JOURNAL OF VIROLOGY, Oct. 2007, p. 11267–11281 Vol. 81, No. 20
0022-538X/07/$08.00+0 doi:10.1128/JVI.00007-07
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Antisense Transcription in the Human Cytomegalovirus Transcriptome▽†

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Received 2 January 2007/Accepted 27 July 2007

Human cytomegalovirus (HCMV) infections are prevalent in human populations and can cause serious diseases, especially in those with compromised or immature immune systems. The HCMV genome of 230 kb is among the largest of the herpesvirus genomes. Although the entire sequence of the laboratory-adapted AD169 strain of HCMV has been available for 18 years, the precise number of viral genes is still in question. We undertook an analysis of the HCMV transcriptome as an approach to enumerate and analyze the gene products of HCMV. Transcripts of HCMV-infected fibroblasts were isolated at different times after infection and used to generate cDNA libraries representing different temporal classes of viral genes. cDNA clones harboring viral sequences were selected and subjected to sequence analysis. Of the 604 clones analyzed, 45% were derived from genomic regions predicted to be noncoding. Additionally, at least 55% of the cDNA clones in this study were completely or partially antisense to known or predicted HCMV genes. The remarkable accumulation of antisense transcripts during infection suggests that currently available genomic maps based on open-reading-frame and other in silico analyses may drastically underestimate the true complexity of viral gene products. These findings also raise the possibility that aspects of both the HCMV life cycle and genome organization are influenced by antisense transcription. Correspondingly, virus-derived noncoding and antisense transcripts may shed light on HCMV pathogenesis and may represent a new class of targets for antiviral therapies.

Human cytomegalovirus (HCMV) is classified within the *Betaherpesvirinae* subfamily, and most humans are infected with HCMV during their lifetime. Like other herpesviruses, HCMV cannot be completely eliminated by the immune system and remains either as a low-level persistent infection or in a quiescent latent state for the lifetime of the infected person. Although infections in immunocompetent adults are usually benign, considerable morbidity is seen in congenitally infected infants and in persons with compromised immunity (reviewed in reference 63). HCMV infections are especially important in transplant patients, who may suffer graft loss, vascular disease, and other manifestations including hepatitis and pneumonitis (reviewed in 37, 63). An increasing number of studies have also raised the possibility that HCMV infections may be linked to some chronic diseases, including atherosclerosis, autoimmune diseases, and cancer (reviewed in reference 74). Unfortunately, no effective vaccine is currently available to prevent or ameliorate HCMV infections.

Progress in developing effective antiviral drugs and vaccine candidates will likely rely upon detailed knowledge of viral gene products and how they function in pathogenetic processes. For this reason, there is an intense interest in defining the gene products of HCMV and other herpesviruses. The HCMV genome of 230 kb is among the largest of the herpesvirus genomes. The genome is comprised of two unique regions, known as the unique long (UL) and unique short (US) regions, that are bounded by terminal (TRL or TRS) and internal (IRL and IRS) repeat regions. Although the entire sequence of the laboratory-adapted AD169 strain of HCMV was first available in 1989 (22), the precise number and nature of viral genes and gene products are still in question. After sequencing the HCMV genome, Chee and colleagues predicted approximately 200 open reading frames (ORFs) capable of coding for proteins (5, 22). It was subsequently discovered that the AD169 laboratory strain harbored a deletion of approximately 15 kb relative to clinical isolates. This region was predicted to encode 19 additional ORFs, suggesting that the HCMV genome encoded up to 220 genes (18). More recently, comparison of the HCMV genome with the chimpanzee cytomegalovirus genome led to a revised estimate for the protein-coding genes of AD169 to 145 (27). Likewise, application of an in silico approach based on the Bio-Dictionary gene finder algorithm to define the coding potential of HCMV supported elimination of 37 previously annotated ORFs (61). However, comparison of the AD169 genomic sequence to those of clinical isolates and use of proteomic experimental approaches predict that additional unannotated ORFs exist (62, 83).

Most studies of herpesvirus genomes have focused on protein-coding potentials of these viruses. However, rapid advances in understanding of the role of noncoding gene products and antisense (AS) transcripts are dramatically changing the paradigms applied to gene definitions, gene regulation, and gene functions (reviewed in references 51, 56, 60, and 93). In particular, the application of bioinformatics approaches to analyze expressed sequence databases has revealed that AS transcription is widespread in human and other genomes (reviewed in references 51 and 60).

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† Supplemental material for this article may be found at http://jvi.asm.org/.

▽ Published ahead of print on 8 August 2007.

Sense-antisense (S-AS) transcript pairs have been found in genomes from archaebacteria to humans (58, 79, 94), and a subset of S-AS pairs is recognized to be conserved across species (94). While some pioneering studies have estimated that between 1 and 15% of human or mouse genes were influenced by S-AS pairs (29, 47, 52, 73, 92), more recent estimates of up to 20 to 26% of human genes (24, 94) and 72% of mouse genes (45) suggest that AS-mediated gene regulation may be much more common than previously appreciated. Natural AS transcripts (NATs) are classified as *cis* or *trans* in nature. *cis* NATs are transcribed from opposite strands of the same genomic locus and are predicted to have longer and more perfect complementary sequences for S transcripts than *trans* NATs derived from separate loci. Regulatory functions of NATs are predicted to derive from double-stranded RNA-dependent and -independent mechanisms, including RNA editing, RNA interference, chromatin remodeling, transcriptional interference, and masking of RNA elements involved in splicing, localization, transport, and translation of RNAs (reviewed in references 51 and 60).

In this study, we examined the transcriptional products of HCMV during lytic infection of fibroblasts. Remarkably, of the 604 HCMV cDNA clones analyzed in this study, at least 45% were derived from genomic regions predicted to be noncoding. Of similar interest was our finding that 55% of the cDNA clones in this study were completely or partially AS to known or predicted HCMV genes. Moreover, *cis* NAT pairs were identified or predicted for 56 of the 191 genes currently annotated at the Los Alamos National Laboratory Sexually Transmitted Diseases Sequences Database (STD database) (now at the Oral Pathogens Sequences Database). We conclude that genomic maps based on ORF analyses and other in silico analyses may drastically underestimate the true complexity of viral gene products. In addition, the abundance of AS transcription in the HCMV viral genome raises the distinct possibility that AS-dependent gene regulatory mechanisms influence both viral gene expression and gene organization. These noncoding and AS transcripts may offer new insights into HCMV pathogenesis and may serve as novel targets for developing intervention strategies and treatments for HCMV-related diseases.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts (HFFs), telomerase-immortalized HFF cells (HFF-TEL, a kind gift of Tom Shenk [10]), MRC-5 human fibroblasts, and human umbilical vein endothelial cells (HUVECs) were used in this study. HFF and HFF-TEL cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, while MRC-5 cells were maintained in modified Eagle's medium supplemented with 10% fetal calf serum, 1.7 mM sodium bicarbonate, 1.4 mM sodium chloride, essential and nonessential amino acids, vitamins, and sodium pyruvate at manufacturer-recommended concentrations (Sigma-Aldrich, InVitrogen). All three cell lines were used between passages 2 and 10. HUVECs were isolated from vessels as previously described (71) and propagated in endothelial cell growth medium consisting of M-199 (GibcoBRL) supplemented with 20% fetal bovine serum (HyClone), 22.5 μg/ml bovine brain extract (BioWhittaker, Inc., Walkersville, MD), 12 U/ml sodium heparin (Sigma, St. Louis, MO), and 20 mM HEPES buffer. All growth surfaces for HUVECs were pretreated with human fibronectin (25 μg/ml; Upstate Biotechnology). Cells were passed weekly by brief trypsin digestion at a ratio of 1:4 and used in experiments at passages 5 to 7.

HCMV strain AD169 was obtained from ATCC and was propagated and titrated on MRC-5 cells by plaque assay (87). Cytomegalovirus strain VHL/E, originally isolated from duodenal biopsy material from a bone marrow transplant recipient (86), was propagated in HUVEC as detailed elsewhere (85) to preserve its natural endothelial cytopathogenicity.

Extraction of HCMV genomic DNA. HCMV genomic DNA was extracted as described previously (81). Briefly, confluent HFF-TEL cells in two 175-cm^2 flasks were exposed to 1 PFU per cell of AD169 or Towne strains. The cells were harvested at 72 h postinfection and collected by centrifugation. The cell pellets were resuspended in 5 ml of 150 mM NaCl, 10 mM Tris (pH 7.4), and 1.5 mM $MgCl_2$. After incubation on ice, NP-40 was added to a final concentration of 0.1%. The lysate was centrifuged at 3,700 rpm for 20 min using a Beckman GS-6R centrifuge. The supernatant was collected and brought to a final concentration of 0.2% sodium dodecyl sulfate (SDS), 0.5 mM EDTA, and 50 mM β-mercaptoethanol. After incubation on ice and extraction with phenol-chloroform, the genomic DNA was precipitated with ethanol, resuspended in 1 ml of Tris-EDTA buffer, and treated with RNase (Sigma-Aldrich). The genomic DNA was further purified by centrifugation in a linear 5 to 20% (wt/vol) potassium acetate gradient at 40,000 rpm for 3.5 h at 20°C in a Beckman L7 Ultracentrifuge SW60 rotor. Following centrifugation, the DNA was collected, precipitated with ethanol, and resuspended in 50 μl distilled water. The purified genomic DNA was digested with MseI, followed by phenol-chloroform extraction and ethanol precipitation. The digested genomic DNA was finally resuspended in 50 μl sterile water.

Construction of HCMV AD169 cDNA libraries. RNA was extracted from infected HFF cells cultured in 175-cm^2 flasks under conditions that selected for immediate-early (IE), early (E), or late (L) viral transcripts. For all conditions, cells were exposed to 20 PFU per cell of the AD169 strain of HCMV. To select for IE transcripts, cells were treated with 100 μg/ml of cyclohexamide (Sigma-Aldrich) for 1 h prior to infection and throughout the 24-h infection period, when cells were harvested for RNA isolation. To select for E transcripts, 100 μM ganciclovir (Roche Pharma) was added to the medium after the infection period, and cells were harvested 72 h later. To select for L viral transcripts, untreated infected cells were harvested 72 h after infection. Prior to isolation of total RNA, cells from a small section of the flasks were scraped and collected. Cells were disrupted in SDS-polyacrylamide gel electrophoresis sample buffer (25 mM Tris-Cl, pH 6.8, 2.5% β-mercaptoethanol, 5% glycerol, and 0.5% SDS), boiled, and sonicated. Proteins were separated by denaturing gel electrophoresis and transferred to nitrocellulose (Amersham Bioscience). Efficacies of drug treatments were verified by immunoblot analyses for IE *UL122/123* products, IE1/2 (Rumbaugh Goodwin no. 1203), the early *UL55* product, glycoprotein B (gB) (Rumbaugh Goodwin no. 1201), and the L *UL99* product, pp28 (Rumbaugh Goodwin no. 1207) (data not shown).

Unless otherwise stated, all extractions of total cellular RNA were performed using the TRIZOL reagent (Invitrogen), following the instructions of the manufacturer. Polyadenylated mRNA was isolated using an Oligotex kit (QIAGEN) according to the manufacturer's instruction. cDNA libraries were constructed using two cloning vectors, pAcCMV (96), derived from the pAcSG2 Baculovirus transfer vector (BD Biosciences), and pcDNA3.1(+) (Invitrogen). These vectors were modified by introducing recognition sites for two restriction enzymes, PacI and PmeI, that are absent in the AD169 genome. Specifically, sequences recognized by the PacI and PmeI enzymes were inserted between the StuI and KpnI sites of vector pAcCMV using the following oligonucleotides: 5′ CCTGTTTAAACCTAGGCGG CCGCTTAATTAAGGTAC and 5′ CTTAATTAAGCGGCCGCCTAGGTTTAA ACAGG. The PmeI site at position 1007 in pcDNA3.1(+) was replaced with sequences specifying a PacI cutting site using a site-directed mutagenesis kit (Stratagene) and the following oligonucleotides: 5′ CTAGAGGGCCCGTTTAAT TAAGCTGATCAGCCTCGACTG and 5′CAGTCGAGGCTGATCAGCTTAAT TAAACGGGCCCTCTAG.

cDNA libraries were constructed by following the instruction manual for the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen) with some minor modifications. Briefly, a poly(T)-tailed PacI primer-adapter was used for first-strand cDNA synthesis (5′GCGGCCGC TTAATTAACC$(T)^{15}$). After second-strand synthesis, an EcoRI-PmeI adapter was added to the 5′ end of the cDNA that was generated from the following oligonucleotides: 5′AATTCAGGCCTGTTTAAACG and CGTTTAAACAGG CCTG. cDNA fragments were used in ligation reactions with modified pAcCMV or pcDNA3.1(+) vectors previously digested with the EcoRI and PacI restriction enzymes. Recombinant plasmids harboring cDNA sequences were transformed into XL1-Blue Supercompetent *Escherichia coli* cells (Stratagene).

cDNA library screening by colony hybridization. Random transformed bacterial colonies were picked individually and transferred onto agarose grid plates (approximately 100 clones on each plate). Colonies on grid plates were transferred to Hybond-N+ nylon membranes (82 mm in diameter; Amersham Bioscience) and processed for hybridization as described by Hirsch (40). MseI-digested HCMV genomic DNA was labeled using the DIG High Prime DNA

TABLE 1. Primers for RT-PCR and 5′ and 3′ RACE

Primer name	Sequence (5′–3′)	Genomic position[a]
AS-UL36-37-F	GGTGCCGCATTCCTGCTCCAGA	49677–49698
AS-UL36-37-R	CCTGCGTGTCTTGAACGAGAGTCG	50566–50543
AS-US30-31-F	GCTCCAAGAGCGACATGAGATCG	223730–223708
AS-US30-31-R	GGCGACGCCAAGTACGGTATTCGT	223019–223042
AS-UL61-62-F	CGAGGTTGGGTGTGGCCGGAA	94987–95007
AS-UL61-62-R	CGCTCCTAGGCTCTCGACGCCAT	95612–95590
AS-UL67-F	CCGTCACAGCCCTATGACGCGA	97937–97958
AS-UL67-R	GGTCGTGGTGACGATGATGATGT	98720–98698
AS-IGS-RL2-3-F	CCTCGCACTTCTTCGCTCTCA	2939–2919-or 187459–187479
AS-IGS-RL2-3-R	GGAGTGAAGAGGGTGAAGGCAA	2140–2161 or 188258–188237
AS-TRL8-9-F	CCGTTTCTCCTCAGCTGCCGT	7791–7771-or 182607–182627
AS-TRL8-9-R	CCACAGAGGGAGACGGGACGAAG	7159–7181 or 183239–183217
AS-UL27-28-F	GCTGCTGGGTTGGCAGTGGA	34614–34633
AS-UL27-28-R	GATGAGATCGTAGAGGGCGCGTG	35289–35267
AS-UL70-F	GCTGACGTGCGGTTCGTAGTGT	101764–101785
AS-UL70-R	CGAGCGCGACACAGAGGAGA	102523–102506
AS-UL87-88-F	GCTCGCGCACCGTACCGTCGT	132216–132196
AS-UL87-88-R	CACGGTCGAGAGAAGCGGCTGCA	131608–131630
AS-UL102-F	CGTGACTCGTTAAGCGTTGAGCC	150083–150061
AS-UL102-R	GGAGTGGTCATAACGGCGGTAGTG	149395–149418
AS-TRL3-F	CCAAGCCAGATTTCACGTCCATGAG	3567–3543 or 186831–186855
AS-TRL3-R	GCTTTGAGAGCGAAGAAGTGCGAG	2915–2938 or 187483–187460
AS-UL23-F	CGTTTTCGTGGCAGCGTGTCA	27954–27974
AS-UL23-R	GAAGGACGGGATGCACAGCGT	28566–28546
AS-UL24-F	CGTTGTGGCCGCTGAAACGTC	28985–29005
AS-UL24-R	CGTGTTGTCGCCGGATCAGCA	29601–29581
AS-UL25-F	CGAACGGCTCTGGTTGTTGACC	30979–30958
AS-UL25-R	GGTGGCGACGATAACAGCAGCAG	30467–30489
AS-UL47-F	CGTAGGGCATGAGCGTGCAG	61984–61965
AS-UL47-R	CGGAGGCGTTAAATACCGTGAGC	61388–61410
AS-UL88-F	GCTTTCCGGCCAACATGCCCAG	133032–133011
AS-UL88-R	GGTGGTGGAATGCGGTCAGTTG	132425–132446
gB E1	TCCAACACCCACAGTACCCGT	83769–83749
gB E2	CGGAAACGATGGTGTAGTTCG	83502–83522
GAPDH-F	CCATGGGGAAGGTGAAGGTCGGAGC	
GAPDH-R	GGTGGTGCAGGCATTGCTGATG	
pL8212-5RACEsp1	GCTCTAGACCTTATCCCCATCGTCGTG	29138–29119
pL8212-5RACEsp2	GATCTAGACGACCGCTACATCCGGGTG	29081–29061
pL5312-5RACEsp1	GCTCTAGATGAGGCACACAGCGGTCTT C	29555–29577
pL5312-5RACEsp2	CGTTCACGAAGCGACCCACGAAG	29606–29628
PL222-5RACEsp1	GATCTAGACTTCGTCCTCTTCGGGCAC G	149904–149924
PL222-5RACEsp2	CGTCTAGAACGCTTAACGGTCACG	150066–150083
pE104114-5RACEsp1	GATCTAGAGGGTCCGGGCTTTATGCG	49426–49407
pE104114-5RACEsp2	GCTCTAGAAGGACTTTCTGCGGAACG	49316–49297
pE104114-3RACEsp1	CCGAATTCCCGTAGGTAAAAACCCAC	50904–50924

[a] Nucleotide positions correspond to the AD169 genome sequence under GenBank accession no. NC_001347.

Labeling Detection Starter Kit II (Roche Applied Science). Probes were incubated with the membranes according to the manufacturer's instruction.

DNA sequencing and sequence analysis. Bacterial colonies harboring HCMV-derived cDNA sequences were inoculated into 4 ml LB broth supplemented with 50 μg/ml ampicillin. Plasmid DNA was purified from overnight culture using QIAprep Spin Miniprep kits (QIAGEN). cDNA inserts were sequenced from the 5′ end using the T7 primer for pcDNA3.1(+) and a pAcCMV-specific primer (5′GGAGACGCCATCCACGCTGTTTTGACC) at the OSU Plant-Microbe Genomics Facility. In total, 870 clones were submitted for sequence analysis from the 5′ ends. Sequences were compared to the AD169 genome (GenBank accession no. NC_001347) using mega BLAST (95). Matched AD169 gene sequences were downloaded and aligned to corresponding cDNA clone sequences manually using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (39). Selected clones were subjected to a second round of sequence analysis using primers specific for the 3′ ends of the cDNA inserts for the pcDNA3.1(+) (5′GCACCTTCCAGGGTCAAGGAAG) and pAcCMV (5′GAGGTGCGTCTGGTGCAAAC) vectors. These primers failed to generate sequence from a subset of clones, presumably because of difficulties in reading through poly(A) tracts. Therefore, in some cases, 3′ ends were sequenced using standard poly(T) primers. Selected cDNA inserts were also sequenced using specifically designed internal primers. AD169 genomic positions (accession no. NC_001347) corresponding to the sequences of the cDNA clones were determined using the SPIDEY program for cDNA to genomic alignments (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/).

RT-PCR for detection of AS transcripts. Confluent MRC-5 cells in six-well plastic tissue culture plates were exposed to 2 PFU per cell of the AD169 strain of HCMV. Cells were harvested at 24, 48, and 72 h after infection. Also, confluent HUVEC monolayers in six-well plastic tissue culture plates were inoculated with sonicated cell lysates containing VHL/E of HCMV (1 PFU/cell). To enhance infection efficiency of endothelial cells, plates were centrifuged at 300 × *g* for 30 min at room temperature. Cells were then incubated for an additional 30 min before removal of inoculum, followed by two washes with phosphate-buffered saline and addition of fresh medium. Cells were harvested at 24, 48, 72, 96, and 120 h after infection. Total RNA was isolated and treated with DNase I (Invitrogen). The reverse transcription-PCR (RT-PCR) analysis was performed using a ThermoScript kit (Invitrogen), following the manufacturer's instructions. cDNA synthesis was performed in the first step using total RNA and gene-specific primers at 55°C for 35 min (Table 1, reverse primers). In the second step, PCR was performed using primers specific for the gene of interest. A complete list of PCR primers is given in Table 1. Reactions were carried out at

94°C for 2 min, 30 cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 50 s, and final extension at 72°C for 10 min. RT-PCR products were analyzed by agarose gel (1% [wt/vol]) electrophoresis. No reverse transcriptase and no template controls were run in parallel.

Northern blots. Confluent MRC-5 cells in six-well plastic tissue culture plates were exposed to 2 PFU per cell of the AD169 strain of HCMV. Cells were harvested at 24, 48, and 72 h after infection. Total RNA was isolated and subjected to denaturing agarose gel (1% [wt/vol]) electrophoresis in the presence of formaldehyde. Nucleic acids were transferred to positively charged nylon membranes (Millipore) using a Posi-Blot 30-30 pressure blotter (Stratagene). All probes were labeled using a DIG Northern starter kit (Roche Applied Science) according to the manufacturer's instructions.

Plasmids pE10422 and pE104114, harboring cDNAs with gene sequences in S and AS orientation, were used to generate probes complementary to AS and S transcripts, respectively, of *UL36* (see Table S1 in the supplemental material for a description of cDNA clones). Plasmids pE10422 and pE104114 were linearized with BssHII and NdeI, respectively. RNA probes were generated using T7 polymerase. The AS probe corresponds to nucleotides 49791 to 49577 and 49473 to 48961, while the S probe corresponds to nucleotides 49135 to 50065, of the AD169 genome. Plasmids pL5312 and pL8212, harboring cDNAs with gene sequences in S and AS orientation, were used to generate probes complementary to AS and S transcripts, respectively, of *UL24*. Plasmids pL5312 and pL8212 were linearized with SalI and NcoI, respectively. RNA probes were generated using T7 polymerase. The AS probe corresponds to nucleotides 29806 to 29136, while the S probe corresponds to nucleotides 28949 to 29362, of the AD169 genome.

Plasmid pL222 carrying the AS UL102 sequence was digested with EcoRI and XbaI (corresponding to nucleotides 150184 to 149378 of AD169), and this fragment was inserted into pBluescript II KS+ (Stratagene). The plasmid was linearized with EcoRI to generate a probe complementary to the AS transcripts of UL102 using the T7 promoter or linearized with XbaI to generate a probe complementary to the S transcripts using the T3 promoter.

Plasmid pE1033, carrying the AS RL5 and S RL4 sequences, was digested with BamHI and SalI (corresponding to nucleotides 4555 to 3958 or 185843 to 186440 of AD169) and inserted into pBluescript II KS+. This plasmid was linearized with BamHI to generate a probe complementary to the AS RL4 transcripts using the T3 promoter. Because the S-specific probe generated from this entire fragment exhibited nonspecific binding, this plasmid was linearized with AvaII to generate a probe complementary to the S transcripts of β2.7 using the T7 promoter (corresponding to nucleotides 4555 to 4399 or 185843 to 185999 of AD169).

Plasmid pE10335, carrying the AS UL61 and AS UL62 sequences, was digested with EcoRI and XhoI (corresponding to nucleotides 94467 to 94735 of AD169), and this fragment was inserted into pBluescript II KS+. This plasmid was linearized with EcoRI to generate a probe complementary to the AS UL61 and UL62 transcripts using the T3 promoter. Because the S-specific probe generated from the T7 promoter exhibited nonspecific binding, a second S-specific probe was generated by linearizing plasmid pE10335 with MluI (corresponding to nucleotides 94467 to 95174 of AD169) and using the T7 promoter.

RACE. Rapid amplification of cDNA ends (RACE) was performed to determine 5′ and 3′ ends of the AS clones of UL36 (E) and the 5′ ends of AS clones for UL24 (E) and UL102 (L). Total RNA was isolated from MRC-5 cells in six-well plastic tissue culture plates exposed to 2 PFU per cell of the AD169 strain of HCMV at 48 h (for UL36 RACE) or 72 h (for UL24 and UL102 RACE) after infection. RNA was treated with DNase I (Roche Applied Science). 5′ or 3′ cDNA ends were amplified using the 5′/3′ RACE kit (Roche Applied Science), following the manufacturer's instructions. The products of the RACE reactions were inserted into a TOPO TA vector (Invitrogen) and sequenced at the OSU Plant-Microbe Genomics Facility. Primers used for RACE experiments are listed in Table 1.

RESULTS

Construction and analysis of HCMV cDNA libraries. Three cDNA libraries were generated from primary human fibroblasts infected with HCMV. We chose to use AD169 HCMV for these studies because it is the most extensively characterized with respect to analyses of viral gene products. HCMV, like other herpesviruses, is known to express genes in a temporal and regulated cascade. The most clearly recognized temporal classes are IE, E, and L. In order to make predictions regarding temporal relationships of viral transcripts, RNA was isolated under conditions that selected for expression of IE, E, or L genes as described in Materials and Methods. We employed positive selection using viral genomic DNA as a probe to isolate cDNA clones harboring viral gene sequences. A total of 870 bacterial colonies that were positive by this screening method were individually expanded. Plasmid DNA isolated from each culture was subjected to DNA sequence analysis, and usable sequence data were obtained for 618 clones. After excluding 14 clones (2.3%) that were recombinants of viral gene sequences or viral and cellular gene sequences, 604 clones were subjected to analysis (109 from the IE library, 180 from the E library, and 315 from the L library). A complete list of the cDNA clones and their positions relative to genomic DNA is listed in Table S1 in the supplemental material. We assigned these 604 clones to 184 transcript groups based on the similarity of cDNA sequences.

Many indicators suggest that these libraries accurately reflect temporal regulation, splicing, and abundance of viral gene products. cDNA clones isolated include S sequences overlapping 125 of the 191 unique genes currently annotated for the AD169 strain of HCMV in the STD database, including full-length sequences for 92 annotated genes. Examples of correct temporal expression and splicing of viral genes in these libraries were supported by clones representing the *UL123* and *UL4* genes. The major transcriptional activator (IE72) encoded by the *UL123* gene is known to be expressed during IE times (78). At least 15 cDNA clones isolated from the IE library were full-length and fully spliced transcripts capable of coding for the IE72 protein. Also, transcripts with short and long 5′ untranslated regions (UTRs) for the *UL4* gene have been characterized to accumulate at early and late times after infection, respectively (1, 15, 20). We identified 14 transcripts harboring longer 5′ UTR regions, all of which were derived from the L library. We also identified 15 clones that had shorter 5′ UTRs, the majority of which (9) were found in the E library. In addition, studies from other laboratories suggest that the most abundant transcripts found in infected cells overlap the *TRL4*/*IRL4* genes (TRL/IRL region genes are henceforth referred to as RL) (35), and this was reflected in our libraries. Indeed, 169 clones in our E and L libraries harbored *RL4* gene sequences, 141 of which were classified within the same transcript group (see Table S1, group 148, in the supplemental material). Taken together, these and other indicators suggest that our libraries reasonably reflect the range and abundance of HCMV transcriptional products.

A more detailed comparison of the temporal profiles of transcripts isolated in this study relative to microarray and Northern studies from other laboratories is shown in Table S2 in the supplemental material. This table also lists the clones characterized in this study, organized by gene name, and provides references for other transcript mapping studies performed for HCMV. The primary conclusion from this analysis is that the tentative temporal class assignments made in this study are largely congruent with temporal class assignments made using Northern and microarray analyses. The major difference is that we found many more genes represented in the transcripts of the IE class relative to findings with other methods. Specifically, we identified 45 genes with at least 1 clone isolated in the IE library. For many of these genes, the majority

TABLE 2. Orientation of cDNA clones

Classification	No. (%[b]) of cDNA clones relative to STD database gene map	No. (%[b]) of cDNA clones, revised based on exptl evidence[a]
cDNA clones in only S orientation	257 (43)	271 (45)
cDNA clones completely or partially in AS orientation	347 (57)	333 (55)
cDNA clones in only AS orientation[c]	51 (8)	54 (9)
cDNA clones with both AS and S orientations[d]	296 (49)	279 (46)

[a] Revised analysis based on excluding those AS clones for which we found no experimental evidence for an S transcript.
[b] Relative to total number of clones in the cDNA library.
[c] Clones spanning one or more genes only in AS orientation.
[d] Orientation of gene regions for clones spanning two or more genes.

of clones were isolated at E or L times with only one or a few clones isolated at IE times. There are two non-mutually exclusive explanations to account for these unexpected transcripts found in the IE library: (i) they represent tegument-associated viral transcripts delivered by the virion rather than newly synthesized viral transcripts; and (ii) these IE transcripts reflect leaky control of E and L gene expression in the IE period in HCMV-infected fibroblasts. Although the latter possibility could be due to incomplete blockade of protein synthesis by cycloheximide treatment, the efficacy of drug treatments used to generate the cDNA libraries was verified by measuring viral protein accumulation (data not shown). It is worthy of note that while the cDNA library approach employed in this study is subject to different biases relative to other methods (see Discussion and reference 19), it is free of bias introduced by employing gene-specific probes. Thus, it is possible that a cDNA library approach offers a specific advantage relative to Northern and microarray approaches in capturing minor transcripts available at various temporal phases of infection.

Another unexpected feature of our libraries was the prevalence of transcripts overlapping genes predicted to be noncoding genes. While we expected transcripts overlapping the RL4 gene to be abundant (35), we also isolated numerous cDNA clones from other repeat region genes. We found that 230 of the 604 clones analyzed mapped to the *RL2-RL9* region. We also found 29 clones derived from the *UL61* to *UL68* gene region and 13 clones derived from the *UL106* to *UL111* gene region, also recently revised as likely to be noncoding regions (27). Altogether, we obtained 274 clones (45% of the total) overlapping annotated genes predicted to be noncoding.

AS transcription in the HCMV transcriptome. One of the most striking features of our transcriptome study was the prevalence of transcripts in AS orientation to known or predicted genes. For this analysis, we compared our cDNA sequences to the genomic map of the AD169 strain (GenBank accession no. NC_001347) of HCMV and the STD database. This map includes up-to-date revisions in annotation proposed by Davison and colleagues, including annotation of those genes revised as noncoding (27).

Of the 604 sequences we analyzed, 257 represented one or more genes strictly in the S orientation (Table 2). Remarkably, 347 sequences were partially or completely AS to genes annotated on the STD database map, representing 57% of the cDNA clones isolated in our libraries. Because experimental evidence verifying the existence of gene products derived from a number of viral genes is lacking, we considered the possibility that the only products derived from a subset of these genes are those we identified in our library and that our calculation for the number of AS transcripts could be overestimated. When we excluded those genes for which we could find no evidence in our libraries or in the literature for a product derived from the S orientation of the gene (orphaned AS transcripts; see Table 5), we estimated that 271 clones (45%) represented transcriptional products strictly in one orientation with respect to gene sequences (designated the S orientation) and 333 clones (55%) were completely or partially in AS orientation. The AS sequences fell into two categories: a minority (54 clones) overlapped one or more genes strictly in AS orientation, whereas 279 clones overlapped more than one gene with sequences in both S and AS orientations. ORF analysis of AS clones predicts that these are predominantly noncoding (see Table S1 in the supplemental material). When these clones are included in the calculations for coding and noncoding transcripts, we estimate that up to 49.5% of the clones isolated in this study are noncoding in nature.

A complete list of genes for which we identified S-AS pairs is shown in Table 3. We obtained direct evidence for the existence of S-AS transcript pairs derived from 38 known or predicted viral genes. We also predict that S-AS pairs exist for 18 additional annotated genes listed in Table 4. In these cases, we identified AS but not S transcripts overlapping the indicated gene sequences. Considering that evidence for S products derived from these genes was published by other laboratories, we predict S-AS pairs also exist for these genes. As mentioned earlier, we also identified AS transcripts derived from gene sequences of nine genes but could find no evidence of a corresponding S product in virus-infected cells or in the literature. We designated this group as orphaned AS transcripts (Table 5). Together our analysis indicates that *cis* natural S-AS pairs are generated during infection for at least 56 of the 191 known or predicted unique genes annotated at the STD database for the AD169 strain of HCMV.

The abundance of the S and AS transcripts forming pairs fell into two groups. For most genes, we isolated between one and six clones representing S-oriented transcripts and one and six clones representing AS-oriented transcripts. There were two dramatic exceptions to this finding: the gene region from *UL61* to *UL67* and the gene region from *RL2* to *RL5*. cDNA clones representing transcripts from these two gene regions were among the most abundant in our libraries, and this was reflected in the number of S-AS pairs. For example, we found seven clones overlapping *RL3* gene sequences in the S orientation and 189 clones overlapping *RL3* gene sequences in the AS orientation. One notable feature of both classes of genes was dominance of either S or AS transcripts of a pair. We observed that clones in one orientation outnumbered those in opposite orientations between 1.5- and 41-fold. In fact, an inverse relationship (at least 2:1) in abundances of S and AS transcripts was observed for 30 of the 38 S-AS pairs identified in this study.

Based upon the library in which the clones were isolated, we

TABLE 3. Genes for which *cis* natural S-AS pairs were identified and their properties

Pair	Gene[a]	No. of S clones	Library	No. of AS clones	Library	Predicted temporal association	Predicted classification of AS pairs[b]
1	**UL12**	1	E	2	L	Discordant	Divergent
2	**UL13**	6	IE, L	1	E	Discordant	Divergent, *full overlap*
3	**UL21**	3	IE	2	IE	Concordant	Divergent
4	**UL22A**	2	IE	3	IE	Concordant	Intronic
5	UL23	3	IE, L	1	L	Discordant	Divergent
6	**UL24**	2	L	3	L	Concordant	Divergent, convergent
7	**UL25**	5	L	1	IE	Discordant	Divergent, intronic
8	**UL29**	3	IE	1	L	Discordant	Divergent
9	**UL36**	3	IE, E	1	E	Concordant	Divergent, *full overlap*
10	**UL37**	5	IE, L	1	E	Discordant	Convergent
11	**UL52**	6	E, L	1	L	Concordant	Divergent
12	**UL61**	2	L	10	E, L	Concordant	Divergent
13	**UL62**	3	L	12	E, L	Concordant	Convergent, divergent, full overlap
14	**UL63**	19	E, L	3	L	Concordant	Convergent, divergent, full Overlap
15	**UL64**	4	L	21	E, L	Concordant	Convergent, divergent, full overlap
16	**UL65**	20	E, L	3	L	Concordant	Convergent, divergent, full overlap
17	UL66	1	L	18	E, L	Concordant	Divergent, full overlap
18	UL67	1	L	17	E, L	Concordant	Divergent, full overlap
19	**UL70**	1	L	4	IE, L	Concordant	Divergent, *full overlap*
20	**UL72**	1	L	3	E, L	Concordant	Divergent, *full overlap*
21	UL73	3	E, L	1	L	Concordant	Full overlap
22	**UL74**	1	L	3	E, L	Concordant	*Full overlap,* intronic
23	**UL88**	1	L	2	L	Concordant	Divergent
24	UL89	1	L	2	L	Concordant	Divergent, convergent
25	UL92	1	L	1	L	Concordant	Divergent, intronic
26	**UL102**	2	L	2	L	Concordant	Convergent, divergent,
27	UL108	2	IE, L	1	IE	Concordant	Convergent
28	UL111	6	IE, L	2	IE, L	Concordant	Convergent, full overlap
29	**UL111A**	1	L	2	L	Concordant	Full overlap
30	UL112	5	IE, L	1	L	Concordant	Divergent
31	UL115	4	IE, L	2	L	Concordant	Divergent, intronic, *full overlap*
32	UL116	2	IE	2	L	Discordant	Divergent, intronic, *full overlap*
33	**RL2**	6	E, L	15	E, L	Concordant	Convergent, divergent, full overlap
34	RL3	7	E, L	189	E, L	Concordant	Convergent, divergent, full overlap
35	RL4	167	E, L	4	E, L	Concordant	Convergent
36	**TRS1**	1	IE, E, L	1	L	Concordant	*Full overlap*
37	**US32**	1	L	1	L	Concordant	Divergent
38	**US33**	1	L	4	IE, E, L	Concordant	Convergent, divergent, intronic

[a] Bold indicates sequence analysis confirmed a genuine poly(A) tail of the AS member of the pair.
[b] Italicized "full overlap" designations are tentative because the 3′ ends were not sequenced.

made predictions regarding the temporal association of S and AS transcripts derived from the same gene. Accordingly, 7 S-AS pairs were discordant relative to the library in which they were identified, whereas most (28) were concordant inasmuch as they were isolated from the same library. Keeping in mind that the E library is expected to contain both IE and E transcripts and the L library could contain transcripts expressed at IE, E, or L temporal classes, together these findings suggest that the majority of S-AS pairs are concordantly and inversely expressed during infection.

We also classified S-AS pairs with respect to the nature of the complementary overlapping sequences (Table 3). We used combinations of classification schemes proposed by others (45, 94) divided into one of four categories: full overlap, intronic, convergent, and divergent. Full overlap was defined as one gene sequence being completely contained within the gene sequence of the other member of the pair. Intronic was defined as one gene sequence starting within the intron of the other member of the pair and ending beyond the start of its pair. Divergent was defined as S-AS pairs exhibiting overlap in their 5′ regions in a head-to-head manner. Finally, convergent was defined as S-AS pairs exhibiting overlap in their 3′ regions in a tail-to-tail manner. Each potential pair was assigned to only one category, and the order of stringency was full overlap, intronic, divergent, and convergent. Using this scheme, we identified S-AS pairs that fell into each of these groups. Al-

TABLE 4. Genes with predicted S-AS pairs

Predicted pair	Gene[a]	No. of AS clones	Library	Reference for S gene product
1	**UL2**	3	E, L	19
2	**UL3**	32	IE, E, L	19
3	**UL11**	1	E	41
4	**UL27**	1	L	67
5	**UL30**	1	L	34
6	UL39	1	L	34
7	UL47	1	IE	44
8	**UL48**	4	E, L	4
9	**UL68**	19	E, L	34
10	UL87	1	L	34
11	UL107	2	IE, L	11, 64
12	RL5	150	E, L	34
13	**RL8**	15	IE, E, L	19
14	**RL9**	3	E, L	19
15	**RL10**	1	L	75
16	**RL11**	2	L	2, 54
17	**US29**	3	E, L	19
18	**US30**	2	E, L	19

[a] Bold indicates sequence analysis confirmed a genuine poly(A) tail of the AS member of the pair.

though the least abundant class, intronic pairs were observed for S-AS pairs overlapping seven genes. Convergent overlap was common, with 1 or more S-AS pairs for 14 genes falling into this class. Pairs exhibiting full overlap were also abundant. We found 1 or more S-AS pairs overlapping 19 genes in this class. However, it should be noted that in the absence of 3′ end sequence for all of the cDNA clones, it is possible that the number of genes with S-AS pairs exhibiting full overlap is currently overestimated. Finally, we found that 29 of 38 genes included 1 or more S-AS pairs that were divergent in their overlap. Also, of those genes with multiple S-AS pairs, the divergent class was typically most abundant. Thus, while we observe a diversity of S-AS pair classes derived from HCMV genes, pairs with divergent or head-to-head overlap were most common.

Finally, we classified S-AS pairs according to functional class of known or predicted gene products (Table 6). Several S-AS pairs overlap genes known to be involved in DNA replication and packaging, including those genes (*UL70* and *UL102*) that encode the components of the HCMV helicase-primase complex. We also found S-AS pairs for the viral inhibitors of apoptosis encoded by *UL36* and *UL37* genes and the recently described noncoding β2.7 transcript overlapping the *RL4* gene (69). Additionally, we identified S-AS pairs that overlap genes

TABLE 5. Orphaned AS transcripts

Group	Gene	No. of AS clones	Library
1	UL28	1	L
2	UL79	2	L
3	UL90	1	L
4	US1	3	IE, E
5	US4	2	L
6	US5	2	L
7	US31	1	E
8	US35	4	IE, E
9	J1S	1	L

encoding tegument proteins, glycoproteins, and proteins involved in subversion of immune responses (*UL111A* and *RL11*) (2, 54) or cellular antiviral defense mechanisms (*TRS1* and *IRS1*) (17). Finally, we identified S-AS pairs for at least 32 genes of unknown function, most of which are predicted to be noncoding. This aside, these findings indicate that there is not a clear bias of S-AS pairs for genes of specific functional classes and that S-AS pairs exist for both coding and noncoding genes.

Verification of HCMV AS transcripts. At least two features of a subset of AS clones suggest they cannot represent artifacts of our infection conditions or library construction. First, at least 68 of the clones harboring AS sequences possess genuine poly(A) tails (see Table S3 in the supplemental material). Because not all clones were sequenced from the 3′ ends, it is likely additional clones with genuine poly(A) tails will be identified. These 68 clones constitute at least 1 AS member for 25 of the 38 genes with S-AS pairs identified in this study (Table 3, genes identified in bold) and 13 of 18 genes with predicted S-AS pairs (Table 4). AS clones with genuine poly(A) tails were also found in each of the functional classes listed in Table 6. A second feature is exemplified by the S-AS pair overlapping the *UL36* gene. In this case, we identified three S clones, all of which represented mature, spliced transcripts lacking the *UL36* intron sequences (see Table S1, transcript group 35, in the supplemental material). The AS clone overlapping the *UL36* gene region (transcript group 36) contains this intron sequence, indicating that it could not represent aberrant cloning of the S cDNA product.

Our analysis of *cis* NATs from the HCMV genome relied upon isolation of virally derived transcripts from human foreskin-derived fibroblasts. If this is a truly robust phenomenon, we predict that *cis* NATs will be observed in other infected cell types and in cells infected with other strains of HCMV. To test this, we used RT-PCR to identify AS transcripts in lung-derived human fibroblasts infected with the laboratory-adapted strain AD169 of

TABLE 6. Functional classes of genes with verified or predicted *cis* natural S-AS pairs

Functional class	Genes for which S-AS pairs were identified[a]
DNA replication/packaging	**UL27, UL52, UL70, UL72**, UL89, **UL102**, UL112
Gene expression	IRS1, **TRS1**
Antiapoptotic	**UL36, UL37**, RL4
Glycoproteins	**UL11, UL13, UL22A**, UL73, **UL74**, UL115, UL116, **RL10, RL11**
Subversion of immune or antiviral defense	**UL111A**, IRS1/**TRS1, RL11**
Tegument proteins	UL23, **UL24, UL25**, UL47, **UL48, UL88**
Unknown	**UL2, UL3, UL11, UL12, UL13, UL21, UL27, UL29, UL30,** UL39, **UL61, UL62, UL63, UL64, UL65,** UL66, **UL67, UL68,** UL87, UL92, UL107, UL108, UL111, **RL2**, RL3, RL5, **RL8, RL9, RL10, US29, US32, US33**

[a] Bold indicates sequence analysis confirmed a genuine poly(A) tail of the AS member of the pair.

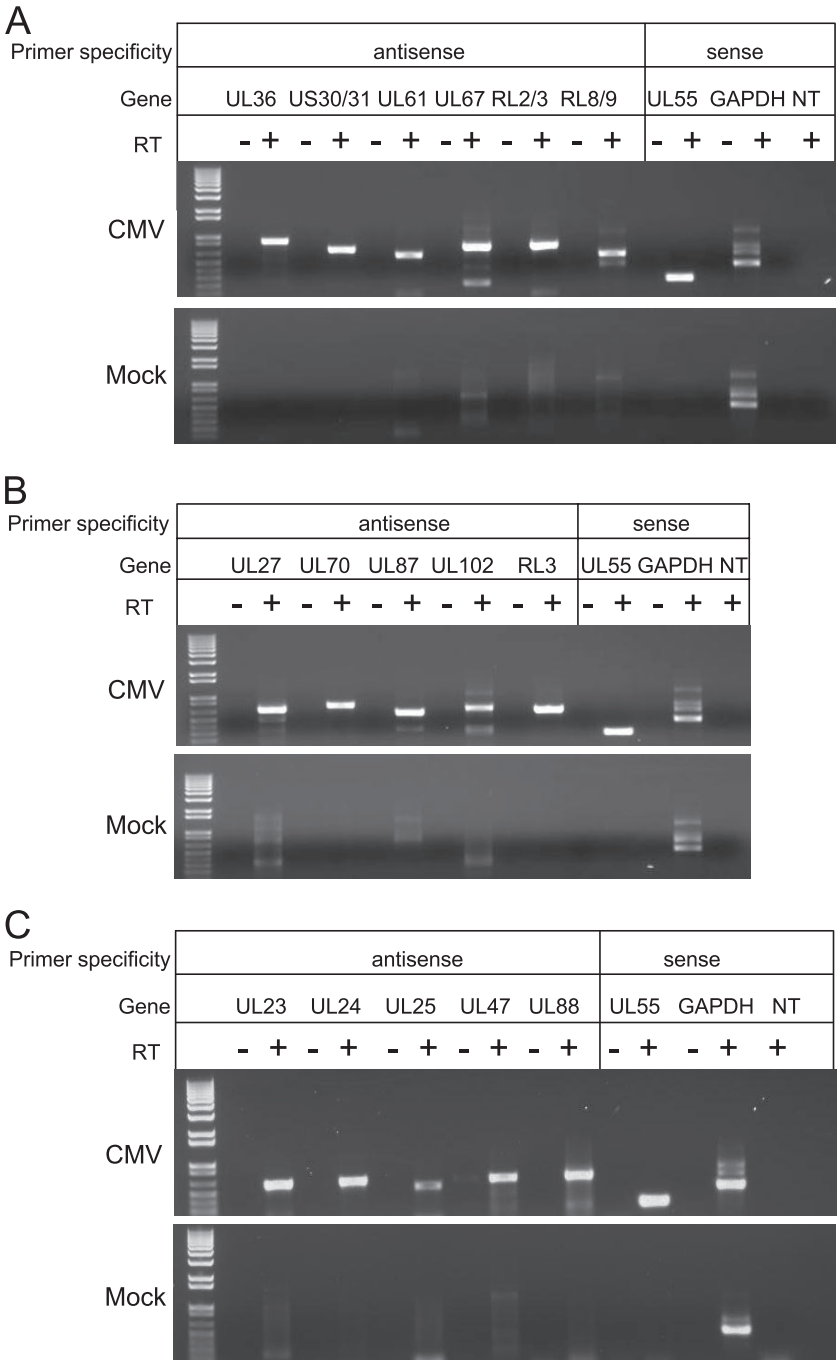


FIG. 1. Verification of virally derived AS transcripts. Digital images of agarose gels used to separate PCR products specific for virally derived AS and S transcripts. Confluent MRC-5 cells in six-well tissue culture plates were exposed to 2 PFU per cell of the AD169 strain of HCMV (top panels) or were mock infected (bottom panels). (A) RT-PCR of AS transcripts identified in the E library using total RNA isolated at 48 h after infection. (B) RT-PCR of AS transcripts identified in the L library using total RNA isolated at 72 h after infection. (C) RT-PCR of AS transcripts specific to tegument genes using total RNA isolated at 24 h after infection (UL47) or 72 h after infection (UL23, UL24, UL25, and UL85). Total RNA was isolated and subjected to RT-PCR using primers specific to virally derived AS transcripts (Table 1). Primers specific for the S transcripts of viral UL55 (gB) or cellular GAPDH were included as positive controls. No-reverse-transcriptase and no-template (NT) controls were run in parallel. At 48 h, the no-template control included primers specific for AS US30/31. At 72 h, the no-template control included primers specific for AS UL87/88. In panel C, the no-template control included primers specific for AS UL23. RT-PCR products were separated by agarose gel (1% [wt/vol]) electrophoresis, and bands were visualized by exposure to UV light.

HCMV and endothelial cells infected with the VHL/E clinical strain of HCMV. We designed primers specific for AS transcripts from both coding and noncoding viral genes. We also utilized primers specific for S-oriented viral UL55 transcripts coding for gB or cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. For specificity controls, RNA isolated from virus-infected and mock-infected cells was analyzed in the presence and in the absence of reverse transcriptase.

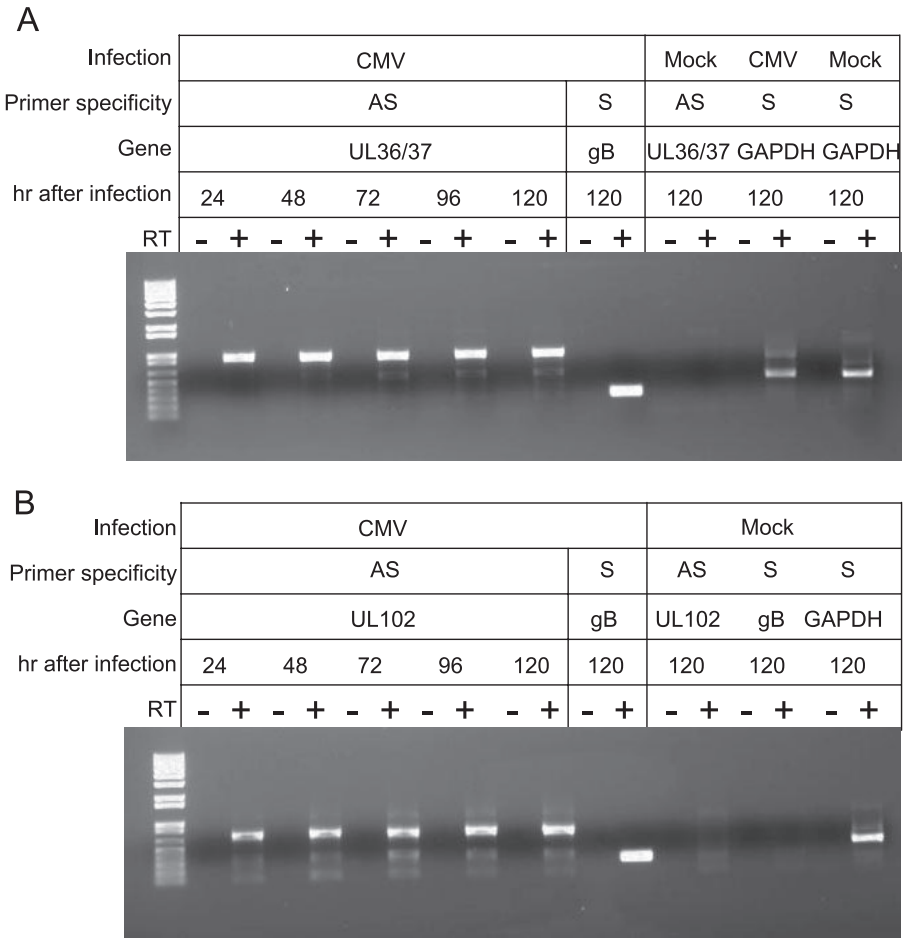
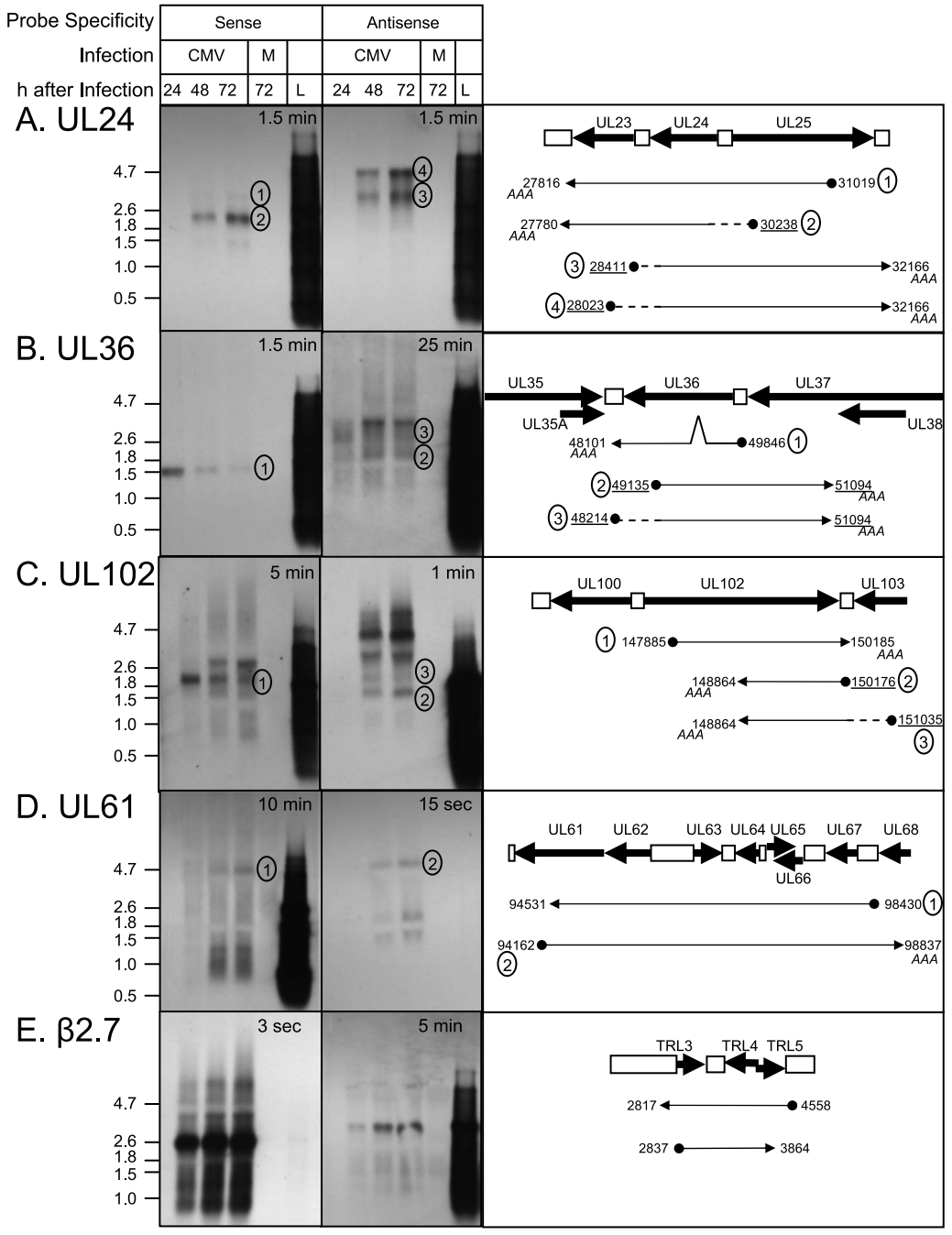


FIG. 2. Verification of AS transcripts in endothelial cells infected with a clinical strain of HCMV. Digital image of an agarose gel used to separate PCR products specific for virally derived AS and S transcripts. Confluent HUVEC monolayers in six-well tissue culture plates were inoculated with the VHL/E strain of HCMV (1 PFU/cell). Cells were harvested at 24, 48, 72, 96, and 120 h postinfection or were mock infected for 120 h. Total RNA was isolated and subjected to RT-PCR using primers specific to virally derived AS transcripts for UL36/37 (A) or UL102 (B). Primers specific for the S transcripts of viral UL55 (gB) or cellular GAPDH were included as positive controls. No-reverse-transcriptase and no-template controls were run in parallel. RT-PCR products were separated by agarose gel (1% [wt/vol]) electrophoresis, and bands were visualized by exposure to UV light.

In the first set of experiments, RT-PCR was used to amplify AS transcripts identified in the E and L libraries using RNA isolated from AD169-infected MRC-5 fibroblasts at 48 and 72 h after infection, respectively. As shown in Fig. 1A, we specifically amplified portions of AS transcripts found in the early library derived from genes known or predicted to be protein coding (*UL36* and *US30*/*31*) and genes predicted to be noncoding (*UL61*, *UL67*, *RL2*/*3*, and *RL8*/*9*). Similarly, we specifically amplified products of expected sizes for AS transcripts derived from genes known to be coding (*UL27*, *UL70*, *UL87*, and *UL102*) and predicted to be noncoding (*RL3*) from RNA isolated at 72 h after infection (Fig. 1B). Finally, we specifically amplified products of expected sizes for AS transcripts derived from genes specifying tegument proteins (*UL23*, *UL24*, *UL25*, *UL47*, *and UL88*) (Fig. 1C). Except for the cellular GADPH, we could not amplify these transcripts from mock-infected cells. The absence of bands of predicted sizes in reactions conducted without reverse transcriptase ensures that these results do not reflect contamination of viral DNA in our reactions. We conclude from these experiments that virally derived AS transcripts are generated during lytic HCMV infections of fibroblast cells and that these AS transcripts do not reflect DNA contamination during the cDNA library construction.

In the second set of experiments, we analyzed two specific AS transcripts derived from the *UL36*/*37* gene region and the *UL102* gene in primary endothelial cells infected with the VHL/E strain of HCMV. Because lytic replication in endothelial cells infected with VHL/E proceeds much more slowly than in fibroblasts infected with laboratory-adapted strains, we performed RT-PCR over a 120-h time course (Fig. 2). Specific AS transcripts were amplified at each time point examined for these two gene regions. We conclude that generation of *cis* NATs is a common feature of infection by both laboratory-adapted and clinical isolates of HCMV and that *cis* NATs occur during infection of clinically relevant cell types. Also, while cycloheximide and ganciclovir were used to generate the IE and E libraries, all of these experiments were performed in the absence of any drug treatment. This rules out the possibility that these AS transcripts represent aberrant transcriptional


Probe Specificity
Sense
Antisense
Infection
CMV
M
h after Infection
24 48 72
72
L
A. UL24
1.5 min
4.7
2.6
1.8
1.5
1.0
0.5
UL23
UL24
UL25
27816
31019
27780
30238
28411
32166
28023
AAA
B. UL36
25 min
UL35
UL36
UL37
UL35A
UL38
48101
49846
49135
51094
48214
C. UL102
5 min
1 min
UL100
UL102
UL103
147885
150185
148864
150176
151035
D. UL61
10 min
15 sec
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UL63
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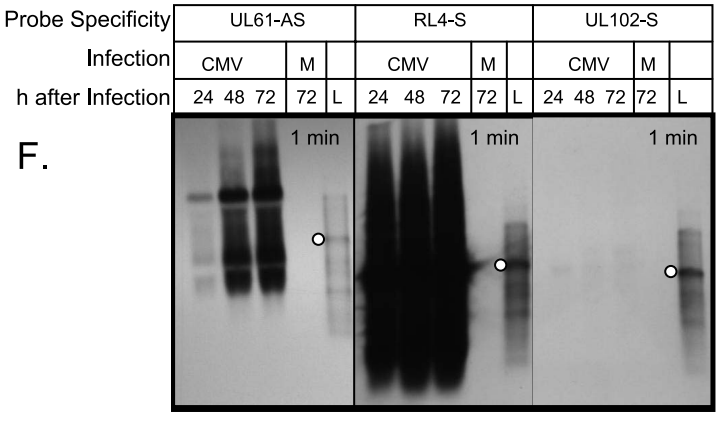
Probe Specificity
UL61-AS
RL4-S
UL102-S
Infection
CMV
M
h after Infection
24 48 72
72
L
F.
1 min

products that accumulate in cells exposed to protein synthesis or viral polymerase inhibitors.

To more carefully analyze the relationship between accumulation of S-AS pairs, we performed Northern analyses using RNA from AD169-infected MRC-5 cells at various times after infection with probes specific to either S or AS products of the *UL24*, *UL36*, *UL102*, *UL61*, and *RL4/5* genes (Fig. 3). We identified two S and three AS clones overlapping the *UL24* gene in our libraries. By using a probe specific to the S transcripts of *UL24*, we identified a major band between the 1.8- and 2.6-kb markers beginning at 24 h after infection and increasing in abundance through 72 h after infection and a larger, weaker band just over 3 kb expressed with the same temporal profile (Fig. 3A). These bands correspond to predicted sizes of clones pIE811 and pL5312, described in Table S1 in the supplemental material. RACE was performed to verify the 5′ ends of pL5312. We identified genomic positions of 30238 and 30029 as initiation positions for transcripts that are 432 and 223 bp larger than clone pL5312. Using a probe specific for the AS transcript of UL24, we identified two major bands between 3.0 and 4.0 kb at 48 and 72 h after infection. The largest AS UL24 clone isolated was 3.2 kb (pL8212). By RACE analysis, we identified two transcript initiation sites, at genomic positions 28411 and 28023, predicting transcripts of 3,755 and 4,143 bp in this region, that correspond well with the two bands observed by Northern blotting. Together these findings confirm the presence of *cis* NATs derived from the *UL24* gene in infected cells and validate our prediction of concordant expression and our finding of similar abundance of the UL24 *cis* NATs.

We identified three S clones and one AS clone overlapping the UL36 gene. As shown in Fig. 3B, we identified a band of the expected size (1.6 kb) for the spliced S product of the *UL36* gene (80a) that corresponds to the clones listed in transcript group 35 in Table S1 in the supplemental material. Also as expected, we could identify this band throughout the 72-h infection period, though it was most abundant at 24 h after infection. In our E library, we isolated one clone (pE104114) of approximately 1.9 kb that was AS to *UL36*. When we used a probe specific for the AS transcript, we identified only a weak band of 1.9 kb at 48 and 72 h after infection. We also observed a larger, more abundant band at 48 and 72 h. RACE was performed in an attempt to identify the boundaries of this larger transcript. In this analysis we confirmed the boundaries of the AS clone pE104114 and provided evidence for a transcript initiating at genomic position 48214, predicting a 2,880-bp transcript AS to UL36. This may represent the larger, more abundant AS transcript identified on the blot. However, because of the limitation in resolving the bands in this size range, we cannot exclude the possibility that this represents the faint band observed between the two major bands on this blot. In either case, we predict that there exists an additional AS transcript derived from the *UL36* gene. This analysis also confirms our prediction of concordant and inverse expression for the *UL36*-derived *cis* NATs.

Similar to that observed for the UL36 AS transcripts, we found that expression of the S UL102 transcript precedes expression of the AS UL102 transcripts (Fig. 3C). We identified a 2.3-kb S transcript in our library represented by clone pL2211. An additional larger S transcript is also observed by Northern analysis, consistent with that reported previously (73a). RACE analysis confirmed the 5′ end of AS clone pL222 and identified another, larger AS transcript of 2.1 kb initiating at genomic position 151035. Additional, even larger bands were observed by Northern analysis that have yet to be characterized. We predicted that *cis* NATs of *UL102* should be expressed with similar abundance and concordantly. While concordant expression was confirmed, the difference in exposure times required to visualize these transcripts indicates inverse accumulation of the S-AS transcripts.

Finally, we used a Northern blot approach to ascertain whether the abundance of clones in our libraries overlapping the *RL4/5* and *UL61/UL62* genes reasonably reflects the abundance of these transcripts in infected cells (Fig. 3D, E, and F). First we set out to verify the existence of S and AS clones from these gene regions. Clone pL537 was the longest S clone overlapping *UL61* in our library, predicting a band of 3.9 kb, which corresponds well with the largest band observed on this Northern blot (Fig. 3D). Numerous clones overlapped *UL61* in the AS orientation, the longest of which are listed in transcript group 59 (see Table S1 in the supplemental material), which predict a band of 4.6 kb. These clones correspond well with the largest band identified with the AS-specific probe for *UL61* (Fig. 3D). The smaller bands identified by both S and AS probes specific for *UL61* may represent distinct smaller transcripts or degradation products of the larger transcripts isolated in this study. The major 2.7-kb transcript overlapping the *RL4/5* genes has been described previously (35) and represented the major band identified by the S-specific probe (Fig. 3E). However, most or possibly all of the clones overlapping

FIG. 3. Northern blot analysis of S and AS transcripts. Film images of S and AS transcripts analyzed by Northern blotting and schematic depictions of select S and AS transcripts. Confluent MRC-5 cells in six-well tissue culture plates were exposed to 2 PFU per cell of the AD169 strain of HCMV or were mock infected (M). Cells were harvested at 24, 48, and 72 h after infection. Total RNA was isolated, subjected to denaturing agarose gel electrophoresis, and transferred to nylon membranes. Prelabeled RNA molecular mass markers were loaded for each group (L). Membranes were incubated with probes specific for the S and AS transcripts of UL24, 3 μg RNA/lane (A); UL36, 3 μg RNA/lane (B); UL102, 4 μg RNA/lane (C and F); UL61, 5 μg RNA/lane (D and F); or RL4, 4 μg RNA/lane (E and F) as described in Materials and Methods. Exposure times are indicated at the top of each panel. A schematic of transcripts represented by select cDNA clones isolated in our library relative to the genome is shown in the right panels. Gene regions and intergenic regions are depicted by thick arrows and white boxes, respectively. Transcripts cloned in this study are represented as thin arrows below the gene regions. 5′ ends of transcripts are depicted with filled circles. Clones in which we identified the poly(A) tails are indicated (*AAA*). The genomic positions of the 5′ and 3′ ends of the clones in the libraries are shown. Underlined genomic positions are those verified by RACE. Dashed lines represent presumptive transcript sequences based on RACE analysis. Tentative assignment of bands corresponding to clones identified in this study is indicated with circled numbers. In panel F, the relative abundances of transcripts overlapping the RL4 and UL61 gene regions are compared to those of transcripts derived from the UL102 gene region. The white circle indicates the position of the 2.6-kb marker band. No images were altered.

the *RL4* genes isolated in this study appear to be initiated from genomically encoded poly(A) tracts and thus do not represent the full-length 2.7-kb transcript (represented by clone pE103121, depicted in the right panel of Fig. 3E). We identified a band of approximately 3 kb using a probe that would recognize transcripts that are in AS orientation relative to the 2.7-kb transcript. Although the precise boundaries of the AS RL4/5 transcripts (represented by clone pE103210 in the right panel) as well as the *UL61*-derived transcripts have yet to be verified, the different exposures times required to visualize the S and AS transcripts clearly demonstrate inverse expression of the *UL61*-derived AS transcripts relative to the *UL61*-derived S transcripts and inverse expression of *RL4*-derived S transcripts relative to the *RL4*-derived AS transcripts. Finally, we compared the abundance of clones overlapping the *UL61*/*UL62* and *RL4*/*5* gene regions to that derived from the *UL102* gene region (Fig. 3F). In our libraries, we isolated two S UL102 clones, five times as many AS UL61 clones, and 85 times as many S RL4 clones. The abundance of these clones in our libraries correlated with the signal intensities of transcripts that bound the S-specific RL4 probe and the AS-specific UL61 probe relative to the S-specific UL102 probe. Taken together, these experiments prove the existence of *cis* NATs derived from coding (*UL24*, *UL36*, and *UL102*) and predicted noncoding (*UL61 and RL4*) genes. Furthermore, these studies indicate that the representation of S and AS transcripts in our libraries reasonably reflects the abundance and temporal expression patterns of S and AS transcripts generated in HCMV-infected fibroblasts.

DISCUSSION

The HCMV transcriptome. In this study, we isolated and characterized transcripts generated during lytic infection of HCMV-infected fibroblasts. Selecting for cDNA clones representing viral genes and obtaining sequence data on each isolated clone constituted a de facto analysis of the HCMV transcriptome. This analysis revealed two novel findings. First, there was an unexpectedly high percentage of clones derived from genomic regions currently considered to be noncoding (27). While we expected a high prevalence of RL4 transcripts based on reports from other studies (35, 43, 57), additional noncoding transcripts were also represented at high frequencies, especially those from the *UL61-UL67* gene region. Altogether, 45% of the clones isolated in this study were derived from gene regions predicted to be noncoding (27), suggesting further studies of the function and regulation of these gene regions are needed. Second, we identified a striking number of transcripts, predominantly AS, that were not predicted by the STD database annotation of the HCMV genome. We therefore conclude that genomic maps based on ORF analyses and comparisons to related viral genomes drastically underestimate the true complexity of viral gene products.

The discovery of widespread AS transcription also bears upon the use of viral gene arrays for transcriptome analyses and recombinant viruses for gene function studies. Specifically, these findings suggest that tiling arrays using overlapping probes from both DNA strands rather than probes based upon ORF predictions will be required to capture an accurate representation of viral transcripts. Additionally, these findings raise the possibility that AS as well as S gene products could be affected when recombinant viruses harboring mutations or deletions are generated.

There are several factors that could influence the composition of this library that are relevant to the interpretation of these data. First, because highly abundant transcripts are expected to be preferentially represented, we infer that transcripts from genes not represented in our library are of relatively low abundance. While abundance of these transcripts is likely a key factor, we cannot exclude the possibility that artifactual pressures might also influence library composition. For example, it is possible that there were selective advantages for isolating transcripts from gene regions with long or repetitive tracts of adenosines, such as the RL gene region. These transcripts may have been preferentially enriched during the purification of polyadenylated RNAs prior to cDNA library construction. Similarly, genomically encoded poly(A) tracts may have served as primer binding sites during the reverse transcription step of the cDNA library construction. While many cDNA clones appear to have genuine poly(A) tracts, we also found a number of clones, especially from predicted noncoding regions, in which the 3′ end sequence corresponded to a genomically encoded poly(A) tract. Therefore, further studies will be necessary to define precise ends of a subset of transcripts, as well as their relative abundance in infected cells. Despite these caveats, Northern analyses of transcripts derived from single-copy and repeat-region genes suggest that composition of our library reasonably reflects the abundance and temporal expression patterns of transcripts in infected fibroblasts.

AS transcripts of the HCMV genome. Individual AS transcripts have been described for many herpesviruses, including the betaherpesviruses, (6, 48, 82), the gammaherpesviruses, (65, 66, 72), and especially the alphaherpesviruses, (8, 9, 13, 16, 21, 25, 26, 42, 49, 50, 53, 68, 84, 88, 89, 91). In fact, Roizman and colleagues predicted that genes in AS orientation to known herpesvirus genes could be common (16). However, to our knowledge, our study is the first to systematically document *cis* NATs derived from a herpesvirus. We found that at least 55% of the clones analyzed in this study contain sequences in AS orientation overlapping 56 known or predicted genes. These figures are dramatic inasmuch as they suggest that more than half of all virally derived transcripts harbor AS sequences. Nevertheless, three factors suggest that our study may have underestimated AS transcription in HCMV. First, our libraries do not contain all of the S transcripts expressed by HCMV, and thus, it is likely that they do not contain all of the AS transcripts that accumulate during infection. Indeed, at least two previously reported AS transcripts overlapping the HCMV *UL82* and *UL123* genes were not isolated in our libraries (6, 48). Second, we selected for polyadenylated transcripts during construction of our libraries, and studies suggest that a large fraction of AS transcripts are poly(A) negative (46). Finally, we classified only *cis* NATs identified in our libraries. Considering that the HCMV genome includes numerous repeat elements, it is likely that *trans*-derived NATs are also generated during infection.

Relative to eukaryotic genomes, herpesvirus genomes are small and densely populated with genes. In fact, many genes are directly adjacent to one another, and a number of viral

genes overlap each other, often in opposite orientations. These characteristics suggest that the occurrence of S-AS pairs may only reflect the dense organization of viral genes and the relative paucity of intergenic regions relative to larger genomes. Nevertheless, S-AS pairs derived as a consequence of this gene organization may be functionally relevant with respect to regulation of viral genes. This raises the interesting possibility that regulatory consequences of S-AS pairs may constitute one evolutionary pressure influencing function and orientation of adjacent viral genes.

Given these properties of the HCMV genome, it seems reasonable to predict that higher proportions of viral genes would be associated with S-AS pairs than would be the case for genes of eukaryotic genomes. In this study, we found that at least 29% of the currently annotated 191 HCMV genes are associated with S-AS pairs. While this figure is similar to estimates of 22 to 26% (24, 94) of human genes associated with S-AS pairs, it is lower than the estimate of 72% of mouse genes (45) predicted to be influenced by S-AS pairs. These preliminary findings suggest that the proportion of HCMV genes involved in the generation of S-AS pairs is similar to that observed for the human genome, despite the striking differences in sizes and structures of these genomes. Another common feature between mammalian and viral S-AS pairs is the nature of the complementary sequences. Our analyses indicate that most S-AS pairs overlap either in 5′ or 3′ regions, with intronic organization as the least frequent class of S-AS pairs, and this is similar to reports by other groups for the human genome (52, 92, 94). One prediction from this type of overlap is that the potential regulatory consequences of these S-AS pairs relate to function, accessibility, or processing of the UTRs of viral transcripts (80). Since posttranscriptional processing of HCMV viral transcripts has been described previously (7, 14, 30–33, 55, 76, 90), it will be of interest to determine whether such events are influenced by AS transcripts. Also, in mammalian genomes, S-AS pairs appear to be overrepresented among genes involved in genomic imprinting (45), metabolic, catalytic, and cell organization functions (94), and translational regulation (24). However, our findings indicate that there is not a clear functional bias among HCMV genes for which S-AS pairs were identified.

A key question raised by our findings is whether the virally derived S-AS pairs have regulatory consequences during lytic or latent viral infections. AS transcripts can impact viral gene expression though multiple mechanisms, such as influencing splicing, editing, stability, localization, and translation of transcripts (51, 60). In addition, double-stranded intermediates generated from direct interaction of S-AS pairs may lead to gene regulation by RNA silencing or chromatin remodeling (51, 60). S-AS pairs involved in regulatory functions are predicted to be expressed concordantly and to accumulate in an inverse manner (23). Indeed, coexpressed and inversely expressed S-AS pairs not only are more frequent in the human genome than would be expected by chance but are also evolutionarily conserved (80). Our results predict that the majority of HCMV S-AS pairs are expressed with just such a profile, and we provide experimental evidence for concordant expression and inverse accumulation for S-AS pairs derived from the *UL36*, *UL102*, *UL61*/*UL62*, and *RL4*/*5* gene regions.

Another possibility is that AS transcripts may serve as primary transcripts for microRNAs (miRNA), as was recently suggested for the AS latency-associated transcripts of herpes simplex virus and Marek's disease virus (12, 38). Several studies have identified putative or validated miRNAs of HCMV (28, 36, 64). Interestingly, a number of miRNAs that have been predicted or validated are derived from the AS strand of viral genes, including the *UL31*, *UL53*, *UL70*, *UL102*, *UL114*, *UL150*, and *US29* genes (36, 64). In support of this possibility, we identified AS transcripts from several of these genes, including *UL70*, *UL102*, and *US29*. While further studies are required to establish regulatory roles for these AS transcripts, we predict that these AS transcripts may dramatically alter our understanding of viral gene regulation during both lytic and latent infections.

To summarize, the remarkable accumulation of noncoding and AS transcripts during infection suggests that currently available genomic maps based on ORF and other in silico analyses may drastically underestimate the true complexity of viral gene products. These findings also raise the possibility that aspects of both the HCMV life cycle and genome organization are influenced by AS transcription. These noncoding and AS transcripts may offer new insights into HCMV pathogenesis and may serve as novel targets for developing intervention strategies and treatments for HCMV-related diseases.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical efforts of Pete Zimmerman, RuthAnn Weimer, Abbey Wright, and Mary Sivulich in cDNA library construction.

This work was supported by grant AI51411-03 from the National Institutes of Health.

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