

## Phylogenetic analysis of short enteroviral sequences from patients with chronic fatigue syndrome

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This study used phylogenetic analysis based on a region of the 5' non-translated region (5'NTR) of a variety of enteroviral sequences to compare sequences associated with chronic fatigue syndrome (CFS) and those from enteroviruses causing acute infections. Direct sequencing of PCR products was used to obtain the nucleic acid sequences from CFS patients. The inferred phylogenetic tree identified three groupings, one correlating

with the diagnosis of CFS. The analysis identified a close relationship between the chronic fatigue enteroviral sequences, and showed that 19/20 were distinct from previously described enteroviruses. These results suggest there is persistence of enterovirus infection in some CFS patients and indicate the presence of distinct novel enterovirus sequences.

### Introduction

Human enteroviruses are established agents in a wide spectrum of clinical conditions many of which involve the musculoskeletal and nervous systems (Melnick, 1990). Most individuals encounter multiple enterovirus infections. Some of these viruses may be capable of persistence and could potentially be pathological agents in some cases of chronic fatigue syndrome (CFS) (Gow *et al.*, 1991; Cunningham *et al.*, 1991; Archard *et al.*, 1988). The suggested link between enteroviruses and CFS has been investigated by serological methods and by nucleic acid analysis, and several studies have shown that a significant proportion of patients complaining of CFS have markers for enterovirus infection (Yousef *et al.*, 1988; Archard *et al.*, 1988; Cunningham *et al.*, 1990; Preedy *et al.*, 1993).

The polymerase chain reaction (PCR) allows the rapid detection of specific sequences using specific oligonucleotide primers. The 5' non-translated region (5'NTR) of the enterovirus genome is an ideal target for detection by PCR, being highly conserved between viruses of this family (Chang *et al.*, 1989; Hughes *et al.*, 1989; Iizuka *et al.*, 1987; Jenkins *et al.*, 1987; Lindberg *et al.*, 1987; Rivera *et al.*, 1988; Ryan *et al.*, 1990; Toyoda *et al.*, 1984). Therefore, analysis based on the detection of this region allows for the identification of the majority of enteroviruses with the exception of some echoviruses and coxsackie A viruses (Gow *et al.*, 1991; Zoll *et al.*, 1992).

In this study we examined serum samples and throat swabs from patients identified as suffering from CFS between January 1992 and January 1994 by PCR using primers specific to the 5'NTR of the enteroviruses. For a comparison, serum samples were obtained at the same time from patients with no diagnosis of fatigue. The amplified enteroviral PCR products from the study group were directly sequenced and compared with those obtained from individuals without fatigue, and with all published enteroviral sequences available from GenBank.

### Methods

**Clinical specimens.** Patients had been referred to the outpatient clinic of the Department of Infection and Tropical Medicine, Ruchill Hospital, Glasgow, UK, by their general practitioner for assessment of their fatigue state. These individuals were drawn from the West of Scotland and attended between January 1992 and January 1994. There were no epidemiological features which linked this cohort of patients. The specimens used in this study were obtained from patients with a diagnosis of CFS which fulfilled the Oxford criteria (Sharpe *et al.*, 1991) and whose serum was enterovirus PCR positive.

Serum samples from a group of comparison patients were also examined. This group consisted of individuals whose samples had been received from the routine virology service provided at the Regional Virus Laboratory, which were obtained during the study period and were age and sex matched with the CFS patient group. In addition echovirus (echovirus types 3, 4, 7, 9, 11 and 20) isolates identified by the Regional Virus Laboratory (Grist *et al.*, 1979) and typed by standard neutralization assays (Minor & Bell, 1990) were used for comparison purposes.

**RNA extraction.** RNA was extracted from the samples using the 'Glassmax' RNA extraction kit as supplied by Gibco BRL using a modified version of the manufacturer's instructions. Briefly, 200 µl of serum or throat swab sample (in viral transport medium) was

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transferred to an Eppendorf with 450 µl of denaturing solution [guanidinium isothiocyanate-mercaptoethanol (10%)], 25 µl of RNA guard (1/20 dilution in water) (Gibco BRL), 25 µl of ribosomal RNA (1/40 dilution in water) (Gibco BRL) and vortexed. Ethanol (420 µl; 100%) (BDH) was then added to the reaction mixture, which was vortexed and centrifuged at 15000 r.p.m. for 5 min. The pellet of nucleic acid was retained and re-suspended in 450 µl of binding solution and 40 µl sodium acetate solution (as supplied). The resultant solution was transferred to a Glassmax cartridge and centrifuged at 15000 r.p.m. for 1–2 min to allow the nucleic acid to bind to the cartridge and to elute the carrying solution. The cartridge was then washed three times with RNA wash and twice with 80% ethanol. After the final wash the cartridge was given an additional centrifugation to remove any ethanol, and the nucleic acid was eluted-off by centrifugation with 25 µl DEPC-treated water heated to 65 °C. The extracted RNA was stored at –70 °C until required.

**PCR amplification.** Before PCR amplification the viral RNA was reverse transcribed to cDNA following the methods outlined by Gow *et al.* (1991) with the substitution of 1 µl of random hexamers (0.05 Units/ml) in place of the specific oligomers, in a 20 µl reaction mix. For first round PCR, 1 µl of each primer (40 pmol/µl), P1 and P4, was used in a 100 µl reaction containing 0.5 µl of *Taq* polymerase (1 unit/µl), 2 µl of each dNTP (100 µmol/µl) and 15 µl of reverse transcribed (RT) product.

In addition, 5 µl of RT product was used in a similar reaction with ABL1 and ABL2 primers which code for the Ablason tyrosine kinase gene and are indicative of successful RNA extraction (Hermans *et al.*, 1988). All of the serum samples from which sequence were derived were Ablason RNA positive.

Thirty-five cycles of 1 min at 94 °C, 1 min at 55 °C and 1.7 min at 72 °C were performed using a Techne PHC-1 thermocycler and the amplified products run-out on a 2.5% agarose gel.

An aliquot of first round product (3 µl) was then amplified in a second round (nested PCR) to give a product for sequencing. The second PCR reaction was similar to the first using primers P6 and P9 instead of P1 and P4. The reaction mixture was subjected to 25 cycles as described above and the products run-out on a 2.5% agarose gel stained with ethidium bromide. All experiments were conducted with positive and negative controls to exclude false-positive and negative results. All samples and controls were assayed blind to eliminate bias.

The nested PCR product (20 µl) was purified using a GeneClean kit (BIO101 Inc.). The final purified DNA was eluted in 20 µl of sterile distilled water.

**Oligonucleotide primers.** Oligonucleotide primers were obtained from Oswel DNA services (Edinburgh, UK) and were HPLC purified.

One set of primers (P1 and P4) was used to amplify a section of the 5'NTR from nucleotide positions 63–477 producing a fragment approximately 414 bp in length. The second set ('nested' primers) (P6 and P9) amplified a section of this first round product from nucleotide positions 169–434, producing a 265 bp segment (Zoll *et al.*, 1992).

P1 5' CGGTACCTTTGTGCGCCTGT 3'  
P4 5' TTAGGATTAGCCGCATTCAG 3'  
P6 5' GCACTTCTGTTACCCC 3'  
P9 5' TCAATAGACTCTTCGCAC 3'

**Sequencing the PCR amplification product.** All sequencing reactions were carried out using a Sequenase version 2.0 sequencing kit (US Biochemical).

Gene-cleaned DNA (10 µl) was mixed with 5 µl of each of the primers P6 and P9 and incubated at 94 °C for 2 min. It was mixed again, and incubated at 70 °C for 3 min, 94 °C for 45 s and 37 °C for 10 min. This primer-template DNA mixture (8 µl) was then added to the following for the labelling reaction: 2 µl of reaction buffer, 2 µl of Sequenase enzyme (1/8.5), 2 µl of labelling mixture (1/10) (dCTP,

dGTP and dTTP), 1 µl DTT (0.1 M) and 1 µl [<sup>35</sup>S]dATPαS (25 µCi/µl). The reagents were mixed and 3.5 µl of this was immediately added to 2.5 µl of each termination mix (pre-warmed to 37 °C for 1 min). The reaction mixture was incubated at 37 °C for 3–5 min, after which 4 µl of stop solution was added to each tube.

After heating the tubes at 95 °C for 2 min, 4 µl of each reaction was loaded onto a 6% sequencing gel. The gels were run for approximately 3 h at a constant 1500 V.

The gels were then fixed in acetic acid-methanol, washed and transferred to chromatography paper. This was then exposed to photographic film and developed after approximately 4 days depending on the activity of the radioactive nucleotide.

**Analysis of sequences.** The sequences derived were analysed by programs available in the University of Wisconsin Genetics Computer Group (GCG) sequence analysis package, version 7.0 (Devereux *et al.*, 1984).

Phylogenetic trees were generated using the Felsenstein PHYLIP package (version 3.4, June 1991; Felsenstein, 1988) as described by Chan *et al.* (1992) with the addition of DNA parsimony phylogenetic analysis.

Alignment of the nucleotide sequences was carried out using PILEUP (GCG). After this the programs COMPACT and TOPHYLIP (A. Wright, Glasgow University, UK) were used to truncate the sequences and eliminate deletions/insertions in order to concentrate on core sequence information and to eliminate undue weight given to these deletions/insertions.

To establish the phylogenetic relationships of the sequences obtained in this study with enteroviral sequence data already published, the latter were obtained from GenBank and transferred to the GCG package.

## Results

In the group of patients who had been referred for assessment of fatigue 44/238 serum samples and 29/175 throat swabs samples were positive by enteroviral PCR assay. In this study the PCR products from the serum of 20 of these patients all with a diagnosis of CFS were used [five males and 15 females, age range 23–72 years (mean age 38.5 years) with a duration of symptoms of 12–60 months (average duration of symptoms 23.8 months)]. Of the non-CFS comparison group, sera of 3/130 'healthy' individuals were positive for the enteroviral PCR assay and in addition PCR products were obtained from four patients complaining of anterior chest pain with a presumptive diagnosis of myocarditis.

There was no evidence for an epidemiological link between any of the CFS patients other than referral to the same hospital clinic. CFS patient samples (13 serum samples and seven throat swab samples), six echoviruses (isolated and typed in the Regional Virus Laboratory, Ruchill) and seven non-CFS samples were selected, successfully amplified and sequenced in both directions using upstream and downstream primers. Reproducibility of PCR and sequence analysis was established in three ways. Firstly, two independent RNA extractions were carried out on the same serum sample and sequenced in different laboratories. One was sequenced at the Regional Virus Laboratory, Ruchill where the

	193	229
Sample taken 30/8/93	ATCAATAAACTGCTCACGCGGTCAAGGAGAAAACG	
Sample taken 02/6/94	-----	
Throat Swab 30/8/93	-----TT-----	
Coxsackievirus B3	-----G-----T-----G--	
		285
TCCGTTACCCGACTGACTACTTCGAGAAACCCAGTAACACCATGGAGATTGCGAAGC		
-----		
-T-----T--G-CA-----A-----T-----G---AG---AG--T		
		342
GTTTCGCTCAGCACACCCCCAGTGTAGATCAGTCCGATGAGTCACCGCATTCCTCCAC		
-----		
-----G-----		
-----TA-----GT-----		
		399
AGGCGACTGTGGCGGTGGCTGCGTTGGCGGCCTGCCCATGGGGCAACCCATGGGATG		
-----		
-----CN---		
-----A---		
G-----C-----A-----C-		
403		
CTTC		
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--CT		

Fig. 1. Nucleotide sequences of the partial 5'NTR of enterovirus isolates from CFS patient no. 7. Differences between the sequences from the CFS patient and a published coxsackievirus B3 sequence are shown. Differences are printed in full; N refers to a mixed population of both G and C nucleotides at this position. Numbers refer to nucleotide positions with respect to the complete genome of coxsackievirus B3 (Klump *et al.*, 1990). The overall comparison shows a 99.05% identity between the two serum samples taken 10 months apart, 98.10% identity between the throat swab and serum samples taken at the same time and 87.68% identity between the first serum sample and the published coxsackievirus B3 sequence (Klump *et al.*, 1990) from the same region.

study was carried out and the other at a second laboratory in Glasgow using a different sequencing technique (Cancer Research Campaign Laboratories, Gartnavel Estate, Glasgow; sequencing was carried out using an Applied Biosystems 373 automated sequencer). The sequence from the two laboratories was identical, with the exception of a single base change. Secondly, sequence was obtained from serum and throat swab samples of a patient simultaneously; thirdly, sequential samples were obtained from a small number of patients who were enterovirus PCR positive. The sequence comparison is shown in Fig. 1. From this comparison it can be seen that the sequences are clearly reproducible and the CFS sequences were substantially different from coxsackievirus B3 which was used as a representative enterovirus sequence. Consistency of sequence obtained over time from a particular patient is evidence for persistent virus infection.

The sequences obtained are closely related, coming from a conserved region of a single family of viruses, and thus the alignment programs are likely to have made an optimal choice of sequence alignment. Despite the variability in sequences there is a high degree of homology between viral sequences which makes further phylogenetic comparisons valid.

Felsenstein (1988) describes three main families of inferring phylogenies. These are (1) parsimony and

compatibility methods, (2) distance methods and (3) maximum likelihood methods. There is no one method which has been shown to be optimal for inferring phylogenetic trees from alignments. Therefore the consensus of opinion in the recent literature has been to carry out all three methods to derive inferred trees of each. If each of the different methods yields the same tree or at least a virtually identical tree then this is thought to be reliable (Saitou & Imanishi, 1989; Chan *et al.*, 1992).

Fig. 2 illustrates the tree produced by parsimony analysis of the CFS patient sequences in relation to the non-CFS and published sequences. This shows that there are three groups. Group I comprises poliovirus types 1, 2 and 3, enterovirus 70 as well as coxsackieviruses A21 and A24. Group II comprises the coxsackie B virus group, coxsackievirus A9 and echoviruses 3, 4, 7, 9, 11 and 20, 6/7 non-CFS patients and one CFS patient sequence. The third Group (III) comprises 19/20 sequences obtained from the CFS patients and one non-CFS patient. The two other phylogenetic analysis methods produced virtually identical patterns (data not shown).

Group I, the poliovirus group, demonstrated an overall similarity and the inclusion of coxsackievirus A21 was expected since this group has been described as being serologically related (Hughes *et al.*, 1989). The sequence analysis carried out here, however, relates to a non-

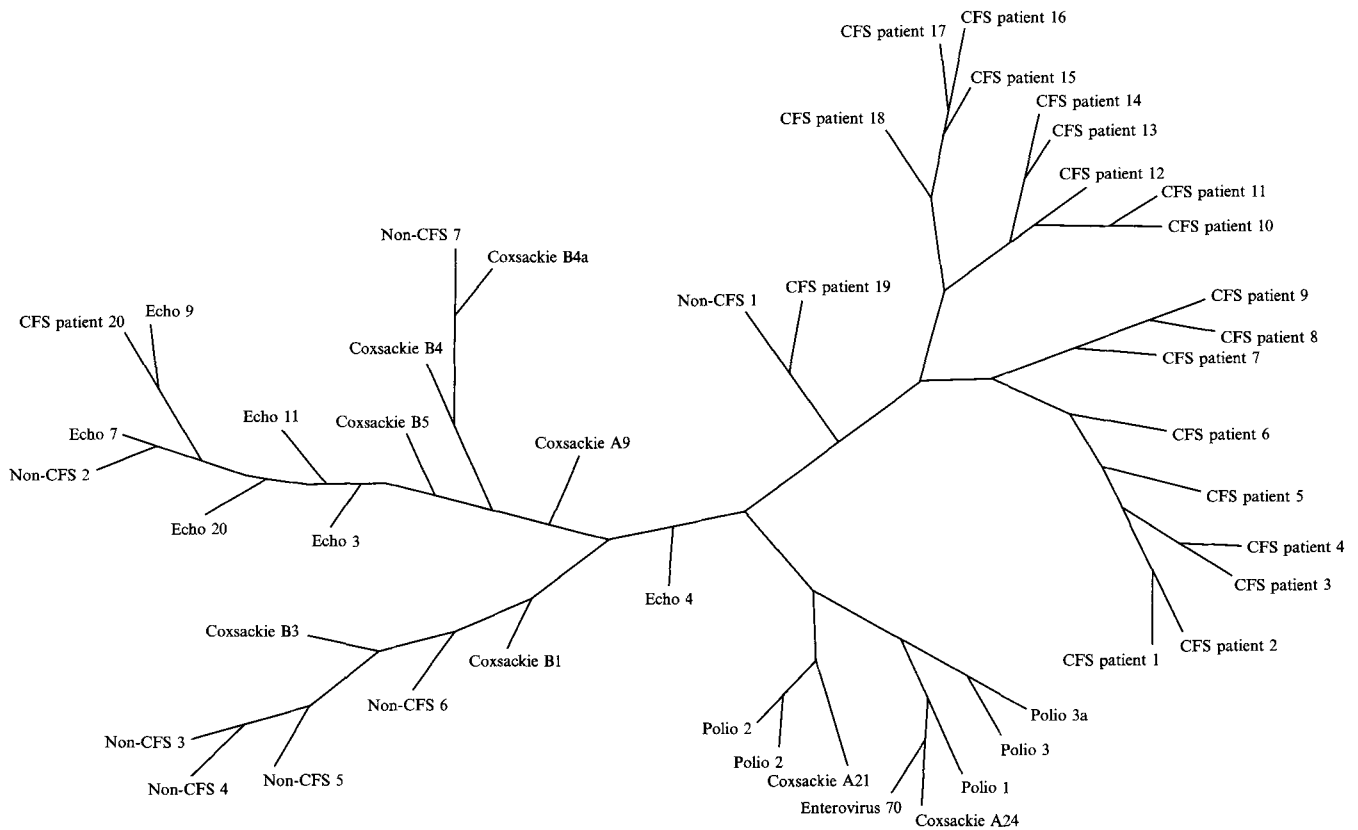


Fig. 2. Phylogenetic tree produced by parsimony analysis (PHYLIP/DNAPARS) based on the nucleotide sequence of a short region of the 5'NTR of enteroviral isolates. References: coxsackievirus A9 (Chang *et al.*, 1989); coxsackievirus A21 (Hughes *et al.*, 1990); coxsackievirus A24 (Supanaranond *et al.*, 1992); coxsackievirus B1 (Iizuka *et al.*, 1987); coxsackievirus B3 (Klump *et al.*, 1990); coxsackievirus B4 (Jenkins *et al.*, 1987); coxsackievirus B5 (Zhang *et al.*, 1993); enterovirus 70 (Ryan *et al.*, 1990); polioviruses 1, 2 and 3 (Toyoda *et al.*, 1984). Echoviruses were isolated, typed and sequenced in the Regional Virus Laboratory, Ruchill Hospital. CFS and non-CFS patient sequences were derived as described in Methods.

coding region of the enterovirus genome as opposed to the serologically important regions.

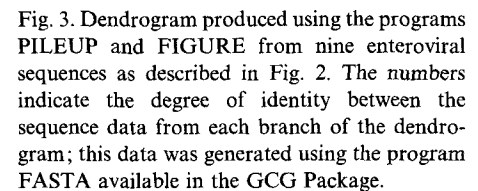
Group II, which included the coxsackie B viruses, echoviruses and coxsackievirus A9 also grouped as expected, since coxsackievirus A9 has been described as being closely related to the coxsackie B virus group as have some echoviruses (Chang *et al.*, 1989). In addition this group contained 6/7 enteroviral sequences obtained from individuals without CFS and one from an individual with CFS. This may indicate that these patients were infected with coxsackie/echo-like viruses.

Group III consisted of the sequences obtained from the CFS patients and comprises a distinct grouping, separate from each of the other groups. In addition to the sequences obtained from CFS patients there was one sequence obtained from a non-CFS patient. This provides evidence for a distinct enteroviral type being associated with some cases of CFS.

Another method of describing the relationships between sequences uses a dendrogram format which gives a numerical measure of relationships. Fig. 3 shows a

dendrogram of nine sequences, three from each of Groups I, II and III. The diagram shows that the sequences from each group map together, as predicted from the phylogenetic tree. The dendrogram program uses the entire nucleotide sequence of the PCR product (265 bp) and therefore gives a more complete indication of the relationships, and also gives support to the phylogenetic tree analysis shown in Fig. 2.

Analysis of the 264 bp fragment amplified in the described PCR assay demonstrated that CFS patient sequences were similar to the known published enteroviral sequences (using the GCG program FASTA, see Table 1), and unrelated to all other available sequences in the GenBank database (sequence homology less than 50%). Comparisons were therefore also made with related viruses such as swine vesicular disease virus (SVDV) (Inoue *et al.*, 1989), Theiler's murine encephalomyelitis virus (TMEV) (Law & Brown, 1990), foot-and-mouth disease virus (FMDV) (Clarke *et al.*, 1987), Mengo virus (Palmenberg & Duke, 1993); echovirus 22 (Hyypia *et al.*, 1992); hepatitis A virus (Najarian, 1985);



other than SVDV (83.2% sequence identity), which is known to be closely related to coxsackievirus B5 (Zhang *et al.*, 1993).

## Discussion

Enteroviruses have been increasingly implicated in the pathology of CFS. The percentage of CFS patients with laboratory findings indicative of enterovirus presence differs from study to study (Clements *et al.*, 1995; Gow *et al.*, 1991, 1994). Serological studies in particular have yielded contradictory evidence for enteroviral association with CFS (Miller *et al.*, 1991; Yousef *et al.*, 1988) and there has never been a direct link made between any particular enterovirus type and CFS. We have recently shown that there is no correlation of the coxsackie neutralization result and the detection of enteroviral sequences by PCR in serum of CFS patients (Nairn *et al.*, 1995).

In this study we used phylogenetic analysis to describe the relationships between the 5'NTR enteroviral

sequences identified in CFS patients and known enteroviruses. Three different strategies were used to deduce phylogenetic trees independently (as suggested by Felsenstein, 1988). From the data presented the sequences derived from all but one of the individuals suffering from CFS were separate and distinct from other known enteroviruses and from enteroviruses typed and subsequently sequenced in this laboratory. There are, however, many enteroviruses with no published sequence data from the 5'NTR. Therefore one possibility from the data presented here would be that the sequences obtained from the CFS patients are from known enteroviruses whose sequence is not yet available. Alternatively, the CFS sequences may indicate the presence of novel enteroviruses, or they could represent sequences from known enteroviruses with abnormal 5'NTRs. The latter situation might lead to the establishment of a persistent infection in some CFS patients. Evidence for persistence is demonstrated in one patient from whom the enteroviral sequences obtained from specimens taken 10 months apart had only a single base change.

The enteroviral sequences from CFS patients form a distinct group phylogenetically; however, as the sequence analysed here is short and without corroboration from other regions (preferably coding regions) the tentative group described here cannot be defined further. A CFS-like sequence was obtained from a non-CFS patient indicating that the distribution of these sequences is not limited to patients with a diagnosis of CFS. When compared with the behaviour of known enteroviruses, which frequently cause asymptomatic infection, this is not unexpected. The predicted secondary structures [using MFOLD and PLOTFOLD programs from the GCG package (data not shown)] for the CFS patient enteroviral sequences conform to the typical stem-loop patterns that have been described for other enteroviruses, suggesting a conserved functional role for this area (Rivera *et al.*, 1988). The predictive value of these computer programs by themselves in determining secondary structure is a matter for current debate and they should be used with caution. It is worth noting that the enteroviral sequences obtained from patients without CFS were dissimilar to the sequences obtained from the CFS patients and close to the published sequences of coxsackieviruses and echoviruses. In addition, Gow *et al.* (1994) have recently published a short enteroviral sequence identified from a muscle biopsy from a CFS patient. The sequence although very short (approx. 80 bp) demonstrated a high degree of homology with the enteroviral sequences obtained from the CFS patients presented here (data not shown) and dissimilar to the known coxsackie B viruses (Gow *et al.*, 1994). This may provide corroborating evidence for the presence of a novel type of enterovirus associated with CFS.

In many CFS patients the course of the disease typically starts with an acute infection which may be the acute stage of an enteroviral infection. The syndrome is then subsequently typified by long-term fatigue with possible viral persistence. The establishment of persistence may be a function of changes in the virus 5'NTR. An altered 5'NTR has been shown to affect the productive cycle since it is essential for a variety of functions including the initiation of translation, being the site of the ribosome landing pad (Jang *et al.*, 1988). The atypical enteroviral 5'NTR sequences identified in CFS patients described here may, as a result, lead to a persistent infection rather than a typical acute infection. However, although there is evidence that mutations in the 5'NTR can affect virus replication some mutations have been shown to have no discernible effect (Pilipenko *et al.*, 1992).

The existence of atypical enteroviral type 5'NTR sequences associated with CFS raises the possibility that some cases of the disease syndrome are caused and perpetuated by a novel enterovirus. Further studies are in progress, in particular the detection of the coding regions for viral structural genes.

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## References

- ARCHARD, L. C., BOWLES, N. E., BEHAN, P. O., BELL, E. J. & DOYLE, D. (1988). Post viral fatigue syndrome: persistence of enterovirus RNA in muscle and elevated creatine kinase. *Journal of the Royal Society of Medicine* **81**, 326–329.
- BAE, Y.-S., EUN, H.-M. & YOON, J.-W. (1989). Genomic differences between diabetogenic and nondiabetogenic encephalomyocarditis virus. *Virology* **170**, 282–287.
- CHAN, S. W., MCOMISH, F., HOLMES, E. C., DOW, B., PEUTHERER, J. F., FOLLETT, E., YAP, P. L. & SIMMONDS, P. (1992). Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. *Journal of General Virology* **73**, 1131–1141.
- CHANG, K. H., AUVINEN, P., HYYPIÄ, T. & STANWAY, G. (1989). The complete nucleotide sequence of coxsackievirus A9; implications for receptor binding and enterovirus classification. *Journal of General Virology* **70**, 3269–3280.
- CLARKE, B. E., BROWN, A. L., CURREY, K. M., NEWTON, S. E., ROWLANDS, D. J. & CARROLL, A. R. (1987). Potential secondary and tertiary structure in the genomic RNA of foot and mouth disease virus. *Nucleic Acids Research* **15**, 7067–7079.
- CLEMENTS, G. B., MCGARRY, F., NAIRN, C. & GALBRAITH, D. N. (1995). The detection of enterovirus specific RNA in serum: the relationship to chronic fatigue. *Journal of Medical Virology* **45**, 151–166.
- CUNNINGHAM, L., BOWLES, N. E. & ARCHARD, L. C. (1991). Persistent virus infection of muscle in post-viral fatigue syndrome. *British Medical Bulletin* **47**, 852–871.

- CUNNINGHAM, L., BOWLES, N. E., LAINE, R. J. M., DUBOWITZ, V. & ARCHARD, L. C. (1990). Persistence of enteroviral RNA in chronic fatigue syndrome is associated with the abnormal production of equal amounts of positive and negative strands of enteroviral RNA. *Journal of General Virology* **71**, 1399–1402.
- DEVEREUX, J., HAEGERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.
- FELSENSTEIN, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics* **22**, 521–565.
- GOW, J. W., BEHAN, W. M. H., CLEMENTS, G. B., WOODALL, C., RIDING, M. & BEHAN, P. O. (1991). Enteroviral RNA sequences detected by polymerase chain reaction in muscles of patients with post viral fatigue syndrome. *British Medical Journal* **302**, 692–696.
- GOW, J. W., BEHAN, W. M. H., SIMPSON, K., MCGARRY, F., KEIR, S. & BEHAN, P. O. (1994). Studies on Enterovirus in patients with chronic fatigue syndrome. *Clinical Infectious Diseases* **18** (suppl. 1), S126–129.
- GRIST, N. R., BELL, E. J., FOLLET, E. A. C. & URQUHART, J. E. D. (1979). *Diagnostic Methods in Clinical Virology*, 3rd edn. Oxford: Blackwell.
- HERMANS, A., GOW, J. & SELLERI, L. (1988). bcr-abl oncogene activation in Philadelphia chromosome positive acute lymphoblastic leukemia. *Leukemia* **2**, 628–633.
- HUGHES, P. J., NORTH, C., MINOR, P. D. & STANWAY, G. (1989). The complete nucleotide sequence of coxsackievirus A21. *Journal of General Virology* **70**, 2943–2952.
- HYYPÄ, T., HORSNELL, C., MAARONEN, M., KALKKINEN, N., AUVINEN, P., KINNUNEN, L. & STANWAY, G. (1992). A distinct picornavirus group identified by sequence analysis. *Proceedings of the National Academy of Sciences, USA* **89**, 8847–8851.
- IZUKA, N., KUGE, S. & NOMOTO, A. (1987). Complete nucleotide sequence of the genome of coxsackie B1. *Virology* **156**, 64–73.
- INOUE, T., SUZUKI, T. & SEKIGUCHI, K. (1989). The complete nucleotide sequence of swine vesicular disease virus. *Journal of General Virology* **70**, 919–934.
- JANG, S. K., KRAUSSLICH, H. G., NICKLIN, M. J. H., DUKE, G. M., PALMENBERG, A. C. & WIMMER, E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *Journal of Virology* **62**, 2636–2643.
- JENKINS, O., BOOTH, J. D., MINOR, P. D. & ALMOND, J. W. (1987). The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the Picornaviridae. *Journal of General Virology* **68**, 1835–1848.
- KLUMP, W. M., BERGMANN, I., MULLER, B. C., AMEIS, D. & KANDOLF, R. (1990). Complete nucleotide sequence of infectious coxsackie B3 cDNA. *Journal of Virology* **64**, 1573–1583.
- LAW, K. M. & BROWN, T. D. K. (1990). The complete nucleotide sequence of the CDV11 strain of Theilers murine encephalomyelitis virus (TMEV). *Nucleic Acids Research* **18**, 6707–6708.
- LINDBERG, A. M., STALHANDSKE, P. O. K. & PETTERSSON, U. (1987). Genome of coxsackie B3. *Virology* **156**, 50–63.
- MELNICK, J. L. (1990). Enteroviruses: polioviruses, coxsackie viruses, echo viruses and newer enteroviruses. In *Virology*, pp. 549–605. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press.
- MILLER, N. A., CARMICHAEL, H. A., CALDER, B. D., BEHAN, P. O., BELL, E. J., MCCARTNEY, R. A. & HALL, F. C. (1991). Antibody to coxsackie B virus in diagnosing post viral fatigue syndrome. *British Medical Journal* **302**, 140–143.
- MINOR, P. D. & BELL, E. J. (1990). Picornaviridae. In *Principals of Bacteriology, Virology and Immunity*, 8th edn, pp. 323–356. Edited by L. H. Collier & M. C. Timbury. London: Edward Arnold.
- NAIRN, C., GALBRAITH, D. N. & CLEMENTS, G. B. (1995). A comparison of coxsackie B neutralisation and enteroviral PCR in chronic fatigue patients. *Journal of Medical Virology* (in press).
- NAJARIAN, R. (1985). Primary structure and gene organization of human hepatitis A virus. *Proceedings of the National Academy of Sciences, USA* **82**, 2627–2631.
- PALMENBERG, A. C. & DUKE, G. M. (1993). GenBank accession no. L22089.
- PILIPENKO, E. V., GMYL, A. P., MASLOVA, S. V., SVITKIN, Y. V., SINYAKOV, A. N. & AGOL, V. I. (1992). Prokaryotic like cis elements in the cap independent internal initiation of translation on Picornavirus RNA. *Cell* **68**, 119–131.
- PREEDY, V. R., SMITH, D. G., SALISBURY, J. R. & PETERS, T. J. (1993). Biochemical and muscle studies in patients with acute onset post-viral fatigue syndrome. *Journal of Clinical Pathology* **46**, 722–726.
- RIVERA, V. M., WELSH, J. D. & MAIZEL, J. V. (1988). Comparative sequence analysis of the 5' noncoding region of the enteroviruses and rhinoviruses. *Virology* **165**, 42–50.
- RYAN, M. D., JENKINS, O., HUGHES, P. J., BROWN, A., KNOWLES, N. J., BOOTH, D., MINOR, P. D. & ALMOND, J. W. (1990). The complete nucleotide sequence of enterovirus type 70: relationships with other members of the Picornaviridae. *Journal of General Virology* **71**, 2291–2299.
- SHARPE, M. C., ARCHARD, L. C., BANATVALA, J. E., BORYSIWICZ, L. K., CLARE, A. W., DAVID, A., EDWARDS, R. H. T., HAWTON, K. E. H., LAMBERT, H. P., LANE, R. J. M., McDONALD, E. M., MOWBRAY, J. F., PEARSON, D. J., PETO, T. E. A., PREEDY, V. R., SMITH, A. P., SMITH, D. G., TAYLOR, D. J., TYRRELL, D. A. J., WESSELY, S., WHITE, P. D., BEHAN, P. O., ROSE, F. C., PETERS, T. J., WALLACE, P. G., WARRELL, D. A. & WRIGHT, D. J. M. (1991). A report on chronic fatigue syndrome: guidelines for research. *Journal of the Royal Society of Medicine* **84**, 118–121.
- STANWAY, G. (1984). The complete nucleotide sequence of a common cold virus: human rhinovirus 14. *Nucleic Acids Research* **12**, 7859–7875.
- SUPANARANOND, K., TAKEDA, N. & YAMAZAKI, S. (1992). The complete nucleotide sequence of a variant of coxsackie virus A24, an agent causing acute haemorrhagic conjunctivitis. *Virus Genes* **6**, 149–158.
- TOYODA, H., KOHURA, M., KATAOKU, Y., SUGANUMA, T., OMATA, T., IMURA, N. & NOMOTO, A. (1984). Complete nucleotide sequences of all three poliovirus serotype genomes. *Journal of Molecular Biology* **174**, 561–585.
- YOUSEF, G. E., BELL, E. J. & MANN, G. F. (1988). Chronic enterovirus infection in patients with post viral fatigue syndrome. *Lancet* **i**, 146–149.
- ZHANG, G., WILSDEN, G., KNOWLES, N. J. & MCCAULEY, J. W. (1993). Complete nucleotide sequence of a coxsackie B5 virus and its relationship to swine vesicular disease virus. *Journal of General Virology* **74**, 845–853.
- ZOLL, G. J., MELCHERS, W. J. G., KOPECKA, G., JAMBROES, G., VAN DER POEL, H. J. A. & GALAMA, J. M. D. (1992). General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. *Journal of Clinical Microbiology* **30**, 160–165.

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