JOURNAL OF VIROLOGY, Mar. 1995, p. 1734–1740 Vol. 69, No. 3
0022-538X/95/$04.00+0
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Human Cytomegalovirus UL102 Gene

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Received 25 October 1994/Accepted 14 December 1994

We have identified and characterized the transcript for the human cytomegalovirus (HCMV) UL102 gene. The UL102 gene product is proposed to encode the primase-associated factor. The primase-associated factor is one of the three components of the helicase-primase complex, along with UL105 (helicase) and UL70 (primase). In order to characterize the UL102 transcription unit we used single-stranded antisense RNA probes to identify an abundant 2.7-kb transcript originating from the UL102 region. This transcript can be initially detected at 24 h postinfection and in the presence of phosphonoformic acid but not in the presence of cycloheximide. A 2.7-kb cDNA clone containing this transcript was isolated from a 72-h HCMV (strain Towne) cDNA library. Sequence analysis of this clone revealed a continuous unspliced transcript between the region of UL101X and UL102; the only in-frame translational stop codon is 2,619 bp downstream from the first ATG in the message. Genome sequencing of the UL102 region from strains AD169 and Towne revealed that the UL101X stop codon TAA was actually TAC and that the cDNA and genomic sequences were in agreement. The cDNA clone starts 5 nucleotides (nt) upstream of the UL101X ATG, continues through the putative ATG of UL102, and ends 97 nt downstream of the putative termination codon of the UL102 open reading frame. Primer extension analysis indicated a transcriptional start site 23 nt upstream of the UL101X open reading frame.

Human cytomegalovirus (HCMV) requires eleven distinct loci for origin-dependent DNA replication (25, 26). These loci were identified by using a cotransfection-replication assay similar to the procedure employed with herpes simplex virus type 1 (HSV-1) and Epstein-Barr virus (EBV) (11, 34). Some of these loci contain genes that were previously shown to be homologs to HSV-1 replication genes on the basis of nucleotide sequence homology (3). The products of these genes, shown in Table 1, are as follows: a DNA polymerase (UL54), a polymerase accessory protein (UL44), a single-stranded DNA-binding protein (UL57), a primase (UL70), a helicase (UL105), and a primase-associated factor (PAF) (UL102). Other genes identified encode either known regulatory proteins, UL36-38, IE1/IE2, and IRS1, or HCMV early-kinetic-class genes having unknown functions with respect to HCMV DNA replication, UL112-113 and UL84.

Although many of these genes were previously characterized with respect to the analysis of transcripts and in some cases the identification of protein products, for example, UL112-113, IE1/IE2, UL54, UL36-38, UL84, UL44, and UL57 (4, 9, 10, 12, 13, 15, 18, 19, 33), others remain undefined. The least well defined of the HCMV replication genes are those that encode the proposed helicase-primase complex. UL70 and UL105 of HCMV were shown to have significant nucleotide sequence homology to HSV-1 UL52 (primase) and UL5 (helicase), respectively (3, 20).

HCMV genes UL101 and UL102 were initially identified as candidate homologs for the genes encoding HSV-1 replication proteins UL9 (origin-binding protein) and UL8 (PAF), respectively. These assigned roles were based exclusively on their similar genomic positions as determined by comparison with HSV-1 (3, 21, 22). However, cotransfection of plasmids in a replication assay using subgenomic fragments of HCMV revealed that UL101 was not required for origin-dependent DNA replication but UL102 was absolutely necessary (25). From these same studies, it was also determined that in addition to the requirement for the UL102 open reading frame (ORF), a large upstream region (735 nucleotides [nt]) was also necessary, possibly indicating a potential additional essential coding region. Upon closer examination of this upstream region there appeared to be a small ORF comprising 223 nt just upstream of the putative ATG for UL102. This ORF, which we will refer to as UL101X, is in the same reading frame context as UL102.

The PAF is an essential protein for herpesvirus origin-dependent DNA replication (11, 25, 34). The HSV-1 UL8 protein has been purified and demonstrated to form a complex with the helicase and primase subunits and presumably is situated at the head of the replication fork (23). Although in vitro assays have shown it to be dispensable for helicase activity (2), recent studies have demonstrated that the UL8 protein acts to increase the efficiency of primer synthesis by UL5/UL52 (32). Together, these three proteins exhibit helicase, DNA-dependent ATPase/GTPase, and primase activities (5, 6, 27).

One goal of our laboratory is to eventually express and characterize all HCMV replication proteins; we also plan to further study the overall mechanism of viral replication with the intent of developing antisense oligonucleotides as antiviral agents. To this end, it is essential that transcripts encoding putative HCMV replication proteins be identified and mapped. In this report we identify the transcript encoding the candidate homolog for the HCMV PAF. With DNA probes, we isolated and sequenced a cDNA clone containing this message. Primer extension and Northern (RNA) analyses have identified the putative 2.7-kb PAF transcript and the 5′-transcriptional start site for this mRNA. The UL102 transcript is an unspliced message that originates 20 nt upstream of the UL101X ATG. DNA sequence analysis showed that the UL102 transcript contains only one in-frame translational stop codon, 2,619 bp downstream from the first ATG within the message, with the potential to encode a 100-kDa protein. Genomic sequencing of both Towne and AD169 confirmed that the absence of the UL101X translational stop codon (TAG) in the UL102 cDNA was correct and revealed that the previously reported stop codon for UL101X in AD169 was an error in the published sequence (3).

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TABLE 1. Herpesvirus genes previously reported to be required for origin-dependent DNA replication[a]

HCMV gene	HSV-1 gene	EBV gene	Proposed product
UL54	UL30	BALF2	Polymerase
UL44	UL42	BMRF1	Processivity factor
UL105	UL5	BBLF4	Helicase
UL70	UL52	BSLF1	Primase
UL101-102[b]	UL8	BBLF2/3	PAF
UL57	UL29	BALF2	ssDNA-binding protein[c]
UL84			
UL112-113			
IE1/IE2			Transactivator
UL36-38			Transactivator
IRS1			Transactivator

[a] Putative HSV-1 and EBV homologs are shown along with their respective proposed protein products.
[b] The HCMV gene investigated here (the putative PAF).
[c] ssDNA, single-stranded DNA.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts were used for all experiments and were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HCMV strain AD169 (American Type Culture Collection) was used for all experiments involving Northern and primer extension analyses. HCMV strain Towne (American Type Culture Collection) was used to isolate genomic DNA from this virus in order to sequence the region corresponding to AD169 UL101-102 and UL101X. Where noted, cycloheximide (200 μg per ml) treatment was performed by preincubation of cells for 1 h followed by infection, and phosphonoformic acid treatment (300 μg per ml) was performed at the time of infection.

HCMV (AD169) nucleotide sequence coordinates are from Chee et al (3).

DNA constructs. The following subgenomic HCMV DNAs were made from the *Hin*dIII R fragment (nt 142994 to 149645) (Fig. 1, *Hin*dIII cleavage map). Plasmid p101-102 was made by cleaving *Hin*dIII-R with *Asc*I (nt 146384) and *Hin*dIII (nt 149645), filling in the resulting 3.3-kb fragment with Klenow and ligating it into *Sma*I-cleaved pBluescript SK(−) (Stratagene, La Jolla, Calif.). Riboprobes were generated from the following constructs. Plasmid p101Aribo was made by cleaving p101-102 with *Nru*I (nt 146502) and *Eco*RI (vector) and ligating the resulting 116-bp fragment into *Sma*I-*Eco*RI-cleaved pBluescript SK(−). This construct contains the HCMV genomic sequence from the *Asc*I site (nt 146384) to the *Nru*I site. Plasmid p101Bribo was made by cleaving p101-102 with *Nru*I (nt 146502) and *Xba*I (nt 146605) and ligating the resulting 103-bp fragment into *Sma*I-*Xba*I-cleaved pBluescript SK(−). Plasmid p102Aribo was made by cleaving p101-102 with *Mlu*I (nt 147055) and *Kpn*I (nt 147618) and ligating the resulting 561-bp fragment into *Sma*I-*Kpn*I-cleaved pBluescript SK(−). Plasmid p102Bribo was made by cleaving p101-102 with *Eco*RV (nt 148825) and *Hinc*II (nt 148153) and ligating the resulting 250-bp fragment into *Sma*I-cleaved pBluescript SK(−). Plasmid p102Cribo was made by cleaving p101-102 with *Age*I (nt 149470) and *Hin*dIII (nt 149645), filling in the resulting 174-bp fragment with Klenow, and ligating it into *Sma*I-cleaved pBluescript SK(−).

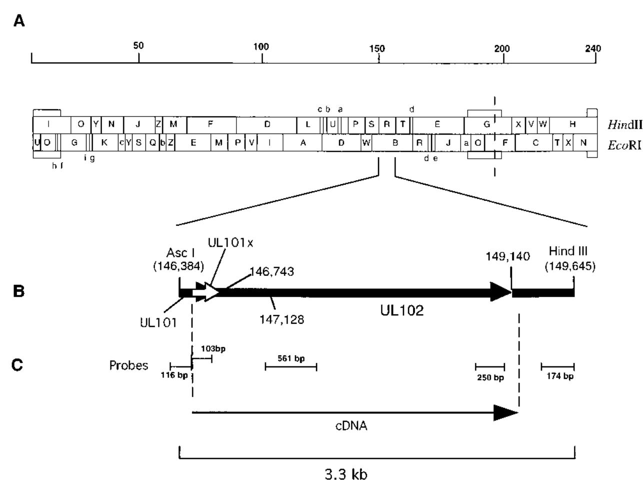

FIG. 1. Physical map of HCMV genome and location of UL101X-102. (A) Physical map of the HCMV genome showing *Hin*dIII and *Eco*RI cleavage sites. (B) Subgenomic *Asc*I-*Hin*dIII fragment previously demonstrated to be required for origin-dependent DNA replication. Indicated are the relative positions and nucleotide sequence coordinates of ORFs UL101, UL101X, and UL102. (C) Probes used to define transcript boundaries and to screen HCMV (Towne) cDNA library. Also indicated is the position of a cDNA encoding the putative PAF that was isolated from a 72-h-postinfection HCMV cDNA library.

Northern analysis. Total cellular RNA was prepared by the following procedure as described previously (28). Infected or mock-infected cells were lysed directly on a 6-cm-diameter dish with 500 μl of 2% sodium dodecyl sulfate (SDS)–200 mM Tris-HCl (pH 7.5)–1 mM EDTA, lysates were transferred to a 1.5-ml microcentrifuge tube, and 150 μl of ice-cold precipitation buffer (42.9 g of potassium acetate, 11.2 ml of acetic acid, water to 100 ml) was added. The tubes were vortexed and iced for 2 min and centrifuged for 5 min (at room temperature), and the supernatants were transferred to fresh tubes and extracted twice with 300 μl of chloroform and isoamyl alcohol (24:1). RNA was precipitated with 0.65 ml of ice-cold isopropanol and pelleted for 5 min at 14,000 × *g*. Pelleted RNA was resuspended in 50 μl of 100% formamide and stored at −80°C.

Ten micrograms (approximately 10 μl) of RNA was electrophoresed on a 1.0% agarose gel containing 6% formaldehyde and then transferred to a Zeta-probe nylon membrane. The filter was hybridized with single-stranded RNA probes (riboprobes), constructs described above, in hybridization buffer (1.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 1% SDS, 50% formamide, 0.5% nonfat dried milk, 100 μg of denatured salmon sperm DNA per ml) at 60°C for 16 h. The blots were then washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS (wt/vol) twice for 15 min at room temperature; this step was followed by washing with 0.1× SSC–0.1% SDS (wt/vol) twice for 45 min at 60°C. The blots were then exposed to X-Omat AR (Kodak) X-ray film at −80°C for 24 h.

Primer extension. The 20-base oligonucleotide 5′-CGAACAGGGTGTAC GGGTGG-3′, end labeled with ^{32}P, was mixed with 5 μg of infected or uninfected total-cell RNA in a volume of 10 μl, heated to 80°C, and quick-chilled on ice. To this mixture, reverse transcriptase buffer was added to make a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 200 U of SuperScript II RNase H− reverse transcriptase (Bethesda Research Laboratories, Inc., Bethesda, Md.) in a 20-μl volume. This primer extension mixture was incubated at 42°C for 30 min. Samples were then treated with RNase A, phenol extracted and ethanol precipitated, resuspended in gel loading buffer heated to 80°C, and electrophoresed on a 7 M urea sequencing gel. This same primer was used for dideoxy sequencing and electrophoresed on the same gel in order to determine the exact start site of the transcript.

Screening of cDNA library and dideoxynucleotide sequencing. A Towne (74-h-postinfection) cDNA library was a generous gift from E. Mocarski (14). cDNA inserts were ligated into the mammalian expression vector pME18S (31).

Colonies were plated on petri dishes (150 by 15 mm) at a density of approximately 600 to 800 colonies per dish. The colonies were screened by using an agarose gel-purified random-primer-labeled probe encoding the 5′ end of the UL102 ORF, *Mlu*I (nt 147055)-*Kpn*I (nt 147618). Positive colonies were picked, replated, and rescreened with the same probe. DNA from positive clones was prepared by Qiagen (Chatsworth, Calif.) column purification and cleaved with *Xho*I to release the cloned fragment, and those subclones with inserts were subjected to DNA sequencing with Sequenase (U.S. Biochemicals, Cleveland Ohio).

Initial sequencing was performed by using forward (5′-GCTGCGGAATTC CGG-3′) and reverse (5′-ACTGGTAGGTATGGA-3′) primers that were complementary to vector sequences; internal primers were then used once it was confirmed that the clones contained the correct DNA sequence. Sequence information was generated and confirmed by sequencing in both directions. Determination of open reading frames and manipulations of sequence data were done with Hitachi software MacDNAsis Pro (National Biosciences, Plymouth, Minn.) and the Gene construction kit (Textco, West Lebanon, N.H.).

Nucleotide sequence accession number. The nucleotide sequence of HCMV UL102 cDNA has been submitted to GenBank under the accession number U18289.

RESULTS

Screening of cDNA library and identification of the UL102 transcript. Figure 1 illustrates the location within the HCMV genome of the 3.3-kb *Asc*I-*Hin*dIII fragment that encodes ORFs UL101X and UL102. This fragment, when cotransfected with other HCMV essential genes, can complement HCMV OriLyt-dependent DNA replication (25, 26). We used hybridization probes complementary to ORFs UL101X and UL102 to elucidate the transcripts originating from this region of the genome.

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AD169 GENOMIC GGCGCGCCCG CGCCGATGAC GACAGGCTCG CGGGTCGTTA AATACTACGA TGGGAGCCGC CGCGGCTCAC GACGCGGTTT GAGCACGTCC GGGCGGTCGG
UL102 cDNA    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

TGAAAAAAGA CCCCGCGGGC CTTCGCGACT CTCTTCTGTC CGAGGATGAC CGCTCAGCCG CCGCTGCACC ACCGCCACCA CCCGTACACC CTGTTCGGGA 146,583
---------- ---------- ----GCGACT CTCTTCTGTC CGAGGATGAC CGCTCAGCCG CCGCTGCACC ACCGCCACCA CCCGTACGCC CTGTTCGGGA
mRNA START                                 UL101X

CCAGCTGTCA TCTCAGCTGG TACGGCCTTC TAGAGGCCTC GGTGCCTATC GTACAATGTC TGTTTTTGGA TCTGGGTGGC GGCCGTGCCG AGCCGCGGCT
CCAGCTGTCA TCTCAGCTGG TACGGCCTTC TGGAGGCCTC GGTGCCCATC GTACAATGTC TGTTTTTGGA TCTGGGTGGC GGCCGTGCCG AACCGCGGCT
                                          UL101X GENOMIC
                                               STOP
TCACACGTTC GTGGTGCGCG GTGACCGTCT ACCGCCGGCT GAGGTGCGTG CTGTGCATCG CGCCAGCTAG GGTGCGCTGG CCTCGGCCGT GACTACGGAC 146,783
TCACACGTTC GTGGTGCGCG GTGACCGTCT GCCGCCGGCT GAGGTGCGTG CTGTGCATCG CGCCACGTAC GCCGCGCTGG CCTCGGCCGT GACTACGGAC

GCCGATGAGC GTCGGCGCGG CCTAGAGCAG CGTAGCGCCG TGTTGGCGCG CGTGTTGCTA GAAGGCAGCG CGTTAATCCG CGTGTTGGCG CGCACCTTCA
GCCGACGAGC GCCGGCGCGG CCTAGAGCAG CGTAGCGCCG TGTTGGCGCG CGTGTTGCTA GAAGGCAGCG CGTTAATCCG CGTGTTGGCG CGCACCTTCA

CGCCGGTGCA GATTCAGACG GACGCTAGTG GCGTGGAGAT TTTGGAGGCC GCACCGGCAC TGGGCGTGGA AACCGCAGCG CTGTCGAACG CGCTTAGTCT 146,983
CGCCGGTGCA GATTCAGACG GACGCTAGCG GCGTGGAGAT CTTGGAGGCC GCGCCGGCAT TGGGCGTGGA AACCGCAGCG CTGTCGAACG CGCTTAGTCT

TTTCCACGTA GCCAAGCTAG TGGTCATCGG CTCGTATCCC GAAGTGCACG AGCCGCGTGT GGTCACGCAT ACCGCGGAAC GCGTCTCCGA AGAGTATGGC
TTTCCACGTA GCCAAGTTAG TGGTCATCGG CTCGTATCCC GAAGTGCACG AGTCGCGTGT GGTCACGCAT GCCGCGGAAC GCGTCTCCGA AGAGTATGGC
                                          UL102

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AGGCCGTCCT GGCACGCCTC TTTGAGGTGC GCGAGGTGTG TTTTTTGCGC ACCTGTCTGC GTCTGGTCAC GCCCGTCGGT TTCGTGGCCG TGGCAGTGAC

CGACGAGCAG TGTTGTTTAT TGCTGCAGTC GGCCTGGACT CACCTTTACG ACGTGCTTTT CCGTGGTTTC GCTGGGCAGC CGCCGCTACG CGACTACCTG 147,338
CGATGAGCAG TGTTGTTTGT TGCTGCAGTC GGCCTGGACT CACCTTTACG ACGTGCTTTT TCGTGGTTTC GCTGGGCAGC CGCCGCTACG CGACTACCTG

GGGCCGGACC TCTTTGAGAC GGGCGCCGCC CGTTCTTTCT TTTTTCCCGG TTTCCCGCCC GTGCCCGTCT ACGCGGTCCA CGGTCTGCAC ACGTTAATGC
GGGCCGGACC TCTTTGAGAC GGGCGCTGCC CGTTCTTTCT TTTTTCCCGG TTTCCCGCCC GTGCCCGTCT ACGCGGTCCA CGGTCTGCAC ACGTTAATGC

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GTCACTCACC GCCATTCTAG CGGCGGCGGT GGTAGTGGCG TGGGCGACGC GGACCACGCG AGTGGCGGCG GTCTAGATGC CGCTGCCGGG AGTGGTCATA
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CGTGGAGGTC TGGGCGCGAG AGTTATTGTC GTCCTCCACC GCTTCCACCA CCGCTTGTTC TTCTTCTTCC GTTCTCTCCT CCGCCTTGCC GTCCGTCACT 148,938
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                                                       UL102 STOP

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GCGTTGAGCA CGTCTAGCAG GCCCAGGCCC ACGGGCCAAC CGTCCACGGT CTTGCGCTCG GTCAGCTTGA GGCTGAACGA GTGTGCCTCG TCCTGACCGG

TAAGGCGGAA AAAGAAGCGT GCTACCAGCT GCAGGCAGGT ATGCCGCGTC TGCTGGAAGA GCACGAAGGT AGCGGGCACG TACTGCACAA TGTGCGGCTC
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B          10         20         30         40         50         60
   MTAQPPLHHR HHPYALFGTS CHLSWYGLLE ASVPIVQCLF LDLGGGRAEP RLHTFVVRGD

           70         80         90        100        110        120
   RLPPAEVRAV HRATYAALAS AVTTDADERR RGLEQRSAVL ARVLLEGSAL IRVLARTFTP

          130        140        150        160        170        180
   VQIQTDASGV EILEAAPALG VETAALSNAL SLFHVAKLVV IGSYPEVHES RVVTHAAERV

          190        200        210        220        230        240
   SEEYGTHAHK KLRRGYYAYD LAMSFRVGTH KYVLERDDEA VLARLFEVRE VCFLRTCLRL

          250        260        270        280        290        300
   VTPVGFVAVA VTDEQCCLLL QSAWTHLYDV LFRGFAGQPP LRDYLGPDLF ETGAARSFFF

          310        320        330        340        350        360
   PGFPPVPVYA VHGLHTLMRE TALDAAAEVL SWCGLPDIVG SAGKLEVEPC ALSLGVPEDE

          370        380        390        400        410        420
   WQVFGTEAGG GAVRLNATAF RERPAGSDRR WLLPRCRVTT ATVKTTSWKS ARSTGGAHPP

          430        440        450        460        470        480
   SDDATFTVHV RDATLHRVLI VDLVERVLAK CVRARDFNPY VRYSHRLHTY AVCEKFIENL

          490        500        510        520        530        540
   RFRSRRAFWQ IQGLLGYISE HVTSACASAG LLWVLSRGHR EFYVCDGYSG HGPVSAEVCV

          550        560        570        580        590        600
   RTVVDCYWRK LFGGDDPGPT CRVQESAPGV LLVWGDERLV GPFNFFYGNG GAGGSPLHGV

          610        620        630        640        650        660
   VGGFAAGHCG GACCAGCVVT HRHSGGGGGS GVGDADHASG GGLDAAAGSG HNGGSDRVSP

          670        680        690        700        710        720
   STPPAALGGC CCAAGGDWLS AVGHVLGRLP ALLRERVSVS ELEAVYREIL FRFVARRNDV

          730        740        750        760        770        780
   DFWLLRFQPG ENEVRPHAGV IDCAPFHGVW AEQGQIIVQS RDTALAADIG YGVYVDKAFA

          790        800        810        820        830        840
   MLTACVEVWA RELLSSSTAS TTACSSSSVL SSALPSVTSS SSGTATVSPP SCSSSSATWL

          850        860        870        880        890        900
   EERDEWVRSL AVDAQHAARR VASEGLRFFR LNA*...... .......... ..........
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FIG. 2. (A) Nucleotide sequence of a cDNA clone isolated from a 72-h-postinfection cDNA library encoding the putative PAF. The HCMV (AD169) published genomic nucleotide sequence from the *Asc*I (nt 146383)-to-*Hin*dIII (nt 149645) fragment is shown above the cDNA sequence. Base pair mismatches are shown as outlined and underlined letters. Start and stop codons are also indicated. (B) Predicted amino acid sequence from the first ATG to the first in-frame stop codon within the cDNA.

A 72-h-postinfection HCMV strain Towne cDNA library was screened in order to identify the cDNA clone encoding the putative PAF. We initially used the restriction fragment *Mlu*I (nt 147055)-*Kpn*I (nt 147618). This fragment was complementary to the UL102 putative 5′ end. This probe was selected because its location would ensure the selection of presumably full-length cDNA clones. By using this probe, several positive clones were identified. Plasmid DNA from positive clones was cleaved with *Xho*I, which releases the cDNA insert from the vector, and analyzed by gel electrophoresis (30). Clones that were shown to have inserts that were larger than approximately 2 kb were selected for sequencing. Initially, approximately 15 positive cDNA clones greater than 2 kb were identified from screening about 10,000 colonies. All clones were sequenced with respect to their 3′ and 5′ ends. The 3′ ends of all these cloned inserts were virtually identical, terminating approximately 100 nt beyond the putative translational stop site of UL102 (30). However, the 5′ ends of the cDNA inserts varied in length. Of these cDNA inserts, two were about 2.6 kb in length. Initially, these two were partially sequenced with respect to their 3′ and 5′ ends and determined to have identical 3′ ends. The two clones, p102CD1 and p102CD2, were subsequently fully sequenced and also shown to have identical internal sequences. However, p102CD2 had a 5′ sequence that extended further upstream. The analysis of this sequence is presented below, and the full sequence is shown in Fig. 2.

Sequence analysis of cDNA encoding the putative PAF. Plasmid p102CD2 was sequenced by using the dideoxy chain termination method as described in Materials and Methods (1).

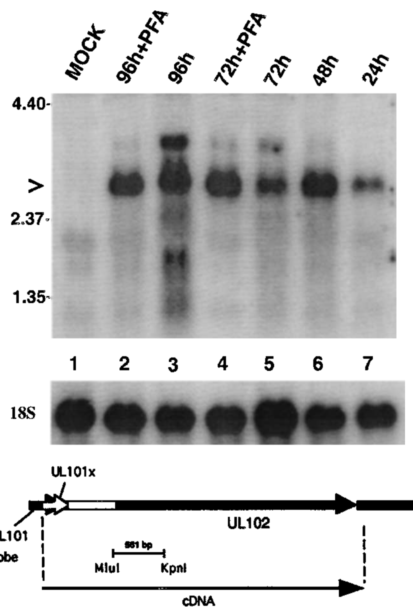

FIG. 3. Northern blot of HCMV (AD169) infected total-cell RNA. Single-strand specific antisense riboprobes were used to probe infected total-cell RNA at various time points. Infected human foreskin fibroblast RNA was harvested at various times postinfection and in the presence or absence of PFA. Ten micrograms of total-cell infected or uninfected (mock) RNA was hybridized with a probe (*Mlu*I-*Kpn*I) 561 bp long which contained coding sequences extending from the putative ATG of the UL102 ORF to 469 nt into the ORF (Fig. 2). This probe detected an abundant 2.7-kb RNA (arrow) as early as 24 h postinfection and in the presence of PFA (lanes 7 and lanes 2 and 4, respectively). Steady-state 18S RNA levels are shown above diagram. The numbers on the left are sizes, in kilobases.

Initial 5′-end sequencing revealed that the p102CD2 cDNA insert originated 5 bp upstream from the putative ATG for the UL101X ORF. Subsequent full-length sequencing determined that the UL102 mRNA was unspliced and extended 96 nt beyond the putative stop codon for the UL102 ORF (Fig. 2). Internal sequencing revealed that the stop codon present in the genomic DNA of strain AD169 at nt 146744 was absent in the cDNA (Fig. 2). This prompted us to resequence the AD169-cloned fragment (pUL101-102) and Towne DNA corresponding to this region. Sequence analysis of the AD169 plasmid or Towne genomic DNA in the region of a putative UL101X stop codon revealed that the guanosine residue in the genomic in-frame stop codon, TAG (nt 146746), is actually a cytosine, changing this codon to a TAC (30). This codon sequence now encoded the amino acid tryptophan, and consequently the only stop codon in the same reading frame was now at nt 149321 in the genomic DNA. Figure 2 shows that the total length of the cDNA is 2,723 bp. The cDNA encodes a 2,621-bp ORF having the capacity to code for a protein containing 873 amino acids, corresponding to a molecular mass of approximately 100 kDa. No apparent polyadenylation signal is present in the genomic sequence or the 3′ end of the cDNA; also, it did not contain a poly(A) tract indicative of polyadenylated messages. The predicted protein sequence for the largest ORF is shown in Fig. 2B. Figure 2 also shows that there are three other ATGs in the same reading frame context present at nt 612, 957, and 2346 of the cDNA. A nucleotide sequence comparison revealed 97% homology between the UL101 cDNA (Towne) and AD169 genomic DNA.

The UL102 transcript is a 2.7-kb message. The characterization of the cDNA clone discussed above allowed us to de-

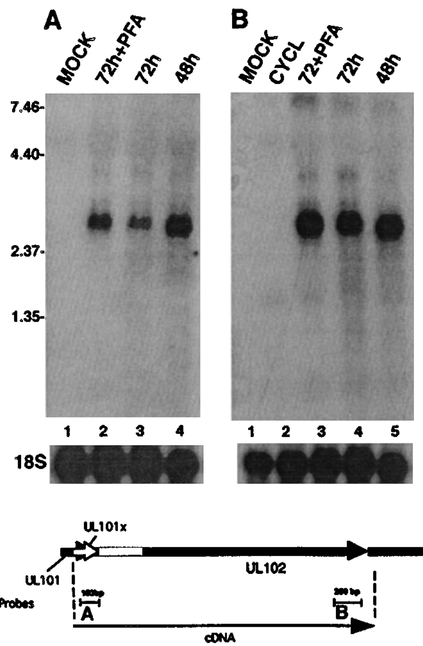

FIG. 4. Autoradiogram of Northern blots hybridized with upstream and downstream regions of UL102. Infected total-cell RNA was hybridized to a 103-bp *Nru*I (nt 146502)-*Xba*I (nt 146605) fragment containing the UL101X ORF putative coding region (see Materials and Methods) (A) and a 250-bp *Eco*RV (nt 148825)-*Hin*cII (nt 148153) probe encoding the 3′ end of the UL102 ORF (B) (see Materials and Methods). Figure 2 shows probe positions in the context of the UL101X and UL102 ORFs. Mock, uninfected RNA; cycl, cycloheximide. The numbers on the left are sizes, in kilobases.

sign hybridization probes to be used in Northern analysis. Figure 3 shows the result of a Northern blot using a 561-bp DNA fragment to generate a riboprobe as described in Materials and Methods. This fragment contains a sequence that is complementary to a region that includes the putative ATG for UL102 (Fig. 3). This probe detected an abundant 2.7-kb message at 24 h postinfection (Fig. 3, lane 7) and in the presence of PFA (Fig. 3, lanes 2 and 4). Northern blots of infected cell RNA isolated at earlier time points failed to detect this transcript (30). A minor larger RNA species was also detected beginning at 72 h postinfection and was most abundant at 96 h (Fig. 3, lanes 3 and 5); however, its abundance was considerably diminished at 96 h postinfection in the presence of PFA (Fig. 3, lane 2). Other experiments have confirmed that this larger transcript is not detected in the presence of PFA (30). This larger transcript is probably a late viral transcript, because of its detection only after 48 h and its sensitivity to PFA. Other smaller bands seen in infected RNA samples are probably the result of nonspecific binding of the riboprobe. These bands are also seen in the mock-infected RNA lanes. The filter was rehybridized with a probe specific for the 18S RNA as an internal control to determine the relative amounts of RNA loaded.

Hybridization probes complementary to regions within the UL101X ORF made from p101Bribo (103 bp) and to the 3′ end of UL102 made from p102Bribo (250 bp) were also used in Northern blots (see Materials and Methods). Figure 4A shows the result of a Northern blot when a probe that is complementary to the 5′ end of UL101X was used. This probe detected the same 2.7-kb RNA species as that seen in Fig. 3. When a probe complementary to the 3′ end of UL102 was used, the same result was observed (Fig. 4B). Figure 4 also

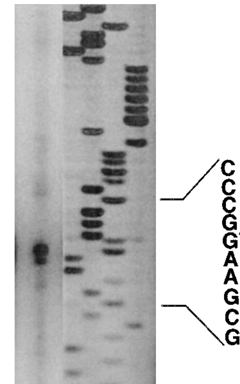

FIG. 5. Primer extension analysis of the putative primase-associated transcript. HCMV infected total-cell RNA was hybridized to a synthetic 20-base oligonucleotide primer (5′-CGAACAGGGTGTACGGGTGG-3′) 36 nt downstream of the 5′ end of the putative 2.7-kb PAF cDNA. The homologous sequence is shown at the right, and the asterisk indicates the transcriptional initiation site which was determined to be 15 nt longer than the 5′ end of the UL102 cDNA.

demonstrates that this transcript is not detected in the presence of cycloheximide (Fig. 4B, lane 2). Probes that were complementary to the region upstream of the putative ATG of UL101X (116 bp) (Fig. 2) or to a region downstream of UL102 (174 bp) (Fig. 2), beyond the 3′ terminus of the PAF cDNA clone, failed to detect the 2.7-kb RNA species (30). The results of these studies indicated that the size of the transcript encoding the putative PAF is consistent with the size and location of the cDNA clone isolated and that the temporal regulation of this gene is consistent with that of an early-kinetic-class gene.

Primer extension analysis was performed in order to determine the location and sequence of the 5′ end of the transcript. Infected and uninfected total-cell RNA was hybridized to an oligonucleotide primer, end labeled with ^{32}P, complementary to a region corresponding to bases 38 to 57 of the cDNA sequence. Figure 5 shows the result of this experiment and shows that the PAF RNA initiates 52 bp upstream of the oligonucleotide primer and extends 15 bp upstream of the 5′ end of the cDNA clone.

Inspection of the UL102 gene upstream region. Primer extension analysis revealed the UL102 mRNA start site, and as a consequence of this finding, the upstream untranscribed region was also elucidated. The boundaries of a promoter for the UL102 gene were somewhat determined by the size of the upstream region within the clone previously shown to complement DNA replication (25). It was previously reported that the *Asc*I-*Hin*dIII genomic clone was sufficient, in the presence of other required genes, to replicate cloned *oriLyt* in the cotransfection-replication assay (25). On the basis of this information, we therefore assumed that a promoter or at least a portion of a promoter region was present within this clone. The upstream region from nt +1 to −124 (*Asc*I-*Nru*I), relative to the transcriptional start site, is shown in Fig. 6. This sequence contains no identifiable consensus TATA sequences; however, there is a TAAATA sequence at nt −82. Multiple GC-rich repeats (GCCGCCGCGGC) were also identified in addition to two AP2 sequences and an SP1 site at nt −12, −13, and −35, respectively. In addition, an IR1-like sequence is present beginning at nt −95 (16).

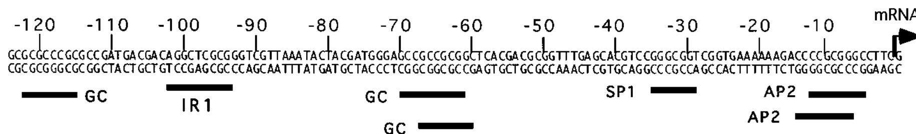

FIG. 6. Nucleotide sequence and identification of consensus transcription factor binding sites in the upstream noncoding region of the UL102 gene. Shown are several GC-rich repeat regions, an SP1 site, two AP2 sites, and an IR1-like region.

DISCUSSION

The goal of this study was to elucidate the structure of the HCMV transcript encoding the putative candidate homolog for PAF. An HCMV genomic fragment containing the ORF UL102 was initially identified, by using the cotransfection-replication assay, as being essential for origin-dependent DNA replication (25). This genomic fragment contained a large upstream region. When the region was deleted or frameshifted, the fragment did not retain the ability to complement origin-dependent DNA replication. This observation indicated a possible additional required ORF or the presence of an RNA originating within this region. Initial inspection of this upstream region revealed a small ORF that we refer to in this report as UL101X (25). Results presented here identify the PAF transcript and a putative promoter. This is consistent with previous data reported from the origin-dependent DNA replication assay indicating an essential region upstream of the UL102 ORF.

Inspection of the cDNA nucleotide sequence corresponding to the PAF transcript revealed that a 2.7-kb RNA species originated from the region of the HCMV (AD169) genome from nt 146510 to 149241. The size of this cDNA matched exactly the size of the RNA detected on Northern blots using probes from this region. Sequence analysis of this clone indicated that the UL102 mRNA was unspliced and initiated just upstream of the UL101X ORF. As for UL101X, the cDNA sequence revealed that the reported genomic stop codon (TAG) was actually TAC, and therefore this ORF no longer existed in the cDNA. Subsequent genomic sequencing of Towne and AD169 also showed that the reported stop codon for UL101X was an error in the published sequence (3). As a consequence of this finding and the corroborating Northern analysis and primer extension analysis data, we conclude that the UL102 transcript is a 2.7-kb transcript and encodes an ORF capable of producing a protein having a molecular mass of around 100 kDa. Within the UL102 transcript there is the capacity to encode several smaller proteins of 617, 556, and 279 amino acids. These ORFs are all in frame with respect to the stop codon at the end of UL102. At this time we do not know which ATG(s) is being used to make the putative PAF. However, evidence from previously published results seem to indicate that the first ATG encodes the first amino acid for this protein. Frameshifts in the region between the first ATG and the UL102 ATG failed to replicate cloned *oriLyt* in the replication assay (25). In addition, preliminary immunoblot results indicate that an infected cell protein with a molecular mass of about 100 kDa is detected by using peptide antibodies made to the upstream coding region (30).

As stated in Results, inspection of the upstream untranscribed sequence failed to reveal a TATA-like sequence, although a TAAATA sequence is present at nt −82. Also present are several GC-rich repeat regions and SP1 and AP2 consensus sequences. This region was subcloned to show that it responds to HCMV infection and that a possible promoter may exist in this upstream region (30).

The 3′ end of the UL102 transcript and gene contains no identifiable polyadenylation signal. The cDNA terminates 96 nt downstream of the only in-frame translational stop codon TAA (Fig. 2). The closest downstream consensus polyadenylation signal is over 5,800 bp away at nt 154972. It is unlikely that this signal is used, and it would be inconsistent with Northern analysis data and other data presented here. The cDNA library used was constructed from poly(A)-purified RNA. It is possible that this RNA was selected because of long A tracts (7 adenines in a row) at the 3′ end of the sequence. In addition, the *Asc*I-*Hin*dIII genomic clone is capable of complementing DNA replication; therefore, efficient expression of the UL102 transcript must be possible without any apparent polyadenylation signal (25). Several other cDNAs that we isolated with the same probe had identical 3′ ends. Another line of evidence indicating that this is the true 3′ end is the observation that hybridization probes downstream of the end of the cDNA failed to detect the 2.7-kb transcript.

The evidence that UL102 encodes the PAF is based on positional alignments between this gene and UL8 of HSV-1, and more recently, amino acid sequence similarities have been found between UL102 and the other herpesvirus PAF genes (11). In HSV-1, UL8 encodes a 75-kDa protein that is absolutely essential for HSV-1 DNA replication (24). In one study it was shown that the UL8 protein acts to increase the efficiency of primer utilization through stabilization of the interaction between primer and template (29). Another study has implicated the UL8 gene product in increasing the efficiency of primer synthesis (32). The HSV-1 helicase-primase complex includes two other proteins, UL5 (helicase) and UL52 (primase) (6–8, 17). The two other proposed subunits of the HCMV helicase-primase complex, UL105 and UL70, show significant homology to their HSV-1 counterparts (3, 20). EBV counterparts are BBLF4 (helicase) and BSLF1 (primase). The candidate EBV PAF gene consists of two ORFs, BBLF2 and BBLF3, that are presumably spliced into a single transcript (11). The protein encoded by BBLF2/3 in infected cells has not been identified or characterized. However, the predicted molecular mass of the EBV PAF is approximately 82 kDa. This is slightly smaller than the predicted molecular mass of the HCMV homolog.

The results presented here have aided our understanding of this important HCMV replication gene and how it relates to the equivalent gene in other herpesviruses. In addition, some information about the regulation of this gene will also allow us to study how transactivators contribute to the replication process.

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