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

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A subgenomic restriction fragment from cDNA prepared from Coxsackie B2 virus (CVB2) RNA was subcloned into a riboprobe vector allowing the production of enteroviral group-specific RNA probes complementary to either the positive (genomic) or negative (template) strand of enteroviral RNA. These riboprobes were used to follow productive infection of cultured cells by CVB2; as expected, positive strand RNA was synthesized in approximately 100-fold excess over negative strand. RNA was extracted from muscle biopsy samples from patients with chronic fatigue syndrome and probed for the presence of enteroviral RNA. In cases where enteroviral RNA was detected the amounts of positive and negative strands of enteroviral RNA were approximately equal, in contrast to the situation in lytic infection of cultured cells. This suggests that enterovirus persistence in muscle is due to a defect in control of viral RNA synthesis.

Enteroviruses, especially the Coxsackie B viruses, have been associated with a range of acute and chronic diseases of heart or skeletal muscle (Melnick, 1982). This association has arisen principally from retrospective serology (Christensen et al., 1986; Travers et al., 1977) as rarely can infectious virus be isolated or virus-specific antigens be detected by immunofluorescence in the affected tissue (Morgan-Capner et al., 1984). However, DNA hybridization studies have established that persisting enteroviral RNA can be detected in the affected tissue in a proportion of cases of patients with certain chronic muscle diseases (Bowles et al., 1986, 1987; Archard et al., 1988).

In some patients where enteroviral RNA can be demonstrated in the affected tissue, a continuing humoral immune response against normal viral antigens cannot be detected (Archard et al., 1988; Bowles et al., 1989). This, together with the documented failure to isolate infectious virus or detect virus-specific antigens, suggests that the progression to chronic disease is associated with the selection of defective virus. We have used single-stranded virus-specific RNA hybridization probes to compare the relative abundance of virus RNA species in persistent infections with those in cytopathogenic infection in vitro.

A 1 kb subgenomic cDNA from Coxsackie B2 virus (CVB2) RNA including sequences encoding the viral RNA-dependent RNA polymerase was subcloned into a riboprobe vector (pGEM 3Z) in either orientation, downstream of the SP6 promoter. In this way, probes complementary to either virus genomic (positive strand) RNA or template (negative strand) RNA could be synthesized by in vitro transcription by SP6 RNA polymerase. The nucleotide sequence of the RNA-dependent RNA polymerase is highly conserved between enterovirus serotypes (Stalhandske et al., 1984) and so probes derived from this region are enterovirus group-specific (Bowles et al., 1989). ³²P-labelled RNA probes were synthesized by transcription of linearized template DNA according to standard protocols (Promega Biotec). Both constructs yielded similar quantities of single-stranded RNA in control reactions, as determined by agarose gel electrophoresis and ethidium bromide staining (data not shown). The level of incorporation of [³²P]CTP was similar in transcription from either construct.

An analogous riboprobe, 7B6, was used to quantify the
total amount of RNA extracted from muscle biopsy samples. This control probe, which represents a cellular mRNA whose function in unknown, has been shown to be cell cycle-independent (Kaczmarek et al., 1985). Doubling dilutions of RNA extracted from normal muscle were blotted and probed with 7B6-specific riboprobe to establish that the autoradiographic signal obtained is proportional to the amount of RNA blotted. Autoradiographic development was quantified by scanning densitometry and the integrals under peaks were plotted as a function of RNA concentration; a linear relationship was obtained (Bowles et al., 1989).

Subconfluent monolayers of LLCMK2 cells (rhesus monkey kidney) were infected with CVB2 at 10^5 or 10^4 p.f.u./ml or mock-infected. Virus was adsorbed at 37 °C for 1 h and non-adsorbed virus was reduced by washing. Cytoplasmic RNA was isolated at various times post-infection by NP40 lysis (Berger, 1987), phenol and chloroform extraction and ethanol precipitation. RNA was redissolved in water, denatured with formaldehyde (Bowles et al., 1986) and blotted onto nitrocellulose filters (Schleicher & Schuell) using a slot blot apparatus (Minifold I; Schleicher & Schuell). The filters were prehybridized at 55 °C in 50% deionized formamide, 2 × SSC, 0-5% SDS, 2-5 × Denhardt's solution, 100 μg/ml each of sonicated salmon sperm DNA and Escherichia coli tRNA, for 5 h. The 32P-labelled probe was added to the prehybridization solution and allowed to hybridize at 55 °C overnight. The filters were washed in 1 × SSC/0-1% SDS followed by 0-5 × SSC/0-1% SDS and 0-1 × SSC/0-1% SDS at 70 °C for 30 min each wash. The filters were autoradiographed using presensitized Hyperfilm MP (Amersham) for various periods.

The 7B6 probe, which measures total RNA, shows that the amount of RNA prepared and immobilized from each sample was approximately equal (Fig. 1 a). Hybridization with the probe complementary to the genomic virus RNA strand gave an autoradiographic signal by 6 h post-infection, and this subsequently increased with time. Cells infected with the 10-fold lower dilution of virus gave a concomitantly lower signal (Fig. 1 b). The probe complementary to virus template RNA gave higher non-specific signals in hybridizations to RNA from all samples. However, after densitometric scanning and allowance for this background, a significant signal was detected by 8 h post-infection (Fig. 1 c). Comparison of the signals obtained with either probe showed that the positive sense virus genomic RNA was present in about 100-fold excess over the negative, template strand. This is compatible with previously published data (Rotbart et al., 1988).

Skeletal muscle samples were obtained by needle biopsy from patients diagnosed clinically as having chronic fatigue syndrome (CFS). Patients complained of excessive muscle fatigueability following an assumed or proven viral infection. Most patients fulfilled the criteria of the Centers for Disease Control for diagnosis of CFS (Holmes et al., 1988) and symptoms had been present for at least 6 months. RNA was extracted from muscle biopsy samples by digestion with proteinase K/SDS in the presence of human placental ribonuclease inhibitor and deproteinization as described previously (Bowles et al., 1986). After selective denaturation and slot blotting in triplicate, RNA was hybridized with the viral plus or minus strand-specific probes or the control probe, labelled by the incorporation of [32P]CTP, and the blots were autoradiographed. In this experiment, RNA from multiple samples of muscle from eight patients was
blotted: samples from four patients were positive for enteroviral RNA by comparison with the 7B6 control probe (Fig. 2). This is a higher proportion than seen previously (for example Archard et al., 1988) and is probably due to the small numbers in this particular study. Multiple, post mortem samples of muscle from four individuals included on the same blots were negative. In our cumulative experience, 152 control samples of human muscle (108 normal biopsy samples, taken to exclude various conditions; 28 pathological biopsy samples including 24 inclusion body myositis; two samples obtained from orthopaedic surgery and samples from 14 post mortem procedures) have all proved negative for enteroviral RNA.

In the experiments reported here, all samples positive for enterovirus-specific RNA had approximately equal amounts of genomic and template RNA (Fig. 2). This contrasts with asymmetric virus RNA synthesis in productive infection (Fig. 1), suggesting that the molecular basis of persistent enterovirus infection in chronic, non-inflammatory muscle disease is the generation of mutant virus defective in the control of viral RNA synthesis.

These data are the first demonstration of persistence of defective virus in clinical samples from patients with CFS. Enteroviral RNA replication is mediated by the virus encoded RNA-dependent RNA polymerase via a replication intermediate comprising a positive sense genomic strand and a negative template strand. Primer-dependent synthesis of the negative strand is initiated at the 3' end of the positive strand RNA. This newly synthesized strand is thought to encode a polymerase recognition sequence at its 3' end with a higher affinity than the sequence at the 3' end of the positive strand, resulting in asymmetric synthesis of an excess of positive strand, genomic RNA over negative, template strand (Perez-Bercoff, 1978). In contrast to the in vitro situation, enterovirus variants associated with persistent non-inflammatory infection of muscle no longer exhibit this asymmetry and synthesize approximately equimolar amounts of positive and negative strands of enteroviral RNA.

There are several possible explanations for these observations. An alteration in the specificity of the polymerase for the transcription initiation sites due to a mutation in either the polymerase or its recognition sequences would result in a loss of control of viral RNA replication. Whatever the molecular mechanism, equimolar synthesis of the two complementary strands of virus RNA is likely to result in inhibition of translation of virus-specific gene products, explaining the failure to assemble infectious progeny virus or to attract an inflammatory response. Synthesis of negative strand RNA has been proposed as a mechanism of latency in herpesvirus infections (Croen et al., 1987).

We are currently investigating the effects of persistence of enteroviral RNA on cellular gene expression leading to muscle dysfunction.
References


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