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# Successful transient introduction of *Leishmania* RNA virus into a virally infected and an uninfected strain of *Leishmania*

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**ABSTRACT** **Viruses of *Leishmania* have recently been identified and characterized. These viruses are consistently double-stranded RNA viruses of ≈5 kb. To date, they have not been reported to exist outside their protozoan host, nor have they been shown to be infectious. We report here the ability to transiently transfer these viruses to two strains of *Leishmania*, one previously infected and one that did not previously carry a virus. A PCR-based assay was used to detect viral negative-stranded RNA. Input RNA was ruled out as the source of template because a replication-incompetent (UV inactivated) virus was not detectable after transfer into *Leishmania*.**

Viruses of protozoan parasites have been isolated in a variety of systems. The first report was of a virus from *Entamoeba histolytica* (1). Subsequently, Wang and Wang (2) reported the presence of a double-stranded (ds) RNA virus in *Giardia lamblia*. dsRNA viruses have been isolated in *Trichomonas vaginalis* (3) and most recently in *Leishmania* (4, 5). To date, all of the viruses have shown a number of similarities, and all have a single, linear, dsRNA genome of 5–6 kb. They all appear to have spherical or icosahedral capsids. The role that these viruses play in growth or virulence of the parasite remains to be elucidated. However, irrespective of their role in these areas, they may well prove to be useful as probes for the unique gene expression mechanisms found in protozoa (6).

Viruses of *Leishmania* have been identified in 12 strains of *L. braziliensis* or *L. guyanensis* (7) and are designated *Leishmania* RNA virus 1 (LRV1; Totiviridae). Much of our initial work on *Leishmania* focused on the viral RNA-dependent RNA polymerase. In LRV1, the putative transcriptase and replicase were detected in virus-infected whole-cell extracts from the promastigote stage of the parasite and in virus purified from both CsCl and sucrose gradients (8, 9). More recently, the entire sequence for LRV1-1 was reported by Stuart and colleagues (10).

Transfection of *Leishmania* with foreign genes has been reported both transiently (11) and stably (12, 13). RNA transfection has so far been unsuccessful. Therefore, viral infection may have some advantages over transfection for expression of foreign genes primarily because LRV1 is thought to replicate entirely in the cytoplasm (ref. 14; T. L. Cadd and J.L.P., unpublished observation). There is no need for the transplicing of the miniexon or any other nuclear event. Ultimately, virally encapsidated RNA may prove to be the only efficient method to transfer RNA. Whether the lack of successful RNA transfer into kinetoplastids reflects transferring the RNA into cells or degradation of RNA, viral infection circumvents these problems. Here we show that an exogenous RNA virus, LRV1, can be transferred transiently into strains of *Leishmania*.

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All of the current data suggest that LRV1 persistently infects its host, with its replication cycle under strict control (15). Our ultimate goal is to introduce LRV1 into an uninfected strain of *Leishmania*. However, if LRV1 infection depends on a closely coordinated host–virus relationship, a reasonable first step toward this goal would be to superinfect an infected strain of *Leishmania* with a related strain of LRV1. LRV1-1 and LRV1-4 are two related viruses from two different strains of *L. guyanensis*, CUMC1-1A and M4147, respectively. Here we report that we have introduced LRV1-1 into M4147 and that the LRV1-1 genome was replicated but that the virus itself did not persist. Finally, we have shown that infection of an uninfected strain of *Leishmania* supports viral infection, does not require electroporation, and persists longer but also transiently.

## MATERIALS AND METHODS

**Parasite Strains and Cell Culture.** MHOM/BR/80/MCUMC1-1A and MHOM/BR/75/M4147 (hereafter referred to as CUMC1-1A and M4147) are two strains of *L. guyanensis* infected with LRV1-1 and LRV1-4, respectively. MTAM/BR/80/M6244 is an uninfected strain (hereafter referred to as M6244). Cells were grown in M199 semidefined medium (16) supplemented with 5% fetal bovine serum and 1% fresh, filter-sterilized human urine (17).

**Virus Purification.** This protocol has been described (9) and was modified as follows: LRV1-1 virus was purified from *Leishmania* strain CUMC1-1A by centrifugation through a 10–30% sucrose gradient in 20% (vol/vol) glycerol for 4 hr at 36,000 rpm in a Beckman SW41 rotor. Lysates of $1 \times 10^{10}$ cells were loaded on each 11-ml gradient and virus was recovered from two 1-ml fractions of the virus peak. All virus preparations were passed through a 0.2-μm sterilizing filter before use.

**Electroporation.** Recipient cells ($2.5 \times 10^7$) were washed once and resuspended in 0.20 ml of high-salt electroporation buffer (11). The cells were loaded into 0.2-cm electrogap cuvettes, mixed with 50 μl of purified virus, and exposed to 3.65 kV/cm from a 25 μF capacitor with an average time constant of 0.5 msec (Bio-Rad gene pulser). After electroporation, the cells were incubated on ice for 10 min and then passed into 20 ml of cell culture medium.

**PCR.** Electroporated cells (5 ml) were washed once with TNM (10 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/100 mM NaCl) and lysed in 0.15% Triton X-100 TNM for 15 min on ice. RNA in the lysates was extracted with phenol/chloroform and precipitated with 80 mM NaCl/3 vol of ethanol and then reverse transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) according to the man-

Abbreviations: ds, double stranded; LRV1, *Leishmania* RNA virus 1.

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ufacturer's recommendations. This cDNA was amplified by using *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles ($t_m$, 54°C) again according to the manufacturer's recommendations. LRV1-1 negative-sense RNA was detected in the extracts using the primer LRV1-19 for the reverse transcription reaction and the primers LRV1-19 and LRV1-341 for the PCR. LRV1-4 negative-sense RNA was detected by using the primer LRV4-28 for the reverse transcription reaction and the primers LRV4-28 and LRV4-134 for the PCR (see Fig. 1). The amplified DNA was resolved on 2% agarose gels and visualized with ethidium bromide.

**UV Inactivation of the Viral Polymerase.** Ten-microliter aliquots of sucrose-purified virus in 1.5-ml microcentrifuge tubes were exposed to UV light for 5 min from a Stratagene UV Stratalinker 1800. Viral polymerase activity was assayed with an *in vitro* RNA-dependent RNA polymerase assay as described (5).

## RESULTS

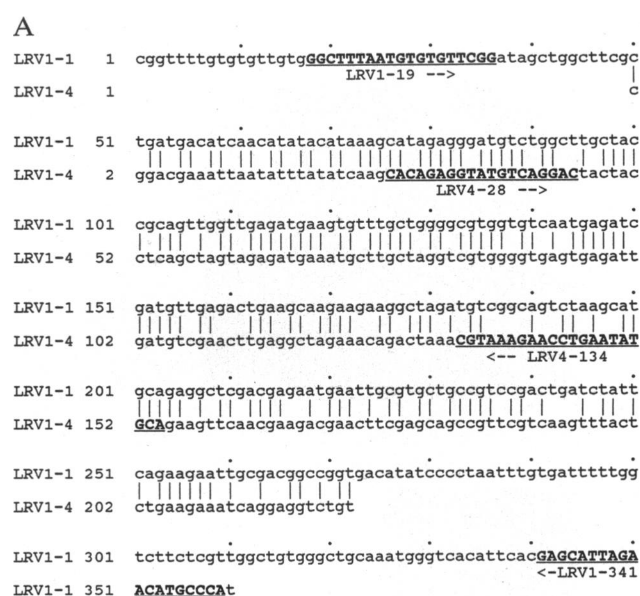
The electroporation conditions for *L. guyanensis* were originally developed for introduction of a neomycin gene (pALT-Neo) by Wirth and colleagues (12). Conditions were modified to suit *L. guyanensis*; of particular importance was the addition of fresh, sterile human urine to the culture medium (17). As a general approach to the detection of viral RNA in parasite extracts, we developed a PCR-based assay that can distinguish between the RNA genomes of both LRV1-1 and LRV1-4. We constructed four oligonucleotides, two for each virus, based on sequence data from a region of the viral genome that varies significantly between the two viruses (Fig. 1*A*). The viral RNA was reverse transcribed using the two upstream primers LRV1-19 and LRV4-28 so that our assay detected only the negative-sense strands of the viral genomes. This assay can quantify the relative amounts of viral RNA in cell extracts (Fig. 1*B*). Normally, viral RNA varies from experiment to experiment. The reason for the variability remains unclear (9). Extracts of M4147 and CUMC1-1A were assayed with these primers and DNA fragments of the expected size were amplified (Fig. 2).

LRV1-1 was isolated from CUMC1-1A and introduced into M4147 cells by electroporation. LRV1-1 RNA was detected in cells electroporated with LRV1-1 up to 4 days after electroporation (Fig. 2*A*). This is in contrast to the cells that were exposed to virus but not to an electric field. LRV1-1 RNA could not be detected in these cells by this assay 24 hr after electroporation. Also, more LRV1-1-specific DNA was amplified from the 1-hr extracts made from the LRV1-1 electroporated cells than from parallel extracts made from the cells exposed to virus but not an electric field. From these data, we presumed that we had introduced LRV1-1 RNA into M4147 cells.

A third band, which appeared inconsistently, migrated between the bands for LRV1-1 and LRV1-4. Based on controls of promastigote CUMC1-1A and M4147 cells, it is found associated with the reaction of primers for LRV1-4 (Fig. 2*A*). Its origin is unknown. Arrows pointing to the expected PCR products are present to prevent any confusion.

A culture of M4147 cells that had been electroporated with LRV1-1 was passed 1:40 and allowed to grow to stationary phase for a total of 10 days after electroporation. LRV1-1 RNA could no longer be detected in this culture (Fig. 2*B*).

To confirm this and to determine whether LRV1-1 replicates within M4147, we electroporated UV-treated LRV1-1 into M4147. An RNA-dependent RNA polymerase reaction has been characterized for both sucrose- and CsCl-purified virus by an *in vitro* polymerase reaction (8, 9). Sucrose-purified virus has been shown to synthesize both single-stranded RNA and dsRNA by use of an RNase protection experiment in which salt concentrations were varied (9). The



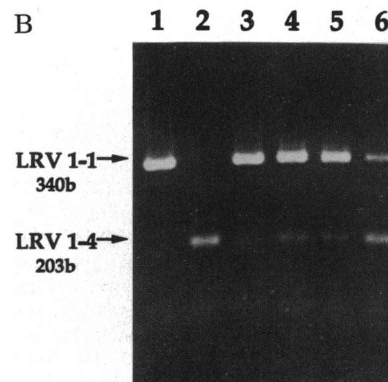




FIG. 1. (*A*) Location of primers for virus-specific RNA PCR. Primer pairs specific for LRV1-1 and LRV1-4 within viral open reading frame 2 are underlined and their 5′ to 3′ orientation is indicated with an arrow. LRV1-1 sequence is according to Stuart *et al.* (10); LRV1-4 sequence is from unpublished data. (*B*) Sensitivity test of the PCR assay. Decreasing quantities of CUMC1-1A cell extracts were added to a constant quantity of M4147 extract. The PCR assay was carried out as described. Lanes: 1, CUMC1-1A extract alone; 2, M4147 extract alone; 3, M4147 extract and CUMC1-1A extract (1:1); 4, M4147 and CUMC1-1A (1:0.2); 5, M4147 and CUMC1-1A (1:0.1); 6, M4147 and CUMC1-1A (1:0.01). b, Bases.

characteristic band of viral dsRNA was absent from an autoradiogram of a polymerase assay of an aliquot of the LRV1-1 exposed to UV for 5 min, indicating that 5 min of exposure is sufficient to eliminate the replicase function of the viral polymerase (Fig. 3*A*). UV treatment also significantly inhibited the transcriptase function of the polymerase, as indicated by the decrease observed in the amount of single-stranded RNA synthesis after exposure to radiation. It is important to note that, as discussed below, the input dsRNA genome is not affected by the UV treatment, only polymerase functions.

M4147 cells were electroporated with UV-treated and untreated LRV1-1 in parallel. In this experiment, detectable levels of untreated LRV1-1 RNA persisted in M4147 cells up through 24 hr after electroporation, while no LRV1-1 RNA could be detected in extracts made at the same time point from cells electroporated with UV-treated virus (Fig. 3*B*). Together these data strongly support the idea that we have successfully introduced LRV1-1 into M4147 cells and that the virus replicated but did not persist.

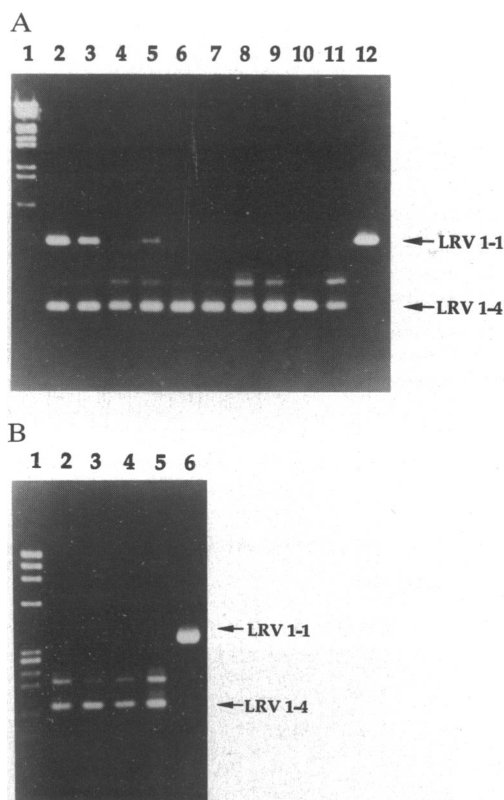



FIG. 2. Transient introduction of LRV1-1 into M4147. (*A*) LRV1-1 virus was introduced into M4147 cells by electroporation (lanes 2, 5, and 8) and parallel preparations of cells were either exposed to virus but not exposed to an electric field (lanes 3, 6, and 9) or exposed to an electric field but with a sterile 30% sucrose/20% glycerol solution in place of virus (lanes 4, 7, and 10). RNA extractions were performed on aliquots taken after 1 hr (lanes 2–4), 24 hr (lanes 5–7), and 4 days (lanes 8–10). These RNA extracts were assayed for the presence of LRV1-1 and LRV1-4 by PCR, as were extracts of M4147 (lane 11) and CUMC1-1A (lane 12). Lane 1, λ phage DNA *Hin*dIII digest. (*B*) After 4 days, electroporated cells and controls were passed 1:40 and allowed to grow to stationary phase; 0.80% of these cultures were harvested as extracts and assayed by PCR as described above. Lanes: 1, φX phage DNA *Hae* III digest; 2, with virus and electroporation; 3, with virus and without electroporation; 4, without virus and with electroporation; 5, M4147 extract as described above; 6, CUMC1-1A extract as described above.

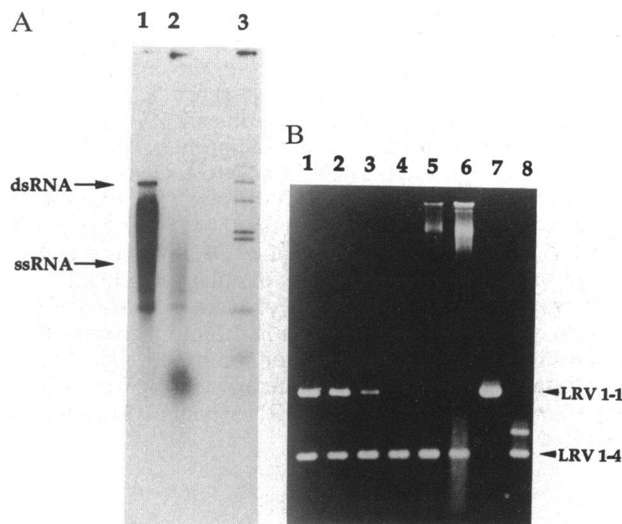



FIG. 3. LRV1-1 viral replicase is necessary for replication in M4147. (*A*) Viral polymerase was inactivated by exposing the virus to UV light. Lanes: 1, untreated virus; 2, UV inactivated virus; 3, λ phage DNA *Hae* III digest. ss, Single stranded. (*B*) UV inactivated virus (lanes 2, 4, and 6) was introduced into M4147 cells by electroporation in parallel with untreated virus (lanes 1, 3, and 5) and RNA extracts were made from these cells after 1 hr (lanes 1 and 2), 24 hr (lanes 3 and 4), and 4 days (lanes 5 and 6). These RNA extracts were assayed for the presence of LRV1-1 and LRV1-4 by PCR, as were extracts of M4147 (lane 7) and CUMC1-1A (lane 8).

Encouraged by our success at introducing LRV1-1 into the infected strain M4147, we performed an identical experiment, attempting to introduce LRV1-1 into the uninfected strain of *L. guyanensis*, M6244. To our surprise, LRV1-1 RNA could be detected in all of the RNA extracts made up through 4 days after electroporation in both the experiment and in the control that was exposed to virus but not to an electric field (Fig. 4*A*). The RNA could be detected for up to 4 days. However, over time it was lost, just as in the infected strains (Fig. 4*B*). Therefore, the results of this experiment support the idea that LRV1-1 virus can be transferred to other strains of *Leishmania*. However, significant differences exist between this result and the previous attempt at viral transfer. The virus persists longer, and electroporation is not required. The implications of this are discussed below.

## DISCUSSION

The use of viruses as vectors has been useful in the expression of many foreign genes in eukaryotes and prokaryotes. To date, this valuable tool has been unavailable for *Leishmania* and the understanding of gene expression in protozoans. We report here the ability to support replication of an exogenous protozoan virus in a virally infected strain as well as an uninfected strain of *Leishmania*.

Our original attempt to transfer the virus was into an already infected strain. Due to the similarities of this viral system with the yeast dsRNA virus system, we presume that there are specific parasite host factors necessary for a productive infection (18). By utilizing an already infected strain we hope to provide those elements as we begin to understand viral infection in promastigotes.

The PCR-based assay that distinguishes between RNA genomes of LRV1-1 and LRV1-4 provides indisputable evidence of the existence of viral transfer to an infected strain. There are two activities that have been found associated with the polymerase. One is a transcriptase associated with dsRNA particles, and the other is a replicase associated with single-stranded particles (14). We chose to address the question of whether or not we had transferred the virus by an assay that detects negative-sense RNA. As shown previously (9), the RNA is synthesized on a preexisting RNA template and is the product of the replicase component of the polymerase. More importantly, replicase function can only be detected after both transcription of plus-strand RNA for mRNA synthesis and synthesis of encapsidating proteins (15). As recently reported, encapsidation is a requirement for the plus strand to be used as a template for synthesis of minus-strand RNA. In other words, the detection of minus-strand RNA is a true measure of gene expression. Both the absence of the diagnostic PCR band when virus was not exposed to an electric field and the presence, although diminishing, of the band when exposed to an electric field up to 4 days after electroporation support the notion that exogenous virus enters and replicates.

Although unlikely, the possibility exists that this PCR assay was able to detect the input RNA. Therefore, it was important to show that replication of virus was necessary to allow detection. Also, as mentioned, transfer into uninfected cells did not require electroporation. Therefore, it was clearly necessary to show that virus must be competent for replica-

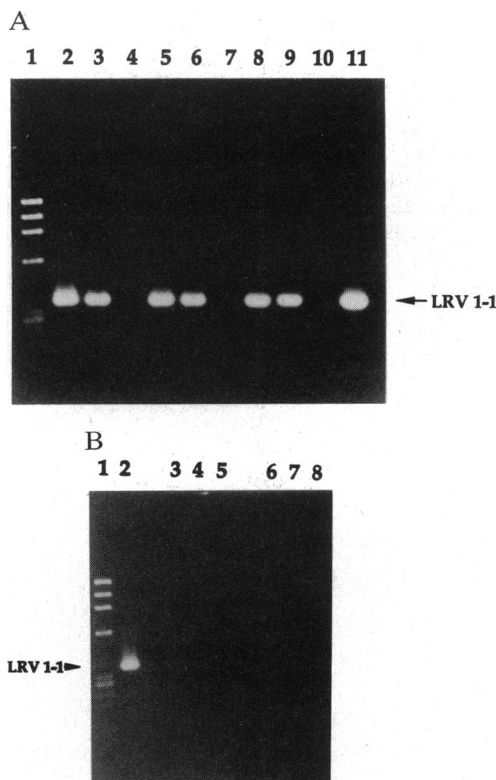



FIG. 4. (*A*) LRV1-1 can replicate in a virally uninfected strain of *Leishmania*. LRV1-1 was introduced into M6244 by electroporation (lanes 2, 5, and 8) and parallel preparations of cells were either exposed to virus but not exposed to an electric field (lanes 3, 6, and 9) or exposed to an electric field but with a sterile 30% sucrose/20% glycerol solution in place of virus (lanes 4, 7, and 10). RNA extractions were performed on aliquots taken after 1 hr (lanes 2–4), 24 hr (lanes 5–7), and 4 days (lanes 8–10). These RNA extracts were assayed for the presence of LRV1-1 by PCR, as was an extract of CUMC1-1A (lane 11). Lane 1, $\phi$X phage DNA *Hae* III digest. (*B*) LRV1-1 is transiently expressed in M6244 cells. Cells were passed 1:20 in M199 medium 4 days postelectroporation and weekly thereafter. PCR assay was carried out on RNA extracted 1 week (lanes 3–5) and 3 weeks (lanes 6–8) postelectroporation. Lanes: 1, $\phi$X phage DNA *Hae* III digest; 2, CUMC1-1A extract; 3 and 6, with virus and electroporation; 4 and 7, with virus and without electroporation; 5 and 8, without virus and with electroporation.

tion in order for the PCR-based assay to detect virus. Replicase incompetent virus generated from UV irradiation, when introduced, could not be detected when exposed to cells and an electric field. It is not surprising that replicase function would be the first function of the polymerase susceptible to UV irradiation because it is the most sensitive of the polymerase activities. We have reported previously that CsCl-purified virus is more labile than sucrose-purified virus and dsRNA synthesis—i.e., replicase function—was frequently undetectable (9). UV inactivation of polymerase functions has been described in a variety of viral systems. Recently, it has been used in combination with 8-azido-ATP to photoaffinity label rotovirus polymerase (19).

It is unlikely that this difference was a manifestation of UV damage done to the viral dsRNA genome that rendered it less accessible to the Moloney murine leukemia virus reverse transcriptase since there was little difference in the amount of DNA amplified after 1 hr from the two different sets of samples. Therefore, we conclude that most of the RNA detected after 24 hr was the *de novo* product of a functioning viral polymerase within the M4147 cells. Specifically, because the assay only detects the negative-sense RNA strand, the RNA detected after 24 hr must be the product of the replicase function of the LRV1-1 polymerase.

It was clear that after 4 days the levels of LRV1-1 RNA in LRV1-1 electroporated cells were just at the limit of detection of our assay. In some experiments, very little LRV1-1-specific DNA was amplified from extracts made after 4 days (Fig. 1*A*; unpublished data), while in other experiments we failed to amplify any LRV1-1-specific RNA at all at that time point (Fig. 3*B*). It is still possible that a small fraction of the LRV1-1 electroporated cells were stably infected with this virus.

Similarly, when electroporation was performed into host cells not previously carrying a virus, we found that sucrose-purified virus could be transferred intact and negative-strand RNA could be detected. The fundamental differences are that the virus apparently can infect without exposure to an electric field and persists longer. Presumably, it may persist longer because there is no competition for host resources or activation of factors to prevent infection may take longer than activation of factors to inhibit superinfection. It is possible that antiviral activities are already functioning in persistently infected cells.

The fact that uninfected cells do not require electroporation for infection is quite interesting. Our initial hypothesis is that there are two modes of entry—one in cells previously infected and one in uninfected cells. Persistent viral infection is well known to modulate gene expression in animal cells. The possibility exists that one form of entry, perhaps receptor-mediated entry, could be eliminated in the presence of a persistent infection. In any case, this result can be pursued further by examining the role viral infection plays in gene regulation of *Leishmania*.

One of the perplexing problems that remains to be addressed is why the virus cannot persist in the new host. Whether in an uninfected strain or in an infected strain, transferring a stable persistent infection is not possible. Irrespective of why exogenous virus does not persist in its new host, these studies provide a tool to analyze viral infection in parasites. The role these viruses play in pathogenicity, or disease, remains to be determined; however, the successful transfer of virus opens additional avenues of investigation.

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