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Transcriptional Patterns of the pCD41 (U27) Locus of Human Herpesvirus 6

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Human herpesvirus 6 (HHV-6) is a lymphotropic herpesvirus, and in vitro, it can productively infect many of the same cell types that human immunodeficiency virus (HIV) infects. Simultaneous infection of T cells by HIV and HHV-6 can lead to both activation of the HIV promoter and acceleration of the cytopathic effects. Several HHV-6 genes have been demonstrated to activate HIV promoter expression. Among them is a cDNA clone, pCD41 (U27), which codes for the HHV-6 DNA polymerase accessory protein. We have now further characterized the transcription pattern in the pCD41 locus and identified at least six RNA species, ranging in size from 1.2 to 4.5 kb. Northern (RNA) blot analyses showed no significant difference in RNA patterns between the HHV-6 variant A (GS) and variant B (Z29) viruses. All the RNA species detected by pCD41 are polyadenylated and polyribosome associated, suggesting that they may be actively engaged in protein synthesis. Cycloheximide and phosphonoacetic acid inhibition assay results indicate that all the pCD41 RNA species belong to the herpesviral early-late family. Using primer extension and S1 mapping techniques, the 5′ and 3′ ends of each transcript were mapped to different positions, and no splicing was observed.

Human herpesvirus 6 (HHV-6) is a lymphotropic herpesvirus that was first isolated from the peripheral blood lymphocytes of patients with lymphoproliferative disorders and AIDS (13, 26, 49, 55). Serological studies have shown that HHV-6 is highly prevalent in the general population and that the seropositivity rate exceeds 90% (24, 25, 30, 49). HHV-6 is the causative agent of exanthem subitum (54) and has been implicated as a cofactor in the pathogenesis of AIDS (28, 35). In vitro, HHV-6 productively infects many of the same cell types as human immunodeficiency virus (HIV), particularly CD4+ human T cells. Although HHV-6 has a primary tropism for CD4+ T cells, HHV-6 also infects and kills CD8+ T cells, natural killer cells, mononuclear phagocytes, and neural cells (19, 33, 34, 48, 53). HHV-6 infection induces the expression of CD4 molecules in the CD8+ T cells which are generally refractory to HIV infection, and these cells become susceptible to HIV infection (34). Coinfection of T cells with HIV and HHV-6 leads to activation of the HIV long terminal repeat (LTR)-directed viral gene expression and can accelerate the cytopathic effects of HIV (14, 33). Furthermore, several HHV-6 genes responsible for trans-activation of the HIV LTR have been identified (10, 21, 22, 27, 37, 39, 59, 60). Besides activating HIV gene expression, HHV-6 also suppresses HIV type 1 replication in vitro (8, 31). Thus, HHV-6 could act as an important cofactor in the pathogenesis of AIDS by regulating the expression of HIV genes and/or by expanding the susceptible host range for HIV infection.

The double-stranded DNA genome of HHV-6 is about 167 kb in size and consists of a 143-kb unique region flanked by 8- to 13-kb direct repeats (17, 26, 42). The genome can potentially encode over 102 proteins (17). Using monoclonal antibodies, we previously identified an HHV-6(GS) cDNA clone, pCD41, now known as U27, which encodes a 41-kDa nuclear phosphoprotein (p41) (9). This protein is conserved among all HHV-6 strains and shows strong homology with the human cytomegalovirus (HCMV) UL44 gene coding for the ICP36 family of early-late-class phosphoprotein (4, 9). Its location in the HHV-6 genome is analogous to the location of UL44 in the HCMV genome. The HCMV UL44 gene product is an accessory protein for HCMV viral DNA polymerase and plays an important role in viral replication (15). HHV-6 p41 has been shown to bind DNA with the same affinity as HCMV UL44 and hence is likely to be essential for HHV-6 replication (4). Recently, we showed that both pCD41 cDNA clone and its genomic clone, pGD41, can trans-activate HIV LTR-directed gene expression in CV-1 cells, suggesting that the pCD41 gene locus may be involved in gene regulation as well as potentially in viral DNA replication. The pCD41 locus encodes two open reading frames (ORFs), ORF A and ORF B. The p41 protein is encoded by ORF A, and a 27-kDa protein can potentially be encoded by ORF B. The C-terminal half of p41 was shown to be responsible for the trans-activation activity, and the NF-κB binding sites on the HIV LTR appear to be important for activation (60). Deletion constructs in the pCD41 cDNA clone suggested that the trans-activation is mediated by the putative gene product from ORF A and that a putative internal promoter can be used to transcribe a protein with trans-activation function. Downstream of this promoter region, there were three ATG codons in the same ORF, and translation in any of these three codons could result in the synthesis of three overlapping proteins of 18, 16, and 14 kDa. In order to determine whether such a transcript exists and whether such truncated trans-activating proteins can be made, it is important to identify the transcripts that are encoded by this gene locus. Multiple RNA species (4.5, 2.6/2.5, 2.1, 1.8/1.7, and 1.2 kb) in HHV-6(GS)-infected HSB-2 cells were found to hybridize to the pCD41 probe (4, 9), suggesting that this gene locus may encode different gene products. Since this gene locus seems to be involved in viral gene regulation, detailed analyses of this region should provide important information on how HHV-6

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regulates its gene expression, replicates its DNA, and interacts with other viruses, especially HHV. In this study, we have shown that at least six different RNA species can hybridize to the pCD41 probe in Northern (RNA) blot analyses and that all of them are transcribed from the same orientation. These RNA species belong to the early-late gene family. They are polyadenylated and associated with polysomies, suggesting that they may be translated. Our RNA mapping results suggest that the 2.3- and 1.5-kb RNA species could encode p41 protein in the ORF A and that the 1.2-kb RNA may encode a protein of about 17 kDa within ORF B.

**MATERIALS AND METHODS**

Plasmids, clones, cells, and viral infection. The construction of pCD41 and pGD41 encoding the HHV-6-p41 protein has been described previously (9). The 3.1-kb HHV-6 immediate-early (IE) gene cDNA clone was kindly given by F. Neipel (50). Human T-cell lines HS-2 (ATCC CCL 121), Molt-3 (ATCC CRL 1552), and J-Hahn were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. HHV-6 (GS) and HHV-6 (292) were kindly given to us by R. Gallo and P. Pellegrino (25, 32, 46). HHV-6 (292) was propagated in either HSB-2 or J-Hahn cells and HHV-6 (292) was propagated in Molt-3 or J-Hahn cells. Cocultivation was carried out at a ratio of 1.5 infected cells/uninfected cells. After incubation at 37°C for 5 h or overnight, the cells were spun down and suspended in fresh medium with 10% fetal bovine serum. Three days after infection, the media were replaced. The infected cells were harvested when at least 75% of them displayed cytopathic effects. To make high-titer virus stock, well-infected cells were pelleted and frozen by use in 1/10 of the original volume of medium. After three freeze-thaw cycles, the cell debris was spun down at 3,000 × g for 10 min and the supernatant was either used immediately or kept at −80°C for later infection.

**DNA labeling.** To generate the single-strand DNA (ssDNA) probes, the pCD41 EcoRI insert was cloned into M13mp18 in different orientations. The phages were purified by phenol/chloroform extraction, and ssDNA was isolated by phenol-chloroform (1:1) extraction and ethanol precipitation (5). Double-strand and ssDNA probes were labeled by the random primed labeling method with a NEN kit (New England Biolabs). The oligonucleotides were labeled by T4 DNA kinase with [γ-32P]ATP (50 Ci/mmol) at 37°C for 1 h, and the cell clumps were dispersed every 15 min. Thirty-five milliliters of fresh medium with 100 µg of CHX per ml was then added and incubated at 37°C for another 5 h. The cells were harvested, and RNAs were isolated by the method through a 5.7 M CsCl cushion. For the untreated control, cells were treated similarly except that no CHX was added. For PAA treatment, 4 × 106 HSB-2 cells were treated with 300 µg of PAA per ml for 2 h prior to the addition of 2 µg of Polybrene per ml and then incubated at 37°C for another hour. The cells were spun and suspended in 5 ml of 10× TCDD/5×106 cells of virus stock with 300 µg of PAA per ml. The clumps were dispersed every 15 min. After 1 h at 37°C, the cells were washed twice with RPMI 1640 medium with PAA and then suspended in 40 ml of fresh medium with serum and PAA. Cells (20 ml each) were harvested at 12 and 24 h, and total RNA was isolated by the CsCl cushion method as described earlier. For the untreated control, no PAA was added.

**PCR and cloning.** All the primers for PCR were designed by using the pCD41 or pGD41 sequences published previously (9). The primer corresponding to the position of either the pCD41 or the pGD41 sequence, Primers for amplification and cloning of probe 1 are as follows: T3 primer, 5'-AAACCTTACACTAGGG-3', and P611 primer, 5'-CATGATAATGTTTGCGTAC-3' (positions 612 to 591 of pCD41).

**Preparation of total RNA and of poly(A) and non-poly(A) RNAs.** Total RNA was extracted with commercial Trizol reagent (GIBCO-BRL) or guanidinium-isoniocydtate as described elsewhere but with slight modification (7). Briefly, the cells were suspended in guanidinium-isoniocydtate lysis buffer (4 M guanidinium-isoniocydtate, 20 mM sodium acetate [pH 5.2], 0.1 mM dithiothreitol [DTT], 0.5% N-lauroylsarcosine) and passed through a 3-mI syringe in the presence of 5 µg/ml protease (Mutazap-A). The lysate was loaded onto a 5.7 M CsCl (prepared in deionized water) column and centrifuged for 18 h in a Beckman SW41 rotor at 35,000 rpm and 18°C. The RNA pellet was suspended in 360 µl of TES (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% sodium dodecyl sulfate [SDS]) solution and recovered by three ethanol precipitations. Poly(A) RNA was separated from the total RNA by the PolyATtract mRNA isolation system III (Promega). The separation was carried out by following the procedures recommended by the manufacturer and retaining the RNA pellet as the non-poly(A) RNA fraction. After ethanol precipitation, the RNA was then used for various studies.

**Preparation of cytoplasmic and nuclear RNAs.** Cell-free HHV-6-GS-infected HSB-2 cells were harvested at 5 days postinfection (p.i.) and suspended in lysis buffer at a density of 106 cells/ml (25 ml, pH 7.4), 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), 1% sodium citrate, and 5% Denhardt solution at 4°C. After hybridization, the membrane was washed twice with 20× SSC-0.1% SDS at room temperature and then three times with 0.5× SSC-0.1% SDS at 5°C. After hybridization, the membrane was washed with 20× SSC-0.1% SDS at room temperature. The membrane was then exposed to Kodak XAR-5 film either with intensifying screens at −80°C or without screens at room temperature. RNA size was calculated by using its mobility with the commercial 0.16- to 1.77-kb and 0.24- to 9.5-kb RNA ladders (GIBCO-BRL).

**Northern blot analyses.** Northern blotting was carried out as described elsewhere (7). Briefly, the RNA samples were separated in a 1.5% formaldehyde agarose gel and then transferred to a supported nitrocellulose membrane (Schleicher & Schuell). The membrane was baked at 100°C for 2 h in a vacuum, then soaked in 20× SSC containing 25 µg/ml of salmon sperm DNA and then heated to 80°C for 15 min and then allowed to cool to room temperature. The membrane was then incubated with 5× SSC-0.1% SDS at room temperature and then three times with 0.5× SSC-0.1% SDS at 5°C. After hybridization, the membrane was washed with 20× SSC-0.1% SDS at room temperature. The membrane was then exposed to Kodak XAR-5 film either with intensifying screens at −80°C or without screens at room temperature. RNA size was calculated by using its mobility with the commercial 0.16- to 1.77-kb and 0.24- to 9.5-kb RNA ladders (GIBCO-BRL).

**Primer extension and S1 mapping.** Total RNA (10 µg) was digested with 10 µg of PstI- and SphI-labeled primer and then coprecipitated with ethanol. The pellet was washed with ether, dried briefly, and suspended in 20 µl of buffer (80 mM Tris-HCl [pH 8.3], 40 mM KCl). The mixture was heated to 90°C, slowly cooled to 60°C, and then incubated at 52°C for 30 min. Reverse transcriptase (25 µl of 400 mM Tris-HCl, 25 µl of 400 mM dNTPs, 1 µl of 300 mM MgCl2, 2.5 µl of 25 mM MgCl2, 2 µl of 0.01% SDS, and 5 µl of 2× MgCl2 dNTP, 10 mM dNTP) was added, and the mixture was incubated at 22°C for 1 h. After digestion with 50 µl of RNase A mixture (100 µM NaCl, 10 mM Tris-HCl [pH 8.0]), 1 µl EDTA, and 15 µg of RNA, the sample was extracted once with phenol-chloroform (1:1), precipitated by...
ethanol, and then analyzed on a 6% sequencing gel. A sequence reaction using the same labeled primer was run alongside to determine the size of the primer extension products. The gel was then dried and exposed to Kodak XAR-5 film with intensifying screens at ~80°C.

The S1 protection assay was carried out as described elsewhere (7). Briefly, 5 x 10^6 cpm of labeled probe was mixed with 20 μg of total RNA, and the mixture was ethanol precipitated. The RNA pellet was briefly dried and then suspended in 20 μl of S1 hybridization buffer (80% formamide, 40 mM PIPES [piperazine-N,N’-bis(2-ethanesulfonic acid), pH 6.4], 400 mM NaCl, 1 mM EDTA). The mixture was heated to 65°C for 10 min and annealed overnight at 30°C. S1 digestion buffer (300 μl) (0.24 M NaCl, 50 mM sodium acetate [pH 4.5], 4.5 mM ZnSO4, 6 μg of denatured salmon sperm DNA, 150 U of S1 nuclease [Boehringer Mannheim]) was added, and the mixture was incubated for different times at 30°C. After addition of 80 μl of S1 stop solution (4 M ammonium acetate, 20 mM EDTA pH 8.0), 20 μg of denatured salmon sperm DNA per ml, 1 ml of ethanol was added to precipitate the DNA. The protected products were then separated on a 6% sequencing gel, using a sequencing reaction as marker.

DNA sequence analysis. Sequence data were obtained with the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals) based on the dideoxynucleotide termination method (47). Sequence analyses were carried out with the Genetics Computer Group, Inc., sequence analysis package (Madison, Wis.).

RESULTS

HHV-6 variants A and B have similar transcriptional patterns in the pCD41 region. Two major subgroups of HHV-6 viruses, variants A and B, have been identified (1, 2, 6, 23, 32, 51). To study the gene expression of the pCD41 gene locus, we first determined the transcriptional patterns for both variants. Total RNA was isolated from either variant A HHV-6(GS)-infected HSB-2 cells or variant B HHV-6(Z29)-infected Molt-3 cells when at least 70% of the cells displayed cytopathic effects. For HHV-6(GS)-infected HSB-2 cells (Fig. 1, lane 2), six RNA bands with estimated sizes of about 4.5, 2.7, 2.3, 1.8, 1.5, and 1.2 kb were detected by the 2.1-kb pCD41 probe, with the 2.3-kb RNA species being most abundant (Fig. 1, lane 2). For HHV-6(Z29)-infected Molt-3 cells, the pCD41 hybridization signals were much weaker than those of HHV-6(GS), even though an equal amount of RNA was loaded. The overall pCD41 RNA pattern in HHV-6(Z29)-infected Molt-3 cells is similar to those seen in the HHV-6(GS)-infected HSB-2 cells, although the 2.3-kb RNA seems to be more predominant (Fig. 1, lane 6).

To further determine whether these pCD41 RNA species are polyadenylated, total RNA was separated into poly(A) - and non-poly(A) fractions, and Northern blot analyses were performed. Several poly(A) + RNAs with lengths of 4.7, 2.7, 2.3, 1.9, 1.5, and 1.2 kb were detected in HHV-6(GS)-infected HSB-2 cells (Fig. 1, lane 3). Six distinct non-poly(A) RNA species (4.5, 2.5, 2.1, 1.7, 1.4, and 1.1 kb) were also detected (Fig. 1, lane 4). The non-poly(A) RNAs are likely to be the immature mRNAs, because they are about 100 to 200 bases shorter than the 4.7-, 2.7-, 2.3-, 1.9-, 1.5-, and 1.2-kb poly(A) + mRNAs, respectively (Fig. 1, lanes 3 and 4). For the 4.5- and 1.8-kb RNA species, the mRNAs in the poly(A) + fractions (lane 3) seem larger than those in the total RNA fraction. This could be due to rRNA interference during Northern blot analysis.

pCD41 RNAs are transcribed from one orientation. Since up to six RNAs were detected in this region, it was important to determine their orientations. Labeled ssDNA probes (+/−) were prepared as described in Materials and Methods and used in Northern analyses. Hybridization signals were detected only with the labeled complementary strand of pCD41 (minus strand) probe (data not shown), suggesting that all pCD41 RNAs are transcribed from the same DNA strand in the same orientation as the pCD41 clone.

All the RNAs are polyribosome associated. To determine whether the pCD41 RNA species can potentially be translated, HHV-6(GS)-infected HSB-2 cells were solubilized by Nonidet P-40 treatment and separated into cytoplasmic and nuclear fractions to determine the subcellular localizations of pCD41 RNAs. The RNA species could be detected in both cytoplasmic and nuclear fractions (data not shown). The association of the mRNA with polyribosomes was further analyzed by a combination of sucrose gradient and Northern analyses. The cytoplasmic fraction from HHV-6(GS)-infected HSB-2 cells was centrifuged through a 20 to 47% sucrose gradient in the presence of either 5 mM MgCl2 or 10 mM EDTA. Figure 2A shows the sedimentation profile, and Fig. 2B shows the results of Northern analysis with pCD41 used as probe. In the presence of MgCl2, the 2.7-, 2.3-, 1.8-, 1.5-, and 1.2-kb RNA species could clearly be detected in the polyribosomes (fractions 2 to 4) and monosomes (fractions 6 to 8) fractions. A faint band at the position of 28S RNA was also detected in those fractions. It is unclear whether the signal is from the 4.5-kb RNA species or from nonspecific hybridization with 28S rRNA. In contrast, in the presence of EDTA, the disruption of both polyribosomes and monosomes resulted in release of the ribosome-associated pCD41 RNAs; the hybridization signals were mainly detected at the ribosomal subunit fractions (Fig. 2B, lower panel, fractions 9 to 11), and only a very weak signal remained in the monosome fractions (fractions 6 to 8), perhaps because of incomplete disruption of monosomes. Our results suggest that all the pCD41 RNA species are associated with polyribosomes and have the potential to be actively translated.

Kinetic analysis of pCD41 mRNA expression. Herpesvirus gene expression can be classified as IE, early, and late (20). Expression of early genes is independent of viral DNA replication, and early genes are involved in gene regulation and DNA replication. The late genes are dependent on viral DNA replication and encode structural proteins (20). For the pCD41 gene locus, the intact pCD41 transcript (2.3-kb species) has been suggested as an early-late gene (9), while the other species have not been studied. It is possible that some of the smaller transcripts identified in this study belong to other classes. To classify these RNA species, two inhibitors, CHX and PAA, were used.

The IE transcripts are independent of other viral protein synthesis and are initiated immediately upon virus entrance. Therefore, CHX, a translation inhibitor, will have no effect on the transcription of IE genes but will inhibit IE-dependent viral

FIG. 1. Northern blot analyses of HHV-6-infected T cells. (A) HHV-6 vari- ant A strain GS-infected HSB-2 cells. (B) HHV-6 variant B strain Z29-infected Molt-3 cells. Lanes 1 and 5, uninfected cells as negative control; lanes 2 and 6, total RNA from infected cells; lane 3, poly(A) + RNA purified from total RNA with an oligo(dT) column; lane 4, unbound fraction (pass) from the oligo(dT) purification. The size of each RNA (in kilobases) is estimated from coelectrophoresed RNA ladder and is indicated on the left.

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early and late genes transcription. To determine whether or not pCD41 RNA expression is IE dependent, HHV-6(GS)-infected HSB-2 cells were treated with CHX for 6 h, and total RNAs were analyzed (50). Because of the inefficiency of HHV-6 cell-free infection, poly(A)⁺ RNA was isolated from 100 µg of total RNA to enhance the hybridization signal (Fig. 3A). One of the characterized HHV-6 IE genes, IE 1.1, was used as a control (50). For the IE 1.1 gene, only the 3.6-kb IE 1.1 transcript could be seen clearly in both CHX-treated and untreated cells, although a slight reduction in signal was observed in the treated samples. For the pCD41 panel, with the same amount of total RNA, no signal differentiable from that of the uninfected cell was detected (Fig. 3A, lanes t). This result indicates that very little expression from the pCD41 locus occurred at 6 h p.i. Thus, none of the pCD41 RNAs could be classified as an IE gene. However, the 4.5-, 2.3-, and 1.8-kb RNA species were detectable in the poly(A)⁺ RNA (Fig. 3A, lanes m), but after prolonged exposure, this could be due to incomplete inhibition by CHX. To confirm that an equal amount of RNA was loaded in each sample, the membranes were stripped and then rehybridized with a β-actin probe. No difference was observed between untreated and treated RNA samples.

PAA can inhibit the replication of HHV-6 DNA and prevent late-gene expression (3, 9, 12). To determine whether any pCD41 species are expressed in late infection, total RNAs were isolated from HHV-6(GS)-infected HSB-2 cells treated with PAA and then analyzed by Northern blotting (Fig. 3B). At 12 and 24 h p.i., all the different pCD41 RNAs species could be detected in both PAA-treated and untreated controls, but the intensity of the signal was much lower in treated cells than in the untreated control. This indicates that expression of all the different pCD41 RNAs is significantly but incompletely inhibited by PAA treatment. The incomplete inhibition also suggests that some transcription of the pCD41 locus can occur prior to viral DNA synthesis. For IE 1.1 gene expression (which should be independent of viral DNA syntheses at the early infection), the expression of the 3.6-kb transcripts was also slightly inhibited by PAA. β-Actin transcript was again used as an internal control, and no inhibition of the actin RNA
was observed. The early initiation and incomplete inhibition by PAA suggests that all the RNA species in the pCD41 locus should be classified as early-late transcripts. This observation corresponds well with the HCMV UL44 early-late gene expression (11, 29). The expression of the HCMV 51-kDa protein encoded by UL44 begins prior to that of most other viral proteins but requires preceding viral proteins and DNA replication (11, 29). The synthesis of the p41 protein at this time corresponds to its role as an accessory protein for viral DNA replication.

**Mapping the pCD41 RNA species.** In order to determine the approximate position of each pCD41 transcript, six pCD41 fragments (EcoRI-MstII [I], MstII-AsuII [II], AsuII-NsiI [III], NsiI-PvuII [IV], PvuII-BglII [V], and BglII-EcoRI [VII]) were used as probes for Northern blot analyses. In addition, one PCR product, T3-612 (I), was used to detect possible transcription initiated upstream of the pCD41 gene locus (Fig. 4 and 5). When the intact p41 cDNA was used as a control in hybridization, the RNA species detected (Fig. 4, lanes 2 and 10 to 12) were similar to those observed earlier (Fig. 1, lanes 1 to 4). The hybridization pattern of probe I is different from that of pCD41 (Fig. 4, lane 3). Besides the 4.5- and 2.7-kb RNAs, two new RNA species with molecular sizes of 3.0 and 0.8 kb were also detected. The newly detected RNA species could be the transcripts of other ORFs upstream of pCD41 and initiated from outside the pGD41 locus. These transcripts are unlikely to encode either the trans-activating protein or the viral accessory protein and were not pursued further in this study. Other RNA species detected by the pCD41 probe, such as the 2.3-, 1.8-, 1.5-, and 1.2-kb RNA species, did not hybridize to probe I, suggesting that they are initiated within the pCD41 region. When probe II was used for hybridization, four RNA species (4.5-, 2.7-, 2.3-, and 1.5-kb RNAs) could be detected (Fig. 4, lane 4). Since the 2.3- and 1.5-kb RNA species were not detected by probe I, this result indicates that the 2.3- and 1.5-kb RNA species may be initiated within the probe II region (Fig. 4 and 5). Hybridization with probes III (Fig. 4, lane 5) and IV (Fig. 4, lane 6), gave similar patterns, and the 1.8-kb RNA was detected in addition to those observed with probe II, suggesting that the 1.8-kb RNA species is initiated within the probe III region. Hybridization with probe V (Fig. 4, lane 7) showed a pattern similar to that of the entire pCD41 insert probe, and all the RNA species were detected. The hybridization patterns for probes VI and VII were also very similar: all poly(A) RNAs except the 1.5-kb RNA were observed (Fig. 4, lanes 8 and 9). This suggests that the 1.5-kb RNA encodes only the ORF A region of pCD41 (Fig. 4 and 5). Fragment VII is only 150 bp in length and contains the poly(A) signal; hybridization of the 2.7-, 2.3-, 1.8-, and 1.2-kb RNAs with this probe suggests that these RNAs have a common poly(A) signal.

The hybridization results allowed approximate mapping of all the RNA species in the pCD41 locus except the 4.5-kb RNA. Hybridization of the 4.5-kb RNA with all the probes indicates that this RNA is transcribed through the pCD41 and possibly the pGD41 region (Fig. 4 and 5). The 4.5-kb RNA, which is about twice the size of the pCD41 transcript, must be initiated far beyond our pCD41 and pGD41 loci. Thus, it is difficult to further narrow down its putative 5' and 3' ends with the probes employed here. In addition, a 4.5-kb RNA is unlikely to encode either the 41-kDa accessory protein or the
trans-activating protein. The initiation site of this transcript was not pursued further.

**Mapping the 5' end of the pCD41 RNA species.** On the basis of fragment hybridization results and the size of each RNA, four primers were designed for primer extension and S1 mapping assays in order to map precisely the various pCD41 RNA species (Fig. 4 and 5). To determine the specificity of each primer for the different pCD41 RNA species, Northern blot analyses were conducted with the labeled primers as probes.

The primer PG612 hybridization pattern is different from that obtained with probe I (Fig. 6B; Fig. 4, lane 3). Three RNA species of 4.5, 3.0, and 2.7 kb were detected with primer PG612, but the 0.8-kb RNA species was not detectable. This suggests that the 0.8-kb RNA species terminated within probe I and upstream of the primer. It is interesting that both the 3.0- and 2.7-kb RNAs could be detected when the 22-bp primer PG612 was used as probe. Probe I hybridization results suggest that the signal intensity between two RNAs is similar and that nearly equal amounts of the two RNA species persist in the total RNA (Fig. 4, lane 3). However, in the primer PG612 hybridization, the relative intensities of 3.0- and 2.7-kb RNAs are very different. Sequence analyses have shown that the 3' end of the U28 gene is located in the primer PG612 region and could partially hybridize to the primer PG612. Therefore, our primer hybridization data confirm that the 3.0-kb RNA is the U28 gene transcript, and it was not pursued further in this study. Besides the 4.5- and 2.7-kb RNAs, other RNA species detected by pCD41 were not found by the PG612 primer, confirming that all of them were initiated downstream of the PG612.

To map precisely the 5' end of the 2.7-kb RNA, primer extension and S1 mapping assays were carried out with PG612 primer and S1 probe (Fig. 6A). Two distinct protected bands at nucleotides (nt) 229 and 255 of pGD41 were detected by S1 analysis (Fig. 6C, lane 8). In the primer extension assay, two similar bands were also observed (Fig. 6C, lane 6). Both primer extension and S1 mapping results indicate that the 2.7-kb RNA may have two start sites located at nt 229 and 255 of pGD41 and that no splicing occurs in this mapped region.

Primer PC123 hybridized to the 4.5-, 2.7-, 2.3-, and 1.5-kb RNAs, suggesting that the 5' ends of the 2.3- and 1.5-kb RNA species are located upstream of the primer (Fig. 5 and 7B). To precisely map the 5' ends of these RNAs, primer extension and S1 mapping were carried out with the PC123 primer and the S1 probe (Fig. 7A). Two predominant bands at positions +13 and +14 were observed with the primer extension assay. This suggests that the 2.3- and 1.5-kb poly(A) RNAs initiate from the same sites at positions +13 and +14 of the pCD41 sequence (Fig. 7C, lane 6). These RNA initiation sites were further confirmed by S1 analysis. Although several bands were observed with the S1 mapping result, the most intense bands were at the same positions as those shown by the primer extension assay (Fig. 7C, lane 8). The mapping of the 5' end for both 2.3- and 1.5-kb RNA species by both primer extension and S1 protection suggests that no splicing had occurred in the analyzed region.

To map the 5' end of 1.8-kb RNA, a second primer, PC565, was used (Fig. 5 and 8A). This primer hybridized to 4.5-, 2.7-, 2.3-, and 1.5-kb RNA species in addition to the 1.8-kb RNA (Fig. 8B). The hybridization of 1.8-kb RNA to primer PC565

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**FIG. 5.** Schematic representation of the organization and transcription pattern of the pCD41 locus. Numbers indicate the nucleotide positions according to the pCD41 or pGD41 sequence. The locations of the RNA start sites are indicated in front of each RNA species; for the 2.7-kb species, the RNA starts at positions +229/255 with respect to the pGD41 sequence; the 2.3-kb species starts at RNA nt +13 with respect to the pCD41 sequence. The putative ORFs encoded by the RNA are shown by shaded boxes; the AUGs represent the potential start sites, and their nucleotide locations with respect to the pCD41 sequence are indicated. ▲ AUG codon located on the RNA that cannot initiate any functional protein. Arrowheads indicate the primers that were used to analyze the 5' end of each RNA. Multiple A's at the 3' end of the RNA indicates polyadenylation.
but not primer PC123 suggests that the 5' end of the 1.8-kb RNA is between the two primers (Fig. 5). A band at position +468 and a very faint band at position +443 were detected by primer extension assay (Fig. 8C, lane 6). One intense band and two weaker bands were detected by S1 mapping (Fig. 8C, lane 8). Since the primer extension band corresponds to the intense S1 protection band at position +468, its presence suggests that the 1.8-kb RNA was initiated at position +468 on the pCD41. The very faint band at position +443 did not correspond to any bands detected by S1 and is likely to have been caused by premature termination during primer extension. Besides the +468 primer extension band, a weaker band at approximately position +13 was also detected by primer PCS65 (data not shown). This corresponds to the start sites of the 2.3- and 1.5-kb RNAs mapped by PC123 and indicates that no splicing occurs upstream of PCS65.

The last primer, PC1449, hybridized to all the poly(A)^+ RNA species but not to the 1.5-kb RNA (Fig. 5 and 9B). Since it is the only tested primer that hybridized to the 1.2-kb poly(A)^+ RNA, this primer was used in primer extension studies to map the 5' end of the 1.2-kb RNA (Fig. 9A and C). Because of interference by other predominant RNA species, higher background bands were seen in the primer extension assay and no consistent predominant band was detected. Since no definite band was detected by primer extension, S1 analysis was performed. Two bands at positions +1294 and +1295 were consistently detected in the S1 mapping assays, suggesting that the 5' end of the 1.2-kb poly(A)^+ RNA is likely to be at position +1294 or +1295.

**Mapping the 3' end of the 1.5-kb RNA species.** The 1.5-kb RNA species can hybridize to probe V but not to downstream probes VI and VII, suggesting that the 3' end, poly(A) site, or splice site may be located within the probe V region (Fig. 4 and 5). To determine the locations of the 3' ends of 1.5-kb RNA, S1 analyses were used (Fig. 10). Multiple protected bands were observed with the HHV-6(GS)-infected RNA (lanes 2 and 4) but not with the negative control RNA (lanes 1 and 3). The longest protected band is 128 bases long and is located at positions +1348 on the pCD41 sequence. Since sequence analyses did not reveal any splice donor sites within this region, it is likely that the protected site is the 3' end of the 1.5-kb poly(A)^+ RNA rather than a splice donor site.

**DISCUSSION**

The herpesvirus DNA polymerase accessory proteins, such as the UL42 protein in herpes simplex virus type 1, the BMRF1 gene product of Epstein-Barr virus (EBV), and the UL44 gene product in cytomegalovirus, have been shown to be very important for viral replication (10, 15, 18, 38, 41, 44, 45, 52) and to be involved in gene regulation. For example, the EBV
BMRF1 gene fragment was reported to activate transcription of the simian virus 40 promoter (40); antisense mRNA to the UL44 transcripts was shown to have an affect on both viral replication and β1 gene expression in an astrocytoma cell line (45). For the analogous U27 gene in HHV-6, we have demonstrated that its gene product p41 protein of HHV-6 can transactivate the HIV promoter (60). Thus, these accessory proteins could play important roles in viral gene regulation and DNA replication.

Gene expression in herpesvirus accessory gene loci appears to be extremely complex. Multiple transcripts have been detected in the herpesviral DNA polymerase accessory protein gene loci (4, 9, 29, 41, 43, 56, 57). Generally, three or four nonspliced RNA species of different sizes were found to initiate at different positions. The largest and smallest RNAs are minor species, and their functions are unclear; the predominant medium-size RNAs are the coding transcripts for accessory protein. For example, for the EBV BMRF1 locus, three different sizes of RNA (3.5, 2.7, and 1.5 kb) were found to hybridize to the BMRF1 cDNA, which encodes the 50-kDa EBV accessory protein (10, 43). In the HCMV UL44/ICP36 locus, four RNA species of about 8.0, 4.8, 3.2, and 2.0 kb were detected by Northern hybridization (29, 41, 56, 57). In this study, the HHV-6 accessory gene locus was extensively studied, and at least six different sizes of RNA species could be detected. It appears that the multiple transcriptional patterns in these gene loci may be important for viral gene regulation or expression. Therefore, further investigation of these RNA functions is very important for understanding regulation in this accessory protein region.

Our total RNA blot results have shown that at least six different RNA species could be detected by the pCD41 probe from both HHV-6 variant A or B virus-infected cells. Although the relative amounts of RNA expressed by the two variants are slightly different, their transcriptional patterns are very similar. The RNA pattern shown in this study supports our previous observation (9) but differs slightly from that in a report by Agulnick et al. (4), who detected only the 2.7-, 2.1-, and 1.7-kb RNA species for the pCD41 locus. These could be due to the use of different probes in the Northern analyses. Our probe, pCD41, contains both ORF A and ORF B regions, while theirs contains only the ORF A, the p41 coding region.

We have identified several RNA species (2.7, 2.3, 1.8, 1.5 and 1.2 kb) that can potentially encode the HHV-6 accessory and trans-activation protein. Other species, such as 4.5-, 3.0-, and 0.8-kb RNAs, are either expressed beyond the pCD41 locus or initiated far upstream from the pCD41 region and were therefore not extensively studied in this work. Unlike other pCD41 transcripts, the 2.7-kb RNA initiates from two different positions (at nt 229 and 255) in the middle of the large
One putative TATA box sequence (224-TATTAAA-230) is located at the optimal position for the RNA initiated at nt 255. However, no such sequences could be found upstream of the RNA initiation site at nt 229, although an AT-rich region exists in that region. These results imply that viral specific or other unidentified sequences may be important for transcription initiation at nt 229. Since several AUGs (positions +251 and +287) immediately downstream of both initiation sites of the 2.7-kb RNA cannot encode either the U28 ORF or the p41 protein, and since no splicing occurs in the 2.7-kb RNA, it is unlikely that this RNA can encode either U28 or the p41 protein, and the function of this RNA is still unclear. However, we cannot eliminate the possibility that an AUG further downstream (at position +63 of ORF A; Fig. 5) may be used to translate part of the p41 ORF. The 2.3- and 1.5-kb RNAs initiate at nt +13 or +14 of the pCD41 sequence, one nucleotide upstream of a previous report mapping a 2.5-kb RNA expressed from this region (58). While the 2.3-kb RNA can potentially encode for both ORF A and ORF B, it is unclear whether it is a bicistronic mRNA that can encode both ORFs. Although the 1.5-kb RNA initiates at the same position as the 2.3-kb RNA, it terminates earlier and therefore encodes only ORF A. Therefore, our mapping results suggest that the 2.3- and 1.5-kb poly(A)+ RNAs are the most likely transcripts for the p41 protein, which contains both accessory and trans-activation functions. The potential AUG at position +63 could be the initiation codon for the p41 protein. The 1.8-kb RNA does not seem to code for any protein, because the first two AUGs (positions +571 and +581) downstream of its 5′ end can encode only a few amino acids, although this RNA is polyribosome associated. However, it is still possible that internal AUGs further downstream could be used to encode truncated forms of the p41 protein, as suggested by our previous study (60). This RNA did not initiate from any of the TATA sequences as predicted earlier (60), and no typical promoter sequence was found in its upstream sequences. This suggests that the expression of this RNA may be regulated differently, possibly by viral transcriptional factors.

Because there are six overlapping RNA species in a 2.1-kb region, it is difficult to map certain RNA species precisely, especially the 1.2-kb RNA. The primers or probes that were used for the detection of 1.2-kb RNA also hybridized to the other RNAs, such as the predominant 2.3-kb species, causing lower specific signals and higher nonspecific background. Thus, for this RNA, only consistent bands shown in different experiments were considered. Although no TATA box sequence was found upstream of the 1.2-kb RNA, an AP2 binding site is present at nucleotide positions 1248 to 1257 of pCD41. A protein of 168 amino acids can potentially be encoded by the 1.2-kb poly(A)+ RNA, but so far, no HHV-6-specific or homologous gene product from other herpesviruses has been identified.

The DNA fragment hybridization results suggest that 2.7-, 2.3-, 1.8-, and 1.2-kb RNA species use the same poly(A) signal sequence. For the 1.5-kb RNA, the 3′ end was mapped to position +1348, and it probably uses a different poly(A) signal site. Sequence analyses have shown a poly(A) signal-like sequence (TATAAA) about 20 bases upstream of the mapped position (Fig. 10C). It is possible that this rare poly(A) signal has a lower efficiency and was used to generate a minor 1.5-kb transcript. A rare poly(A) signal (ATTAAA) is used by the EBV BMRF1 gene, which encodes the EBV accessory protein (10). Because of the overlap between the 3′ end of the 1.5-kb RNA and the 5′ end of the 1.2-kb RNA, it is also possible that the initiation or transcription of the 1.2-kb RNA may interfere with the transcription of the 2.3-kb RNA and cause early termination of the 2.3-kb transcript, leading to generation of the 1.5-kb RNA.

Our data and previous results suggest that the p41 protein could be encoded by either the 2.3- or the 1.5-kb RNA. Recently, the promoter for the 2.3- or 1.5-kb RNA was characterized to determine how this protein is regulated (58). This promoter is almost silent without viral infection but is highly active in infected cells. This finding suggests that the transcription of the p41 RNA is regulated by viral proteins. Our kinetic studies have shown that the RNAs should be classified as the early-late gene and supported the idea that the p41 promoter is under viral regulation. Besides the viral factor(s), four different cellular factors (C1 through C4) have been reported to bind to the p41 promoter in a mobility shift assay (58). The results indicate that in infected cells, transcriptional stimulation of the pCD41 promoter requires C1 and C2 binding activities and possible C3 and C4 elimination. Therefore, besides the viral regulators, cellular factors may play a very important role in the regulation of this gene locus. The CHX inhibition results indicate that pCD41 RNA synthesis is not detectable at 6 h p.i., when IE genes are expressed. In addition, the PAA inhibition results show that all the pCD41 RNA species are significantly but incompletely inhibited by PAA treatment at 12 and 24 h p.i. All those data suggest that the continued syntheses of all the pCD41 RNAs depend on viral DNA replication. This observation corresponds well with the HCMV UL44 ear-
ly-late gene expression. (29) The expression of a 51-kDa HCMV UL44 protein begins prior to that of most other viral proteins but requires preceding viral proteins and DNA replication. Furthermore, we have shown that in the presence of PAA, p41 protein is significantly reduced in amount but not abolished in the HHV-6 infected cells (9). Because both the accessory protein and HIV LTR trans-activating protein have been mapped to the pCD41 ORF A region, further understanding of the regulation of the accessory gene locus may provide important information about viral replication and regulation of viral gene expression.

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