Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors

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Chronic fatigue syndrome (CFS) is a serious systemic illness of unknown cause. A recent study identified DNA from a xenotropic murine leukemia virus-related virus (XMRV) in peripheral blood mononuclear cells (PBMCs) from 68 of 101 patients (67%) by nested PCR, as compared with 8 of 218 (3.7%) healthy controls. However, four subsequent reports failed to detect any murine leukemia virus (MLV)-related virus gene sequences in blood of CFS patients. We examined 41 PBMC-derived DNA samples from 37 patients meeting accepted diagnostic criteria for CFS and found MLV-like virus *gag* gene sequences in 32 of 37 (86.5%) compared with only 3 of 44 (6.8%) healthy volunteer blood donors. No evidence of mouse DNA contamination was detected in the PCR assay system or the clinical samples. Seven of 8 *gag*-positive patients tested again positive in a sample obtained nearly 15 y later. In contrast to the reported findings of near-genetic identity of all XMRVs, we identified a genetically diverse group of MLV-related viruses. The *gag* and *env* sequences from CFS patients were more closely related to those of polytropic mouse endogenous retroviruses than to those of XMRVs and were even less closely related to those of ecotropic MLVs. Further studies are needed to determine whether the same strong association with MLV-related viruses is found in other groups of patients with CFS, whether these viruses play a causative role in the development of CFS, and whether they represent a threat to the blood supply.

xenotropic murine leukemia virus-related virus | murine leukemia virus-like virus | viral gag gene sequence | polytropic | mouse mitochondria DNA PCR

Chronic fatigue syndrome (CFS) is a debilitating disorder defined solely by clinical symptoms (1) and the exclusion of other diseases; its distribution is wide and its cause is unknown. In many instances, the illness starts suddenly with an infectious-like syndrome. A number of objective immunological and neurological abnormalities have been found more often in patients with CFS than in healthy controls or in patients with other fatigue-inducing illnesses (2). Various microbial and viral infections have been implicated as possible triggers of CFS, including human herpesvirus-6, Epstein–Barr virus, enteroviruses, parvovirus B19, and the bacteria that cause Lyme disease and Q fever (2). However, no single agent has been associated with a large fraction of cases.

A recent study reported that a high percentage of patients with CFS are infected with a mouse leukemia retrovirus that has been designated xenotropic murine leukemia virus-related virus (XMRV) (3), a virus first identified in samples of human prostate cancer tissue about 4 y ago (4). However, two subsequent studies failed to find an infectious murine leukemia virus (MLV)-related virus in German prostate cancer patients (5, 6), and four recent studies from Europe and the United States have failed to detect XMRV or an MLV-related viral gene sequence in the blood of CFS patients using PCR (7–10).

In the mid-1990s, we obtained serum and whole-blood samples from CFS patients for the investigation of possible mycoplasmal infections (11). Whole-blood, peripheral blood mononuclear cell (PBMC), and plasma samples from 37 CFS patients in the mycoplasma studies were maintained in frozen storage at −80 °C. Twenty-five patients were from an academic medical center and 12 were referred by community physicians. Repeat blood samples were obtained from the academic medical center patients: four samples were obtained 2 y later and similarly kept in frozen storage, eight were obtained ~15 y later, in 2010, and processed for XMRV/MLV-related virus testing without being frozen.

By nested PCR assays targeting the MLV-related virus *gag* gene, using both the previously described primer sets (3, 4) and an in-house–designed primer set with highly conserved sequences from different MLV-like viruses and XMRVs, we examined DNA prepared from the blood samples of these 37 CFS patients for the presence of MLV-like virus *gag* gene sequences. In addition, RNA was prepared from the deep-frozen plasma samples of these patients and analyzed by RT–PCR assay. DNA extracted from frozen PBMC samples of 44 healthy volunteer blood donors was tested in parallel.

Results

MLV-Related Viral *gag* Gene Sequences Detected in the Blood of CFS Patients. By nested PCR assays, targeting the mouse retrovirus *gag* gene using either the previously reported PCR primer sets (first round: 419F/1154R; second round: GAG-I-F/GAG-I-R) (3, 4) or our in-house–designed PCR primer set (first round: 419F/1154R; second round: NP116/NP117) (Fig. 1), we detected a high frequency of MLV-related virus *gag* gene sequences in patients with CFS. The NP116/NP117 is an internal primer set with highly conserved sequences in different MLV-like viruses and XMRVs (Fig. S1). After the first round of nested PCR using primer set 419F/1154R, gel electrophoresis revealed positive PCR-amplified products with the predicted size of ~730 bp in 21 of 41 PBMC or whole-blood samples from 37 CFS patients (Fig. 1A). The nested PCR results produced by the second round of amplification using either the internal primer set GAG-I-F/GAG-I-R (with a predicted size of an ~410-bp product) or the internal primer set NP116/NP117 (with a predicted size of an ~380-bp product) were essentially identical. Overall, samples from 32 of 37 (86.5%) CFS patients revealed positive amplification products with the correct predicted sizes in the nested PCR (Fig. 1B). Of the 25 CFS patients who had been rigorously evaluated at the academic medical center, 24 (96%) were positive. On repeated testing 2 y
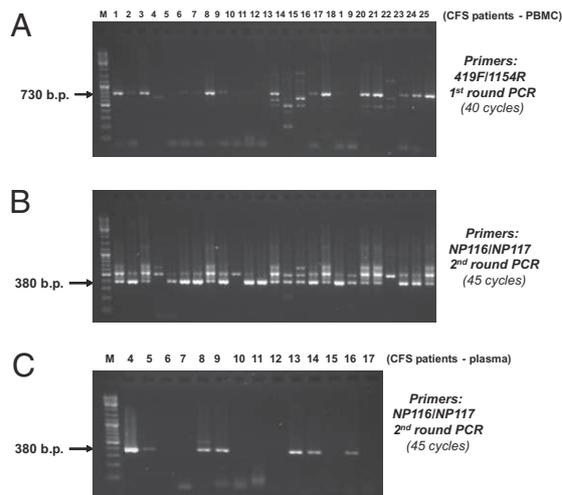
Fig. 1. MLV-related *gag* gene sequences detected in blood DNA from CFS patients. (*A*) Results of PBMC DNA from CFS patient samples 1–25 (of 41 samples examined) amplified after the first round of nested PCR using a previously published primer set (419F/1154R): targeting *gag* gene. (*B*) Results of PBMC DNA from the 25 CFS samples after completing the second round of nested PCR using an in-house–designed PCR primer set (NP116/NP117). (*C*) MLV-related *gag* gene RNA sequences are detected in plasma of CFS samples by RT-nested PCR. Results of RT-nested PCR for RNA derived from the plasma samples of CFS patients 4–17 are shown. The positions of expected sizes of the "positive" PCR amplicons are indicated by arrows. M, DNA ladder size markers. All positive PCR amplicons with the expected size have been confirmed by DNA sequencing.

later of four of the academic center patients, all four remained positive. On repeated testing of eight academic center patients ~15 y later (in 2010), seven remained positive. All PCR products with the correct predicted size were retrieved from the gel and analyzed by DNA sequencing. Their DNA sequences were all confirmed to be those of MLV-related virus *gag* genes. The alignments of all of the sequences obtained from PCR products of ~730 bp are shown in Fig. S1. All of the positive PCR products amplified from the CFS patients' samples using primer set 419F/1154R were 746 bp in length. All of the positive PCR products amplified from the patients' blood samples using primer set GAG-I-F/GAG-I-R and primer set NP116/NP117 were 413 and 380 bp in length, respectively.

In 42% of samples, we also detected and sequence-confirmed the presence of MLV-related viral RNA in the frozen plasma samples of these CFS patients, using an RT–PCR assay (Fig. 1*C*). With one exception, all of the patients who tested positive for viral RNA *gag* gene sequences in the plasma samples also tested positive in the DNA prepared from PBMCs and/or whole blood. On the other hand, only about half of the patients with MLV-related virus *gag* gene sequences detected in PBMC DNA also had viral *gag* RNA sequences detected in the plasma.

MLV-Related Viral *gag* Gene Sequences Detected in the Blood of Healthy Volunteer Blood Donors. DNA originating from 44 healthy volunteer blood donors was tested in parallel by nested PCR (Fig. 2). The nested PCR testing using the MLV-related virus *gag* gene-specific primer sets could produce many side products from human DNA (Figs. 1 and 2). We sequenced all of the PCR-amplified DNA bands from the 44 control samples of blood donors having molecular sizes close to that of the predicted PCR products from the target XMRV *gag* gene in the first round of PCR (Fig. 2*A*) and in the second round of nested PCR (Fig. 2*B*). After the first round of amplification in nested PCR, a positive PCR product with the predicted size of ~730 bp was found in PBMC DNA from 1 of 44 blood donor controls (lane 4, Fig. 2*A*). This ~730-bp PCR product amplified from the blood donor (BD22) was confirmed by DNA
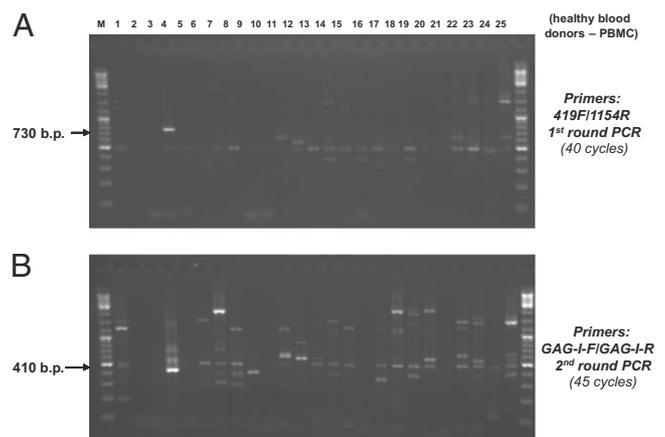

Fig. 2. MLV-related *gag* gene sequences detected in normal blood donors by nested PCR. (*A*) Results of PBMC DNA from blood donors 1–25 (of 44 donors examined) amplified after the first round of nested PCR using primer set 419F/1154R. Lane 4: PBMC DNA from BD22 has a positive target PCR amplicon confirmed by sequencing. (*B*) Results of PBMC DNA from the 25 normal blood donors after the second round of nested PCR using PCR primer set GAG-I-F/GAG-I-R (4). Sequencing of the PCR bands with size ~413 bp revealed that lane 4 (BD22), lane 7 (BD26), and lane 9 (BD28) were MLV-like virus *gag* gene sequences; lane 8 (BD27) was a human sequence. The positions of expected sizes of the positive PCR amplicons are indicated by arrows. M, DNA ladder size markers.

sequencing as an MLV-related virus *gag* gene sequence of 745 bp (Fig. S1). Overall, we found 3 of 44 (6.8%) blood donors' PBMCs (BD22, BD26, and BD28) to be positive for the MLV-related virus *gag* gene sequences by completing both rounds of nested PCR (Fig. 2*B* and Fig. S2).

MLV-Related Viral *env* Gene Sequences Detected in the Blood of a CFS Patient and a Healthy Blood Donor. PBMC DNA from all of the CFS patients and healthy blood donors was also tested by PCR, targeting various regions of the MLV-related viral *env* gene. The MLV-related viral *env* gene segment of 240 bp was amplified and confirmed by sequencing from one healthy donor (BD-26) by a semi-nested PCR using the primer set 5922F/6273R in the first round of amplification and 5922F /6173R in the second round of amplification. The MLV-related viral *env* gene segment of 206 bp was amplified and confirmed by sequencing from 1 CFS patient by a nested PCR using primer set 5922F/6273R in the first round of amplification and 5942F/6159R in the second round of amplification (*SI Materials and Methods*).

Phylogenetic Analyses of MLV-Related Virus *gag* and *env* Gene Sequences. Multiple sequence alignment (MSA) and phylogenetic analysis of the MLV-related virus *gag* gene sequences amplified from 21 CFS patient samples and one blood donor (BD22) are shown in Fig. S1 and Fig. 3*A*, respectively. There were three different MLV-related retroviral *gag* gene sequences identified by PCR in the blood samples of the CFS patients and a fourth variant was detected in blood donor BD22. The sequences in all four variants were more closely related to the sequences of polytropic mouse endogenous retroviruses (mERVs) than to those of XMRVs. Although variations were observed, the majority (18/21, 86%) of CFS patient samples had the same viral *gag* gene sequence (CFS type 1), whereas 2/21 had a different, but similar, viral *gag* gene sequence (CFS type 2), and a third distinct sequence (CFS type 3) was found in the remaining CFS case. Phylogenetic analysis using the 746-nt sequences obtained revealed that CFS type 1, CFS type 2, and CFS type 3 formed a cluster that is clearly separable from the cluster formed by the newly reported XMRVs (Fig. 3*A*). Interestingly, the 745-nt virus *gag* gene sequence of donor BD22 (with a 1-nt deletion in the alignment) could not be included in either the cluster of CFS type 1/CFS type 2 or the cluster of
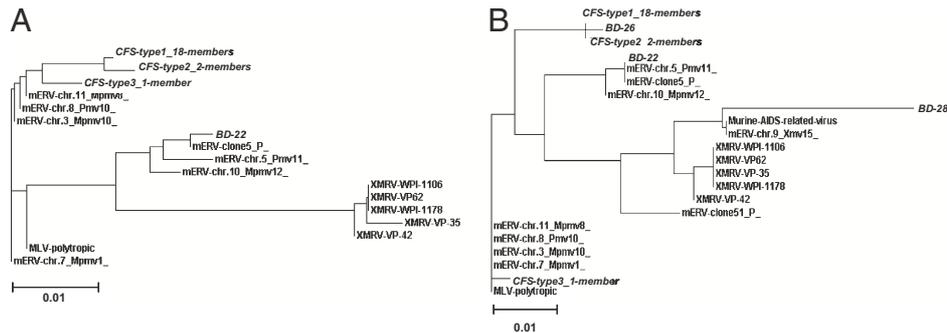
Fig. 3. Phylogenetic trees corresponding to the MSAs shown in Figs. S1 and S2 were generated by the ClustalW2 program using the neighbor-joining method (*Materials and Methods*). (*A*) Phylogenetic analysis based on the 746-nt *gag* gene nucleotide sequences amplified from blood samples of CFS patients and BD-22 of the corresponding MSA in Fig. S1. (*B*) Phylogenetic analysis based on the 380-nt *gag* gene sequences amplified from blood samples of CFS patients and healthy blood donors using the primer set NP116/NP117 of the corresponding MSA in Fig. S2.

XMRVs. The viral *gag* gene sequences of CFS type 3 and blood donor BD22 appear to be phylogenetically more closely related to polytropic mERVs or modified polytropic mERVs (Fig. 3*A*).

Sequence analysis of the shorter fragments of the viral *gag* gene amplified from blood of 36 out of 41 CFS patient samples and 3 out of 44 blood donor samples after the second round of nested PCR similarly confirms that there are significant variations among the MLV-like *gag* gene sequences. Fig. S2 shows sequence alignment of the 380-nt segments of viral *gag* genes amplified from three blood donors (BD22, BD26, and BD28), patients with CFS types 1–3, XMRVs, and other closely related mERVs/polytropic MLVs. As an example, the viral *gag* gene sequence identified in BD28, but not sequences of BD22 and BD26, has a prominent 21-nt deletion that is uniquely present in polytropic mERV clone 51 (Fig. S2). In phylogenetic analysis, the 380-nt segment of the *gag* gene sequence found in BD26 appears to be closely related to those of CFS types 1 and 2. However, similar to the analytic result with the 746-nt product, the 380-nt *gag* gene sequences of BD22 and BD28 again cannot be included in either the cluster of CFS type 1/CFS type 2 or the cluster of XMRVs (Fig. 3*B*). On the other hand, when the protein sequences coded by the *gag* gene sequences identified in the CFS patients and blood donors are compared with those of a wider range of exogenous and endogenous MLVs, they are most similar to those of polytropic MLVs and XMRVs (Fig. S3). A ClustalW Gag protein tree again reveals that MLV-like virus *gag* proteins have much more similarity to those of modified-polytropic and polytropic mERVs or to those of XMRVs, but are very different from those of exogenous ecotropic MLVs (Fig. 4).

The sequence alignment and the phylogenetic analysis of the MLV-related virus *env* gene sequences obtained from both the CFS patient and healthy blood donor revealed that they were also more closely related to those of polytropic or modified polytropic MLVs than to those of XMRVs (Fig. S4).

Testing for the Presence of Mouse DNA in Patient and Blood Donor Samples Positive for MLV-Like *gag* Gene Sequences. Mouse DNA contains endogenously many closely related proviruses of MLVs. Hence, contamination of the blood samples or reagents by mouse DNA could have produced falsely positive PCR results. Although we took great precautions to prevent potential contamination in the laboratory, and although multiple negative controls were always included in each assay, we took additional steps to confirm that no mouse DNA had contaminated the assays or the clinical samples prepared in this study. We estimated that there were about 200–1,800 mitochondrial DNA (mtDNA) copies per mammalian cell. A highly sensitive PCR assay targeting mouse-specific mtDNA was developed (*Materials and Methods*) to exclude any possible minute mouse DNA contamination in the assay system and in the clinical samples with positive amplified *gag* gene products.

The first round of the semi-nested PCR (40 cycles) used primer set mt15982F/mt16267R and could detect 10 fg of mouse DNA in the presence of 35 ng of human background DNA. By comparison, when studied in parallel under the same assay conditions, the first round of nested PCR (40 cycles) against the MLV *gag* gene required ~10 pg of mouse DNA to detect viral gag gene sequences (Fig. 5*A*). Thus, the first round of mouse-specific mtDNA PCR assay could detect a positive mtDNA signal at a mouse DNA concentration 1,000-fold below the concentration required to detect a positive *gag* signal. The second round of mouse mtDNA semi-nested PCR, using primer set mt16115F/mt16267R, could consistently amplify the target 153-bp amplicon from 2.5 fg of mouse DNA mixed with 35 ng of human DNA (Fig. 5*B*). By comparison, the second round of the nested *gag* gene PCR produced positive ~400-bp amplicons from 500 fg of mouse DNA mixed with 35 ng of human background DNA in the reaction. Thus, the second round of mouse mtDNA semi-nested PCR had a sensitivity hundreds-fold higher than that of the second round of the MLV *gag* gene nested PCR in amplifying mouse DNA.

Using this highly sensitive PCR assay for mouse-specific mtDNA, we examined all of the blood samples that were found positive for MLV-like virus *gag* gene sequences from both CFS patients and healthy controls for evidence of mouse DNA contamination. PBMC DNA (30–40 ng) from the CFS patients and the healthy blood donors, as well as serial dilutions from 50 to 1 fg of mouse DNA mixed with 35 ng of human DNA as the
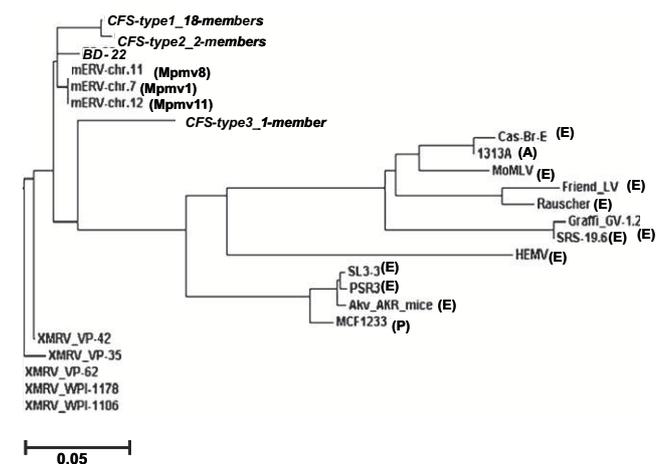

Fig. 4. Phylogenetic analysis of protein sequences based on the alignment shown in Fig. S3. CFS types 1, 2, and 3 and BD-22 and MLVs *gag* protein sequences are compared. Gag protein sequences starting from the AUG initiation codon are aligned with those of relevant endogenous as well as exogenous MLVs. Sequences of MLVs are referred to as polytropic (P), ecotropic (E), amphotropic (A), or modified polytropic (Mpmv). MoMLV, Moloney murine leukemia virus; HEMV, hortulanus endogenous murine virus.
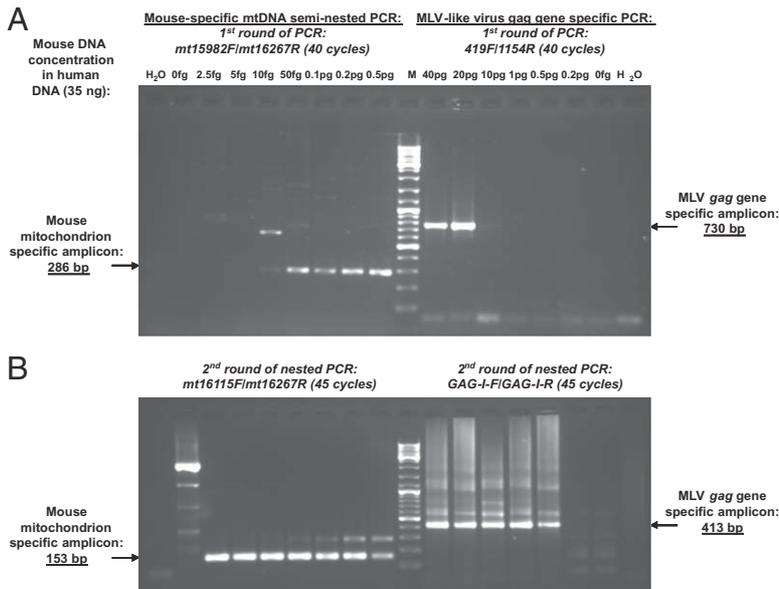
Fig. 5. Comparison of sensitivity in amplifying mouse DNA by the semi-nested PCR targeting mouse-specific mtDNA and by the nested PCR targeting MLV-like virus *gag* gene. Serial dilutions of mouse spleen DNA (from 40 pg to 2.5 fg) were spiked into 35 ng of total human PBMC DNA and compared in parallel for the mouse DNA detection sensitivity of the two PCR assays. (*A*) In the first round of the mtDNA-specific PCR assay, 10 fg or more of mouse DNA could be detected in the presence of 35 ng of human DNA by producing the 286-bp target product. In the first round of MLV *gag* gene nested PCR assay, 10 pg or more of mouse DNA could be detected in the presence of 35 ng of human DNA by producing the ~730-bp target product. (*B*) In the second round of mouse-specific mtDNA semi-nested PCR, the 153-bp target amplicon could consistently be amplified from 2.5 fg of mouse DNA. In the second round of *gag* gene-specific nested PCR, the 413-bp target product could be amplified from 0.5 pg or more of mouse DNA. Lane 0 fg: 35 ng of human DNA without spiking any mouse DNA. Lane H₂O: No DNA template. M: 100-bp DNA ladder mix. Primers and PCR cycle numbers used in each round of amplification for both of the assays are shown at the top of each gel.

positive templates, were tested in parallel. No mouse DNA was found in the PCR mix nor in the blood samples of CFS patients and blood donors that tested positive for the MLV-like virus *gag* gene sequences. Fig. 6 shows the results of the two rounds of mouse-specific mtDNA semi-nested PCR testing in DNA from PBMCs of four CFS patients (patients 8, 17, 20, and 25) with positive 746-bp amplicons in the first round of the nested PCR targeting the MLV-like virus *gag* gene, as well as from three blood donors (BD22, BD26, and BD28) who tested positive and two donors (BD21 and BD23) who tested negative for MLV-like *gag* gene sequences.

Discussion

Detection of MLV-Related Nucleic Acid Sequences. Our laboratory detected MLV-related virus *gag* gene sequences in DNA from PBMC and whole-blood samples from 32 of 37 (86.5%) CFS patients, compared with 3 of 44 (6.8%) volunteer blood donors, using a two-round nested PCR. Following only one round of PCR amplification, 21 of the 41 CFS patients' DNA samples were found positive compared with only 1 of 44 donor samples. In every instance throughout these studies, the "positive" result by PCR (an amplicon of the predicted size) was confirmed by sequencing.

In four CFS patients from whom two samples were obtained, 2 y apart, the *gag* gene sequences were detected on both occasions. Further, *gag* gene sequences were still detectable in seven of eight CFS patients from whom fresh samples were obtained ~15 y after they were initially found to be MLV *gag* gene positive. In one *gag*-positive CFS patient and one *gag*-positive blood donor, MLV-related *env* gene sequences also were detected by PCR. However, we were unable to PCR amplify and determine the MLV-related *env* gene sequences in the majority CFS patients, possibly because of the low copy number and the greater genetic variability in the *env* gene compared with the *gag* gene.

In the CFS patients, plasma samples revealed MLV-related virus *gag* gene sequences in 42% when tested by RT–PCR for viral RNA. Whereas all but one patient whose plasma tested positive for viral RNA also tested positive in PBMCs for viral DNA, only half of the cases in which MLV-related virus *gag* gene sequences were detected in PBMCs had detectable RNA sequences in plasma. Thus, accurate determination of the prevalence of these agents in patients and donors requires cellular DNA for analysis.

Sequence Variability. Previous reports of XMRV isolates from patients with CFS and with prostate cancer and from individuals in different geographic locations have described very similar nucleic acid sequences (3, 4, 12), a feature believed to be a unique

characteristic of XMRVs (13). However, our analysis revealed three different types of MLV-related virus *gag* gene sequences in CFS patients. In all three groups, the sequences were more closely related to the sequences of polytropic mERVs than to XMRVs and were more distant from the sequences of ecotropic MLVs (Fig. 3). Moreover, viral *gag* gene sequences with significant variations from both the cluster of CFS type 1/CFS type 2 and the cluster of XMRVs were identified in at least two blood donors (BD22 and BD28); phylogenetic analysis revealed the latter sequences to be more closely related to those of polytropic or xenotropic mERVs (Fig. 3*B*). It is unclear whether the sequence variations of the viral genes identified in the CFS patients and healthy blood donors have any significance in viral pathogenesis or disease development.

The MLV-like virus *gag* gene sequences in the CFS patients and blood donors had a deletion of 9 nt in the 5′ *gag* leader region and did not have the 24-nt deletion in this region reported in XMRVs. Internal deletions of 9 nt similar to what we have identified in the CFS patients and the blood donors are known to be present in the region that encodes the glycosylated Gag protein (GlycoGag) in some infectious endogenous (ecotropic) MLVs and exogenous (xenotropic) MLVs, such as AKV and DG-75 (14, 15). Many previous studies have shown that the nonstructural GlycoGag of MLVs plays a critical role in viral pathogenesis and in vivo infectivity (16–19). In this context, the MLV-like virus *gag* gene sequences identified in most of our CFS patients (both CFS type 1 and CFS type 2) appear to have an intact GlycoGag in-frame with the matrix and are consistent with the gene sequences of infectious MLVs. Unfortunately, the sequences presently obtained in the study are still a bit short and lack the alternative start codon CUG. Slight extension of the 5′ leader sequence will be needed to confirm the intact nature of GlycoGag.

Could Our PCR Results Have Been Falsely Positive? Voisset and coauthors (20) recently reviewed the pitfalls encountered in the identification of new retroviruses ("rumor viruses"). False-positive results can occur for a variety of reasons. Viral gene sequence-specific PCR primers can nonspecifically amplify nucleic acid sequences that differ from the target sequence. For this reason, we sequenced every positive PCR product (every amplicon of the predicted size) and confirmed MLV-related gene sequences in every instance.

Although contamination must always be a concern for any PCR-based study, several pieces of evidence argue against the possibility that the PCR products identified in our study are the
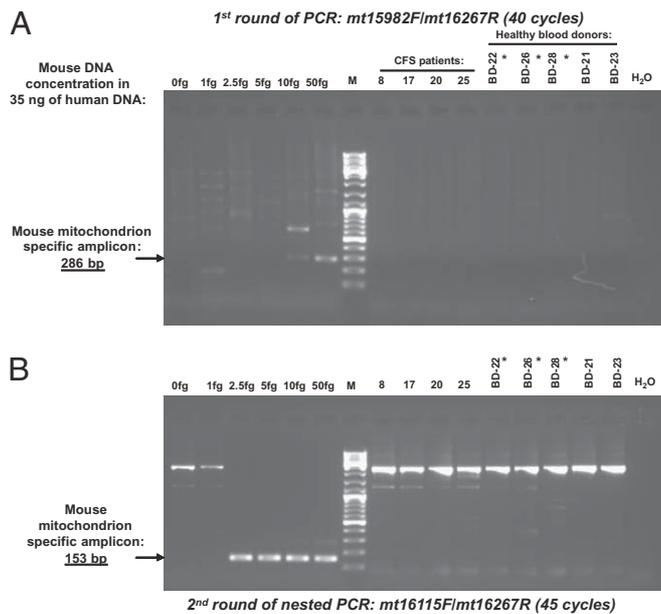
Fig. 6. Testing of CFS patients' and healthy blood donors' samples positive for MLV-like *gag* gene sequences for the presence of mouse DNA contamination using the semi-nested PCR assay targeting mouse-specific mtDNA. Serial dilutions of mouse DNA were spiked into 35 ng of human DNA and used as the controls of the assay sensitivity. The first round of mouse mtDNA semi-nested PCR (*A*) detected 10 fg of mouse DNA, and the second round of the semi-nested PCR (*B*) detected 2.5 fg of mouse DNA in the presence of 35 ng human background DNA. No evidence of mouse DNA contamination could be found by either round of mouse mtDNA semi-nested PCR in the PBMC DNA (35 ng) of CFS patients (patients 8, 17, 20, and 25); three blood donors (BD-22, BD-26, BD-28) tested positive and two blood donors (BD-21 and BD-23) tested negative for the MLV-like virus *gag* gene sequences. Healthy blood donors' samples positive for MLV-like *gag* gene sequences are labeled by asterisks. Lane 0: 35 ng of human DNA without spiking any mouse DNA. Lane H₂O: No DNA template. M: 100-bp DNA ladder mix.

result of laboratory contamination. First, every clinical sample that tested positive for the MLV-like virus *gag* gene sequences was tested for evidence of mouse DNA contamination using a semi-nested PCR for mouse-specific mtDNA that was exponentially more sensitive in detecting mouse mtDNA than MLV-related *gag* sequences (Fig. 5). Any detection of MLV *gag* that was caused by contamination with mouse DNA also would have detected mouse mtDNA by PCR, thereby identifying the *gag* result as falsely positive. In fact, no positive signal was detected by the mtDNA semi-nested PCR assay in any of the reaction mixtures or in the DNA of clinical samples examined in the study, thus excluding possible contamination by mouse DNA.

Second, we addressed the possibility that the clinical samples or the assay system might have been contaminated. The blood samples were obtained in clinical laboratories that never worked with mice or retroviral vectors and were drawn through sterile needles into vacuum tubes that remained unopened until testing. The laboratory in which PCR testing was performed also had never worked with murine cells, tissues or serum samples, or MLV vectors. Finally, because repeated entry into samples would increase the chance of contamination, we emphasize that sample vials from both patients and normal donors had never been entered before our testing.

Third, there were at least six different MLV-related *gag* gene sequences amplified from the blood samples of CFS patients and blood donors. Typically, contamination would be manifest as the same sequence in all or most samples. Moreover, the sequences that we observed all had significant variations from the previously reported exogenous MLVs or viral vectors. Furthermore, during this study more than 300 negative controls set up for

multiple PCR amplification assays targeting the MLV *gag* gene were performed, and all were negative.

Finally, a new set of blood samples was recently obtained from 8 of the original 25 patients followed in an academic medical center. Testing of the repeat blood samples, ~15 y after the index sample, showed that seven of eight patients examined had detectable MLV-*gag* gene sequences. Significant variations of MLV-like virus *gag* gene sequences amplified from the freshly obtained blood samples were identified as would be expected in retroviral infections, but not from contamination.

The ultimate proof of low-grade infection by MLV-related viruses in humans may rely on demonstrating the integration of the viral genes into the human genome (20). The identification of provirus integration sites will take more time and effort to investigate, given that we estimate only one virus gene copy in every 400–4,000 nucleated PBMCs. Also, previous work with XMRV indicates that integration sites are quite variable (21) and the same may be true for the polytropic mouse endogenous retroviruses, which are predominant in this study.

Why Have Other Studies Come to Different Conclusions? Although we find evidence of a broader group of MLV-related viruses, rather than just XMRV, in patients with CFS and healthy blood donors, our results clearly support the central argument by Lombardi et al. (3) that MLV-related viruses are associated with CFS and are present in some blood donors. However, four recent studies have failed to confirm the PCR results reported by Lombardi (7–10). There are various possible explanations for this disparity. As stated in the reports, there could be a difference in the prevalence of these infectious agents in CFS patient populations in different geographic areas. This argument is somewhat less plausible since the publication of a recent negative study with subjects from the United States (10). Nevertheless, the heterogeneity in *gag* gene sequences that we observed suggests that geographic differences in different MLV-related viruses may be considerable and could affect both the sensitivity and the specificity of molecular amplification using standard primer sets.

Indeed, it is possible that the PCR primers used in various studies may have different sensitivity in detecting the diverse group of MLV-related virus *gag* gene sequences that we found in the clinical samples. The 5′ *gag* leader sequence of previously described XMRVs represents the most divergent segment of the XMRV genome in comparison with the genomes of the other MLVs (4). In particular, there is evidently a unique 15-nt deletion in the 5′ *gag* leader region in all of the XMRVs previously identified in patients with prostate cancer and CFS (3, 4). To detect XMRVs in human samples with better sensitivity and specificity, some studies used a PCR primer spanning this unique deletion as the "XMRV-specific" primer (6). However, none of the viral *gag* gene sequences amplified from the blood samples of CFS patients and blood donors in our study has this particular deletion (Fig. S1). As a consequence, such primers might have been insensitive in detecting the MLV-related *gag* gene sequences that we have identified.

Finally, it is also quite possible that there is heterogeneity in the patients diagnosed with CFS in different studies. CFS is a syndrome defined exclusively by a group of nonspecific symptoms and thus has an ill-defined phenotype. Future studies should adhere to consensus case definitions such as that developed by the Centers for Disease Control and Prevention (CDC) (1). Conversely, putative "healthy" control subjects should explicitly deny the presence of those symptoms that constitute the case definition of CFS. Furthermore, even bona fide cases of CFS may have different viral or other etiologies.

Further Considerations. The finding of XMRV or MLV sequences in persons with CFS or other diseases does not constitute definitive proof of viral infection. However, in the study of Lombardi et al. (3) and studies reviewed subsequently by Silverman et al. (22) the evidence for XMRV infection in humans not only involved detection of viral nucleic acids using PCR, but also reported the detection of

viral antigens, detection of anti-viral antibodies, the ability to culture the virus in a prostate cancer cell line, the detection of gamma retrovirus particles by electron microscopy, and transmission of infection to macaques. In sum, none of the four studies that have failed to confirm the PCR evidence reported by Lombardi et al. (3), nor our own study, has attempted to fully replicate that study.

It remains to be shown that the association that we have found, using the methods that we have described, can be generalized to a larger group of patients with CFS. Indeed, we suspect that the association will be lower in CFS cases identified through community-based surveys, as contrasted to cases seen at academic medical centers. Even if subsequent studies confirm an association between MLV-like viruses and CFS, that will not establish a causal role for these viruses in the pathogenesis of this illness. For example, such a high frequency of infections with MLV-related viruses in patients with CFS could reflect an increased susceptibility to viral infections due to an underlying CFS-related immune dysfunction, rather than a primary role for these viruses in the pathogenesis of CFS.

Finally, the finding of MLV-related virus *gag* gene sequences in nearly 7% of healthy volunteer blood donors in our study and of XMRV in 3.7% of healthy controls in the study of Lombardi et al. (3) raises additional issues. The possibility that these agents might be blood-transmitted and pathogenic in blood recipients warrants extensive research investigations of appropriately linked donor–recipient cohorts.

Materials and Methods

Samples from CFS Patients and Healthy Controls. Initially, we tested 41 whole-blood samples that had been obtained for culture isolation of mycoplasmal agents in the mid-1990s. We maintained whole-blood, PBMC, and plasma samples from CFS patients in frozen storage at −80 °C. Of the 41 patient samples, 29 were collected from 25 patients by one of us (A.L.K.) at the Chronic Fatigue Research Center, Brigham and Women's Hospital (Boston, MA). Four of the CFS patients also had blood obtained on a second occasion ∼2 y later. Most of the patients were from the New England area; none were related, and virtually none had any regular social contact. Each of the 25 patients was systematically evaluated with a standardized history (supplemented by a patient questionnaire), physical examination, and battery of laboratory tests. Each met the 1988 CDC criteria for CFS, and 21 also met the 1994 CDC criteria. The average age of the patients at the time of venepuncture was 44.4 y; 4 were male and 21 were female. All of the patients signed informed consent documents approved by the Institutional Review Board of Brigham and Women's Hospital. A new set of blood samples was obtained in 2010 from 8 of the original 25 patients followed in the academic medical center. The blood samples were processed for PCR study without first being frozen. The other 12 samples from CFS patients were sent by individual clinicians taking care of patients in the mid-1990s who were given

the diagnosis of CFS. We do not have details regarding the methodology by which the referring clinicians established the diagnosis of CFS. The samples had also been sent in the mid-1990s and stored at −80 °C. Frozen PBMC samples from 44 normal blood donors from the Washington, DC, area were collected in 2003–2006 and stored at the Department of Transfusion Medicine, Clinical Center, National Institutes of Health. All patient and control samples were coded and tested in parallel. Details of the preparation of blood samples and DNA/RNA isolation are described in *SI Materials and Methods*.

XMRV/MLV *gag* Nested PCR. The nested PCR for the *gag* gene was performed according to the protocols described previously (3, 4) with minor modifications. Three primer sets used in the study are as follows: 419F and 1154R (3), GAG-I-F and GAG-I-R (4), NP116 forward, and NP117 reverse. The NP116/NP117 was an in-house–designed primer set based on the highly conserved sequences found in different MLV-like viruses and XMRVs (Fig. S1). Primer sequences and details of PCR sensitivity and specificity quality controls can be found in *SI Materials and Methods*.

Phylogenetic Analysis. To generate the neighbor-joining phylogenetic tree, the viral *gag* gene sequences obtained from blood samples of patients with CFS, normal blood donors, as well as all of the closely related MLV *gag* gene sequences selected from the National Center for Biotechnology Information (NCBI) database by BLAST querying with the *gag* gene sequences obtained in the PCR study (*SI Materials and Methods*) were aligned with ClustalW2 (http://www.ebi.ac.uk/tools/clustalw2) using default settings. The analysis produced the same phylogenetic trees with or without consideration of the sequence gaps in alignment.

Mouse Mitochondrial DNA Assay. The complete mtDNA sequences of humans and mice were downloaded from GenBank and aligned using ClustalW. Sequence alignment revealed the 439 bp of the 3′ end of mouse mtDNA (beyond 15,862 bp, according to the coordinates of BALB/c mouse; accession no. AJ512208) were not present in human mtDNA. Primer sets were designed for a semi-nested, mouse-specific mtDNA PCR based on the sequence in this region of mouse mtDNA using Primer-Blast from NCBI. The external PCR primers (*SI Materials and Methods*) were designated as mt15982F and mt16267R, which would produce a predicted amplicon of 286 bp. The internal primers of the semi-nested PCR were designated as mt16115F and mt16267R, which would produce a predicted amplicon of 153 bp from mouse mtDNA. PCR system and setup were the same as for the *gag* gene-nested PCR study. However, PCR conditions were slightly different: 4 min at 94 °C; (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C) × 40–45 cycles; 10 min at 72 °C.

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Mouse retroviruses and chronic fatigue syndrome: Does X (or P) mark the spot?

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Five years ago, a retrovirus resembling a murine leukemia virus (MLV) was found in patients with prostate cancer (1), and last year, a similar gammaretrovirus was identified in patients with chronic fatigue syndrome (CFS) (2). The agent was named xenotropic MLV-related virus (XMRV), because its *env* gene was nearly identical to that of xenotropic MLV, an infectious endogenous MLV that preferentially infects cells from foreign species, including humans (Fig. 1*A*) (3). The two reports struck a common chord, because the viral sequences found in prostate cancer and CFS were nearly identical. A second common theme emerged in reports from Europe that XMRV was rarely found, if at all, in prostate-cancer samples or patients with CFS; however, other investigators confirmed the presence of XMRV in prostate-cancer samples from North America (4). Although a recent report from the Centers for Disease Control and Prevention (CDC) did not find a link between XMRV and CFS (5), distinct MLV-related sequences are found in serial samples collected from the mid-1990s to 2010 from patients with CFS reported in the study by Lo et al. (6) in PNAS. However, the reasons for the current geographical restriction and the source of the infection are baffling.

Originally, a viral etiology of familial prostate cancer was pursued in patients with RNase L deficiency, an antiviral endoribonuclease that relays the antiviral IFN response (7). Because modulated RNase L activity has been linked with CFS, similar case-control studies were conducted, and XMRV was found in 67% of CFS patients (2). Since then, XMRV has been found in prostate-cancer patients with no link to RNase L deficiency (4) and in 3.7% of healthy donors in the original CFS study (2). Lo et al. detect MLV-related sequences in 88% of their CFS samples and 6.8% of control blood donors (6). If verified, this perplexing frequency of detecting MLV-like sequences in blood donors suggests a more widespread source of infection.

The *gag* gene sequences identified by Lo et al. (6) share 96.6% homology with XMRV, but they seem divergent in a region that has long puzzled virologists. Over 30 y ago, extracellular glycosylated
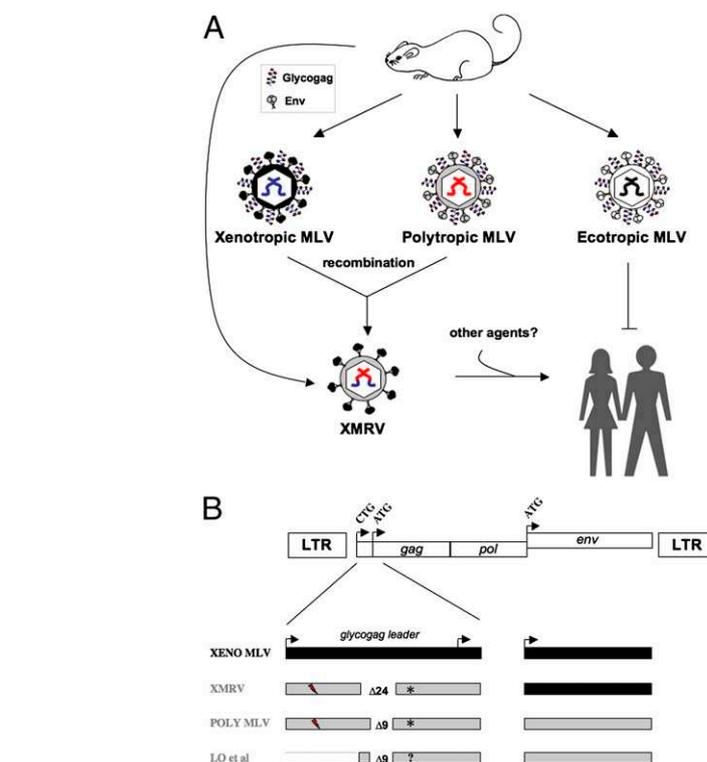

Fig. 1. MLV-related sequences in CFS patients. (*A*) Virions are represented with color-coded genomic RNA caged in hexagonal *gag*-encoded capsids, with glycogag and the *env*-encoded envelope (Env) glycoproteins represented as zigzag lines, circles, and sticks, respectively. Env determines tropism for mouse and not human cells (ecotropic), nonmouse cells (xenotropic), or both cell types (polytropic). XMRV seems to be a xenotropic/polytropic recombinant that may both lack glycogag (see below) and require complementation with another infectious agent to spread in humans. (*B*) Schematic MLV proviral DNA genome with the *glycogag*, *gag*, and *env* CTG and ATG translation initiation codons. The xenotropic (black) or polytropic (gray) origin for *env*, when known, and the glycogag leader sequence (upstream of the *gag* ATG) are shown with the corresponding in-frame 24- (xenotropic) or 9-bp (polytropic) deletion and a frameshift (lightning) that leads to a stop codon (star). Sequences reported in PNAS (6) do not allow predictions on glycogag production.

forms of the MLV Gag proteins were identified (8) and found to be translated from an alternative CTG initiation codon in-frame with the conventional *gag* ATG to append a type II leader sequence to the Gag precursor (9) (Fig. 1*B*). Glycosylated forms of Gag, or glycogag, can be incorporated in virions (10) and are required for efficient viral release (11), spread, and pathogenesis (12, 13) (Fig. 1*A*). Intriguingly, the hepatitis B virus harbors a similar precore region that encodes the e antigen (HBeAg) in-frame with the core protein, and natural mutants with stop codons preventing expression of HBeAg have been reported to arise during the

natural course of infection (14). Similarly, the 24-bp in-frame deletion common to all XMRV isolates located in the glycogag leader sequence maintains the ORF, whereas the ORF is eventually occluded by an upstream point deletion (Fig.1*B*). The sequences reported by Lo et al. (6) with a 9-bp deletion are distinct from that

of XMRV. Although the available sequence also maintains the glycogag ORF, sequence information on the CTG region and availability of the ORF is still outstanding.

Lo et al. (6) report that the MLV-related sequences that they identify correspond to polytropic and not xenotropic MLV sequences that differ in their Env host range. Both xenotropic and polytropic MLV Env bind the polymorphic Xpr1 receptor to mediate cell entry, although with different efficiencies in laboratory and wild-mouse strains (15–18). Therefore, identifying the precise origins and relatedness of both these MLV-related agents found in humans becomes of paramount importance to clarify the source and epidemiology of infection and ultimately, the potential role in disease. Indeed, XMRV genomes (1, 2) are actually hybrids between polytropic endogenous MLV sequences for their 5′ half up to approximately the middle of *pol* and xenotropic MLV for their 3′ half that harbors *env*. The glycogag leader of XMRV matches 100% of a polytropic endogenous sequence of the 129×1/SvJ laboratory mouse strain (accession no. AAHY01591888.1), a commonly used strain for gene knock-outs in embryonic stem cells. However, the *glycogag/gag* sequences reported by Lo et al. (6) best match endogenous polytropic sequences of the C57BL/6J laboratory strain of *Mus musculus*, with 99% nucleotide homology, and the *env* sequences share 97% homology with similar endogenous polytropic MLV and mink cell focus-forming viruses.

Env cross-dressing between different MLV, known as pseudotyping, is a common feature in dually infected hosts (19), allowing MLV to extend their original tropism. Indeed, the multiple origins of these xenotropic sequences, the hybrid nature of the XMRV genome, and the occlusion of the otherwise necessary glycogag ORF underscore the potential complementation and recombinational events that may lead to their transmission into humans. Interestingly, MLV glycogag can both increase the production of HIV-1 (11) and efficiently substitute for Nef to reestablish HIV-1 spread (20). These observations suggest a scenario in which retroviruses, MLV-related agents, and potentially, other viral agents may cross-complement to promote coinfection and enable pathogenicity.

The current data suggest that a variety of xenotropic and polytropic MLV can be found in North Americans with and without disease. To add to this bewilderment, it is likely that more than one environmental agent impacts on the development of both CFS and prostate cancer. At this juncture, it would seem reasonable to conduct extensive case-control studies in North America, as suggested by Lo et al. (6), using coded control samples from subjects with inflammatory disease to determine the frequency of MLV infection in patients with CFS. The potential transmission of MLV-related sequences from human to human should also be epidemiologically evaluated.

As we currently lack postulates to prove a causal association with a prevalent agent and a chronic disease with genetic predisposition, it would also be appropriate to conduct interventional studies. Indeed, the *Helicobacter pylori* hypothesis of peptic ulcer disease was only accepted after Barry Marshall showed that bacterial eradication with antibiotics cured peptic ulcer disease (21). Studies to gain proof of principle have been performed with antivirals in other chronic, idiopathic diseases linked to retroviral infection, such as primary biliary cirrhosis associated with mouse mammary tumor virus, another possible murine zoonosis (22). Trials using a combination of reverse transcriptase inhibitors led to significant improvements in clinical, histological, and biochemical outcomes in these patients, albeit with some evidence of viral resistance to therapy (23). Such studies are now feasible for CFS, because reverse-transcriptase inhibitors, such as tenofovir and emtricitabine, and the integrase inhibitor raltegravir can inhibit XMRV (24).

The caveats for conducting clinical trials in patients with CFS and MLV infection are that the potential benefits of treatment should outweigh the risks; also, studies should be conducted as randomized controlled trials with meaningful and feasible endpoints using robust therapies. At this juncture, studies to establish proof of principle are justified to determine whether safe antiviral regimens can impact on CFS and to determine whether xenotropic or polytropic MLV is causally associated with this debilitating disease.

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