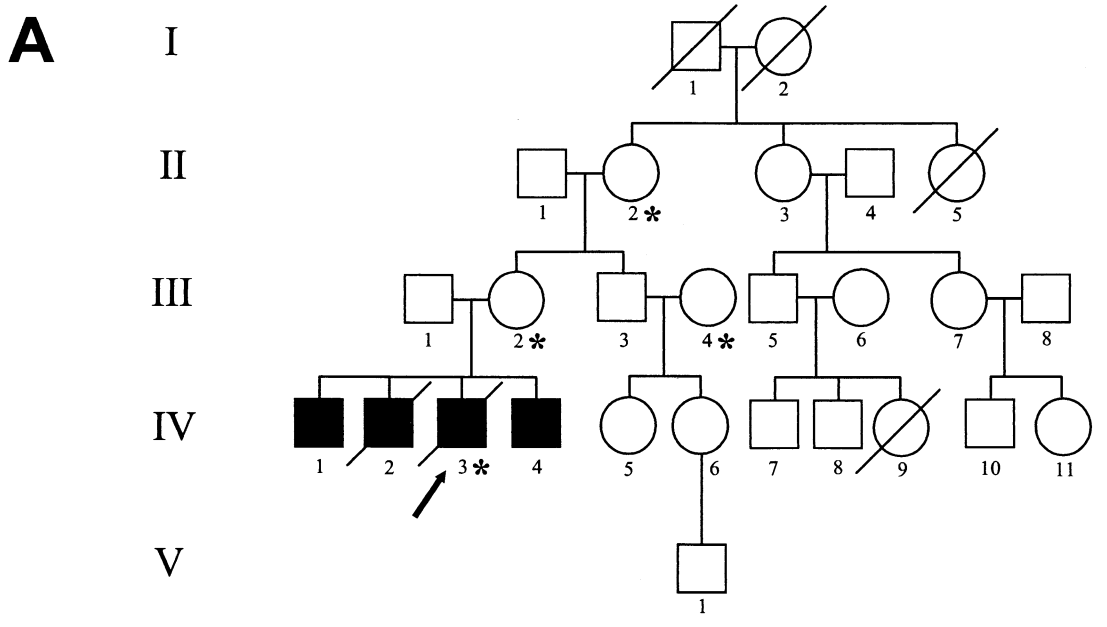

Figure 1. Pedigrees of family 1 **(A)** and family 2 **(B).** An **arrow** indicates the probands in each family. **Solid symbols** indicate clinically affected individuals, and those tested for the A4300G mitochondrial deoxyribonucleic acid mutation have an **asterisk**. In family 1, there is a less extensive family tree, and from the pedigree, either an X-linked or autosomal-recessive pattern of inheritance is possible. However, there was no clinical or pathologic evidence of Fabry disease in either family.

Table 1. Echocardiographic Features of Affected Family Members From Families 1 and 2

		Two-Dimensional Echocardiogram				
		LVWT				
Subject No.	Age (yrs)/Gender	VS (mm)	PW (mm)	LVEDD (mm)	LVESD (mm)	EF (%)
Family 1						
IV-01	16/M	8	12	55	34	Normal
IV-02	1/M*	11	13	40	36	10
IV-03	5/M*	11	12	57	54	5
IV-04	6/M	9	8	38	24	Normal
Family 2						
III-15	45/F	18	13	46	36	Normal
IV-01	34/M	18	11	69	60	10
IV-03	39/F	16	13	47	33	Normal
IV-05	36/M	18	23	47	33	Normal
IV-08	31/F	17	17	40	24	Normal
IV-09	22/M	27	25	52	34	Normal
	23	25	16	82	72	17
IV-20	27/M	19	16	49	33	Normal
	28	18	13	68	56	40
IV-23	30/M	13	16	52	33	Normal
V-05	18/F	13	15	45	32	Normal
V-07	17/F	13	16	46	33	Normal
V-08	14/M	12	15	46	32	Normal
V-13	10/M	12	7	45	31	Normal
V-14	10/F	12	15	45	32	Normal
V-20	10/F	12	9	46	32	Normal

*Age at time of death. Family 1: The measurements shown for subject nos. IV-01 and IV-04 represent the most recent data, whereas those for IV-02 and IV-03 were taken on admission during the final illness. Family 2: Two values are given for subject nos. IV-09 and IV-20, indicating the rapid decline in cardiac function observed in these patients.

LVEDD = left ventricular end-diastolic diameter; LVESD = left ventricular end-systolic diameter; LVWT = left ventricular wall thickness; PW = posterior wall; VS = ventricular septum; other abbreviations as in Table 1.

patients' heart DNA samples was determined using 28 M13-tailed oligonucleotide primer pairs (20). Overlapping polymerase chain reaction (PCR)-amplified fragments were sequenced with BigDye terminator cycle sequencing chemistries on an Applied Biosystems (Foster City, California) 377 automated DNA sequencer. Sequence data were analyzed using Navigator and Factura software (Applied Biosystems) and compared with the revised Cambridge reference sequence (21).

Quantification of mutated mtDNA. The levels of mutated mtDNA in skeletal muscle, heart, cultured cells, and circulating lymphocytes from members of both families were determined by PCR/restriction fragment length polymorphism (RFLP) analysis. Two different strategies were employed. The first, originally described by Casali et al. (14), was used to determine the amount of mutated mtDNA in members of family 2. In the second, a 239–base pair (bp) fragment encompassing the mutation site was amplified with the forward primer L4084 (positions 4084–4103) 5′-GTCACCAAGACCCTACTTCT-3′ and the reverse mismatch primer H4322 (positions 4322–4302) 5′-GGGGGTTTAAGCTCCTAT**G**AT-3′ (mismatch base shown in bold). Samples were subjected to 30 cycles of amplification with an annealing temperature of 56°C; the final extension proceeded for 8 min. After the addition of 30 pmol of each primer, 5 μCi (alpha-^{32}P)-deoxycytidinetriphosphate (3,000 Ci/mmol), and 1 U *Taq* polymerase, the PCR reactions were subjected to an additional cycle of amplification. Labeled products were precipitated and equal amounts (1,000 to 2,000 counts) digested with 10 U *Hinf*I (Roche Molecular Biochemicals, Lewes, United Kingdom). Restriction fragments were separated by 12% nondenaturing polyacrylamide gel electrophoresis, dried onto a support, and analyzed with ImageQuant software (Molecular Dynamics, Eugene, Oregon) following exposure to a PhosphorImager (Molecular Dynamics). The mismatch primer creates an additional *Hinf*I recognition site in the PCR product in the presence of the mutation. On digestion, a single *Hinf*I site in the wild-type product generates fragments of 180 and 59 bp. The A4300G mutation generates an additional recognition site cleaving the 180-bp fragment into two smaller products of 158 and 22 bp.

High-resolution Northern blot analysis. Total cytosolic RNA was isolated from solid tissues (~200 mg; frozen in liquid nitrogen and ground to a fine powder), cultured myoblasts, and fibroblasts (1 to 2 $\times$ 10^6 cells) using Trizol reagent (Life Technologies). Large RNA species were precipitated by the addition of 10 mol/l LiCl, allowing smaller RNAs (5S tRNA) to be precipitated from the resulting supernatant. Small RNAs (1 μg) were denatured (90°C for 5 min) and separated through a 13%, 8 mol/l urea denaturing polyacrylamide gel using 1× Tris Borate

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Table 2. Clinical Characteristics of Members of Family 2

Subject No.	Age (yrs)/Gender	Clinical Symptoms	ECG	Two-Dimensional Echocardiogram
II-01	90/F	None	Normal	NA
III-01	59/F	None	TWI	NA
III-02	54/F	CD	NA	NA
III-03	56/F	Dyspnea	LVH, TWI	NA
III-04	54/F	None	TWI	NA
III-05	59/M	Dyspnea	LVH, TWI	NA
III-13	48/F	None	Normal	LVH
III-14	46/F	None	Normal	Normal
III-15	45/F	Dyspnea, CP	LVH, TWI	LVH
III-16	33/F	CD	NA	NA
IV-01	34/M	NYHA class IV	LVH	LVH and dilation
IV-02	32/F	None	Normal	Normal
IV-03	39/F	Dyspnea	LVH, TWI, short PR interval	LVH
IV-04	37/F	None	Normal	Normal
IV-05	36/M	CP	LVH	LVH
IV-07	26/F	None	Normal	Normal
IV-08	31/F	Dyspnea	LVH, TWI	LVH
IV-09	23/M	Dyspnea	LVH, TWI	LVH and dilation
IV-16	32/F	None	Normal	Normal
IV-20	28/M	Dyspnea	LVH	LVH and dilation
IV-21	25/F	None	Normal	Normal
IV-23	30/M	None	LVH	LVH, PW
IV-24	28/F	None	Normal	Normal
IV-25	23/F	None	Normal	Normal
IV-26	23/F	None	Normal	Normal
IV-27	19/F	None	Normal	Normal
V-01	13/M	None	Normal	Normal
V-02	8/M	None	Normal	Normal
V-03	14/F	None	Normal	Normal
V-04	6/F	None	Normal	Normal
V-05	18/F	None	NA	LVH
V-06	16/F	None	Normal	Normal
V-07	17/F	None	NA	LVH
V-08	14/M	Normal	NA	LVH
V-12	12/F	Normal	NA	Mild AR
V-13	10/M	Normal	LVH	LVH, septal
V-14	10/F	Normal	LVH	LVH
V-20	10/F	Normal	LVH	LVH

The data presented reflect the most recent clinical, electrocardiographic, two-dimensional echocardiogram results.

AR = aortic regurgitation; CD = cardiac death; CP = chest pain; ECG = electrocardiogram; F = female; LVH = left ventricular hypertrophy; M = male; NA = not applicable; NYHA = New York Heart Association; TWI = T-wave inversion; PW = posterior wall.

EDTA (TBE) as a running buffer. Separated samples were electroblotted onto GeneScreen-plus membranes (NEN Dupont, Steverage, United Kingdom) in 0.25× TBE and immobilized by ultraviolet cross-linking. Regions of mtDNA encompassing the $tRNA^{Ile}$ and $tRNA^{Leu(UUR)}$ genes amplified by PCR were used as probes for Northern blots. The mt-$tRNA^{Ile}$ probe was amplified using the forward primer L4152 (positions 4152–4171) 5′-CGACCAACT-CATACACCTCC-3′ and the reverse primer H4328 (positions 4328–4308) 5′-AAATAAGGGGGTTTAA-GCTCC-3′. The mt-$tRNA^{Leu(UUR)}$ probe was amplified using the forward primer L3200 (positions 3200–3219) 5′-TATACCCACACCCACCCAAG-3′ and the reverse primer H3353 (positions 3353–3334) 5′-GCGATT-AGAATGGGTACAAT-3′. Purified PCR products (QIAquick PCR purification columns, Qiagen, Crawley, United Kingdom) were radiolabeled with (alpha-^{32}P)-deoxycytidinetriphosphate (3,000 Ci/mmol) by the random-primer method, and unincorporated nucleotides were removed by gel filtration through a Sephadex G-50 DNA grade column (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out at 42°C overnight in a solution of 5× SSPE, 50% formamide, 10% dextran sulfate, 5× Denhardt's solution, and 1% sodium dodecyl sulfate (SDS) containing 2×10^6 cpm radiolabeled probe. After hybridization, two 15-min washes were performed at room temperature with 2× SSPE, followed by a 15-min wash at 65°C with 2× SSPE and 2% SDS. Blots were subjected to PhosphorImager analysis and the radioactive signal for the mt-$tRNA^{Ile}$ probe (69 bp) normalized to that of the mt-$tRNA^{Leu(UUR)}$ probe (75 bp) for each sample.

RESULTS

Histochemical and biochemical analyses of respiratory chain enzymes. Histologic examination of postmortem and explanted hearts failed to show significant myofiber disarray. Histochemical analysis of heart tissue from both probands revealed large numbers of cells devoid of COX activity, which was particularly evident when the sections were dual-stained for both COX and SDH activity. The number of COX-negative cells was higher in the left than in the right ventricle (Figs. 2A to D). However, the activities of both SDH and COX were normal in skeletal muscle from the proband of family 1, and there were no other histochemical features suggestive of mitochondrial disease, such as the subsarcolemmal accumulation of mitochondria (ragged-red fibers) (Fig. 2E).

The results of the biochemical analysis of respiratory chain enzyme activities for Patient no. IV-03 of family 1 are reported in Table 3. There was a severe defect in the activities of both complexes I and IV in heart mitochondria, with normal activity of complex II. The activities of individual respiratory chain complexes were entirely normal in the muscle (Table 3) and cultured skin fibroblasts from this family, as well as in the fibroblasts and MyoD-converted myoblasts from family 2 (data not shown).

Molecular genetic analysis. SEQUENCE ANALYSIS OF THE ENTIRE MITOCHONDRIAL GENOME. There was no evidence of mtDNA rearrangements or mtDNA depletion on Southern blot analysis of DNA from the heart (data not shown). The sequence of the entire mitochondrial genome amplified using DNA extracted from both proband hearts showed, in addition to the A4300G mutation, a number of neutral polymorphisms (3,22) that were clearly different between the two families. Consequently, these two families are certainly unrelated. Surprisingly, family 2 showed 10 sequence changes (A3358T, C6336T, T7657C, A8440G, G8790A, A13434G, T13500C, A14062G, G14305A, G14323A) that are not registered on the MITOMAP data base. The presence of such a large number of sequence variants in mitochondrial heart DNA prompted us to also sequence mtDNA from skin fibroblasts, where identical changes were seen. This confirmed not only that the A4300G mutation is the only mtDNA defect common to the two families, but also that a second mitochondrial mutation was not contributing to the expression of the cardiac-specific phenotype.

DEMONSTRATION OF HOMOPLASMIC A4300G MUTATION. We assessed the level of the A4300G mutation in all available DNA samples from both families using PCR-RFLP analysis. The presence of the mutant G4300 allele creates an additional recognition site for the restriction enzyme *Hinf*I, permitting the detection of heteroplasmic mtDNA at this site. The mutation was homoplasmic in every tissue investigated from Patient no. IV-03 of family 1 (Fig. 3), with no wild-type mtDNA visible even after prolonged exposure of the dried gel to the PhosphorImager

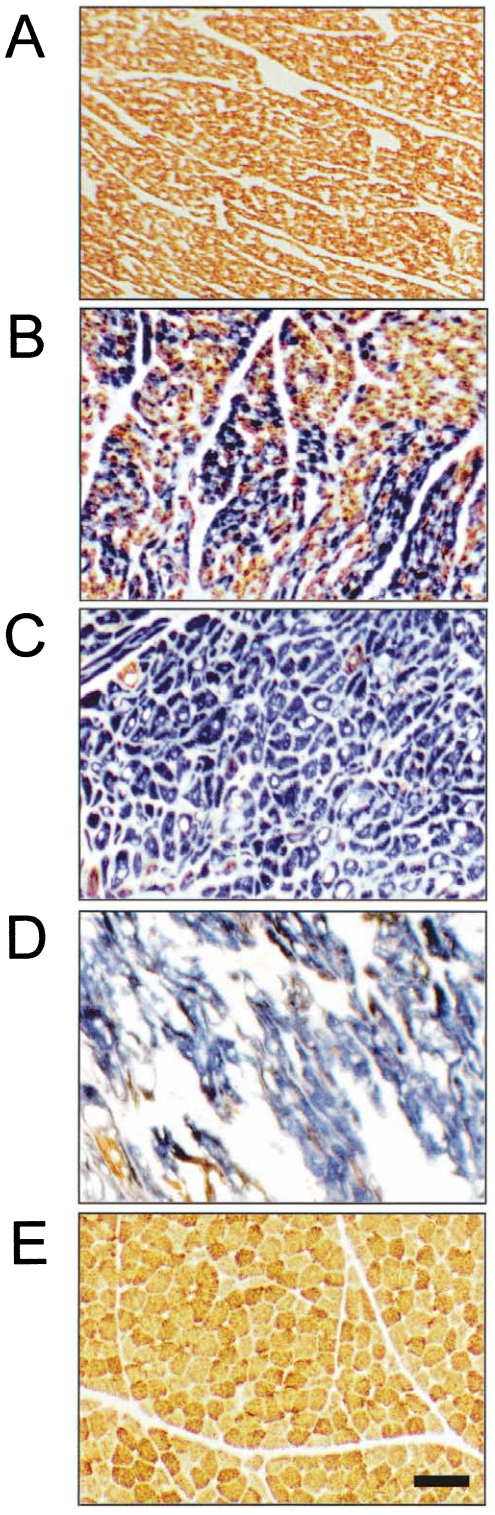

Figure 2. Histochemical analysis of skeletal muscle and cardiac tissue. **(A)** Age-matched control heart sample dual-stained for cytochrome *c* oxidase (COX) and succinate dehydrogense (SDH) activity. **(B)** Right ventricular sample from Patient no. IV-03 (family 1) dual-stained for COX and SDH activity, highlighting the distribution of COX-negative **(blue)** cells. **(C)** Left ventricular sample from Patient no. IV-03 (family 1) dual-stained for COX and SDH activity. **(D)** Left ventricular sample from Patient no. IV-01 (family 2) dual-stained for COX and SDH activity. **(E)** A transverse section of skeletal muscle from the proband in family 1 (IV-03) reacted for COX activity, showing a normal staining pattern. The **scale bar** represents 200 μm.

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Table 3. Respiratory Chain Complex Activities in Skeletal Muscle Mitochondria and Cardiac (Left Ventricle) Mitochondria From the Index Case (Patient IV-03) of Family 1

	Skeletal Muscle		Cardiac Muscle	
Complex	Controls (n = 15)	Patients	Controls (n = 8)	Patients
Complex I/CS	0.240 ± 0.060	0.317	0.254 ± 0.074	0.015
Complex II/CS	0.320 ± 0.088	0.413	0.281 ± 0.036	0.196
Complex IV/CS	1.34 ± 0.39	1.48	2.34 ± 0.54	0.059
Complex I/II	0.74 ± 0.12	0.77	0.88 ± 0.18	0.077
Complex IV/II	4.31 ± 1.36	3.57	8.08 ± 1.90	0.302

Control data are presented as the mean value ± SD. Enzyme activities are expressed as nmol NADH oxidized min/U citrate synthase (CS) for complex I; nmol 2,6-dichlorophenol-indophenol reduced min/U citrate synthase for complex II (succinate: ubiquinone-1 reductase); and the apparent first-order rate constant.s/U citrate synthase for complex IV ($\times 10^3$).

plate. The 4300G allele was also homoplasmic in blood cells from the patient's mother, maternal grandmother, and also a maternal cousin (data not shown). Our findings in family 1 prompted us to reinvestigate the A4300G mutation in family 2, previously reported by Casali et al. (14,15) as heteroplasmic. We screened blood and fibroblast DNA from 23 maternal relatives by PCR-RFLP analysis (Fig. 1). The mutation was homoplasmic in every individual, both symptomatic and asymptomatic, contrary to the earlier report (15) (Fig. 4, RFLP patterns of three representative family members shown). This highlights the importance of using appropriate controls in the analysis of mtDNA mutations (23) and raises concerns about reports of mutations within single families.

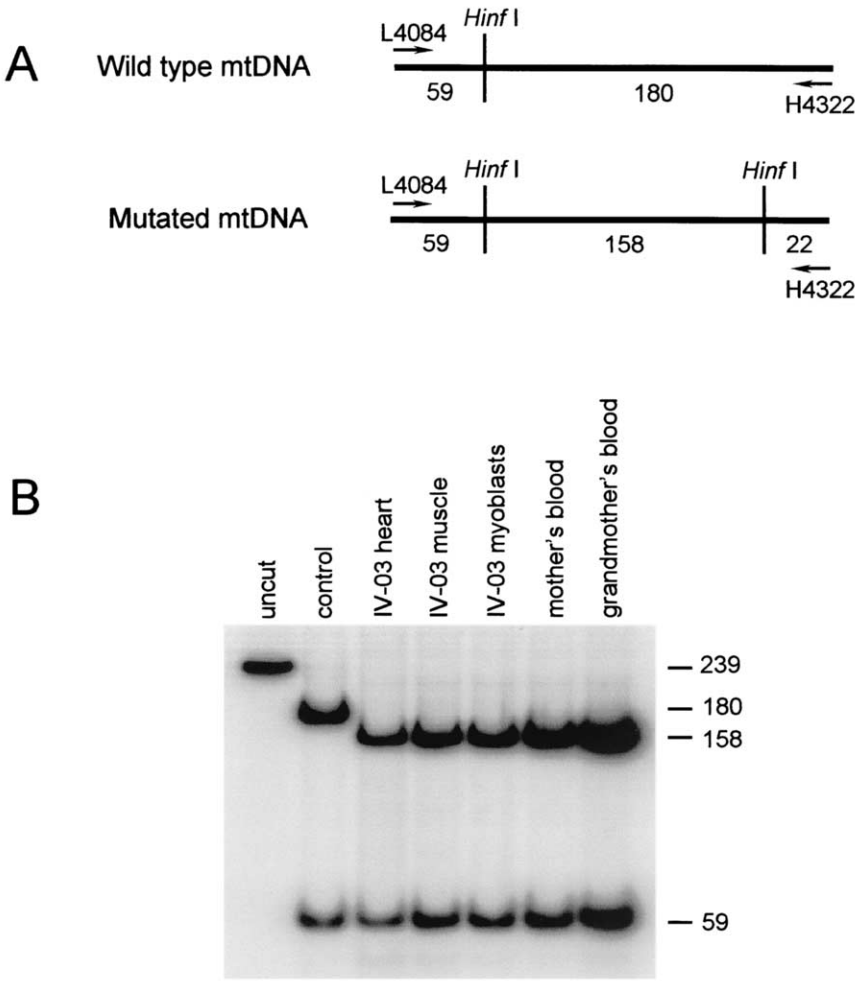

Figure 3. Quantification and distribution of the A4300G mutation in family 1. **(A)** Scheme of the restriction fragment length polymorphism (RFLP) analysis performed. A single *Hinf*I site cuts the wild-type polymerase chain reaction-amplified product of 239 bp into two fragments of 180 and 59 bp. In the presence of the A4300G mutation, a mismatched reverse primer creates a new *Hinf*I recognition site that cuts the 180-bp fragment into two smaller products of 158 and 22 bp. **(B)** Labeled products generated by the RFLP analysis were separated through a 12% nondenaturing polyacrylamide gel. Fragment sizes (bp) are shown on the **right**. mtDNA = mitochondrial deoxyribonucleic acid.

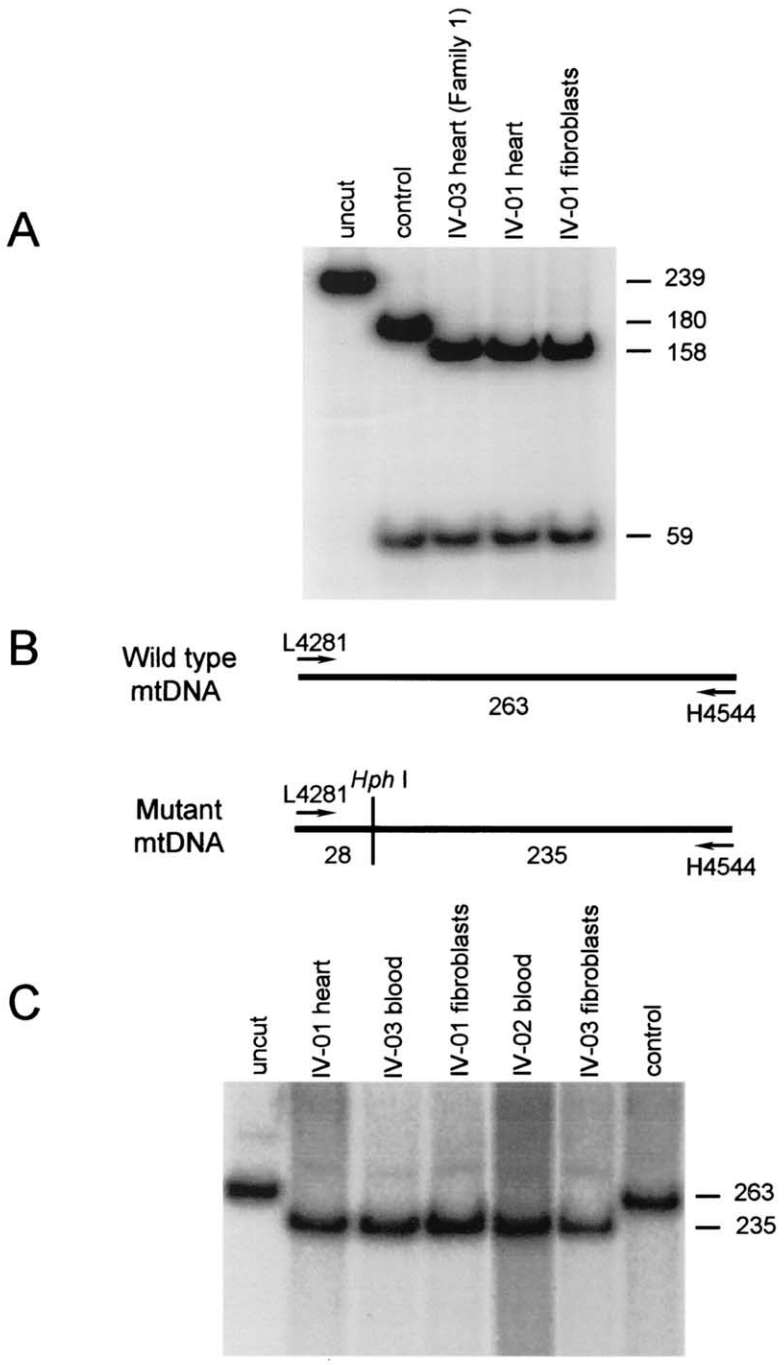

Figure 4. Quantification and distribution of the A4300G mutation in family 2. **(A)** Labeled products generated by the same restriction fragment length polymorphism (RFLP) analysis described in Figure 3, showing the homoplasmic mutation in the heart deoxyribonucleic acid of both probands (IV-03 in family 1 and IV-01 in family 2) and in the fibroblasts of the proband (IV-01) from family 2. Fragment sizes (bp) are shown on the **right**. **(B)** Scheme of the alternative RFLP analysis first described by Casali et al. (14). In combination with the A4300G mutation, a mismatched forward primer creates a new *Hph*I recognition site that cuts the 263-bp polymerase chain reaction-amplified product into two smaller products of 235 and 28 bp. In this assay, wild-type mitochondrial deoxyribonucleic acid (mtDNA) remains uncut. **(C)** Representative gel of samples from three family members showing labeled products generated by the RFLP analysis described earlier. A total of 23 maternal relatives were screened by this assay and shown to be homoplasmic for the A4300G mutation. Fragment sizes (bp) are shown on the **right**.

DETERMINATION OF STEADY-STATE MT-TRNA^{ILE} LEVELS IN BOTH FAMILIES. To investigate the effect of the A4300G mutation on the processing of mt-tRNA^{Ile} from its precursor, the steady-state level of mt-tRNA^{Ile} was determined in tissues from affected individuals by Northern blot hybridization. There was a marked decrease (5% to 10% of controls) in the quantity of the mature mt-tRNA^{Ile} transcripts from LV tissue of both probands compared with

controls (Fig. 5). However, the mt-tRNA^{Ile} gene did not show any obvious size change in the mutant form. A similar decrease (10% to 15% of controls) was also observed in skeletal muscle, a clinically unaffected tissue, from the proband of family 1, when compared with steady-state levels of mt-tRNA$^{Leu(UUR)}$ in the same samples. Cultured skin fibroblasts and myoblasts from both families expressed the molecular defect to a lesser extent (~40% and 50% of control values).

DISCUSSION

We have described the detailed clinical, histochemical, biochemical, and molecular genetic investigation of two families with maternally inherited HCM that revealed a homoplasmic mutation in the mt-tRNA^{Ile} gene. The mutation affects a base pair in the secondary structure of mt-tRNA^{Ile} in a region of the gene that shows high evolutionary conservation. The clinical features of this cardiomyopathy are distinct from those of familial autosomal-dominant HCM. All affected patients from both families presented with nonobstructive HCM without symptoms of other system dysfunction. There was a mildly asymmetric pattern of LV hypertrophy, with some patients showing a prominent thickening of the posterior wall and others of the ventricular septum (Table 1). The illness often had an adverse clinical course, with LV dilation and failure, even at a young age. There were two childhood deaths in family 1 and two deceased members, a heart transplant recipient at age 34 years and a 23-year-old patient with heart failure waiting for heart transplant in family 2. An additional member of family 2 (Patient no. IV-20) with a LV cavity size within the upper normal limits, showed LV dilation and decreased ejection fraction on a six-month follow-up echocardiogram (Table 1). Although rapidly evolving cardiac failure was observed, neither ventricular arrhythmias nor sudden death were noted in these families.

Numerous heteroplasmic point mutations within mt-tRNA genes have been reported as causing disease, several in association with a number of cardiac muscle disorders (12,13). These include pathologic changes at A4317G (24), C4320T (25), A4295G (26), and A4269G (27) within the mt-tRNA^{Ile} gene. Although the A4300G mutation was initially judged to be heteroplasmic when first described (14), our present studies of these two families not only confirm the importance of the mt-tRNA^{Ile} gene as a "hot spot" for mitochondrial cardiomyopathy mutations, but also highlight the role that homoplasmic mt-tRNA mutations can play in causing isolated cardiomyopathy. This has important implications for the diagnosis of these conditions. For example, homoplasmic mutations in mtDNA previously reported as neutral polymorphisms could be pathogenic, especially if associated with tissue-specific disorders.

The results of our biochemical and molecular studies in these two families strongly support the pathogenic role of the homoplasmic A4300G mtDNA transition. There were very low activities of respiratory chain complexes I and IV (both contain mitochondrially encoded polypeptide subunits) in heart tissue and a marked decrease in the amount of mature cardiac mt-tRNA^{Ile} (~10% of controls). Surprisingly, comparably low steady-state levels of mt-tRNA^{Ile} were also observed in skeletal muscle, even though only the heart was biochemically and clinically affected. The presence of additional local factors, such as ventricular hemodynamic load or expression of cardiac-specific genes, acting independently or synergistically with the homoplasmic mtDNA mutation could explain the difference in phenotypic expression between cardiac and skeletal muscle. The finding of a marked difference in histochemical COX activity, even between the left and right ventricles, reinforces this hypothesis. The expression of several cardiac genes may vary between the left and right ventricles, both in normal conditions and in response to differences in load and local stress (28–30). The energy demands placed on the LV are much greater than those placed on the right ventricle, and the severity of the mitochondrial defect in the LV of these hearts would be sufficient to cause heart failure.

Homoplasmic mtDNA mutations present many challenges in understanding the molecular mechanism of disease, particularly as they predominantly appear to present in a tissue-specific manner—for example, LHON (6) and sensorineural deafness (8,9). The former is a mitochondrial DNA disorder in which the vast majority of patients present solely with optic neuropathy. The other feature in common between patients with LHON mutations and our families is that in both there was a very sudden onset of symptoms. In LHON, this presents with acute or subacute visual loss and in our patients with cardiac failure. Although it seems likely that the cause of the acute symptoms is an energy crisis in specific tissues, dissecting out the important genetic and environmental factors in LHON has proved extremely difficult. In some patients with sensorineural deafness, there is a clear association between a point mutation (A1555G) in the 12S mt-rRNA gene and toxicity to aminoglycosides (8), an obvious environmental factor. Such a situation, however, is not evident in our patients or in the majority of families with LHON.

Conclusions. Our extensive studies of these families have confirmed that the A4300G mutation is causative because of the mitochondrial biochemical defects in the affected tissue. Although rare compared with heteroplasmic changes, pathogenic, homoplasmic mtDNA mutations have been reported previously in association with various tissue-specific presentations, in particular the optic nerve in patients with LHON and sensorineural deafness (8,11). We believe that in individual patients, similar homoplasmic changes are not being pursued as potential causes of disease because either we do not recognize a particular phenotype or the mtDNA change seen was found to be homoplasmic and thus thought unlikely to be pathogenic. On this basis, it is

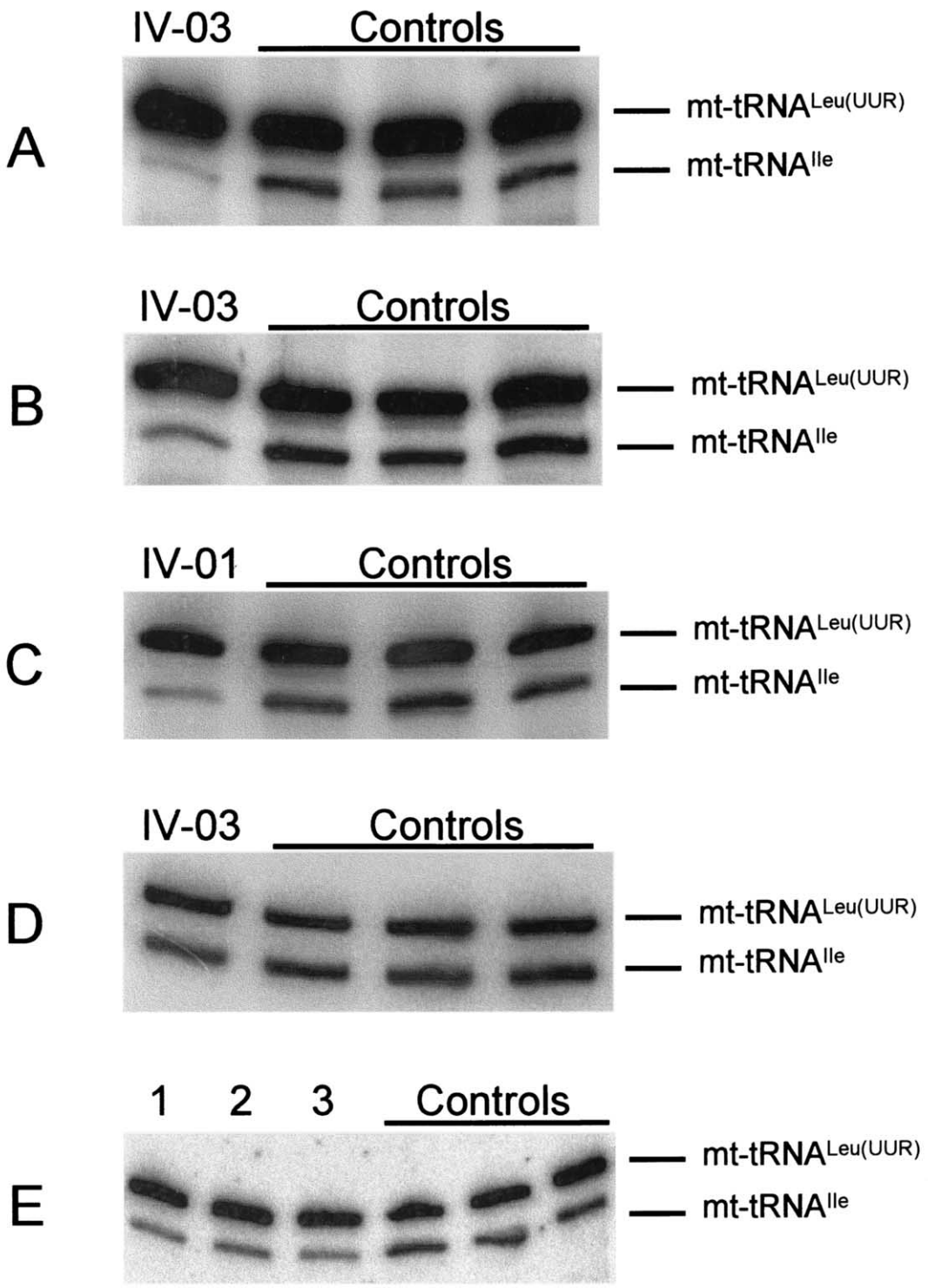

Figure 5. Determination of steady-state mitochondrial transfer ribonucleic acid gene for isoleucine (mt-tRNA^{Ile}) levels in solid tissues and cultured cells using high-resolution Northern blots. Small ribonucleic acids (1 μg) were separated through a 13%, 8 mol/l urea denaturing polyacrylamide gel, electroblotted onto membranes and hybridized with radiolabeled probes specific for mt-tRNA^{Ile} and mt-tRNA$^{Leu(UUR)}$ transcripts as described in Methods: High-resolution Northern blot analysis. Together with the patient samples, each panel shows three appropriate control samples prepared from the identical tissue or cell type. **(A)** Left ventricle of Patient no. IV-03 (family 1). **(B)** Skeletal muscle of Patient no. IV-03 (family 1). **(C)** Left ventricle of Patient no. IV-01 (family 2). **(D)** Cultured myoblasts of Patient no. IV-03 (family 1). **(E)** Cultured skin fibroblasts of patient no. IV-03 (family 1; **lane 1**), Patient no. IV-01 (family 2; **lane 2**), and Patient no. IV-03 (family 2; **lane 3**).

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possible that homoplasmic mt-tRNA mutations are greatly under-reported as causes of mitochondrial disorders (31), and they may play a more important role in the development of cardiomyopathy than previously thought.

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Reprint requests and correspondence: Prof. Douglass M. Turnbull, Department of Neurology, The Medical School, Framlington Place, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH, United Kingdom. E-mail: d.m.turnbull@ncl.ac.uk.

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