Failure to Confirm XMRV/MLVs in the Blood of Patients with Chronic Fatigue Syndrome: A Multi-Laboratory Study

Graham Simmons,[1] Simone A. Glynn,[2] Anthony L. Komaroff,[3] Judy A. Mikovits,[4] Leslie H. Tobler,[1] John Hackett Jr.,[5] Ning Tang,[5] William M. Switzer,[6] Walid Heneine,[6] Indira K. Hewlett,[7] Jiangqin Zhao,[7] Shyh-Ching Lo,[8] Harvey J. Alter,[9] Jeffrey M. Linnen,[10] Kui Gao,[10] John M. Coffin,[11] Mary F. Kearney,[12] Francis W. Ruscetti,[12] Max A. Pfost,[4] James Bethel,[13] Steven Kleinman,[14] Jerry A. Holmberg,[15] Michael P. Busch,[1]* for the Blood XMRV Scientific Research Working Group (SRWG)†

Murine leukemia viruses (MLVs), including xenotropic-MLV–related virus (XMRV), have been controversially linked to chronic fatigue syndrome (CFS). To explore this issue in greater depth, we compiled coded replicate samples of blood from 15 subjects previously reported to be XMRV/MLV–positive (14 with CFS) and from 15 healthy donors previously determined to be negative for the viruses. These samples were distributed in a blinded fashion to nine laboratories, which performed assays designed to detect XMRV/MLV nucleic acid, virus replication, and antibody. Only two laboratories reported evidence of XMRV/MLVs; however, replicate sample results showed disagreement, and reactivity was similar among CFS subjects and negative controls. These results indicate that current assays do not reproducibly detect XMRV/MLV in blood samples and that blood donor screening is not warranted.

Previously unknown murine leukemia virus (MLV)–like sequences were identified in, and implicated as a potential infectious cause of, human prostate cancer in 2006 (*1*). These sequences appeared to be closely related to xenotropic MLV (X-MLV) and were termed X-MLV–related virus, or XMRV. In 2009, similar viral sequences were identified in a cohort of patients with chronic fatigue syndrome (CFS) (*2*). In that study, XMRV could be directly cultured from both peripheral blood mononuclear cells (PBMCs) and plasma from the majority of patients with CFS, and XMRV sequences were detected by means of polymerase chain reaction (PCR) and reverse transcription–PCR (RT-PCR) (*2*, *3*). Furthermore, evidence of an immune response to MLVs was observed in patient plasma (*2*, *3*). In an independent study, other patients with CFS were reported to harbor MLV-related virus sequences, but not XMRV, in PBMC and plasma (*4*). These sequences were derived from viruses resembling polytropic MLVs (P-MLVs) rather than X-MLVs. Both studies identified XMRV/P-MLV in the majority (67 to 86%) of patients with CFS but also in substantial numbers of healthy controls, including blood donors (4 to 7%) (*2*, *4*).

Subsequent studies cast doubt on the association between XMRV/P-MLVs and CFS, and indeed on the detection of XMRV/P-MLVs in human populations [reviewed in (*5*)]. Many—although not all (*6*, *7*)—of these negative studies focused on nucleic acid detection and/or serology and did not include cell-culture assays for virus (*8*–*11*). Several additional findings raised uncertainty about the high rates of XMRV/P-MLV in patients with CFS that had been described in the two seminal papers: (i) Clinical samples and PCR reagents were found to be contaminated by XMRV and mouse DNA containing endogenous MLVs (*12*); (ii) XMRV and P-MLV lack the sequence diversity that would be expected to arise after transmission, infection, and repeated cycles of replication of a retrovirus in humans (*13*, *14*); and (iii) evidence was presented that strongly suggested that XMRV originated in the early 1990s through recombination of endogenous MLVs after serial passage of a human prostate xenograft in laboratory mice (*15*). It was postulated that this laboratory passage resulted in the generation of several prostate cancer cell lines harboring integrated XMRV sequences that produced high levels of infectious virions. These XMRV-infected cell lines were subsequently widely disseminated and probably produced inadvertent XMRV contamination of laboratories and reagents (*15*).

We report here the results of a comprehensive study in which multiple laboratories analyzed the same blood samples for XMRV/P-MLV. These blood samples, which were drawn from persons who were previously reported to be XMRV- (*2*) or P-MLV–positive (*4*) and from blood donors who previously tested negative for XMRV, were aliquoted into replicate tubes and assembled into coded panels together with replicates of experimentally prepared positive control samples. The testing was performed fully blinded so as to remove bias. These samples were tested by nine laboratories using highly sensitive and previously validated nucleic acid, serological, and culture assays (tables S1 to S5) for XMRV and other MLVs (*16*). The two laboratories that had previously found an association for the MLVs with CFS participated in this study (*2*, *4*). All nine laboratories used XMRV/P-MLV nucleic acid amplification testing (NAT), serological, and/or culture assays of their own choosing, which were incorporated into parallel or serial testing algorithms in order to generate final results. The majority of laboratories included assays to detect mouse DNA contamination either on all samples or on all NAT-positive samples.

Fourteen patients with CFS, together with one person reporting contact with a CFS patient (*17*)—all of whom were previously reported to be XMRV/P-MLV–positive by at least one method (table S6)—were enrolled into the study at two clinical sites by using institutional review board–approved protocols and consents (henceforth referred to as the XMRV/P-MLV cohorts). Per study protocol, none of the 15 subjects were on antiretrovirals, but several subjects later disclosed that they were taking other antivirals (such as valacyclovir), and two were on immunosuppressive medications (the latter are indicated in table S6). In the case of the P-MLV–like viruses described by Lo and colleagues (*4*), only PCR detection had been performed in the original study; four of five patients enrolled into the current study were reported to be P-MLV–reactive on the archived samples from the original cohort study and on a second sample collected 15 years later (2010), whereas one patient was PCR-positive only on the original archived sample (*4*). The Whittemore Peterson Institute (WPI) patient cohort was more intensively characterized as positive by means of PCR, serology, and/or culture, although none of the study subjects tested positive in all assays at all time points (table S6).

To minimize introduction of potential contaminants, we took extensive precautionary measures during the collection of specimens and the laboratory processing of blood and preparation of sample aliquots (*17*). Blood specimens were collected by independent phlebotomists, shipped to the central laboratory (*17*), and processed into coded PBMC, plasma, and whole-blood (WB) aliquots. Similarly, 15 control specimens from blood donors (n = 12 specimens) or laboratory controls (n = 3 specimens) that had been established as negative for XMRV and MLVs by means of

[1]Blood Systems Research Institute and University of California, San Francisco, San Francisco, CA 94118, USA. [2]Transfusion Medicine and Cellular Therapeutics Branch, National Heart, Lung, and Blood Institute, National Institutes of Health (NIH), Bethesda, MD 20892, USA. [3]Harvard Medical School, and Brigham and Women's Hospital, Boston, MA 02115, USA. [4]Whittemore Peterson Institute, University of Nevada, Reno, NV 89557, USA. [5]Abbott Laboratories, Abbott Park, IL 60064, USA. [6]Division of HIV/AIDS Prevention, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA. [7]Office of Blood Research, U.S. Food and Drug Administration (FDA), Rockville, MD 20852, USA. [8]Office of Cellular, Tissue and Gene Therapies Review, FDA, Bethesda, MD 20892, USA. [9]Department of Transfusion Medicine, NIH, Bethesda, MD 20892, USA. [10]Gen-Probe, San Diego, CA 92121, USA. [11]Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02111, USA. [12]National Cancer Institute–Frederick, Frederick, MD 21702, USA. [13]Westat, Rockville, MD 20850, USA. [14]Department of Pathology, University of British Columbia, Victoria, BC V9A4W4, Canada. [15]U.S. Department of Health and Human Services, Rockville, MD 20852, USA.

*To whom correspondence should be addressed. E-mail: mbusch@bloodsystems.org

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PCR, serology, and culture by multiple laboratories were collected, processed, and aliquoted in parallel (*17*). Lastly, a separate facility in the central laboratory prepared and characterized stocks of the XMRV-infected human cell line 22Rv1 (*15*, *18*) and supernatant, which were used to spike samples so as to create a set of low-level positive controls (*17*).

A total of 11 NAT, five serology, and three culture assays were performed on the samples (*17*). The WPI laboratory did not report culture assay results because their target cells had become contaminated with mycoplasma. Other than this, all sites reported results on all distributed and coded sample aliquots to the central laboratory. The results were then decoded and compiled into analysis data sets specific to the panels.

Few positive NAT results were reported other than on the coded spiked positive control replicate aliquots (Table 1 and table S7). Six of seven laboratories that performed NAT on three sample types (plasma, PBMC, and WB) reported no positive result for coded clinical samples (XMRV/P-MLV cohorts or negative controls), whereas these laboratories detected XMRV in 100% of the spiked controls (Table 1). These laboratories included those that used the most sensitive XMRV/P-MLV assays available, according to our previous blinded analytical sensitivity performance study (*16*). The U.S. Food and Drug Administration (FDA)/Lo laboratory failed to detect MLV-like sequences using the same nested PCR assay as previously published, in either the known negative controls or in the XMRV/P-MLV cohort samples. The samples scored as negative by this laboratory included the replicate samples from five patients with CFS reported as P-MLV–positive in their previous study, four of whom had also tested positive on a second specimen collected more than a decade after the archived CFS cohort panel (*4*).

The only positive NAT results on some of the replicates from clinical samples were reported by WPI. The WPI assays appeared less sensitive than those used by the other laboratories, based on the fact that only three of five plasma and four of five PBMC-spiked positive control replicates were scored as positive by WPI (Table 1 and table S7). However, two plasma clinical aliquots were reported as positive in the WPI nested RT-PCR *gag* assay. These samples were from two different negative controls, and only one out of the three replicates was positive in each case. Sequencing of the excised bands revealed 1 to 3 base changes, compared with XMRV derived from 22Rv1 [supporting online material (SOM) text]. A clinical PBMC sample, derived from one of the nine WPI CFS patients, was also positive in WPI's nested *gag* PCR assay. However, only one of two PBMC replicates for this individual was positive, and all replicates of plasma and WB from this patient were reported as negative by WPI. All positive samples tested negative for mouse DNA contamination as assessed with mouse mitochondrial DNA PCR (*4*). Reactivity rates did not significantly differ between samples from negative controls and the XMRV/P-MLV cohorts ($P > 0.05$) (SOM text and table S10).

In the initial study, Lombardi *et al*. reported that the most effective and consistent method of determining whether an individual was XMRV-positive was by isolation of replication-competent virus through coculture of target prostate cell lines with either patient PBMCs or plasma (*2*, *3*). Although culture results were not reported by WPI in the present study, the National Cancer Institute (NCI)/Ruscetti laboratory also successfully performed virus culture using both plasma and PBMC in the Lombardi *et al*. study (*2*, *3*). Additionally, virus culture was performed by the FDA/Hewlett laboratory, which used two methods, one of which (LNCaP cell culture) was established in their laboratory for this study on the basis of WPI procedures and on-site training by the lead investigators from the WPI and NCI/Ruscetti laboratories; hence, viral culture in this laboratory would be expected to have equivalent sensitivity to the culture method used by Lombardi *et al*. (*17*). Both laboratories successfully detected all five replicates of the spiked positive controls (~10^6 RNA copies/ml). However, although neither of the FDA/Hewlett assays detected confirmed positive cultures in the 30 coded clinical aliquots, the NCI/Ruscetti laboratory reported nine aliquots as positive (Tables 1 and 2). Six of the positive results were from negative control samples (40% positive rate); these six subjects and samples had previously been pedigreed by the same laboratory as culture-negative (*17*). In contrast, only three (20%) of the 15 XMRV/P-MLV–cohort subjects (including 10 subjects who had previously been found to be culture-positive by the WPI and NCI/Ruscetti laboratories) tested positive in the coded panel (table S1). There was no significant difference between the rate of reported positive culture results among negative controls and the XMRV/P-MLV–cohort subjects ($P = 0.43$) (table S8).

Lastly, serology was performed by four laboratories (*17*). Although plasma with human antibodies to XMRV/P-MLVs was not available to produce spiked controls for serology, all four laboratories performed their own internal controls (*17*). Three assays—a Western blot test using purified XMRV [Centers for Disease Control and Prevention (CDC), Atlanta, Georgia] (*19*) and two chemiluminescent immunoassays using recombinant

Table 1. All XMRV/P-MLV assay results from all laboratories. Abbott-M, Abbott Molecular; Abbott-D, Abbott Diagnostics; WB, whole blood; N/A, not applicable. Boldface entries indicate positive results.

Test	Laboratory	Sample type			
		Negative controls*	WPI XMRV/P-MLV subjects*	Lo *et al.* XMRV/P-MLV subjects*	Spiked controls*
NAT/Plasma	Abbott-M	0/15	0/10	0/5	**5/5**
	CDC	0/15	0/10	0/5	**5/5**
	FDA/Lo	0/15	0/10	0/5	**5/5**
	FDA/Hewlett	0/15	0/10	0/5	**5/5**
	Gen-Probe	0/15	0/10	0/5	**5/5**
	NCI/DRP	0/15	0/10	0/5	**5/5**
	WPI	**2/15†**	0/10	0/5	**3/5**
NAT/PBMC	Abbott-M	0/3	0/10	0/5	**5/5**
	CDC	0/3	0/10	0/5	**5/5**
	FDA/Lo	0/3	0/10	0/5	**5/5**
	FDA/Hewlett	0/3	0/10	0/5	**5/5**
	Gen-Probe	0/3	0/10	0/5	**5/5**
	NCI/DRP	0/3	0/10	0/5	**5/5**
	WPI	0/3	**1/10†**	0/5	**4/5**
NAT/WB	Abbott-M	0/15	0/10	0/5	**5/5**
	CDC	0/15	0/10	0/5	**5/5**
	FDA/Lo	0/15	0/10	0/5	**5/5**
	FDA/Hewlett	0/15	0/10	0/5	**5/5**
	Gen-Probe	0/15	0/10	0/5	**5/5**
	NCI/DRP	0/15	0/10	0/5	**5/5**
	WPI	0/15	0/10	0/5	**5/5**
Culture	FDA/Hewlett	0/15	0/10	0/5	**5/5**
	NCI/Ruscetti	**6/15**	**3/10†**	0/5	**5/5**
Serology	Abbott-D	0/15	0/10	0/5	N/A
	CDC	0/15	0/10	0/5	N/A
	NCI/Ruscetti	**8/15**	**3/10**	**2/5†**	N/A
	WPI	**6/15**	**5/10**	**5/5†**	N/A

*Number positive/number tested. A single reactive replicate out of one, two, or three tested for a given individual was considered positive. †No significant association was seen when the reactivity rates of control negatives and XMRV/P-MLV cohort subjects were compared (*P* values are discussed in the SOM text).

XMRV gp70 and p15E (Abbott Diagnostics, Abbott Park, Illinois) (*20*)—failed to detect positive results for any of the coded replicates prepared from the 30 clinical samples. A flow cytometry–based serologic assay run by two laboratories (NCI/Ruscetti and WPI), using mouse cells expressing the spleen focus-forming virus (SFFV) envelope as used in the original Lombardi *et al.* study, reported a number of positive results on samples from both the XMRV/P-MLV cohorts and the negative-plasma controls. The NCI/Ruscetti laboratory reported 13 positive samples, including 8 (53%) from 15 known negatives and 5 (33%) from 15 XMRV/P-MLV cohort subjects (Tables 1 and 2). None of the positive results from the XMRV/P-MLV cohorts or controls were reported for more than one of the uniquely coded replicates, despite the fact that every sample was represented in the panel in duplicate or triplicate (Table 2). There was no significant difference between the proportions of negative controls and XMRV/P-MLV–cohort subjects identified as serology-positive [$P > 0.20$ regardless of how positivity was defined (SOM text and table S9)].

Among all serologic replicates tested, the WPI detected 22 positives, including 10 reactive results among the negative controls and six each in the subjects previously reported as positive by WPI and by FDA/Lo (Table 1 and table S7). Three of the six known negative controls with a positive serology result had at least two of three replicates positive (Table 2). All five patients previously identified as P-MLV–positive by FDA/Lo had a replicate called serology-positive, but only one had both replicates reported as positive. Similarly, for the 10 subjects previously identified as XMRV-positive by WPI, four subjects had one of two replicates reported as serology-positive, whereas both replicates from one patient were reported positive (Table 2). There was no significant difference in the rates of positive WPI serology results between negative controls and XMRV/P-MLV–cohort subjects ($P = 0.27$). There was no statistical agreement between the samples reported as serology-positive by the NCI/Ruscetti and WPI laboratories, despite the fact that they used similar assays (SOM text and tables S9 and S10). Kappa values were calculated for each criterion and for all subjects combined by using standard procedures (*17*, *21*). The kappa values for level of agreement of results between these two laboratories ranged from −0.20 for WPI XMRV/P-MLV–positive subjects (no agreement) to 0.21 for all negative controls combined (fair agreement). However, the most telling kappa value between the WPI and NCI/Ruscetti serology results is the one computed for all subjects combined, which is 0.01, indicating no agreement.

Our study demonstrates that no XMRV/P-MLV assay in any of the nine participating laboratories could reproducibly detect XMRV/P-MLV in 15 subjects (14 with CFS) who had previously been reported as XMRV/P-MLV–infected, usually at multiple time points and often by multiple assays (*2*, *4*). The two laboratories (WPI and NCI/Ruscetti labs) that reported positive results in this study reported similar rates of reactivity among XMRV/P-MLV subjects and known negative control donor samples. The results from both laboratories were inconsistent when their assays were performed in parallel on replicate sample aliquots derived from individual subject specimens. There was also no agreement of reactivity when comparing results between these two laboratories for the 30 blinded XMRV/P-MLV cohorts and control samples. In contrast, assays developed by FDA (Lo and Hewlett), CDC, NCI/Drug Resistance Program (DRP), Abbott Diagnostics, Abbott Molecular, and Gen-Probe—all of which have been designed to detect XMRV and relevant MLVs with high sensitivity and specificity—failed to detect evidence of viral infection in any of the previously positive subjects, including CFS patients, or negative control specimens represented in the study.

Altogether, 15 XMRV/P-MLV cohort subjects were represented in this study, the maximum number of subjects who could be recruited by the cohort investigators (*2*, *4*). Because most patients were selected on the basis of having previously tested positive for XMRV/P-MLV 1 to 3 years ago, it is possible that levels of viremia and/or antibody could have waned by the time samples were drawn in our study; however, this is contradictory to Lo *et al.*'s finding that four of five patients retested positive 15 years later (*4*). The inconsistent reactive results from the two laboratories that previously reported detection of XMRV (NCI/Ruscetti and WPI) and the negative results from all other laboratories, including

Table 2. Results of replicates for assays with positive results (number reactive/number replicates tested). NT, not tested. The kappa for the serology for the negative controls between NCI/Ruscetti and WPI is 0.21. The kappa for the serology for the WPI XMRV/P-MLV subjects between NCI/Ruscetti and WPI is −0.20. The kappa for the serology for the Lo *et al*. XMRV/P-MLV subjects between NCI/Ruscetti and WPI is 0.00. The kappa for the serology for all cohort subjects between NCI/Ruscetti and WPI is −0.08. Boldface entries indicate positive results.

Subject	Assay				
	WPI NAT/Plasma	WPI NAT/PBMC	WPI serology	NCI/Ruscetti serology	NCI/Ruscetti culture
			Negative controls		
1	0/1	0/2	0/2	0/2	0/1
2	0/1	0/1	0/2	0/2	**1/1**
3	0/1	0/1	**2/2**	**1/2**	0/1
4	0/3	NT	**1/3**	**1/3**	0/1
5	0/3	NT	0/3	0/3	0/1
6	**1/3**	NT	0/3	**1/3**	0/1
7	0/3	NT	0/3	0/3	0/1
8	0/3	NT	**2/3**	0/3	0/1
9	0/3	NT	**3/3**	**1/3**	**1/1**
10	0/3	NT	0/3	**1/3**	**1/1**
11	0/3	NT	**1/3**	**1/3**	0/1
12	0/3	NT	**1/3**	0/3	**1/1**
13	0/3	NT	0/3	**1/3**	**1/1**
14	**1/3**	NT	0/3	**1/3**	**1/1**
15	0/3	NT	0/3	0/3	0/1
			WPI XMRV/P-MLV subjects		
1	0/1	0/2	0/2	**1/2**	0/1
2	0/1	0/1	**1/2**	0/2	0/1
3	0/1	0/1	**1/2**	0/2	0/1
4	0/2	0/2	**2/2**	0/2	**1/1**
5	0/1	**1/2**	0/2	0/2	**1/1**
6	0/1	0/2	0/2	0/2	0/1
7	0/2	0/2	0/2	0/2	0/1
8	0/1	0/2	**1/2**	**1/2**	0/1
9	0/1	0/3	**1/2**	0/2	0/1
10	0/1	0/2	0/2	**1/2**	**1/1**
			Lo et al. *XMRV/P-MLV subjects*		
1	0/3	0/2	**1/2**	0/2	0/1
2	0/3	0/2	**1/2**	0/2	0/1
3	0/3	0/2	**1/2**	**1/2**	0/1
4	0/3	0/2	**1/2**	**1/2**	0/1
5	0/3	0/1	**2/2**	0/2	0/1
			Spiked controls		
1	**3/5**	**4/5**	NT	NT	**5/5**

the laboratory that previously reported detection of P-MLV (FDA/Lo), strongly suggest that the positive reactivity in this study represents false positive results because of assay nonspecificity or cross-reactivity (for example, to other endogenous or exogenous retroviruses). However, we cannot definitively exclude the possibility that the levels of XMRV/P-MLV markers in blood may be at or below the limit of detection of all assays and/or fluctuate over time, as recently described in experimentally infected macaque studies (*22*).

On the basis of these findings, we conclude that currently available XMRV/P-MLV assays—including the assays used by the three participating laboratories that previously reported positive results on samples from CFS patients and controls (*2*, *4*)—cannot reproducibly detect direct virus markers (RNA, DNA, or culture) or specific antibodies in blood samples from subjects previously characterized as XMRV/P-MLV–positive (all but one with a diagnosis of CFS) or healthy blood donors. Lastly, our findings are reassuring with respect to blood safety and indicate that routine blood donor screening for XMRV/P-MLV is not warranted at this time.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1213841/DC1
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tRNAs Marked with CCACCA Are Targeted for Degradation

Jeremy E. Wilusz,[1]* Joseph M. Whipple,[2] Eric M. Phizicky,[2] Phillip A. Sharp[1]

The CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase] adds CCA to the 3′ ends of transfer RNAs (tRNAs), a critical step in tRNA biogenesis that generates the amino acid attachment site. We found that the CCA-adding enzyme plays a key role in tRNA quality control by selectively marking structurally unstable tRNAs and tRNA-like small RNAs for degradation. Instead of adding CCA to the 3′ ends of these transcripts, CCA-adding enzymes from all three kingdoms of life add CCACCA. In addition, hypomodified mature tRNAs are subjected to CCACCA addition as part of a rapid tRNA decay pathway in vivo. We conjecture that CCACCA addition is a universal mechanism for controlling tRNA levels and preventing errors in translation.

Although tRNAs require CCA at their 3′ ends for amino acid attachment and correct positioning in the ribosome, CCA is not encoded in nearly all eukaryotic tRNA genes or in many archaeal and bacterial tRNA genes (*1*–*4*). Instead, the CCA-adding enzyme posttranscriptionally adds CCA to the 3′ ends of tRNAs and tRNA-like transcripts (*5*–*8*). Recent work has identified two tRNA-like small RNAs, mascRNA (MALAT1-associated small cytoplasmic RNA) and the MEN β tRNA-like small RNA, that are generated by 3′ end processing of long nuclear-retained noncoding RNAs in human and mouse cells (*9*–*11*) (Fig. 1A). These RNAs are ~70% similar in sequence and are generated by enzymes involved in canonical tRNA biogenesis, including ribonucleases (RNases) P and Z. Although the long noncoding RNAs from which they are processed are expressed at roughly equal levels, the MEN β tRNA-like small RNA is below the threshold of detection by Northern blot analysis (Fig. 1B) (*12*). This suggests that these tRNA-like transcripts are differentially regulated posttranscriptionally. Using a more sensitive 3′ rapid amplification of cDNA ends–polymerase chain reaction (RACE PCR) approach, we detected expression of the MEN β tRNA-like small RNA in vivo (fig. S1). Surprisingly, nearly all sequenced transcripts ended in CCACCA or CCACC, which is not encoded in the genome. This 3′ end modification was not detected on mascRNA (*9*) and thus would be consistent with CCACCA addition as a signal for RNA degradation.

Although the CCA-adding enzyme is thought to terminate after CCA synthesis (*7*, *8*), it was the most likely candidate to catalyze CCACCA addition. We thus expressed and purified His-tagged versions of the CCA-adding enzyme from human (*13*), *Escherichia coli* (*14*), and *Sulfolobus shibatae* (*15*) (fig. S2, A to C) and confirmed that all three enzymes terminate polymerization once CCA has been added to canonical tRNAs (fig. S2, D to F). In contrast, CCACCA or CCACC was added to the MEN β tRNA-like small RNA in vitro (fig. S3), recapitulating the in vivo 3′ RACE results. This indicates that the CCA-adding enzyme catalyzes CCA or CCACCA addition, depending on the characteristics of the RNA substrate.

To determine the sequence elements required for CCACCA addition, we attempted to convert mouse mascRNA from a CCA to a CCACCA target by generating chimeric mascRNA–MEN β tRNA-like transcripts (Fig. 2A and fig. S4A).

[1]Koch Institute for Integrative Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [2]Department of Biochemistry and Biophysics, Center for RNA Biology, University of Rochester School of Medicine, Rochester, NY 14642, USA.

*To whom correspondence should be addressed. E-mail: wilusz@mit.edu