Virology 288, 164–174 (2001)
doi:10.1006/viro.2001.1084, available online at http://www.idealibrary.com on IDEAL®

Effect of the R1 Element on Expression of the US3 and US6 Immune Evasion Genes of Human Cytomegalovirus

Grant C. Bullock,*‡ Philip E. Lashmit,†‡ and Mark F. Stinski*†‡[1]

*Program in Molecular Biology, and †Department of Microbiology, and ‡The Helen C. Levitt Center for Viral Pathogenesis and Disease, College of Medicine, University of Iowa, Iowa City, Iowa 52242

Received April 6, 2001; returned to author for revision June 25, 2001; accepted July 9, 2001

Human cytomegalovirus (HCMV) has several gene products that are important for escape from immune surveillance. These viral gene products downregulate the expression of HLA molecules on the cell surface. The viral US3 and US6 gene products are expressed at immediate-early and early times after infection, respectively. There are two regulatory regions between the US3 and the US6 transcription units. The first region is an NF-κB responsive enhancer that promotes the immediate-early expression of the US3 gene and is designated the R2 enhancer. Upstream of the R2 enhancer is a region designated the R1 element that in transient transfection assays behaves as a silencer by repressing the effect of the enhancer on downstream gene expression (A. R. Thrower *et al., J. Virol.* 1996, 70, 91; Y.-J. Chan *et al., J. Virol.* 1996, 70, 5312). We constructed recombinant viruses with wild-type or mutated R1 elements. The expression of the US3 gene at 6 h after infection and the US6 gene at 24 h was higher when the R1 element was present. The R1 element in the context of the viral genome is not a silencer of US3 or US6 gene expression. The R1 element has multiple effects on the US3 and US6 RNAs. It enhances the level of US3 and US6 mRNA; it determines the 3′-end cleavage and polyadenylation of US6 RNA, and it stabilizes read-through viral RNAs. The potential mechanisms of R1 enhancement of US3 and US6 gene expression are discussed.

INTRODUCTION

Human cytomegalovirus (HCMV), a ubiquitous betaherpesvirus, is the most common infectious cause of birth defects (Yow and Demmler, 1992). During immunosuppression due to acquired immunodeficiency syndrome or chemotherapy after transplantation, the virus can be reactivated from latency and cause serious disease (Alford and Britt, 1990; Ho, 1991). In addition, HCMV infections have been associated with accelerated atherosclerosis and with coronary restenosis following angioplasty (Bruggeman and van Dam-Mieras, 1991; Melnick *et al.,* 1993, 1994; Peersoons *et al.,* 1994; Span *et al.,* 1992). Since the current HCMV vaccines do not protect against reinfection (Plotkin, 1994), determining the mechanisms that allow HCMV to replicate and cause disease in an individual are imperative to the development of a better vaccine.

Similar to other herpesviruses, HCMV persists indefinitely in certain host cells. Following a primary infection, monocytes and hematopoietic progenitor cells of the bone marrow are latently infected with HCMV (Hahn *et al.,* 1998; Kondo *et al.,* 1994, 1996; Minton *et al.,* 1994; Sinclair and Sissons, 1996; Taylor-Wiedeman *et al.,* 1991). Inflammatory cytokines play a role in the reactivation of HCMV from myeloid precursors and monocytes by activating and terminally differentiating them into macrophages and dendritic cells (Hahn *et al.,* 1998; Soderberg-Naucler *et al.,* 1997a,b). In general, reactivation from latency occurs when the immune system is compromised and there is cellular stimulation and differentiation of latently infected cells. The mechanism by which the virus persists in the healthy carrier and the molecular events associated with reactivation of latent virus are not known.

Herpesviruses must be able to evade both innate and acquired immune defenses to invade and persist in the host. Upon primary infection, HCMV is controlled by the innate immune system, but it is not eliminated. HCMV primes the adaptive immune system, and as a result, latently infected people have circulating antibodies and cytotoxic T lymphocytes (CTLs) that can react with HCMV-infected cells (Kern *et al.,* 1999; Landini and La Placa, 1991). During subclinical reactivation, these CTLs recognize viral peptide antigens bound to cell surface HLA molecules and promote clearance of the virus and virus-infected cells. Since HLA molecules are activating receptors for CTLs and inhibitory receptors for natural killer (NK) cells (Ploegh, 1998), their presence on the HCMV-infected cell surface is detrimental when recognized by CTLs but necessary for evading NK cells. Therefore, the HLA molecule represents a vital link between

[1] To whom correspondence and reprint requests should be addressed at Department of Microbiology, College of Medicine, University of Iowa, Iowa City, IA 52242. Fax: (319) 335-9006. E-mail: mark-stinski@uiowa.edu.

0042-6822/01 $35.00

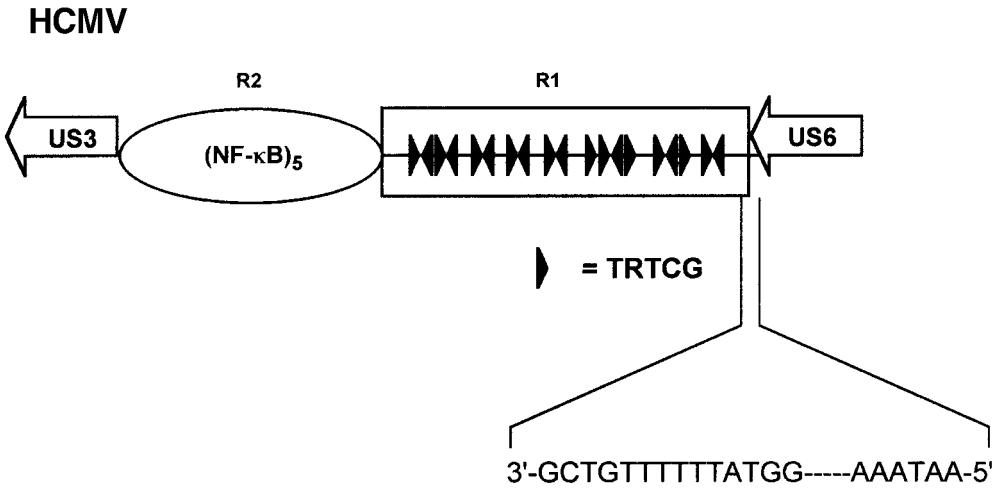

FIG. 1. Diagram of the HCMV Repeat 1 (R1) and Repeat 2 (R2) elements. The pentanucleotide repeats of the R1 element in relation to the R2 enhancer between the immediate-early US3 and early US6 genes are shown. NF-κB designates the five NF-κB binding sites in the 274-bp R2 enhancer. The black triangles represent the pentanucleotide sequence 5′-TRTCG-3′. The pentanucleotide is found 19 times in the 266-bp R1 element. Some pentanucleotides are isolated and others are found as either inverted palindromic repeats or everted palindromic repeats. The polyadenylation signal and the GT box downstream of the US6 gene are indicated.

the HCMV-infected cell and both the innate and the acquired immune systems.

HCMV has evolved several mechanisms for disrupting the surface expression of certain types of HLA molecules (Ploegh, 1998; Wiertz *et al.,* 1997). In both human and murine CMV-infected cells, there is a dramatic decrease in HLA class I expression at the cell surface and a resistance to lysis by CTLs (Barnes and Grundy, 1992; Beersma *et al.,* 1993; del Val *et al.,* 1992; Jones *et al.,* 1995; Warren *et al.,* 1994; Yamashita *et al.,* 1993). A number of HCMV genes are involved in the inhibition of HLA surface expression. Using a series of HCMV deletion mutants, a 7-kb region, encoding 10 genes, was identified as being required for class I downregulation in HCMV-infected cells (Jones *et al.,* 1995). Four of the genes encoded within this region, US2, US3, US6, and US11, reduce class I expression at the cell surface when expressed individually (Ahn *et al.,* 1997; Jun *et al.,* 2000; Lehner *et al.,* 1997). These viral immune evasion genes are sequentially expressed with US3 expression preceding US2, US6, and US11. We are interested in the *cis*-acting elements and the *trans*-acting factors that determine the sequential expression of these viral immune evasion genes.

The genomes of viruses such as adeno-associated virus, simian virus 40, and adenovirus, do not exist *in vivo* as naked DNAs but are in the form of ordered nucleosomal arrays separated by 40- to 50-bp stretches of nuclease-accessible DNA (Dery *et al.,* 1985; Kondoleon *et al.,* 1989; Marcus-Sekura and Carter, 1983; Vayda *et al.,* 1983). Although the genomes of herpes simplex virus and Epstein–Barr virus are in a nucleosomal structure during latency, this structure is altered during productive infection (Deshmane and Fraser, 1989; Dyson and Farrell, 1985).

During reactivation from latency or productive infection, the HCMV IE genes are transcribed first, followed by the early genes. The HCMV strong enhancer-containing promoters such as the major immediate-early and IE US3 promoters have unidirectional effects on downstream transcription in the context of the viral genome. Presumably, there is a chromatin structure inherent to the HCMV genome in the infected cell that adds an additional level of control to viral gene expression at IE times after infection.

Two repeat-containing elements are located between the US3 and the US6 genes (see Fig. 1). The region from nucleotide 194,758 to 194,960 (Chee *et al.,* 1990) or from −84 to −286 upstream of the US3 transcription start site is referred to as the R2 element. The R2 element is an NF-κB responsive enhancer of IE US3 transcription (Chan *et al.,* 1996; Thrower *et al.,* 1996; Weston, 1988). In transient transfection assays, the R2 element activates transcription from the IE US3 promoter as well as heterologous promoters in a position and orientation-independent manner. Immediately after infection with HCMV, the R2 element enhances transcription from the US3 gene, but not from the US6 gene in the context of the viral genome. The mechanism of unidirectional enhancement of transcription is not understood.

The region from nucleotide 195,107 to 195,377 (Chee *et al.,* 1990) upstream of the R2 enhancer is referred to as the R1 element (Weston, 1988). The R1 element is unusual because it contains 19 copies of the pentanucleotide 5′-TRTCG-3′, where R represents a purine nucleotide. These pentanucleotides are found in palindromes arranged as everted repeats (ER) or inverted repeats (IR) and as variably spaced single pentanucleotides (see Fig. 1).

It was proposed initially that the R1 element is a silencer of R2 enhancer-mediated IE US3 transcription

based on transient transfection assays (Chan *et al.,* 1996; Thrower *et al.,* 1996). Since some transcriptional regulatory elements function differently in a chromatin context, transient transfection experiments do not necessarily represent the natural template for viral gene expression. The chromatin structures that form on a plasmid during transient transfection assays are likely different from those on the HCMV genome in infected cells. In addition, the HCMV genome is targeted to a nuclear compartment in the infected cell (Ahn *et al.,* 1998, 1999; Ahn and Hayward, 1997; Ishov *et al.,* 1997).

In this report, we show that the R1 element in the context of the viral genome positively affects the level of expression of the flanking US3 and US6 genes at IE and early times after infection, respectively. When the R1 element was deleted and substituted with nonregulatory heterologous DNA, less gene expression from the US3 gene at 6 h p.i. and the US6 gene at 24 h p.i. was detected. The multiple mechanisms by which the R1 element positively affects US3 and US6 gene expression in the context of the HCMV genome are discussed.

RESULTS

Recombinant viruses without the R1 element

The R1 element of HCMV contains numerous 5′-TRTCG-3′ pentanucleotides arranged as inverted repeats, everted repeats, and monomers (Fig. 1). The R1 element also contains a GT box that may affect cleavage and polyadenylation of upstream transcripts (Fig. 1). To determine the function of the R1 element in the context of the viral genome, recombinant viruses with substitutions of the R1 element were isolated and characterized. The US3 gene was replaced with the CAT gene to facilitate characterization of US3 promoter activity and to prevent deleterious effects to the infected cell due to excessive HLA entrapment in the endoplasmic reticulum by overexpression of the US3 gene product. All recombinant viruses were derived from RVgptR2CAT (Lashmit *et al.,* 1998) by homologous recombination. The R1 element was substituted with two different heterologous DNAs. Because there is considerable read-through RNA in this region of the viral genome (Gretch and Stinski, 1990; Jones and Muzithras, 1991), the R1 element was replaced with H1 to introduce the strong SV40 cleavage and polyadenylation signal. The R1 element was also replaced with H2, a heterogeneous and nonregulatory DNA derived from the plasmid pCAT-basic. Recombinant viruses RV(H1)R2CAT and RV(H2)R2CAT were generated as described under Materials and Methods by replacement of a 266-bp fragment of the R1 element found in RVR1R2CAT with the H1 (269-bp) or the H2 (273-bp) fragment of DNA, respectively. The sizes of the replacement DNAs did not significantly alter the spacing between the US3 and US6 genes. From two separate transfections with the shuttle vectors and the infectious RVgptR2CAT DNA, two to three recombinant viruses of each type were plaque purified and characterized. In separate experiments, we found the steady-state level of US3-CAT RNA to be similar to US3 wild-type RNA at 6 h after infection.

To characterize the recombinant viruses, viral DNAs were digested with *Bam*HI and analyzed by Southern blot hybridization. The size of the expected DNA fragments for each recombinant virus is indicated in Fig. 2. The black bars in Fig. 2 indicate the region to which the [^{32}P]DNA probes hybridize. The R1-DNA probe hybridized exclusively to the R1-DNA fragment. The H1 DNA probe hybridized to the H1 insertion as well as to the CAT gene from which it was derived. The H2 DNA probe hybridized only to the region of H2-DNA substitution. All DNA fragments were of the appropriate size as predicted (Fig. 2). The heterologous DNA substitutions caused no discernible phenotypic differences in viral growth or plaque size (data not shown). Two independent isolates of each recombinant virus behaved similarly in the experiments described below.

Transcription map of US3, US6, and US7

Although the R2 element and the R1 element were designated by Chee *et al.* (1990) to contain the US4 and US5 ORFs, respectively, an analysis of transcription by viral gene microarray detected little to no transcription from these ORFs (Chambers *et al.,* 1999). Using RNase protection assays, we also found little to no transcription from these viral ORFs at early times after infection (data not shown). In addition, traditional promoter elements (TATA boxes) have not been identified upstream of these ORFs by DNA sequence analysis. Last, there is no evidence that these viral ORFs are a part of a spliced mRNA. The transcripts that arise from the US2 to US11 immune evasion genes of wild-type HCMV Towne and AD169 strains have been mapped (Gretch and Stinski, 1990; Jones and Muzithras, 1991; Tenney and Colberg-Poley, 1991; Weston, 1988). There are several different transcripts that contain the US3, US6, and US7 ORFs. Analysis of the steady-state mRNAs from this region is complicated due to alternative 5′-start sites and 3′-end designations. Figure 3 shows the sizes of the transcripts expected from the three recombinant viruses. The predominant unspliced US3 mRNA species is detected in the cytoplasm of virus-infected cells at approximately 3 h p.i. (Tenney and Colberg-Poley, 1991; Weston, 1988). The US6 RNA is detected at approximately 8 h p.i. and slowly reaches the highest steady-state levels at approximately 72 h p.i. Overlapping read-through RNAs from US6 and US7 are detected at late times after infection with US3 specific probes (Tenney and Colberg-Poley, 1991; Weston, 1988).

Since the US3 gene from +8 (194,756) to +210 (194,554) was replaced with the 1657-bp CAT ORF and

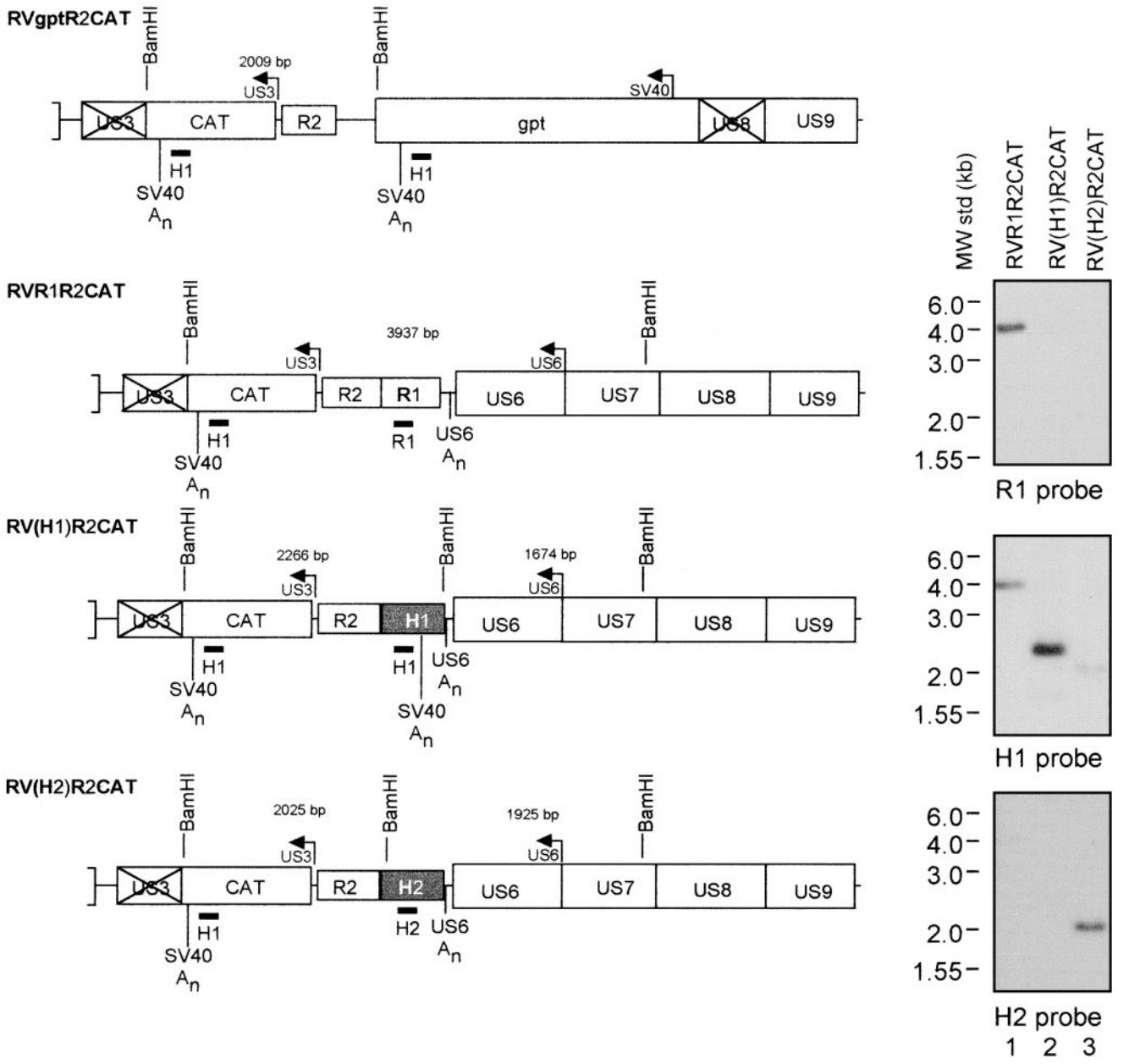

FIG. 2. Recombinant viruses with wild-type R1 or mutant DNA sequences upstream of the R2 enhancer. All recombinant viruses were derived from RVgptR2CAT by selection against the gpt gene product as described under Materials and Methods. In all viruses the US3 gene was replaced by the CAT gene at position +10 relative to the US3 start of transcription. Diagrams of the recombinant viruses from US2 to US9 are on the left. All transcription from this region has the same polarity at early times and is from right to left. The R1 element (located between nucleotides 194,906 and 195,377) was either wild-type in RVR1R2CAT or deleted and replaced with DNAs that were approximately the same size as the deleted R1 fragment. The H1 fragment contains the SV40 late polyadenylation signal and is also present at the end of the CAT gene. The H2 fragment in RV(H2)R2CAT contains nonregulatory DNA taken from pCATbasic. The origins of probes for detection of R1, H1, or H2 DNAs are designated with black bars. The predicted sizes of DNA fragments after *Bam*HI restriction endonuclease digestion of recombinant viral DNAs are indicated in base pairs. Southern blot hybridization of recombinant virus DNA digested with the restriction endonuclease *Bam*HI is on the right. ^{32}P-labeled probes of R1, H1, and H2 DNA sequences are indicated. Lanes: 1 to 3, viral DNAs from RVR1R2CAT, RV(H1)R2CAT, and RV(H2)R2CAT, respectively. Standard molecular size markers are indicated in base pairs.

the 3′UTR of the SV40 small T intron and early polyadenylation signal, the IE US3/CAT mRNA should be approximately 1.8 kb and all early and late read-through RNAs should be increased by approximately 670 bp (Fig. 3).

In wild-type HCMV, the 5′ ends of US6 mRNAs arise from either an early start site at nucleotide 195,970 or a late start site at nucleotide 196,070 (Jones and Muzithras, 1991). The US6 polyadenylation signal at nucleotide 195,378 is more efficient early after infection than late, but many transcripts at late times read through the US6

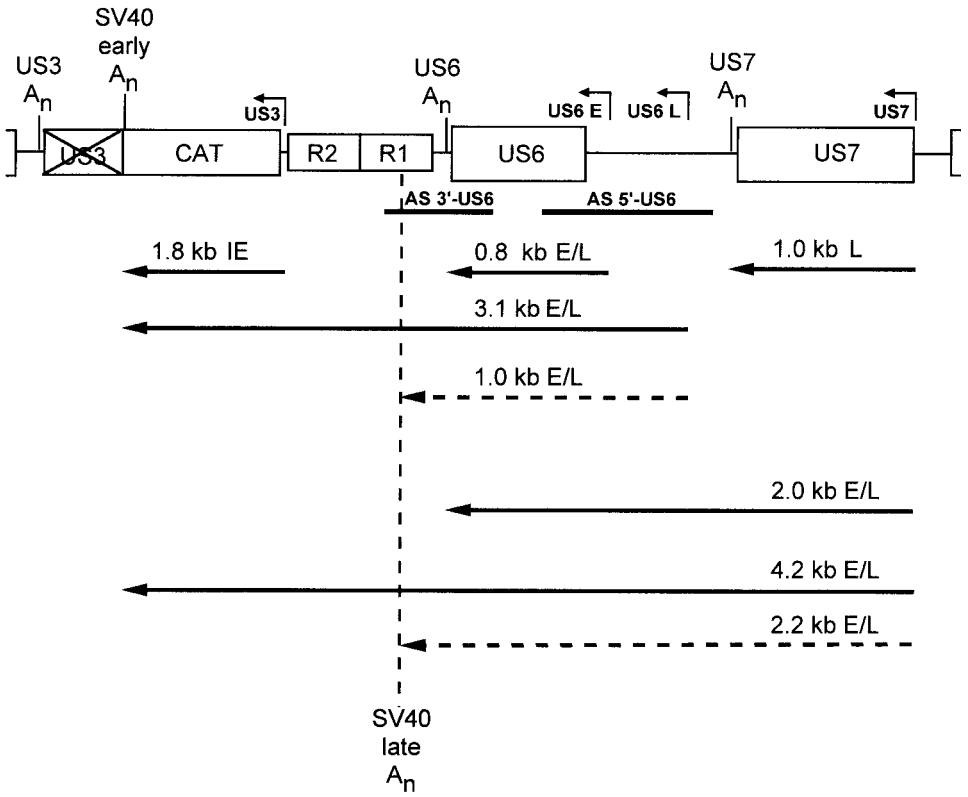

FIG. 3. Map of expected transcripts from the US3, US6, and US7 genes in the recombinant viruses. The sizes of the transcripts are given in kilobases, and the kinetic classes are indicated as immediate early (IE), early (E), or late (L) as described by Gretch and Stinski (1990) and Jones and Muzithras (1991). Bent arrows indicate transcription start sites. Polyadenylation signals are designated by (An). Dashed arrows indicate the expected transcripts from RV(H1)R2CAT that contains the SV40 late polyadenylation signal encoded by the H1 fragment. The origins of the anti-sense (AS) riboprobes used in Figs. 5 and 6 are designated.

polyadenylation signal (Jones and Muzithras, 1991). US7 transcripts also have two different 5′-start sites at early and late times after infection. Downstream of the US6 polyadenylation signal within the R1 element at nucleotide 195,336 is a U-rich sequence (see Fig. 1). It is important to note that the efficient cleavage and polyadenylation is determined by both the hexanucleotide, AAUAAA, and the downstream G-U or U-rich sequences (Goodwin and Rottman, 1992; Levitt *et al.,* 1989).

RV(H1)R2CAT should produce US6 transcripts of 0.8, 1.0, 2.0, and 2.2 kb. The transcripts unique to RV(H1)R2CAT because of the late SV40 cleavage and polyadenylation signal are designated as dashed arrows in Fig. 3. All the other recombinant viruses will produce US6 transcripts of 0.8, 2.0, 3.1, and 4.2 kb (Fig. 3).

Effect of substitutions of the R1 element on US3/CAT expression from recombinant viruses

The US3 gene is the first in a series of immune evasion genes to be expressed after HCMV infection. It is highly induced by 3–5 h after infection and then repressed through the transcription repression sequence (trs) at the transcription start site (Biegalke, 1995, 1997, 1998; Lashmit *et al.,* 1998). To determine the effect of the R1 element on US3/CAT gene expression in the context of the viral genome, permissive cells were infected with RVR1R2CAT, RV(H1)R2CAT, or RV(H2)R2CAT at 5 PFU/cell. Infected cells were harvested and analyzed for CAT activity or CAT RNA at various times after infection as described under Materials and Methods. Similar IE1 RNA levels from all three recombinant viruses detected by RNase protection assay confirmed that the cells were infected with a similar m.o.i. (data not shown).

The relative CAT activity from RV(H1)R2CAT and RV(H2)R2CAT was five- to sixfold lower than RVR1R2CAT at 6 h p.i. (Fig. 4). RNase protection assays detected US3CAT RNA from RV(H1)R2CAT and RV(H2)R2CAT to be approximately threefold lower than RVR1R2CAT (data not shown).

Effect of the R1 element on US6 steady-state mRNAs

To determine the effect of the R1 element on US6 transcription, permissive HFF cells were infected with RVR1R2CAT, RV(H1)R2CAT, and RV(H2)R2CAT at 5 PFU/cell. Cytoplasmic RNA was harvested at 24 and 48 h p.i. and Northern blots were prepared and probed with ^{32}P-antisense 3′-US6 riboprobe (see Fig. 3), as described under Materials and Methods. Equal amounts of RNA

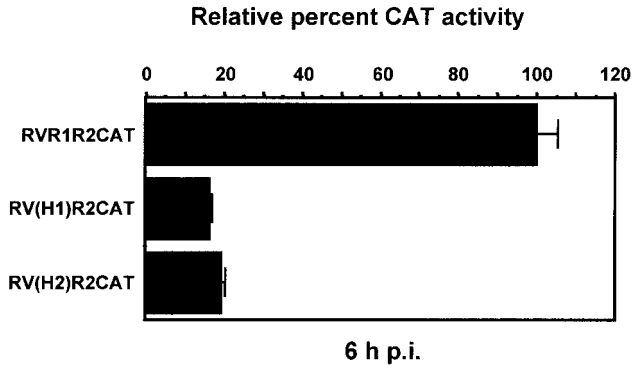

FIG. 4. Expression of the US3-CAT gene in permissive cells infected with recombinant viruses. Relative percentage CAT enzyme activity per microgram of protein was determined as described under Materials and Methods. The recombinant viruses are as described in Fig. 2. Relative CAT activity at 6 h p.i., $n = 5$.

were loaded as determined by UV spectroscopy and confirmed by ethidium bromide staining (data not shown). In addition, the m.o.i. was equal as determined by RNase protection assay for IE1 mRNA (Fig. 5A). At 24 h p.i., RNAs corresponding to the expected 0.8-, 3.1-, and 4.2-kb size classes were detected from RVR1R2CAT (Fig. 5B, lane 2). RV(H1)R2CAT produced abundant amounts of the predicted 1.0-kb RNA (Fig. 5B, lane 3). There was repeatedly little to no viral RNA detected from RV(H2)R2CAT. At 48 h after infection with RVR1R2CAT, RNAs representing the expected size classes of 2.0 and 4.2 kb for US7 RNAs and 3.1 kb for US6 RNA were detected (Fig. 5C, lane 1). The 5.7-kb RNA (Fig. 5C, lane 1) has been detected before (Tenney and Colberg-Poley, 1991). This RNA was detected in all lanes in Fig. 5C with longer exposure times. At 48 h p.i., the 1.0- and 2.0- to 2.2-kb mRNAs were detected from RV(H1)R2CAT, as expected (Fig. 5C, lane 2). The 1.3-kb RNA was not expected and its origin is not understood. A 4.2-kb mRNA was detected, indicating that at 48 h p.i. the 4.2-kb US7 read-through RNA is produced (Fig. 5C, lane 2). Read-through RNAs of 3.1 and 4.2 kb were detected with RV(H2)R2CAT, but these viral RNAs were 12-fold less abundant than RVR1R2CAT (Fig. 5C, compare lane 3 to lane 1).

To compare the levels of US6 steady-state RNAs, cytoplasmic RNAs were analyzed by RNase protection assay as described under Materials and Methods. We used ^{32}P-labeled AS 5′-US6 riboprobe (see Fig. 3) to compare the RNAs from RVR1R2CAT, RV(H1)R2CAT, and RV(H2)R2CAT. The amount of US6 promoter-specified 233 nt early US6 RNA from RV(H1)R2CAT was 50-fold greater than that from RV(H2)R2CAT at 24 h p.i. (Fig. 6, compare lane 3 with lane 4). In addition, the amount of 233-nt early US6 RNA from RVR1R2CAT was 6.5-fold greater than that from RV(H2)R2CAT (Fig. 6, compare lane 2 with lane 4). The 403-nt late US6 RNA was threefold higher with RV(H1)R2CAT than RVR1R2CAT and late US6 RNA from RVR1R2CAT was significantly higher than from RV(H2)R2CAT. The ^{32}P-antisense IE1 probe, which detects spliced IE1 RNA, was used as an internal control. Differences in US6 RNA were calculated relative to the IE1 RNA internal controls. The same m.o.i. was used for the 24 and 48 h p.i. samples.

These data indicate that R1 is a positive element in the context of the viral genome that influences the level of both US3 and US6 gene expression even though these viral RNAs are produced at IE and early times after infection, respectively. With HCMV, there is considerable read-through transcription that increases with time after infection. R1 promotes the accumulation of read-through RNA. A more efficient cleavage and polyadenylation signal similar to that of the SV40 virus stabilizes the US6 mRNAs and decreases the relative amounts of read-through RNA. Viral RNAs from RV(H2)R2CAT lack the R1 element and a cleavage and polyadenylation signal. These viral RNAs are unstable.

DISCUSSION

The HCMV genome has an additional level of transcriptional control that has not been demonstrated in plasmid-based transient transfection assays. When the HCMV genome enters the nucleus of the infected cell, transcription is rapidly initiated from the IE gene promoters in defined nuclear compartments (Ishov *et al.,* 1997). The HCMV MIE and IE US3 enhancer-containing promoters are unidirectional in the context of the viral genome. The genomic elements that determine unidirectional transcription at immediate-early times after infection are not understood. The R1 element is located between the R2 enhancer and the US6 transcription unit (see Fig. 1). We determined how the R1 element functions in the context of the HCMV genome.

Plasmid-based transient transfection assays led to the speculation that the R1 element or the pentanucleotides of the R1 element repress transcription from the US3 enhancer-containing promoter (Chan *et al.,* 1996; Thrower *et al.,* 1996). Since the R1 element functioned as a silencer in plasmid-based transient transfection assays, we predicted that replacement of R1 with heterologous DNA would increase US3/CAT gene expression in the context of the viral genome. In contrast, we found that replacement of R1 with the heterogeneous DNAs resulted in a reduction in the amount of US3/CAT gene expression at 6 h p.i. The difference in relative CAT enzyme activity was five- to sixfold, which represented differences in the levels of steady-state RNA of approximately threefold from the IE US3/CAT gene as determined by RNase protection assay (data not shown). These experiments indicated that the R1 element increases US3/CAT gene expression immediately after infection. The R1 element is likely a multifunctional se-

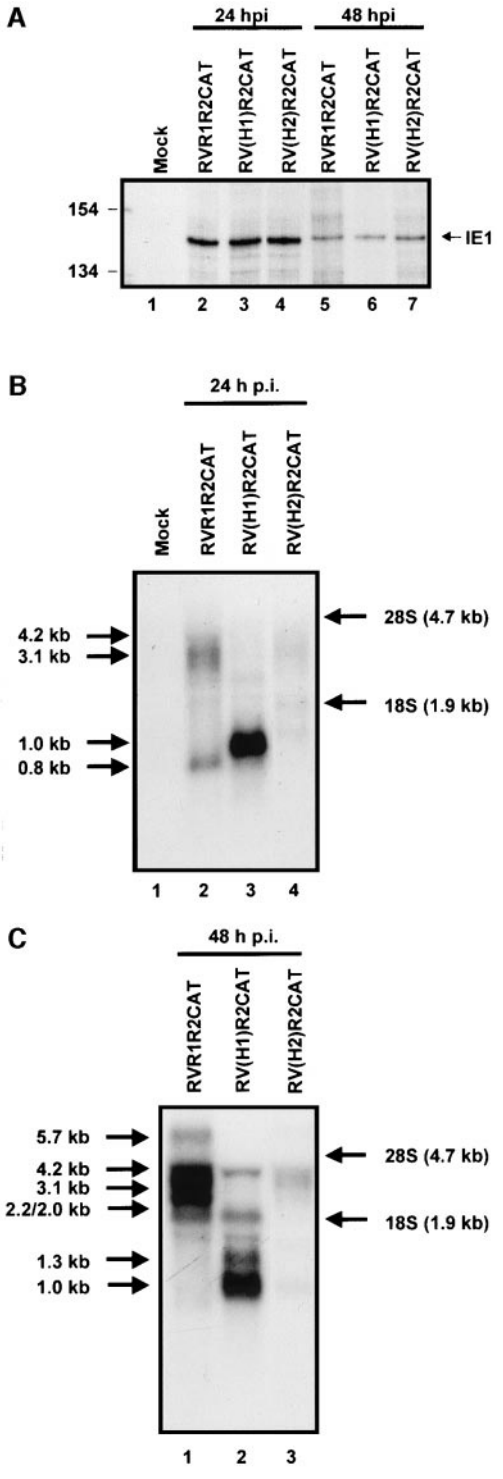

FIG. 5. Northern blot analysis of viral transcripts that either read through or terminate at the US6 cleavage and polyadenylation signal. The riboprobe, AS 3′-US6, shown in Fig. 3, was used to detect US6 transcripts. Cytoplasmic RNA was from mock-infected cells or from cells infected with recombinant virus at 24 and 48 h p.i. The blot was prepared and hybridized to ^{32}P-labeled antisense 3′-US6 RNA (see Fig. 3 for location of AS 3′US6 probe) as described under Materials and Methods. The sizes of the transcripts are given in kilobases. The positions of the 28S and 18S ribosomal RNA are designated. (A) RNase protection assay of IE1 RNA harvested at 24 h (lanes 2 to 4) or 48 h (lanes 5 to 7) to demonstrate equal titer of the recombinant viruses and RNA loading. RHase protection was as described under Materials and Methods. (B) Northern blot of 24 h RNA. Lanes: 1, mock; 2, RVR1R2CAT; 3, RV(H1)R2CAT; 4, RV(H2)R2CAT. (C) Northern blot of 48 h RNA. Lanes: 1, RVR1R2CAT; 2, RV(H1)R2CAT; 3, RV(H2)R2CAT. The positions of the standard DNA molecular weight markers are indicated in kilobases.

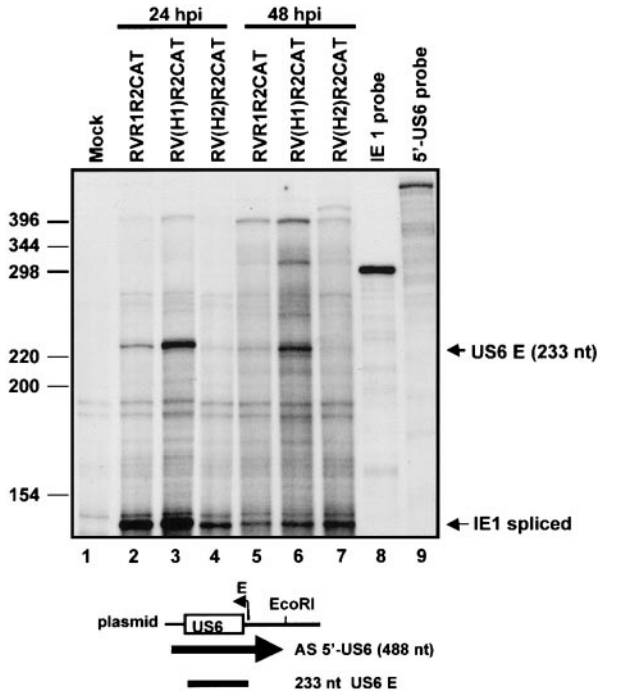

FIG. 6. Comparison of US6 steady-state RNAs in the cytoplasm of human fibroblasts infected with recombinant viruses. Cytoplasmic RNA was isolated from mock-infected or infected cells and compared by RNase protection assay as described under Materials and Methods. The probe was ^{32}P-labeled AS 5′-US6 RNA (see Fig. 3 for location of probe). The positions of standard DNA molecular weight markers are indicated in base pairs. Lanes: 1, mock-infected cell RNA; 2–4, infected cell RNA at 24 h p.i. from RVR1R2CAT, RV(H1)R2CAT, and RV(H2)R2CAT, respectively; 5–7, infected cell RNA at 48 h p.i. from RVR1R2CAT, RV(H1)R2CAT, and RV(H2)R2CAT, respectively; 8, undigested antisense IE1 probe; 9, undigested antisense 5′-US6 probe. The arrow designates RNA that initiates at the early US6 promoter (US6 E). Spliced IE1 RNA is used as an internal control for equal titer of recombinant viruses and RNA loading. A map of the 5′-US6 riboprobe and the protected fragment is shown.

quence that affects RNA transcription, 3′-end processing, and RNA stability at different times after infection.

RNAs that read through the US6 cleavage and polyadenylation signal are not detectable at 6 h (Jones and Muzithras, 1991), but they are abundant at 48 h p.i. Read-through RNAs accumulate as products of primary transcripts when cleavage and polyadenylation is inefficient (Carswell and Alwine, 1989; Mason *et al.,* 1986; Sadofsky and Alwine, 1984). The efficiency of cleavage and polyadenylation is determined by at least two *cis*-acting RNA elements, an upstream conserved hexanucleotide sequence and the downstream G/U- or U-rich sequence (Carswell and Alwine, 1989; Goodwin and

Rottman, 1992; Levitt *et al.,* 1989). There is a U-rich sequence approximately 40 nt downstream of the US6 polyadenylation signal within the R1 element. Replacement of R1 did not alter the US6 hexanucleotide sequence, but the U-rich sequence was deleted. We constructed RV(H1)R2CAT, which contains a particularly efficient signal for cleavage and polyadenylation (Carswell and Alwine, 1989), and found a significant increase in steady-state levels of US6 RNA relative to wild-type levels because the SV40 cleavage and polyadenylation signal presumably converts unstable primary transcripts into more stable mature mRNAs at a faster rate, as previously described (Carswell and Alwine, 1989).

At 48 h p.i., RV(H2)R2CAT produced the 3.1- and 4.2-kb read-through RNAs. This was expected because the H2 insertion did not contain the U-rich element necessary for proper use of the US6 cleavage and polyadenylation site. Since cleavage and polyadenylation occur inefficiently at the end of the US6 gene, most transcripts will read through. However, there was a significant decrease in the relative amount of these transcripts when the R1 element was absent. The 3.1- and 4.2-kb read-through RNAs appeared to be 12-fold more abundant in RVR1R2CAT-infected cells compared to RV(H2)R2CAT-infected cells. The biological function of the read-through RNAs from this region of the viral genome is currently not understood.

The RNase protection data in Fig. 6 confirmed that the R1 element also increased the steady-state levels of US6 mRNA from the early US6 promoter. The R1 element may stabilize US6 mRNAs by increasing the efficiency of their cleavage and polyadenylation through a U-rich sequence (5′-GGUAUUUUUUCUGUCG-3′) located downstream of the polyadenylation signal. In addition, the R1 sequence is present in the cytoplasmic RNAs that read through the US6 cleavage and polyadenylation signal. The steady-state levels of these R1-containing read-through RNAs are reduced when the R1 element is replaced. The R1 element could affect the steady-state levels of the read-through RNAs by increasing both their stability and their transcription. Since the US6 promoter is weak, we were unable to use actinomycin D or nuclear run-off experiments to compare read-through RNA half-lives or to determine the efficiency of transcription initiation at the US6 promoter in our recombinant viruses. Therefore, we cannot use our recombinant viruses to determine how R1 increases US6 gene expression. However, increased read-through RNA does not significantly affect CAT expression when CAT is substituted for the US3 ORF. These data indicate that the intact R1 element in the context of the viral genome is a positive element for the expression of both the US3 and the US6 immune evasion genes. The R1 element is expected to stabilize viral mRNA by increasing the efficiency of 3′-end processing. The R1 element functions in multiple ways to increase both US3 and US6 gene expression during a productive infection.

In summary, we have presented evidence that the R1 element in the context of the HCMV genome increases the expression from both the IE US3 and the early US6 immune evasion genes at immediate-early and early times after infection, respectively. While the biological implications of the multifunctional R1 element require further investigation, we propose that the R1 element, as a whole, is a positive effector of the sequential expression of the viral gene products involved in avoiding the cellular immune system.

MATERIALS AND METHODS

Cell culture

Primary human foreskin fibroblasts (HFF) were cultured as described previously (Stinski, 1978). The same conditions were used to culture the hypoxanthine guanine phosphoribosyl transferase-deficient Lesch–Nyhan fibroblasts (GM02291) obtained from the Coriell Institute for Medical Research (Coriell Cell Repositories, Camden, NJ). The U373 glioma cell line was grown in Dulbecco's modified minimal essential medium (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 100 U penicillin/mL, and 100 μg streptomycin/mL.

Enzymes

All enzymes were used according to the manufacturer's specifications and were obtained from either Bethesda Research Laboratories, Inc. (Gaithersburg, MD), New England BioLabs, Inc. (Beverly, MA), Boehringer Mannheim Biochemicals (Indianapolis, IN), Promega (Madison, WI), or Perkin–Elmer (Norwalk, CT).

Plasmids

The DNA fragment that contains the US2 through US9 genes between the *Nco*I site at 193,671 bp and the *Xho*I site at 198,622 bp was cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) at *Not*I and *Xho*I after the *Nco*I and *Not*I sites were made blunt with the Klenow fragment of *Escherichia coli* DNA polymerase. The US3 gene between the *Nde*I and *Dde*I sites (194,548 to 194,754 bp) was replaced with a 1649-bp chloramphenicol acetyl transferase (CAT) cassette from p7R15R2CAT (Thrower *et al.,* 1996) so that the CAT gene would be transcribed by the US3 promoter. The resulting plasmid was designated pR1R2CAT. The plasmid pH1R2CAT was generated by replacing the 266-bp R1 element from the *Sma*I site at 195,107 to the *Hin*PII site at 195,377 with a 269-bp *Ssp*I/*Bam*HI DNA fragment from pCAT-basic (Promega) after the *Hin*PII and the *Bam*HI sites were made blunt by Klenow polymerase. A 273-bp DNA fragment of pCAT-basic, amplified with the primer pair 5′-CCCCGGCCGTTCGAAGATTCGCGAATGCAGCTGGCA-3′ and 5′-CCTTGGATCCTGAAAATCTCGC-3′, was digested with restric-

tion endonucleases *Bam*HI and *Eag*I and inserted into the same sites in pBluescript II KS+ to generate the plasmid pKS+H2. To generate pH2R2CAT, the 266-bp R1 element from the *Sma*I site to the *Hin*PII site of pR1R2CAT was replaced with a 279-bp *Sma*I/BstB I DNA fragment from pKS+H2. The *Rsa*I (196,141) to *Hin*PII (195,739) fragment of pR1R2CAT was ligated into pBluescript II KS+ at the *Eco*RV and *Cla*I sites to create pKS+5′US6. The *Rsa*I (195,236) to *Hin*DIII (195,800) fragment of pR1R2CAT was ligated into pBluescript II KS+ at the *Hin*dIII and *Eco*RV sites to create pKS+US5/6. All deletions and substitutions in the shuttle vectors were confirmed by dideoxynucleotide sequencing by the University of Iowa DNA facility.

Recombinant viruses

Recombinant viruses were isolated by the method of Greaves and Mocarski (Greaves *et al.,* 1995). Five or ten micrograms of plasmid pR1R2CAT, pH1R2CAT, or pH2R2CAT were linearized by digestion with restriction endonuclease *Xho*I. HFF cells were cotransfected with infectious recombinant RVgptR2CAT DNA (Lashmit *et al.,* 1998) plus one of the above linearized plasmids by the calcium phosphate precipitation method (Graham and van der Eb, 1973). Recombinant viruses were selectively grown and plaque purified from Lesch–Nyhan cells in medium containing 50 μg/mL 2-amino-6-mercaptopurine (6-thioguanine) (Sigma, St Louis, MO). Viral plaques were transferred to HFF cells and recombinant viruses were detected by dot blot hybridization with ^{32}P-labeled US7-specific DNA probe. Virus from wells giving positive hybridization were used to infect HFF cells and plaque-purified a second time. All recombinant viruses had the same growth characteristics and were propagated as described previously (Stinski, 1976).

CAT assay

CAT activities were determined in substrate excess as described by Gorman *et al.* (1982). The acetylated and nonacetylated [^{14}C]chloramphenicol was separated by thin-layer chromatography in a chloroform–methanol (95:5) solvent. The amount of [^{14}C]chloramphenicol acetylation was determined by image acquisition analysis and the protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Richmond, CA).

Southern blot hybridizations

Virus was isolated from the culture medium as described previously (Stinski, 1976). Viral DNA was prepared for digestion with restriction enzyme *Bam*HI and subjected to agarose gel electrophoresis and Southern blot hybridization as described previously (Meier and Stinski, 1997). R1 DNA was prepared by the polymerase chain reaction (PCR) using primers 5′-CCCCATTACACGGCGATAT-3′ and 5′-CGGCTCCTGATGCTGTTTA-3′ to amplify a 342-bp fragment from pR1R2CAT. The 270-bp R1-specific DNA probe was obtained after digestion with restriction endonucleases *Eco*O109I and *Hin*PII. The same primers were used to amplify the 345-bp H1 and 355-bp H2 DNA fragments from pH1R2CAT and pH2R2CAT, respectively. The 269-bp H1-specific DNA probe was isolated by restriction endonuclease digestion with *Eco*O109I and *Bam*HI. The 214-bp H2-specific DNA probe was isolated by restriction endonuclease digestion with *Eco*O109I and *Hin*fI. DNA was put through a QIAquick PCR purification column (Qiagen, Inc, Valencia, CA) and labeled with the Rediprime random prime labeling system (Amersham Pharmacia Biotech, Piscataway, NJ).

Northern blot hybridizations

Cytoplasmic RNA was harvested from uninfected or infected HFFs as described previously (Thrower *et al.,* 1996). Ten micrograms of RNA was fractionated by electrophoresis in 1.5% agarose gels containing 6% formaldehyde and blotted onto Nytran as described previously (Meier and Stinski, 1997). A 381-nt US6-specific (195,236 to 195,548 nucleotides) ^{32}P-labeled antisense riboprobe was synthesized according to the method of Krieg and Melton (1987). Template for riboprobe synthesis was pKS+US5/6 that was linearized by digestion with *Mlu*I. Hybridization of ^{32}P-labeled riboprobe was done at 80°C overnight in 0.5 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, and 7% SDS following prehybridization in the same buffer for 2 h. After hybridization, the Nytran membrane was washed twice in 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), 5% SDS at 80°C for 30 min and then twice in 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), 1% SDS at 80°C for 30 min. The signals were visualized by autoradiography on Hyperfilm MP (Amersham Pharmacia Biotech, Arlington Heights, IL).

RNase protection assay

Riboprobes were synthesized according to the method of Krieg and Melton (1987) and as described previously (Hermiston *et al.,* 1990). The template for the synthesis of the antisense IE1 riboprobe was pGEMIE1 linearized with *Hin*dIII. The template for 5′US6 riboprobe was pKS+5′US6 linearized with *Xba*I. Cytoplasmic RNA was harvested from uninfected and infected HFFs as described previously (Thrower *et al.,* 1996). Ten or twenty micrograms of RNA was hybridized to the ^{32}P-labeled riboprobes at 25 or 45°C overnight. The RNAs protected from RNase T_1 digestion were fractionated in 6% polyacrylamide–urea gels. The signals were visualized by autoradiography on Hyperfilm MP (Amersham Pharmacia Biotech) and quantitated by image acquisition analysis.

ACKNOWLEDGMENTS

We thank members of the laboratory for helpful discussion and Richard Roller for a critical reading of the manuscript. This work was supported by National Institutes of Health (AI13562).

REFERENCES

Ahn, J., Brignole, E. J., and Hayward, G. S. (1998). Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. *Mol. Cell. Biol.* **18,** 4899–4913.

Ahn, J. H., and Hayward, G. S. (1997). The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. *J. Virol.* **71,** 4599–4613.

Ahn, J. H., Jang, W. J., and Hayward, G. S. (1999). The human cytomegalovirus IE2 and UL112–113 proteins accumulate in viral DNA replication compartments that initiate from the periphery of promyelocytic leukemia protein-associated nuclear bodies (PODs or ND10). *J. Virol.* **73,** 10458–10471.

Ahn, K., Angulo, A., Ghazal, P., Peterson, P. A., Yang, Y., and Fruh, K. (1996). Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc. Natl. Acad. Sci. USA* **93,** 10990–10995.

Ahn, K., Gruhler, A., Galocha, B., Jones, T. R., Wiertz, E. J., Ploegh, H. L., Peterson, P. A., Yang, Y., and Fruh, K. (1997). The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity* **6,** 613–621.

Alford, C. A., and Britt, W. J. (1990). Cytomegalovirus. *In* "Virology" (B. N. Fields, D. M. Knipe *et al.,* Eds.), pp. 1981–2010. Raven Press, New York.

Barnes, P. D., and Grundy, J. E. (1992). Down-regulation of the class I HLA heterodimer and beta 2-microglobulin on the surface of cells infected with cytomegalovirus. *J. Gen. Virol.* **73,** 2395–2403.

Beersma, M. F., Bijlmakers, M. J., and Ploegh, H. L. (1993). Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains. *J. Immunol.* **151,** 4455–4464.

Biegalke, B. J. (1995). Regulation of human cytomegalovirus US3 gene transcription by a cis-repressive sequence. *J. Virol.* **69,** 5362–5367.

Biegalke, B. J. (1997). IE2 protein is insufficient for transcriptional repression of the human cytomegalovirus US3 promoter. *J. Virol.* **71,** 8056–8060.

Biegalke, B. J. (1998). Characterization of the transcriptional repressive element of the human cytomegalovirus immediate-early US3 gene. *J. Virol.* **72,** 5457–5463.

Bruggeman, C. A., and M. C. E. van Dam-Mieras. (1991). The possible role of cytomegalovirus in atherogenesis. *Prog. Med. Virol.* **38,** 1–26.

Carswell, S., and Alwine, J. C. (1989). Efficiency of utilization of the simian virus 40 late polyadenylation site: Effects of upstream sequences. *Mol. Cell. Biol.* **9,** 4248–4258.

Chambers, J., Angulo, A., Amaratunga, D., Guo, H., Jiang, Y., Wan, J. S., Bittner, A., Frueh, K., Jackson, M. R., Peterson, P. A., Erlander, M. G., and Ghazal, P. (1999). DNA microarrays of the complex human cytomegalovirus genome: Profiling kinetic class with drug sensitivity of viral gene expression. *J. Virol.* **73,** 5757–5766.

Chan, Y.-J., Tseng, W.-P., and Hayward, G. S. (1996). Two distinct upstream regulatory domains containing multicopy cellular transcription factor binding sites provide basal repression and inducible enhancer characteristics to the immediate-early IES (US3) promoter from human cytomegalovirus. *J. Virol.* **70,** 5312–5328.

Chee, M. S., Bankier, S., Beck, S., Bohni, R., Brown, C. R., Horsnell, T., Hutchison, III, C. A., Kouzarides, T., Martignetti, J. A., Preddie, E., Satchwell, S. C., Tomlinson, P., Weston, K. M., and Barrell, B. G. (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *In* "Current Topics in Microbiology and Immunology," Vol. 154, pp. 125–169. Springer-Verlag, Berlin-Heidelberg.

del Val, M., Hengel, H., Hacker, H., Hartlaub, U., Ruppert, T., Lucin, P., and Koszinowski, U. H. (1992). Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J. Exp. Med.* **176,** 729–738.

Dery, C. V., Toth, M., Brown, M., Horvath, J., Allaire, S., and Weber, J. M. (1985). The structure of adenovirus chromatin in infected cells. *J. Gen. Virol.* **66,** 2671–2684.

Deshmane, S. L., and Fraser, N. W. (1989). During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *J. Virol.* **63,** 943–947.

Dyson, P. J., and Farrell, P. J. (1985). Chromatin structure of Epstein-Barr virus. *J. Gen. Virol.* **66,** 1931–1940.

Goodwin, E. C., and Rottman, F. M. (1992). The 3′-flanking sequence of the bovine growth hormone gene contains novel elements required for efficient and accurate polyadenylation. *J. Biol. Chem.* **267,** 16330–16334.

Gorman, M. C., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Mol. Cell. Biol.* **2,** 1044–1051.

Graham, F. L., and van der Eb, A. J. (1973). A new technique for the assay of infectivity of adenovirus 5 DNA. *Virology* **52,** 456–467.

Greaves, R. F., Brown, J. M., Vieira, J., and Mocarski, E. S. (1995). Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the *E. coli* guanosine phosphoribosyl transferase (gpt) gene. *J. Gen. Virol.* **76,** 2151–2160.

Gretch, D. R., and Stinski, M. F. (1990). Transcription of the human cytomegalovirus glycoprotein gene family in the short unique component of the viral genome. *Virology* **174,** 522–532.

Hahn, G., Jores, R., and Mocarski, E. S. (1998). Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc. Natl. Acad. Sci.* **95,** 3937–3942.

Hermiston, T. W., Malone, C. L., and Stinski, M. F. (1990). Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. *J. Virol.* **64,** 3532–3536.

Ho, M. (1991). "Cytomegalovirus: Biology and Infection." Plenum, New York.

Ishov, A. M., Stenberg, R. M., and Maul, G. G. (1997). Human cytomegalovirus immediate early interaction with host nuclear structures: Definition of an immediate transcript environment. *J. Cell Biol.* **138,** 5–16.

Jones, T. R., Hanson, L. K., Sun, L., Slater, J. S., Stenberg, R. M., and Campbell, A. E. (1995). Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J. Virol.* **69,** 4830–4841.

Jones, T. R., and Muzithras, V. P. (1991). Fine mapping of transcripts expressed from the US6 gene family of human cytomegalovirus strain AD169. *J. Virol.* **65,** 2024–2036.

Jones, T. R., and Sun, L. (1997). Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains. *J. Virol.* **71,** 2970–2979.

Jones, T. R., Wiertz, E. J. H. J., Sun, L., Fish, K. N., and Nelson, J. A. (1996). Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc. Natl. Acad. Sci. USA* **93,** 11327–11333.

Jun, Y., Kim, E., Jin, M., Sung, H. C., Han, H., Geraghty, D. E., and Ahn, K. (2000). Human cytomegalovirus gene products US3 and US6 down-regulate trophoblast class I MHC molecules. *J. Immunol.* **164,** 805–811.

Kern, F., Faulhaber, N., Khatamzas, E., Frommel, C., Ewert, R., Prosch, S., Volk, H., and Reinke, P. (1999). Measurement of anti-human cytomegalovirus T cell reactivity in transplant recipients and its potential clinical use: A mini-review. *Intervirology* **42,** 322–324.

Kondo, K., Kaneshima, H., and Mocarski, E. S. (1994). Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. *Proc. Natl. Acad. Sci. USA* **91,** 11879–11883.

Kondo, K., Xu, J., and Mocarski, E. S. (1996). Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc. Natl. Acad. Sci. USA* **93,** 11879–11142.

Kondoleon, S. K., Kurkinen, N. A., and Hallick, L. M. (1989). The SV40 nucleosome-free region is detected throughout the virus life cycle. *Virology* **173,** 129–135.

Krieg, P. A., and Melton, D. A. (1987). In vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* **155,** 397–414.

Landini, M. P., and La Placa, M. (1991). Humoral immune response to human cytomegalovirus proteins: A brief review. *Comp. Immunol. Microbiol. Infect. Dis.* **14,** 97–105.

Lashmit, P. E., Stinski, M. F., Murphy, E. A., and Bullock, G. C. (1998). A cis-repression sequence adjacent to the transcription start site of the human cytomegalovirus US3 gene is required to down regulate gene expression at early and late times after infection. *J. Virol.* **72,** 9575–9584.

Lehner, P. J., Karttunen, J. T., Wilkinson, G. W., and Cresswell, P. (1997). The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation. *Proc. Natl. Acad. Sci. USA* **94,** 6904–6909.

Levitt, N., Briggs, D., Gil, A., and Proudfoot, N. J. (1989). Definition of an efficient synthetic poly(A) site. *Genes Dev.* **3,** 1019–1025.

Marcus-Sekura, C. J., and Carter, B. J. (1983). Chromatin-like structure of adeno-associated virus DNA in infected cells. *J. Virol.* **48,** 79–87.

Mason, P. J., Elkington, J. A., Lloyd, M. M., Jones, M. B., and Williams, J. G. (1986). Mutations downstream of the polyadenylation site of a Xenopus beta-globin mRNA affect the position but not the efficiency of 3′ processing. *Cell* **46,** 263–270.

Meier, J. L., and Stinski, M. F. (1997). Effect of a modulator deletion on transcription of the human cytomegalovirus major immediate-early genes in infected undifferentiated and differentiated cells. *J. Virol.* **71,** 1246–1255.

Melnick, J. L., Adam, E., and Debakey, M. E. (1993). Cytomegalovirus and atherosclerosis. *Eur. Heart J.* **14,** 30–38.

Melnick, J. L., Hu, C., Burek, J., Adam, E., and DeBakey, M. E. (1994). Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J. Med. Virol.* **42,** 170–174.

Minton, E. J., Tysoe, C., Sinclair, J. H., and Sissons, J. G. (1994). Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. *J. Virol.* **68,** 4017–4021.

Peersoons, M. C. J., Daemen, M. J. A. P., Bruning, J. H., and Bruggeman, C. A. (1994). Active cytomegalovirus infection of arterial smooth muscle cells in immunocompromised rats: A clue to herpesvirus-associated atherogenesis? *Circ. Res.* **72,** 214–220.

Ploegh, H. L. (1998). Viral strategies of immune evasion. *Science* **280,** 248–253.

Plotkin, S. A. (1994). Vaccines for varicella-zoster virus and cytomegalovirus: Recent progress. *Science* **265,** 1383–1385.

Sadofsky, M., and Alwine, J. C. (1984). Sequences on the 3′ side of hexanucleotide AAUAAA affect efficiency of cleavage at the polyadenylation site. *Mol. Cell. Biol.* **4,** 1460–1468.

Sinclair, J., and Sissons, P. J. G. (1996). Latent and persistent infections of monocytes and macrophages. *Intervirology* **39,** 293–301.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997a). Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. *J. Clin. Invest.* **100,** 3154–3163.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997b). Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* **91,** 119–126.

Span, A. H. M., Grauls, G., Bosman, F., Van Boven, C. P. A., and Bruggeman, C. A.. (1992). Cytomegalovirus infection induces vascular injury in the rat. *Atherosclerosis* **93,** 41–52.

Stinski, M. F. (1976). Human cytomegalovirus: Glycoproteins associated with virions and dense bodies. *J. Virol.* **19,** 594–609.

Stinski, M. F. (1978). Sequence of protein synthesis in cells infected by human cytomegalovirus: Early and late virus-induced polypeptides. *J. Virol.* **26,** 686–701.

Taylor-Wiedeman, J., Sissons, P. J. G., Borysiewicz, L. K., and Sinclair, J. H. (1991). Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* **72,** 2059–2064.

Tenney, D. J., and Colberg-Poley, A. M. (1991). Human cytomegalovirus UL36–38 and US3 immediate-early genes: Temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection. *J. Virol.* **65,** 6724–6734.

Thrower, A. R., Bullock, G. C., Bissell, J. E., and Stinski, M. F. (1996). Regulation of a human cytomegalovirus immediate early gene (US3) by a silencer/enhancer combination. *J. Virol.* **70,** 91–100.

Tomazin, R., Boname, J., Hegde, N. R., Lewinsohn, D. M., Altschuler, Y., Jones, T. R., Cresswell, P., Nelson, J. A., Riddell, S. R., and Johnson, D. C. (1999). Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nat. Med.* **5,** 1039–1043.

Vayda, M. E., Rogers, A. E., and Flint, S. J. (1983). The structure of nucleoprotein cores released from adenovirions. *Nucleic Acids Res.* **11,** 441–460.

Warren, A. P., Ducroq, D. H., Lehner, P. J., and Borysiewicz, L. K. (1994). Human cytomegalovirus-infected cells have unstable assembly of major histocompatibility complex class I complexes and are resistant to lysis by cytotoxic T lymphocytes. *J. Virol.* **68,** 2822–2829.

Weston, K. (1988). An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. *Virology* **162,** 406–416.

Wiertz, E. J., Mukherjee, S., and Ploegh, H. L. (1997). Viruses use stealth technology to escape from the host immune system. *Mol. Med. Today* **3,** 116–123.

Wiertz, E. J. H. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L. (1996). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84,** 769–779.

Yamashita, Y., Shimokata, K., Mizuno, S., Yamaguchi, H., and Nishiyama, Y. (1993). Down-regulation of the surface expression of class I MHC antigens by human cytomegalovirus. *Virology* **193,** 727–736.

Yow, M. D., and Demmler, G. J. (1992). Congenital cytomegalovirus disease—20 years is long enough [editorial; comment]. *N. Engl. J. Med.* **326,** 702–703.