

Human Cytomegalovirus Latency-Associated Protein pORF94 Is Dispensable for Productive and Latent Infection

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Human cytomegalovirus latency in bone marrow-derived myeloid progenitors is characterized by the presence of latency-associated transcripts encoded in the *ie1/ie2* region of the viral genome. To assess the role of ORF94 (UL126a), a conserved open reading frame on these transcripts, a recombinant virus (RC2710) unable to express this gene was constructed. This virus replicated at wild-type levels and expressed productive as well as latency-associated *ie1/ie2* region transcripts. During latency in granulocyte-macrophage progenitors, RC2710 DNA was detected at levels indistinguishable from wild-type virus, latent-phase transcription was present, and RC2710 reactivated when latently infected cells were cocultured with permissive fibroblasts. These data suggest pORF94 is not required for either productive or latent infection as assayed in cultured cells despite being the only known nuclear latency-associated protein.

Human cytomegalovirus (CMV), the prototype member of the betaherpesviruses, is a ubiquitous virus that infects a majority of the population (3, 16). CMV is a species-specific pathogen whose replication or latency cannot be studied in a laboratory animal model. Despite this limitation, evidence for a true latent phase has accumulated during the past decade to show that the genome remains in a nonproductive state (1, 7, 9, 11, 14, 15, 21, 24, 25, 28–30) that can be reactivated from experimental (7, 9) and natural (22) latency. CMV latency (20) is associated with both peripheral blood mononuclear cells (1, 22, 24, 25, 28–30) and myelomonocytic lineage granulocyte-macrophage progenitors (GM-PS) (7, 9, 11, 14, 15, 21). Viral DNA is carried as unit-length circles (2) and is present in naturally or experimentally infected cells at similar low-copy-number levels (9, 21). CMV latency-associated transcripts (CLTs) mapping to both DNA strands in the *ie1/ie2* region of the genome are present in experimentally infected GM-PS as well as in bone marrow and granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells from naturally infected adults (7, 11, 21). Sense CLTs are expressed in 2 to 5% of experimentally infected GM-PS (21), but their expression has not been fully evaluated in naturally infected individuals (7). CLTs contain a number of conserved open reading frames (ORFs), including ORF94, whose protein products elicit a serum immune response in naturally infected individuals (11, 12); however, their functions are unknown.

ORF94 (UL126a), the largest of the ORFs encoded by sense CLTs (11), was investigated as a candidate for a role in CMV productive or latent infection. ORF94 consists of the amino-terminal 59 codons of UL126 (5) and an additional 45 codons from exons 2 and 3 of the *ie1/ie2* region, albeit in a reading frame different from that used to encode IE1 and IE2 (10, 11). Of the six latency-associated ORFs, only pORF94 is nuclear when expressed in mammalian cells (K. L. White, J. Xu, and E. S. Mocarski, unpublished results), suggesting a role for

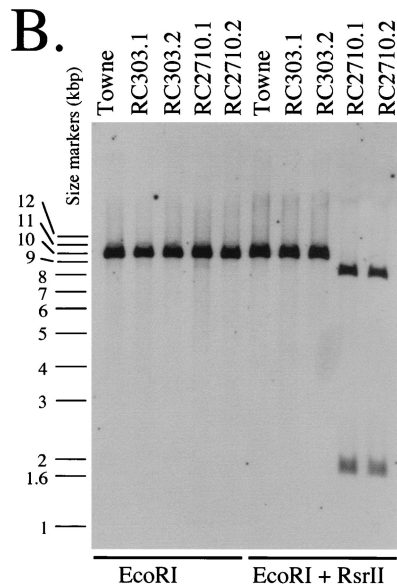
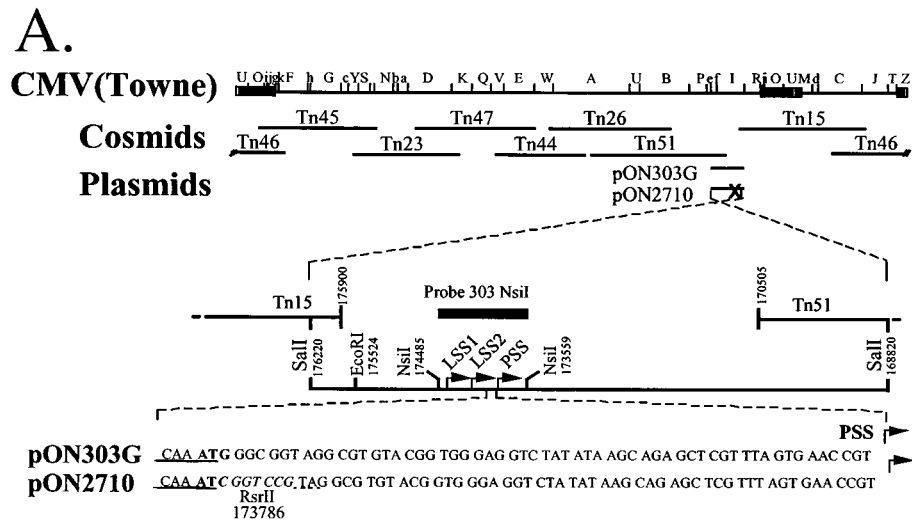
pORF94 in gene regulation, viral genome maintenance, or reactivation.

To address the function of pORF94, we constructed two viruses, a mutant virus (RC2710) and a wild-type control (RC303), and evaluated the biological impact on productive infection in human foreskin fibroblasts (HFFs) and latent infection in GM-PS. The *ie1/ie2* region is transcriptionally complex (27), encoding the major immediate-early transactivators IE1_{491aa} and IE2_{579aa} from a productive-phase start site (PSS) (6, 13, 17, 18, 26), and sense CLTs from two latent-phase start sites (LSS1 and LSS2 [Fig. 1A]) (11). ORF94 overlaps with the CAAT and TATA box elements of the productive-phase promoter such that mutations in this ORF might affect PSS-initiated *ie1/ie2* gene expression (Fig. 1A). Therefore, the ORF94 start codon was mutated by introduction of C to create a +1 frameshift placing a stop codon in frame while at the same time introducing a diagnostic *RsrII* restriction enzyme site, as diagrammed in Fig. 1A. This mutation destroys the only methionine codon available to translate ORF94. pON2710 contains the mutated ORF94 and was constructed by oligonucleotide-directed PCR mutagenesis performed on pON303G (23) as the DNA template with overlapping primers containing the mutation paired with primers outside this region. Primer set 1 (303F [5' CGCCGATAGAGGCGACAT 3'] and *RsrIIR* [5' CGGACCGATTTCGCTCAACGGG 3']) and primer set 2 (303R [5' CCATCACCTATAACATGAGGAAGCG 3'] and *RsrIIF* [5' CGGTCCGTAGGCGTGTACGGTG 3']) were used with the following PCR conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 67°C for 1 min, and 72°C for 2 min, with a final 10-min extension at 72°C. PCR products were cloned into pGEM-T (Promega), joined at the *RsrII* site using *RsrII* and *PstI*, and transferred back into pON303G using *NsiI* to create pON2710. CMV recombinants were constructed (8), using the overlapping cosmids Tn15, Tn23, Tn26, Tn44, Tn45, Tn46, Tn47, and Tn51 and plasmid pON303G or pON2710 (Fig. 1A), to create two independent isolates of each virus, pORF94 mutant (RC2710.1 and RC2710.2) and wild-type (RC303.1 and RC303.2), that were plaque purified before being subjected to further analyses.

The structure of the recombinant viruses was confirmed by DNA blot hybridization analysis to ensure the presence of the ORF94 mutations and restriction enzyme digestion analysis to

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ensure that the genomes were intact. Cosmid-derived viruses were compared to Towne by digestion with *EcoRI* or *EcoRI* plus *RsrII* and hybridization with an ORF94 probe (886-bp *NsiI* fragment from pON303G). As expected, this probe detected a 10-kbp fragment in RC2710 DNA digested with *EcoRI* and two species of 8.3 and 1.7 kbp in RC2710 DNA digested with *EcoRI* plus *RsrII* (Fig. 1B), confirming the introduction of the *RsrII* site in the mutant. Wild-type viral DNA (Towne, RC303.1, and RC303.2) hybridized as expected to a 10-kbp band that was not susceptible to *RsrII* digestion. All viral DNAs were found to generate identical *EcoRI*, *BamHI*, *HindIII*, and *XbaI* fragment patterns analyzed by agarose gel electrophoresis (data not shown), except for the previously demonstrated heterogeneity bordering oriLyt contained within the *EcoRI* E fragment (8). This heterogeneity was derived from different origin structures contributed by either oriLyt-containing cosmid Tn44 or Tn47 and is known to not impact replication efficiency in cosmid-generated viruses (8). RC2710.2 and RC303.1 contained the smaller *EcoRI* E fragment, while RC2710.1 and RC303.2 contained the larger fragment.

FIG. 1. Recombinant virus construction and structure. (A) The top line shows an *EcoRI* restriction map of the CMV (Towne) genome, with the eight cosmids and the plasmids pON303G and pON2710 used to generate recombinant CMV depicted below. The mutation in pON2710 is marked "X." The expanded pON303G/pON2710 region shows the *Sall* fragment of the plasmid endpoints and overlap with adjacent cosmids (Tn15 and Tn51), including relevant restriction sites, and the probe (probe 303 NsiI; filled bar) used in the analysis. The bottom shows nucleotide sequence from the CAAT box (underlined) to PSS sequences of the wild-type (pON303G) and mutant (pON2710), with the *RsrII* site introduced at nt 173786 of the viral genome into pON2710 shown in italics, the pORF94 start codon ATG or mutant ATC in boldface, and the introduced stop codon underlined. (B) Autoradiogram of *EcoRI* (left)- or *EcoRI* plus *RsrII* (right)-digested viral DNA from parental CMV (Towne), two independent isolates of cosmid-derived Towne (RC303.1 and RC303.2), and two independent isolates of cosmid-derived pORF94 mutant (RC2710.1 and RC2710.2), hybridized with the 303 NsiI probe that had been gel purified and random primed with digoxigenin (Boehringer Mannheim) (19). Size markers are indicated to the left.

To determine whether pORF94 altered replication during productive infection, a multiple-step growth analysis of mutant and wild-type virus was performed as described elsewhere (17). HFFs were infected in duplicate at a multiplicity of infection (MOI) of 0.02, and at 1, 3, 5, 7, 9, and 12 days postinfection, cells and supernatant were collected for plaque assay (Fig. 2). RC2710.1 and RC303.1 replicated with similar kinetics and to similar peak titers in HFFs, demonstrating that pORF94 was dispensable for CMV productive replication.

To establish whether the mutations introduced into ORF94 influenced promoter elements controlling the expression of *ie1/ie2* PSS transcripts, we performed RNase protection assays on steady-state RNA from Towne-, RC2710-, and RC303-infected cells (11). Total RNA from HFFs infected at an MOI of 3 harvested at 8 h postinfection (hpi) was evaluated using a probe complementary to nucleotides (nt) -218 to +120, relative to the PSS derived from pON2233 (11), in a pGEM-T-Easy vector. RNA from cells infected with Towne, RC303, or RC2710 protected a 120-nt band (Fig. 3A, upper panel). In contrast, RNA from mock-infected cells was not protected from digestion. All samples contained similar amounts of RNA based on quantification by PhosphorImager analysis of actin transcripts (Fig. 3A, lower panel). PhosphorImager analysis of protected species showed that the levels of RNA initiating from the PSS were the same in both recombinant viruses and only slightly (twofold) higher in Towne-infected cells, indicating that transcription was not significantly altered by the introduced mutations.

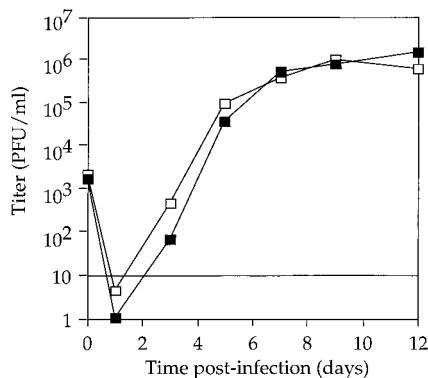


FIG. 2. Growth curves of RC303.1 (filled squares) and RC2710.1 (open squares) following infection of HFFs at an MOI of 0.02 (input virus was plotted at day 0). Cells and media were collected at the indicated time points, and virus yield was measured in duplicate by plaque assay with both values falling within the symbol shown. The detection limit (horizontal line) was 10 PFU/ml.

In addition to the expected 120-nt species in all virus-infected cells, a 338-nt protected band was present, corresponding to RNA originating from promoters upstream of nt -218. Transcripts originating upstream of the PSS have consistently been observed by reverse transcription-PCR (RT-PCR) starting at early times of productive infection (G. Hahn, K. L. White, and E. S. Mocarski, unpublished results) and could originate from LSS1 and LSS2. The RC2710 RNA also protected species of 150 to 170 nt, corresponding to predicted 156- and 172-nt species generated at the sites of mismatched base pairs due to the introduced ORF94 mutations and the wild-

type probe. To further investigate the origin of the upstream transcripts, we performed RNase protection with a larger, 694-nt riboprobe complementary to nt -453 to +120 relative to the PSS, spanning LSS1 (-356) and LSS2 (-292). Following hybridization of total RNA collected at 8 hpi (data not shown) or 48 hpi (Fig. 3B) from HFFs infected at an MOI of 1 with Towne, RC303, or RC2710, and digestion with RNases, we detected 120-nt PSS species and 450-nt protected products specific to all virus-infected HFFs. The size of the 450-nt band was consistent with an expected 476-nt transcript originating from LSS1. Attempts to detect sense CLTs in productively infected HFFs at very early times after infection (2 and 4 hpi) had been uniformly negative (10, 11). However, our data suggest that LSS1 is active at early (8 hpi) and late (48 hpi) times during infection of HFFs and that levels are 20- to 50-fold below PSS levels at late times. Additional analysis of this region has suggested that LSS2 is also active in HFFs infected with Towne (data not shown). Further information derived from rapid amplification of 5' cDNA ends has more accurately mapped LSS1 and LSS2 usage in HFFs (J. M. Lunetta and J. A. Wiedeman, personal communication). No differences were seen in expression of any *ie1/ie2* region transcripts arising upstream of PSS in mutant or wild-type infection.

Two additional bands of 170 and 300 nt were protected by RC2710 RNA (Fig. 3B, upper panel). The sizes of these protected products corresponded to digestion at the mutation resulting in separate 3' (172-nt band) and 5' (299-nt band) portions from LSS1 transcripts. The results of this RNase protection assay demonstrated that the LSS1 transcript levels in Towne and RC303 RNA were similar to the combined LSS1-specific signals of the RC2710 RNA. We conclude that latent start sites are active in productively infected HFFs and are

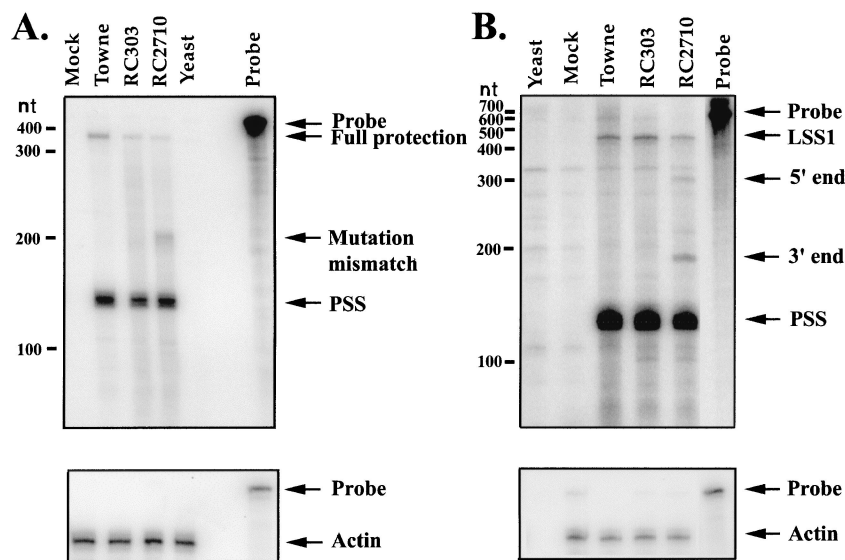


FIG. 3. RNase protection of PSS and LSS in HFFs. (A) PhosphorImage depicting RNase protection of PSS-initiated transcripts in total cellular RNA at 8 hpi at an MOI of 3 (upper panel). The panel shows uninfected cell RNA (Mock), three lanes with CMV-infected cell RNA (Towne, RC303, and RC2710), yeast control RNA (Yeast), and the undigested probe (Probe). Total RNA (5 μ g) was incubated with riboprobe complementary to sequences from nt -218 to +120 relative to PSS. On the right, the 420-nt undigested probe (Probe), 338-nt fully protected band (Full protection), 156- to 172-nt species generated by the presence of the mutations (Mutation mismatch), and the 120-nt PSS protected species (PSS) are shown. Actin control RNase protection (lower panel) with a 334-nt probe (Probe) generated a 245-nt protected band (Actin) used as a loading control. (B) PhosphorImage depicting RNase protection of LSS- and PSS-initiated transcripts in total cellular RNA at 48 hpi at an MOI of 1 (upper panel). The panel shows yeast control RNA (Yeast), uninfected cell RNA (Mock), three lanes with CMV-infected cell RNA (Towne, RC303, and RC2710), and the undigested probe (Probe). Total RNA (20 μ g) was incubated with riboprobe complementary to sequences from nt -453 to +120 relative to PSS. On the right, the 694-nt undigested probe (Probe), 476-nt LSS1 protected band (LSS1), 299-nt (5' end) and 172-nt (3' end) LSS1 protected bands resulting from digestion at the mutation sites, and the 120-nt PSS protected species (PSS) are shown. Actin control RNase protection (lower panel) with a 334-nt probe (Probe) generated a 245-nt band (Actin) used as a loading control. [γ - 32 P]ATP-end-labeled 100-bp ladder (GibcoBRL) was used as a size marker; intensities of bands were determined with the ImageQuant version 2.0 software (Molecular Dynamics), and volume quantitation reports were analyzed at exposures below saturation.

TABLE 1. PCR-ISH^a

Virus	PCR conditions	% CMV DNA-positive cells
RC303	+ <i>Taq</i> Pol	93
	- <i>Taq</i> Pol	<0.0005
RC2710	+ <i>Taq</i> Pol	95
	- <i>Taq</i> Pol	<0.0001

^a GM-P culture 6 was infected separately with RC303.2 and RC2710.2 and analyzed for the presence of CMV DNA by PCR-ISH after 3 weeks of infection.

weaker than the PSS. Disruption of pORF94 does not markedly alter their usage. For CMV, it is possible that a subset of HFFs mount a state of infection that resembles latency rather than the classically demonstrated productive infection. Transcription upstream of the PSS is present in 4% of HFFs at 76 hpi by in situ hybridization (B. Slobedman and E. S. Mocarski, unpublished results). Evaluation of productive- and latent-phase promoter elements in both hematopoietic progenitors and HFFs may provide further insight into regulation in this important region of the genome.

The growth properties of RC2710 suggest that ORF94 is dispensable for productive replication in HFFs. To test whether pORF94 influenced latent infection, we compared the properties of RC2710 and RC303 in GM-Ps, where CMV establishes a latent infection characterized by nuclear association of viral DNA without productive replication (9) and with the expression of transcripts, including sense CLTs predicted to encode pORF94 (11). RC2710- and RC303-infected GM-P cultures were therefore examined for the presence and duration of infectious virus in culture media as well as for presence and quantity of viral DNA in the infected cells. To evaluate establishment of latency in GM-Ps, cell-free virus was measured in culture supernatants by plaque assay on HFFs. For both RC2710 and RC303, input infectious virus was on the order of 10⁸ PFU/culture, decreased to between 5 and 100 PFU/ml at 1 week postinfection, and decreased to below the limit of detection of 1 PFU/ml by 2 weeks postinfection (data not shown). Overall, the total amount of virus detected in the supernatant of GM-P cultures and the rate of virus decline from the supernatant were similar for both viruses. The total number of suspension cells was also determined during passaging and remained equivalent between the cultures infected with RC2710 or RC303 throughout the 3 to 4 weeks of culture (data not shown).

TABLE 2. QC-PCR^a

GM-P culture no.	Day postinfection	CMV genome copy no./cell ^b	
		RC303	RC2710
20 ^c	18	0.2–0.8	0.2–0.8
22 ^c	17	2	2
25 ^d	19	1–3	0.8–2
40 ^d	16	1–4	1–4
45 ^d	15	8	20
46 ^d	18	8	8

^a GM-Ps latently infected separately with RC303 and RC2710 were analyzed for CMV DNA content by QC-PCR.

^b Determined by competition with threefold dilutions of competitor DNA; when competition between the competitor and genomic DNA spanned two dilutions, the data are represented as a range.

^c RC303.2 and RC2710.2 were used.

^d RC303.1 and RC2710.1 were used.

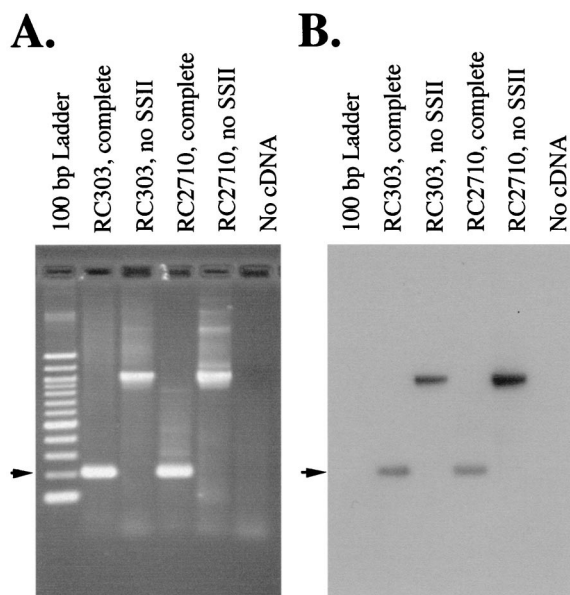


FIG. 4. Detection of sense CLTs by RT-PCR in GM-Ps. (A) Ethidium bromide-stained agarose gel following separation of PCR products amplified by IEP3D and IEP1K for 30 cycles and nested for 30 cycles using IEP2D and IEP1G. (B) Autoradiogram of DNA blot hybridization with α -³²P-end-labeled IEP1H after transfer to a nylon membrane. Lanes (left to right): 100-bp ladder, RC303 and RC2710 amplified with (complete) or without (no SSII) the addition of Superscript II, and a control containing no added cDNA (No cDNA). The spliced cDNA is amplified as a 206-bp product (arrow), and the DNA is amplified as a 1,032-bp product.

We determined the percentage of GM-Ps carrying viral DNA by PCR-driven in situ hybridization (PCR-ISH) (21) and quantity of viral DNA by quantitative-competitive PCR (QC-PCR) (9). PCR-ISH was performed on latently infected GM-Ps using primers IEP3A and IEP3B to amplify viral DNA by PCR prior to hybridization with a probe spanning exon 3 of the *ie1/ie2* region transcripts. As in previous studies (21), PCR amplification was required to detect latent viral DNA in GM-Ps infected with RC2710 or RC303, as samples remained negative when *Taq* DNA polymerase was omitted (Table 1). For samples that were amplified by PCR prior to hybridization, more than 93% of cells were positive for viral DNA regardless of whether the wild-type or mutant virus was used to infect the cultures (Table 1). To examine more precisely the levels of viral DNA in latently infected GM-Ps, we performed QC-PCR (9). The data shown in Table 2 compare RC303 and RC2710 genome copy numbers per cell from several GM-P cultures. These data demonstrate that during latency, the viral genome is maintained at between 0.2 to 20 copies per cell in individual GM-P cultures, with matched samples yielding very similar levels (Table 2). These values were within the range previously described for GM-P cultures and were similar to that detected during natural infection (9, 21). The PCR-ISH and the QC-PCR data together demonstrate that pORF94 is dispensable for establishment as well as maintenance of latency in GM-P cultures. These data suggest that ORF94 does not impact the initial steps of infection leading to establishment of latency in GM-Ps, as cultures infected with RC2710 and RC303 behaved similarly with regard to the amount of infectious virus present in culture media during the first 2 weeks of infection and the percentage of cells capable of supporting a latent infection.

Transcription of sense CLTs during latency is readily detected by RT-PCR above any DNA background (11). Although

viral DNA has been detected in essentially all cells in GM-P cultures (21), sense latent transcripts have been detected in only 2 to 5% of cells (11, 21). RC303- or RC2710-infected GM-P cultures were analyzed for expression of sense CLTs at 2 to 3 weeks postinfection by nested RT-PCR (10, 11) from poly(A)⁺ RNA isolated using the Microfast-Tract poly(A)⁺ isolation kit (Invitrogen) and 30 cycles of PCR for each round of amplification (Fig. 4). DNA blot hybridization with the internal oligonucleotide probe, IEP1M, demonstrates a 206-bp product consistent with the presence of sense CLTs during latent infection. A 1,032-bp species generated from viral DNA was the sole product detected when the reverse transcriptase Superscript II was omitted from the procedure. Four out of six RC303 and three out of six RC2710 cultures were positive, suggesting that pORF94 does not influence the expression of transcripts.

Reactivation of latent GM-PPs was performed as previously described (9) by cocultivation with uninfected HFFs for periods of up to 6 weeks. At the time of coculture, GM-PPs were qualified as latently infected by absence of infectious virus in supernatants and sonicated cells. Monolayers were examined weekly for cytopathic effect, and reactivated virus was observed with 3 to 6 weeks of cocultivation without any significant difference between RC303 and RC2710. These data suggest ORF94 is dispensable for reactivation, but further quantitative evaluation may reveal more subtle effects of ORF94 on reactivation.

pORF94 is an interesting, conserved, nuclear protein that is dispensable in assays for productive and latent infection in cultured cells. We have not consistently been able to detect pORF94 expression in either HFFs or GM-PPs, but the presence of the transcript in both of these cell types and the host humoral response to pORF94 after natural infection (12) argue that this protein is translated during infection but possibly at levels below detection or only under specific conditions. Over 60 CMV genes have been found to be dispensable for growth in cultured HFFs. In addition, the ability of both Toledo and Towne strains to latently infect GM-PPs suggests that the 11 ORFs that differ between these strains are dispensable for latency as well (4, 9, 11). Our work demonstrated that ORF94, a conserved gene on sense CLTs, is also dispensable for experimental latent infection. The large number of CMV genes that are dispensable in culture are nevertheless likely to play important roles in CMV biology. It appears that functions like pORF94 may be revealed only through studies in the natural host or in surrogate primate systems.

All experiments were performed by K.L.W. except for PCR-ISH, which was performed by B.S.; E.S.M. advised both K.L.W. and B.S.

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