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A Subset of Viral Transcripts Packaged Within Human Cytomegalovirus Particles

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A human cytomegalovirus gene array was used to identify a previously unidentified class of viral transcripts. These transcripts, termed virion RNAs, were packaged within infectious virions and were delivered to the host cell on infection. This mechanism of herpesvirus gene expression allows for viral genes to be expressed within an infected cell immediately after virus entry and in the absence of transcription from the viral genome.

Human cytomegalovirus (HCMV), like all herpesviruses, regulates its gene expression in a coordinated cascade (*1*). HCMV genes are classified as immediate early, early, or late, depending on their kinetics of expression and the conditions under which they are transcribed. Regardless of the class of the gene, it has been assumed that all HCMV RNAs present in the infected cell are transcribed from the DNA genome of the infecting virus. Recently, it was noted that RNA copurified with virions (*2*). Using gene array technology, we identified a subset of HCMV transcripts that were packaged within HCMV virus particles and delivered to the host cell upon infection. Delivery of virion RNAs to the host cell allows for their expression immediately after virus entry in the absence of new transcription.

Specific RNAs in virus particles were screened for with an HCMV gene array. The array of polymerase chain reaction (PCR)–amplified fragments includes DNAs corresponding to all 208 open reading frames (ORFs) in the HCMV AD169 genome (*3*, *4*), making it possible to assay most HCMV transcripts in a single experiment. Initially, the arrays were validated by hybridization to radioactive probes prepared by random priming of cosmids, whose inserts collectively included about 90% of the HCMV genome (Fig. 1A). The amount of probe hybridizing to each spot on the array was about the same, confirming that the spots contained similar amounts of target DNA, and the hybridization of each probe was restricted to its cognate set of target DNAs, verifying the specificity of the assay.

To probe the identity of RNA that copurified with virions, we generated radioactively labeled cDNA by reverse transcription of RNA isolated from virus particles and used it to probe the HCMV gene array (*5*). HCMV RNAs corresponding to ORFs UL21.5, UL106-109, TRL/IRL 2-5, TRL/IRL 7, and TRL/IRL 13 were detected (Fig. 1B). UL21.5, originally termed R28070 (*6*), is a late mRNA (*6*, *7*), UL106-109 corresponds to a 5-kb immediate early RNA (*8*), TRL/IRL 2-5 is a 2.7-kb early RNA (*9*, *10*), and TRL/IRL 7 is a 1.2-kb early RNA (*9*, *10*). Analysis of RNA from infected cells confirmed the sizes and accumulation patterns of these RNAs (Fig. 2A, lanes 1 to 4). All of the RNAs accumulated to maximal levels late after infection, when virions are being assembled. The functions of the proteins encoded by these RNAs are unknown.

To confirm the results obtained with the HCMV gene array and to verify that RNA was detected rather than contaminating DNA, we performed a Northern blot assay on RNA from virus particles. Particles were purified and treated with ribonuclease one (RNase One), and deoxyribonuclease I (DNase I)–treated RNA was isolated. Equal portions of virion RNA were either mock-treated or treated with RNase One and then analyzed by Northern blot assay with probes for the RNAs identified in Fig. 1B. The samples were sensitive to RNase One (Fig. 2A, lanes 6 and 7), and the virion RNAs migrated identically to polyadenylated RNAs isolated from infected cells (Fig. 2A, lanes 6 and 8). These observations, together with the fact that the probes were made by reverse transcription with an

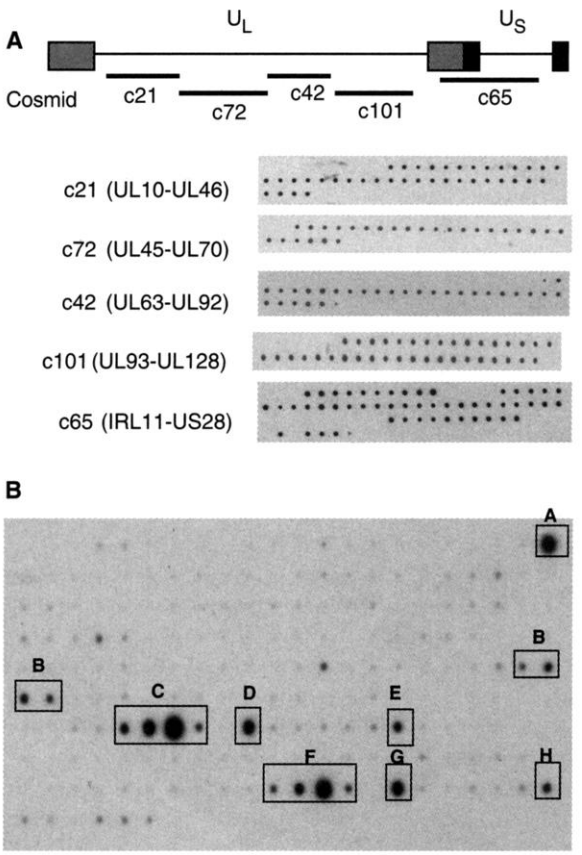

Fig. 1. Characterization of an HCMV DNA array and identification of HCMV transcripts packaged within virus particles. (**A**) HCMV gene arrays (*3*) were probed with individual [^{32}P]-labeled cosmids to confirm specificity of the ORFs spotted on the arrays. (**B**) An HCMV gene array was probed with [^{32}P]-labeled cDNA generated by reverse transcription of DNase I–treated RNA isolated from HCMV particles. The transcripts identified correspond to the predicted ORFs UL21.5 (A), UL106-109 (B), IRL/TRL 2-5 (C and F), IRL/TRL 4 (D and G), and IRL/TRL13 (E and H). Lighter spots observed in the array that are not marked by boxes were not consistently positive in repeated experiments.

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oligo(dT) primer, argue that the gene array detected polyadenylated mRNAs.

Although the results reveal the presence of viral mRNAs within RNase One–resistant particles, they do not show that they are packaged within infectious virions. The mRNAs could be packaged within noninfectious enveloped particles or dense bodies, both of which contaminate partially purified HCMV virion preparations (*11*–*13*), or within a particle that has not been previously described. To verify that viral mRNAs are packaged within infectious virions, we highly purified HCMV particles (*14*). The final step of the purification procedure, centrifugation to equilibrium in a CsCl gradient, produced a single band of particles. Particles were collected from the CsCl gradient, as were equal volumes of gradient material located immediately above and below the particles. The three fractions were then assayed for the presence of viral mRNAs, the presence of the virion protein pp65, and infectivity. UL21.5, UL109, IRL 4, and IRL 7 mRNAs were found to be present within the virion fraction when assayed by reverse transcription PCR (Fig. 2B, lane 3). These mRNAs were not detected in fractions above or below the virion band (Fig. 2B, lanes 1 and 5) or in RNA samples prepared in the absence of reverse transcription (Fig. 2B, lanes 2, 4, and 6). Similarly, the virion protein pp65 (Fig. 2C) and infectivity (*15*) were detected only in the virion fraction.

If the mRNAs are contained inside the virion envelope, they should be delivered to the cell when the viral envelope fuses with the plasma membrane at the start of infection. To ascertain whether the virion mRNAs become cell associated, we infected cells at a multiplicity of 10 plaque-forming units (PFU)/ml either in the presence or absence of actinomycin D. The drug was added to ensure that virus-specific RNA detected in the experiment was delivered by the virus particle and was not the result of new transcription. Cells were harvested at 1.5 hours after infection, RNA was isolated, and samples were assayed by Northern blot. Equal amounts of UL21.5, TRL/IRL 4, and TRL/IRL 7 mRNAs were detected in the presence or absence of actinomycin D (Fig. 3A) and identical results were obtained for UL109 and TRL/IRL 13 (*15*). These results showed that the RNA was delivered by the virus particle and was not the result of new transcription. To confirm that actinomycin D blocked transcription, we also probed blots for the HCMV UL122 and UL123 immediate early mRNAs. As expected, these mRNAs did not accumulate in the presence of the drug (Fig. 3A), as neither transcript was packaged within HCMV virions. The presence of UL83 mRNA was assayed at 1.5 hours after infection. This is a late mRNA that accumulates to high levels at the time when virus particles are assembled. This RNA was not detected within newly infected cells in either the presence or absence of actinomycin D (Fig. 3A), reinforcing the view that a subset of viral RNAs is selected for packaging. The pp65 virion protein had reached the nucleus by 1.5 hours after infection (Fig. 3B), showing that virion constituents had entered the cells in the presence of actinomycin D.

The UL21.5 virion RNA encodes for a 103–amino acid protein that contains a signal sequence (*6*, *16*). The inclusion of UL21.5 RNA in virions would enable its encoded protein to be cotranslationally inserted into the endoplasmic reticulum. This localization probably would not be achieved if the protein, rather than the RNA, was delivered to cells by virions. To test whether UL21.5 protein is targeted to the endoplasmic reticulum and Golgi network, we infected cells with a recombinant adenovirus encoding UL21.5 with the yellow fluorescent protein (YFP) fused at its COOH-terminus and assayed them for YFP fluorescence (*17*). The fusion protein colocalized with the p115 Golgi membrane marker (*18*) to the perinuclear region (Fig. 3C). The colocalization pattern also was observed in HCMV-infected cells with a UL21.5 monoclonal antibody (*15*). Thus, the virion UL21.5 RNA encodes a protein with a leader sequence that localizes to the Golgi network.

The late UL21.5 mRNA is delivered from the virion to the cell during the immediate early phase of infection. Consequently, it should be possible to detect newly synthesized UL21.5 protein at this time. As UL21.5 protein, like its cognate mRNA, is packaged within virions and delivered to infected cells (*15*), metabolic labeling was used to detect the synthesis of UL21.5 protein during the immediate early phase of infection. As mature UL21.5 protein contains no methionine or cysteine residues, a recombinant HCMV virus that expresses a UL21.5-YFP fusion protein was constructed (*19*). The fusion protein contains multiple methionine and cysteine residues within its YFP domain, allowing it to be labeled with [^{35}S] containing amino acids. The recombinant virus packaged the UL21.5-YFP mRNA, and the hybrid RNA was detected in infected cells by 1 hour, well before detectable expression of the most abundant immediate early mRNA (IE1) (Fig. 3D). Furthermore, the steady state level of UL21.5-YFP mRNA was substantially decreased by 4 hours after infection, and this result was in contrast to the immediate early mRNA, whose level was increased at this time (Fig. 3D). To detect

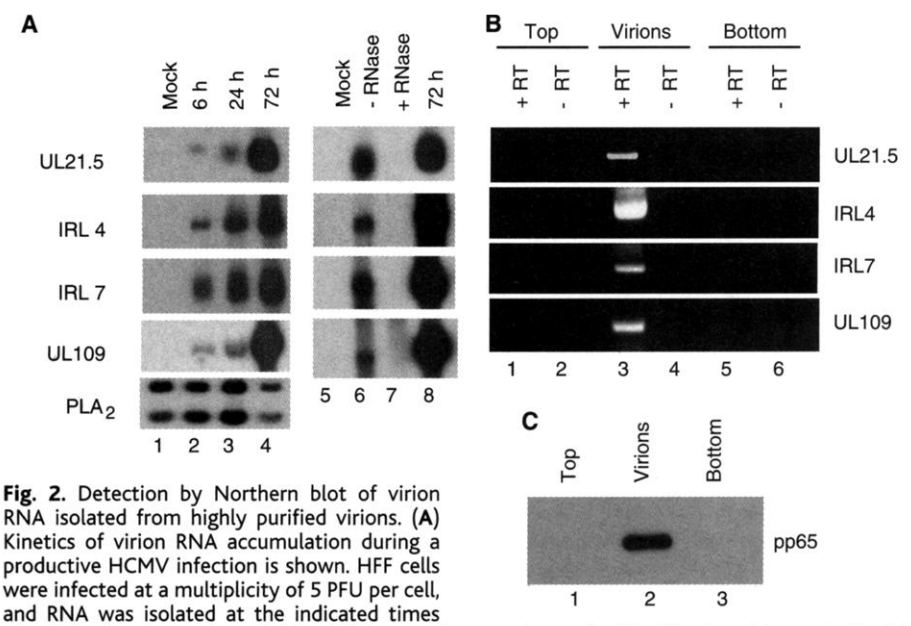

Fig. 2. Detection by Northern blot of virion RNA isolated from highly purified virions. (**A**) Kinetics of virion RNA accumulation during a productive HCMV infection is shown. HFF cells were infected at a multiplicity of 5 PFU per cell, and RNA was isolated at the indicated times after infection. Expression of virion RNA transcripts was determined by Northern blot analysis with [^{32}P]-labeled probes corresponding to the indicated ORFs. RNA (3 μg) isolated from RNase-treated HCMV particles was either treated (+RNase) or mock-treated (−RNase) with RNase One, and samples were analyzed by Northern blot with labeled probes corresponding to the indicated ORFs. As a positive control, 300 ng of RNA isolated from cells at 72 hours after infection was included on the blot. Membranes were also probed for phospholipase A_2 (PLA_2) RNA, which served as a loading control. (**B**) RNA isolated from the virion fraction (Virions), from the region of the gradient above the virions (Top), or from the region of the CsCl gradient below the virion fraction (Bottom) was used in reverse transcription reactions to generate cDNA. cDNA (+RT) or RNA (−RT) was used in PCR reactions to detect viral transcripts with specific primers for each transcript. (**C**) An equal volume of each fraction (Top, Virions, and Bottom) was probed for the HCMV virion protein pp65 by Western blot.

newly synthesized UL21.5-YFP protein, we infected cells with the recombinant virus, labeled them from 0 to 3 hours after infection, and prepared lysates and subjected them to immunoprecipitation using antibody to YFP or the UL21.5 protein. As shown in Fig. 3E, newly synthesized UL21.5-YFP protein was synthesized during the first 3 hours of infection.

Virus-coded mRNAs are present in preparations of highly purified, infectious HCMV particles (Fig. 2B). Association of mRNAs with virions is not random. Rather, a specific subset of mRNAs with no apparent sequence similarity among them is present (Figs. 1B and 3A). How is a specific subset of mRNAs selected for inclusion in virus particles? It is possible that mRNAs are delivered to virions by one or more RNA-binding proteins as the proteins themselves are incorporated. Although we are not aware of reports describing RNA-binding proteins in HCMV virions, the functions of most proteins in viral particles remain incompletely described. Furthermore, a virion RNA-binding protein, US11 (*20*, *21*), has been described in another herpesvirus, herpes simplex virus type 1.

Virion mRNAs appear to express a defined subset of viral functions very early after infection, before the viral genome becomes transcriptionally active. This is a critical stage of infection when the virus will commit to active replication or latency and, at least in the case of replication, when active regulatory systems are established that will control subsequent events in the infected cell. In the case of UL21.5, the inclusion of an mRNA rather than proteins makes it possible to synthesize a protein that is localized to the endoplasmic reticulum and Golgi network. Incorporation of the mature protein lacking its signal sequence into virions would likely preclude its appropriate localization. It is also possible that one or more of the virion mRNAs serves a non–protein-coding function. A noncoding function could be performed within the newly infected cell, as is the case for adenovirus VA RNAs that antagonize the antiviral activity of interferons (*22*). Alternatively, the mRNAs could serve a structural role, perhaps contributing to the assembly and organization of the set of about 25 proteins localized to the tegument domain of the virion.

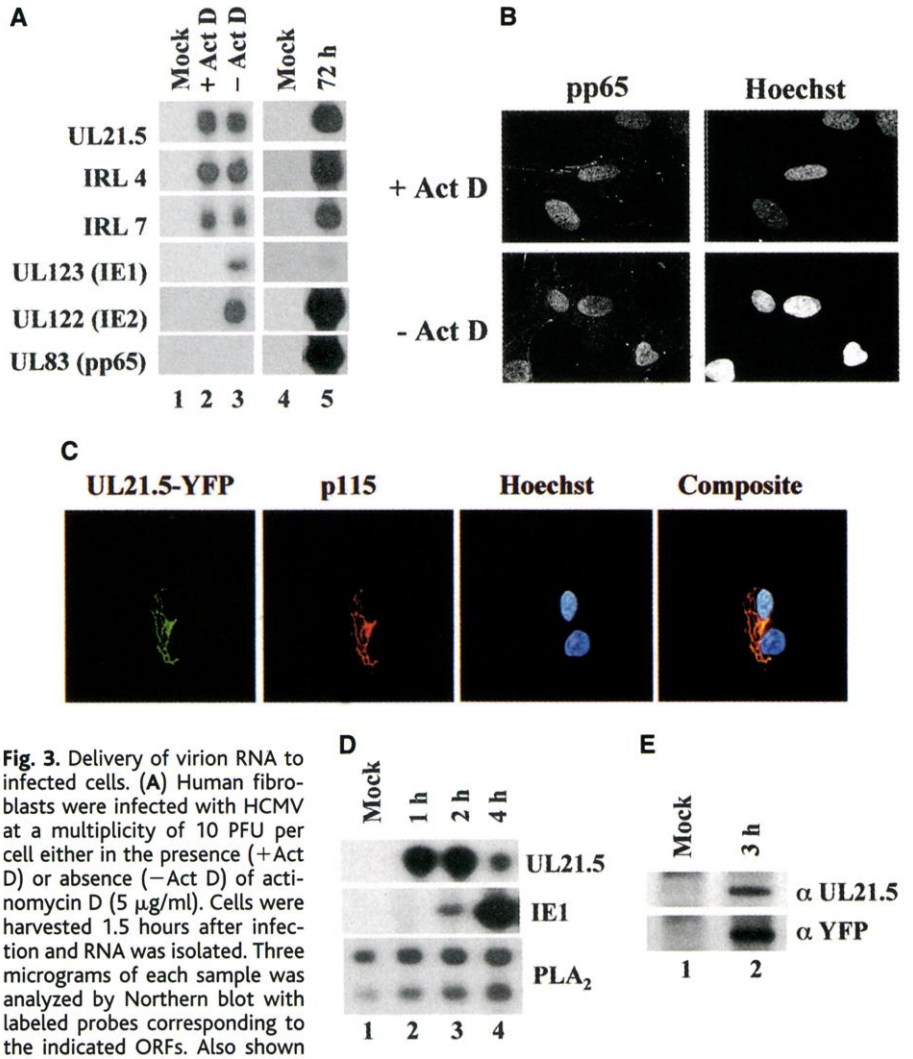

Fig. 3. Delivery of virion RNA to infected cells. (**A**) Human fibroblasts were infected with HCMV at a multiplicity of 10 PFU per cell either in the presence (+Act D) or absence (−Act D) of actinomycin D (5 μg/ml). Cells were harvested 1.5 hours after infection and RNA was isolated. Three micrograms of each sample was analyzed by Northern blot with labeled probes corresponding to the indicated ORFs. Also shown are RNA levels from either mock-infected cells (lane 4) or from cells infected with HCMV for 72 hours (lane 5). (**B**) Human fibroblasts cultured on cover slips were infected with HCMV at a multiplicity of 10 PFU per cell, either in the presence (+Act D) or absence (−Act D) of actinomycin D (5 μg/ml). Cells were fixed at 1.5 hours after infection and stained for the HCMV virion protein pp65 and cellular DNA (Hoechst). (**C**) Subcellular localization of UL21.5. Human fibroblasts cultured on cover slips were infected with a recombinant adenovirus (*17*) expressing a UL21.5-YFP fusion protein (green). Twenty-four hours after infection, cells were fixed in 4.0% paraformaldehyde and stained for a Golgi marker p115 (red) and cellular DNA (blue). (**D**) Detection of the late UL21.5-YFP RNA during the immediate early phase of infection. Human fibroblasts were infected at a multiplicity of 10 PFU per cell with a recombinant HCMV virus (AD169UL21.5-YFP) expressing a UL21.5-YFP fusion protein in place of the wild-type UL21.5 (*19*). Cells were harvested at the indicated times after infection and RNA was isolated. Three micrograms of each sample was analyzed for UL21.5 and IE1 expression by Northern blot. Membranes were also probed for PLA_2 expression to serve as a loading control. (**E**) Detection of newly synthesized UL21.5 protein during the immediate early phase of infection. Human fibroblasts were infected at a multiplicity of 10 PFU per cell with AD169UL21.5-YFP (*19*) in the presence of [^{35}S]methionine and cysteine. Cells were labeled for 3 hours and lysates were collected. Immunoprecitipations were performed with monoclonal antibodies directed against UL21.5 or YFP (Clontech, Palo Alto, California). Precipitates were separated by electrophoresis, and proteins were visualized by autoradiography.

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Malaria Parasite Development in a *Drosophila* Model

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Malaria is a devastating public health menace, killing over one million people every year and infecting about half a billion. Here it is shown that the protozoan *Plasmodium gallinaceum*, a close relative of the human malaria parasite *Plasmodium falciparum*, can develop in the fruit fly *Drosophila melanogaster*. *Plasmodium gallinaceum* ookinetes injected into the fly developed into sporozoites infectious to the vertebrate host with similar kinetics as seen in the mosquito host *Aedes aegypti*. In the fly, a component of the insect's innate immune system, the macrophage, can destroy *Plasmodia*. These experiments suggest that *Drosophila* can be used as a surrogate mosquito for defining the genetic pathways involved in both vector competence and part of the parasite sexual cycle.

Malaria is transmitted from one vertebrate host to another by a mosquito vector. Parasites of the genus *Plasmodium* undergo the sexual portion of their life cycle in a mosquito. The sequence begins when a mosquito, after biting a vertebrate, ingests a gametocyte-infected blood meal and ends with a second blood meal during which sporozoites are passed with the mosquito's saliva into a new vertebrate host (*1*, *2*). The parasites must overcome a number of barriers to develop in a mosquito. Immediately after ingestion, gametocytes emerge from red blood cells and differentiate into male and female gametes. These gametes are fertilized and the resulting zygotes develop into ookinetes within the gut. The ookinetes enter the body cavity (hemocoel) by crossing the gut lining (peritrophic matrix) and the midgut epithelium, where they subsequently lodge on the hemocoel side of the gut epithelium, beneath the basal lamina. The ookinetes then develop into oocysts. Oocysts undergo many rounds of nuclear division and produce thousands of sporozoites that are released into the hemocoel. Sporozoites invade the salivary glands to be injected into a vertebrate host about 2 weeks after the mosquito ingested the first infected blood meal.

The fruit fly *Drosophila melanogaster* has the potential to facilitate experimental analysis of insect stages of the parasite life cycle because the fly is genetically well defined and more easily manipulated than any mosquito. Though mosquitoes and fruit flies have obviously different lifestyles, they are members of the same phylogenetic order and, thus, are expected to share similarities in their physiology. To test whether *Plasmodia* could develop in the fruit fly, we attempted to reproduce *Plasmodium* sexual development in *Drosophila*. Fruit flies were fed blood infected with *Plasmodium gallinaceum*, which causes malaria in chickens. The flies ingested the blood meal, as evidenced by the blood visible in their abdomens, but the parasites were quickly cleared, as shown by the disappearance of *Plasmodium*-specific ribosomal RNA (rRNA) from the flies (Fig. 1). We wondered whether the parasite's failure to develop was caused by an inability of the fly to support *Plasmodium* gametogenesis. We therefore fed ookinetes to *Drosophila*. Ookinetes also failed to establish an infection when fed to the flies (Fig. 1). Again, the *Plasmodium* rRNA could no longer be detected in the parasite-fed flies, suggesting that the parasites were passed in the feces or digested.

The failure of *Plasmodia* ookinetes fed to *Drosophila* to establish an infection is likely to be a consequence of their inability to cross the midgut-associated barriers of the fly. To test this hypothesis, we prepared *P. gallinaceum* ookinetes in vitro from gametocytes obtained from infected chicken blood (*3*) and injected these directly into the hemocoel of adult flies. Under these conditions, *P. gallinaceum* rRNA could be detected in flies 9 days after injection, suggesting that the parasite survived in the fly hemocoel (Fig. 1).

To determine whether the appearance of *Plasmodium* rRNA was due to the development of the parasites or to the presence of arrested ookinetes, we injected ookinetes into flies and into the laboratory host mosquito, *Aedes aegypti*, and compared the parasites by microscopy. We observed *Plasmodium* oocysts in flies 9 days after injection of ookinetes, and the resulting oocysts were similar to those found in *Ae. aegypti* infected by a blood meal (Fig. 2, A, B, C, and D). The *Drosophila* oocysts were found attached to a variety of tissues, including the gut, salivary glands, and fat body. Sporozoites were detected at about the same time in the two hosts (9 days in flies; 11 days in mosquitoes) and were morphologically similar (Fig. 2, E and F). Mosquitoes produced seven times as many sporozoites as

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