Alteration of cellular RNA splicing and polyadenylation machineries during productive human cytomegalovirus infection

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Alternative processing of human cytomegalovirus (HCMV) UL37 pre-mRNA predominantly produces the unspliced UL37 exon 1 (UL37x1) RNA and multiple, lower abundance, alternatively spliced UL37 RNAs. The relative abundance of UL37x1 unspliced RNA is surprising because it requires the favoured use of a polyadenylation signal within UL37 intron 1, just upstream of the UL37 exon 2 (UL37x2) acceptor. Here, it was shown that a downstream element (DSE) in UL37x2 strongly enhanced processing at the UL37x1 polyadenylation site, but did not influence UL37x1–x2 splicing. There was a potential binding site (UCUU) for polypyrimidine tract-binding protein (PTB) at the UL37x1 polyadenylation/cleavage site and its mutation to UGGG reduced both polyadenylation and splicing of UL37x1–x2 minigene pre-mRNA, suggesting a role in both RNA processing events. To determine whether lytic HCMV infection altered the balance of RNA processing factors, which bind to UL37 pre-mRNA cis elements, these were investigated in permissively infected primary and immortalized human diploid fibroblasts (HFFs) and epithelial cells. Induction of polyadenylation factors in HCMV-infected, serum-starved (G0) HFFs was also investigated. Permissive HCMV infection consistently increased, albeit with different kinetics, the abundance of cleavage stimulation factor 64 (CstF-64) and PTB, and altered hypo-phosphorylated SF2 in different cell types. Moreover, the preponderance of UL37x1 RNA increased during infection and correlated with CstF-64 induction, whereas the complexity of the lower abundance UL37 spliced RNAs transiently increased following reduction of hypo-phosphorylated SF2. Collectively, multiple UL37 RNA polyadenylation cis elements and induced cellular factors in HCMV-infected cells strongly favoured the production of UL37x1 unspliced RNA.

INTRODUCTION

The human cytomegalovirus (HCMV) UL37 immediate-early (IE) gene locus encodes several protein isoforms, of which at least one, the UL37 exon 1 protein (pUL37x1), is essential for HCMV growth in humans (Hayajneh et al., 2001a) and in cultured human foreskin fibroblasts (HFFs) (Dunn et al., 2003; Yu et al., 2003). pUL37x1 shares amino-terminal sequences, including its anti-apoptotic domains, with the UL37 glycoprotein, gpUL37, and the UL37 medium protein, pUL37M (Kouzarides et al., 1988; Goldmacher et al., 1999). While the carboxy terminus of gpUL37 is non-essential for HCMV growth in HFFs (Borst et al., 1999), it appears to play a role in HCMV growth in humans, as it does for mouse CMV pathogenesis in vivo (Lee et al., 2000; Hayajneh et al., 2001b).

pUL37x1 is the product of the UL37x1 unspliced RNA and is the predominant UL37 protein produced throughout permissive HCMV infection of HFFs (Mavinakere & Colberg-Poley, 2004a). Nonetheless, gpUL37, the product of the UL37 3–4 kb spliced RNA is also produced, at lower abundance, and is N-glycosylated (Al-Barazi & Colberg-Poley, 1996). Both HCMV UL37 proteins traffic dually into the endoplasmic reticulum (ER) and into mitochondria, where they block the release of cytochrome c following apoptotic signals by recruiting Bax to the mitochondrial outer membrane (Goldmacher et al., 1999; Colberg-Poley et al., 2000; Arnoult et al., 2004; Poncet et al., 2004; Mavinakere & Colberg-Poley, 2004a). gpUL37 is internally cleaved within UL37 exon 3 (UL37x3) sequences in the ER and its amino-terminal (UL37NH2) and carboxy-terminal (gpUL37COOH) fragments dissociate and traffic into distinct
subcellular compartments, mitochondria and the ER (Mavinakere & Colberg-Poley, 2004b).

Alternative processing of UL37 pre-mRNA predominantly generates the UL37x1 unspliced RNA throughout HCMV infection and at least another 10 temporally regulated, lower abundance UL37 spliced RNAs (Kouzarides et al., 1988; Tenney & Colberg-Poley, 1991; Adair et al., 2003). Production of the UL37x1 unspliced mRNA and the early UL38 RNA requires RNA cleavage and polyadenylation at nt 50998 within UL37 intron 1 (Kouzarides et al., 1988; Su et al., 2003a). Pre-mRNA 3′ processing leading to polyadenylation of UL37x1 RNA is partially dictated by the consensus UL37x1 polyadenylation signal, AAUAAA, at nt 51015–51020, which is bound first by cleavage and polyadenylation specificity factor 160 (CPSF-160). Subsequently, the cleavage stimulation factor (CstF) complex binds via CstF-64 to a downstream element (DSE), which is either U- or GU-rich (reviewed by Zhao et al., 1999; Pérez Cañadillas & Varani, 2003) and optimally positioned 14–70 nt downstream of the polyadenylation signal (Gil & Proudfoot, 1987; Chen et al., 1995). Adjacent binding of CstF-64 stabilizes the binding of CPSF to the polyadenylation signal and greatly increases 3′ cleavage (Murthy & Manley, 1992; Takagaki & Manley, 1997) by influencing the position of the cleavage factor, which may be CPSF-73 (Ryan et al., 2004). There is a suitably positioned UL37x2 GU-rich motif, 32 nt downstream of the UL37x1 polyadenylation signal, although it is not known whether this DSE enhances processing at the HCMV UL37x1 polyadenylation site.

Previously, we have shown that processing at the UL37x1 polyadenylation site is in direct competition with the mutually exclusive splicing at partially overlapping cis elements for UL37x1 to UL37x2 (UL37x1–x2) processing (Su et al., 2003b). Although UL37x1 RNA is predominant during HCMV infection, internal polyadenylation signals are usually inefficiently recognized (Niwa et al., 1992) and polyadenylation can be inhibited by an upstream splice donor (Furth et al., 1994; Gunderson et al., 1998). We therefore hypothesized that additional UL37 pre-mRNA cis elements increase the efficiency of processing at the UL37x1 polyadenylation/cleavage site.

Alternative processing of HCMV UL37 pre-mRNA has been accurately reproduced using minigene RNAs (Su et al., 2003a, b). Target 1 carries the UL37x1 polyadenylation signal/cleavage site, UL37 5′ and 3′ splice sites, a suitably spaced DSE in UL37x2 (nt 50974–50983) and a consensus polypyrimidine tract-binding protein (PTB) binding core site (UCUU) at the cleavage site of UL37x1 RNA and within the polypyrimidine tract upstream of UL37x2 (Fig. 1). Therefore, we analysed the UL37 cis elements that determine processing at the UL37x1 polyadenylation site using target 1. Here, we tested whether these DSE and PTB binding sites regulate processing of target 1 pre-mRNA.

In addition to its RNA cis elements, processing of UL37 pre-mRNA is predictably controlled by the relative abundance of negative and positive RNA processing factors during HCMV infection and affinities for their respective cis elements. Previously, we have shown that HCMV infection transiently reduces and then induces CstF-64, PTB and hypo- and hyper-phosphorylated SF2 within 4 h post-infection (p.i.) of permissively infected HFFs (Su et al., 2003b).

PTB (also called hnRNPI) is one of the better-characterized splicing repressors (Wagner & Garcia-Blanco, 2002). In the case of α-tropomyosin, PTB binds to the pyrimidine tract of an acceptor site, blocking U2AF binding and, consequently, prevents splicing (Gooding et al., 1994). The presence of a consensus PTB binding site and its known role in regulating alternative splicing led us to examine alteration of PTB levels during HCMV infection and its role in HCMV UL37 pre-mRNA processing.

Human SF2/ASF is a member of the evolutionarily conserved arginine/serine-rich (SR) family of splicing factors that are essential for constitutive splicing and can regulate alternative RNA splicing (Staknis & Reed, 1994; Jamison et al., 1995; Gravelle, 2000). DNA viruses including herpes simplex virus, vaccinia virus and adenovirus decrease splicing of cellular pre-mRNAs by inducing hypophosphorylation of SF2 (Kanopka et al., 1996, 1998; Huang et al., 2002; Sciabica et al., 2003).

Our goal in these studies was to determine whether changes in the abundance of RNA processing factors during HCMV infection correlated with the processing of HCMV UL37 pre-mRNA. To that end, we examined the abundance of cellular factors throughout the HCMV life cycle to determine whether the induction previously observed at IE times occurred at other times of infection. To assess the cell-type specificity of HCMV infection on key polyadenylation and splicing factors, we examined the abundance of CstF-64, PTB and SF2 during HCMV infection of three different permissive cell types, HFFs, human telomerase reverse transcriptase-life-extended HFFs (hTERT-HFFs) and human telomerase reverse transcriptase-immortalized retinal pigmented epithelial cells (hTERT-RPEs). We used hTERT-RPEs, which can be grown in large numbers and terminally differentiated in vitro (Rambhatla et al., 2002), because they provide a cultured model of HCMV infection of epithelial cells. HCMV replication in epithelial cells is a crucial step for invasion of the human body and dissemination of infection (Coats et al., 2000). To determine the effects of HCMV infection on synchronized cells, we investigated serum-starved G0 HFFs. We determined the functional consequences of the regulation of polyadenylation and splicing factors on the production of UL37x1 unspliced and alternatively spliced UL37 RNA species throughout HCMV infection.

**METHODS**

**Cells.** HFFs were grown in Dulbecco’s MEM (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). hTERT-RPEs were grown in DMEM: Medium 199 (4:1) supplemented with 10% (v/v) FCS. hTERT-HFFs were grown in DMEM: Medium 199 (4:1) supplemented with 10% (v/v) FCS. hTERT-RPEs were grown in DMEM: Medium 199 (4:1) supplemented with 10% (v/v) FCS.
FCS, 2 mM L-glutamine and 1 % (v/v) non-essential amino acids. hTERT-RPEs (Clontech) were grown in DMEM:F-12 (Hyclone) supplemented with 10 % (v/v) FCS, 2 mM L-glutamine and 0.348 % (v/v) sodium bicarbonate. To synchronize cells, HFFs were grown to confluency in 60 mm plates and then serum starved (0–2 % FCS) for 3 days and infected as described previously (Colberg-Poley & Santomenna, 1988). Briefly, synchronized cells were mock-infected, HCMV-infected (m.o.i. of 3) or treated with 10 % FCS for 1 h at 37°C. Inocula were removed and replaced with conditioned 0–2 % FCS medium. Cells were harvested by washing with PBS and lysis in 2 × sample buffer.

Cell transfection, RT-PCR and quantification of alternatively processed target 1 RNAs. HFFs were transfected with target 1 wild-type (wt) or mutant expression vectors as previously described (Su et al., 2003a, b). Site-specific mutants in the UL37 GU-rich sequence (nt 50974–50983) and PTB core binding site (nt 50995–50998) (Fig. 1) were generated using the QuikChange Site-directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing of the complete minigenes (Hayajneh et al., 2001a). Total RNA, isolated from transfected cells, was reverse transcribed and the resulting cDNAs examined for the abundance of alternatively processed target 1 species as previously described (Su et al., 2003b). Briefly, cDNAs were serially diluted and PCR amplified using primers to detect unspliced and polyadenylated (primers 239/240), UL37x1–x2 spliced (primers 176/179) and UL37x1 polyadenylated (primers 265/228) RNAs (Table 1). The level of each species was determined by endpoint dilution and the percentages were calculated by dividing the level of each target 1 species by the sum total of all target 1 RNA species and multiplying by 100. The level of unspliced RNA was deduced by subtracting the level of UL37x1 polyadenylated RNA from target 1 unspliced/polyadenylated RNA.

Production of nuclear extracts. Nuclear extracts were prepared from HeLa cell pellets (2 × 10⁸ cells) (provided by the National Cell Culture Center, Minneapolis, MN, USA) and from HFFs, hTERT-HFFs and hTERT-RPEs (~4 × 10⁶ cells per time point) as previously described (Su et al., 2003b).

Table 1. RT-PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>HCMV sequence (nt)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>176</td>
<td>UL37x1</td>
<td>52271–52251</td>
<td>Su et al. (2003a)</td>
</tr>
<tr>
<td>179</td>
<td>UL37x2</td>
<td>50968–50949</td>
<td>Su et al. (2003a)</td>
</tr>
<tr>
<td>239</td>
<td>UL37x1</td>
<td>52249–52234</td>
<td>Su et al. (2003a)</td>
</tr>
<tr>
<td>228</td>
<td>UL37x1 PA</td>
<td>51108–51014</td>
<td>Su et al. (2003b)</td>
</tr>
<tr>
<td>240</td>
<td>Target 1 junction</td>
<td>52165–52171/GC/51119–51132</td>
<td>Su et al. (2003b)</td>
</tr>
<tr>
<td>265</td>
<td>UL37 intron 1</td>
<td>51116–51095</td>
<td>Su et al. (2003b)</td>
</tr>
<tr>
<td>140</td>
<td>UL37x1</td>
<td>52259–52236</td>
<td>Adair et al. (2003)</td>
</tr>
<tr>
<td>196</td>
<td>UL37x3</td>
<td>50542–50521</td>
<td>Adair et al. (2003)</td>
</tr>
<tr>
<td>264</td>
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<td>52541–52520</td>
<td>Adair et al. (2003)</td>
</tr>
<tr>
<td>298</td>
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<td>120833–120817</td>
<td>This paper</td>
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<tr>
<td>299</td>
<td>UL83 (pp65)</td>
<td>120272–120256</td>
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<tr>
<td>300</td>
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<td>172752–172726</td>
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<tr>
<td>301</td>
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<td>172219–172194</td>
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<td>321</td>
<td>UL122 (IE2) exon 5</td>
<td>170582–170561</td>
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</tr>
<tr>
<td>322</td>
<td>UL122 (IE2) exon 5</td>
<td>170319–170300</td>
<td>This paper</td>
</tr>
</tbody>
</table>

*HCMV genome (GenBank/EMBL accession no. X17403).
RT-PCR and quantification of RNAs from HCMV-infected cells. hpRT-ERTFs (~2.5 × 10^6 cells per time point) were infected with HCMV strain AD169 (m.o.i. of 3). Poly(A)^+ RNA was harvested using the Poly(A) Pure Isolation kit (Ambion). Reverse transcription of 750 ng poly(A)^+ RNA was performed using oligo dT and SuperScript II reverse transcriptase (Invitrogen). Control reactions without enzyme were assayed in parallel. cDNAs (200 ng) were serially diluted in threefold steps and 5-6 μl of each dilution was amplified by PCR with HotStar Taq polymerase (Qiagen) using primers to detect UL37x1, UL37, pp65, IE1 or IE2 (Table 1). Quantification of each RNA species was performed following resolution by gel electrophoresis as previously described (Su et al., 2003b). To minimize experimental variation, the same amount of poly(A) tail was assayed in parallel. cDNAs (200 ng) were diluted in sixfold steps and 5 μl of each dilution was amplified by PCR with HotStar Taq polymerase (Qiagen) using primers to detect UL37x1, UL37, pp65, IE1 or IE2 (Table 1). Quantification of each RNA species was performed following resolution by gel electrophoresis as previously described (Su et al., 2003b).

Western blot analysis. Polypeptides were electro-transferred and assayed by Western blot analysis as previously described (Su et al., 2003b). Primary antibodies included mouse anti-SF2/ASF (32-4500, diluted 1:250; Zymed), mouse anti-CstF-64 [monoclonal antibody 3A7 from T. Shenk (Princeton University, Princeton, NJ, USA) and C. MacDonald (Texas Tech University HSC, Lubbock, TX, USA), diluted 1:50], mouse anti-PTB (32-4800, diluted 1:250; Zymed) and mouse anti-actin (sc-8432, diluted 1:100; Santa Cruz). Reactivity was monitored by chemiluminescence using HRP-conjugated goat anti-mouse (Bio-Rad) and ECL chemiluminescent detection (Amersham).

RESULTS

Delineation of cis elements affecting UL37 pre-mRNA processing at the UL37x1 cleavage/polyadenylation site

UL37x2 DSE (GU-rich sequence). Processing of the UL37 pre-mRNA at the UL37x1 site requires a highly conserved polyadenylation signal (nt 51015–51020), 17 nt upstream of its cleavage site (Kouzarides et al., 1988; Su et al., 2003a, b). Here, we mutated UL37 minigene sequences to determine whether other cis elements affected the efficiency of UL37x1 polyadenylation site utilization. CstF-64 binding to the DSE of U- or GU-rich sequences reflects the efficiency of polyadenylation site usage (MacDonald et al., 1994; Takagaki & Manley, 1997). There was a suitably positioned GU-rich motif within UL37x2 and downstream of the UL37x1 polyadenylation signal (Fig. 1). As large deletions of the U- or GU-rich motif are required to abolish CstF function (Zarkower & Wickens, 1988), we mutated the complete DSE GU-rich sequence (GGUUUUUGUGU to AACCCCCCAG). The target 1 DSE mutant was predicted to prevent downstream binding of CstF-64 and, consequently, reduce polyadenylation at the UL37x1 site. Since this mutation was outside of the RNA cis elements predicted to be required for UL37x1–x2 splicing, we did not anticipate that the target 1 DSE mutant would alter UL37x1–x2 splicing. The efficiency of target 1 DSE mutant RNA processing at the UL37x1 polyadenylation site was dramatically reduced (approx. 75-fold) compared with wt target 1 (Table 2). In contrast, splicing of the target 1 DSE mutant was indistinguishable (onefold) from that of wt target 1. Thus, UL37x2 GU-rich sequences altered the efficiency of UL37 pre-mRNA processing at the UL37x1 polyadenylation site. However, these UL37x2 sequences did not appear to be a site for competition and steric hindrance between polyadenylation and splicing factors for UL37 pre-mRNA.

UL37 consensus PTB binding site. There was a potential PTB core binding site (UCUU, nt 50995–50998) (Perez et al., 1997), which was at the UL37x1 RNA cleavage site and overlapped with the polypyrimidine tract upstream of the UL37x2 acceptor. To determine whether PTB altered processing of UL37 pre-mRNA processing, the UL37 core PTB site was mutated (UGGG) and tested for its effects on target 1 pre-mRNA processing (Table 2). The target 1 PTB mutant had decreased polyadenylation at the UL37x1 site (approx. threefold) and dramatically decreased UL37x1–x2 RNA splicing (approx. 80-fold) (Table 2). The reduction in UL37x1 polyadenylation site usage to undetectable levels by the DSE mutation and by another UL37 PTB mutant was observed in separate experiments (unpublished results). These results suggested that the PTB/polypyrimidine tract site has dual functions in UL37 pre-mRNA processing, at the UL37x1 polyadenylation site and in UL37x1–x2 splicing.

Induction of cellular polyadenylation factors in HCMV-infected HFFs

HCMV infection of permissive HFFs transiently decreases and then dramatically increases the abundance of CstF-64

<table>
<thead>
<tr>
<th>RNA</th>
<th>wt (%)</th>
<th>Mutant (%)</th>
<th>Change (fold)</th>
</tr>
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<tbody>
<tr>
<td>Un</td>
<td>24.7</td>
<td>25</td>
<td>1 ×</td>
</tr>
<tr>
<td>PA</td>
<td>0.3</td>
<td>0.004</td>
<td>75 × ↓</td>
</tr>
<tr>
<td>Sp</td>
<td>75</td>
<td>75</td>
<td>1 ×</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Un, Unspliced target 1 RNA; PA, polyadenylated target 1 RNA; Sp, spliced target 1 RNA.

Table 2. Downstream cis elements influence processing of HCMV UL37 pre-mRNA

The percentage of each UL37 RNA species was calculated by dividing its end-point dilution by the total and multiplying by 100.
and PTB at 4 h p.i. (Su et al., 2003b). We extended these studies to determine whether induction occurred at other phases of the HCMV life cycle. CstF-64 was induced at IE times (4 and 8 h p.i.) and then again at early times (48 h p.i.) (Fig. 2a, top panel). Similarly, the abundance of the potent regulator of alternative splicing, PTB, was transiently decreased at 2, 8, and 24 h p.i. relative to uninfected cell extracts and then subsequently increased at 4 and 48 h p.i. (second panel). At the time of decreased PTB abundance (2, 24, and 96 h p.i.), smaller PTB bands (~25 kDa) were detected at very low abundance.

SF2/ASF is an essential splicing factor whose activity is altered by differential phosphorylation (Xiao & Manley, 1998). During the IE and early times of infection, hypo-phosphorylated and hyper-phosphorylated SF2/ASF species were also biphasically induced in HCMV-infected HFFs (Fig. 2a, third panel, lower and upper bands, respectively). In contrast, there was no notable difference in levels of the nuclear protein, SP-100 (R. Adair and A. M. Colberg-Poley, unpublished results). Equivalent loading of protein samples was monitored by reactivity with an anti-actin antibody (bottom panel).

### Induction of cellular polyadenylation factors in HCMV-infected, G₀ HFFs

To determine whether HCMV infection could alter the balance of polyadenylation factors in resting, synchronized cells, we examined these during HCMV infection of serum-starved (G₀) HFFs. HCMV infection of G₀ HFFs induced CstF-64 within 24 h p.i. until late times of infection (Fig. 2b, top right panel). A similar pattern of induction was observed for induction of PTB (Fig. 2b, second right panel). In contrast, hypo-phosphorylated SF2/ASF rapidly increased in abundance within IE times of HCMV infection (Fig. 2b, third left panel). Equivalent protein loading was monitored using an anti-actin antibody (Fig. 2b, bottom panel).

### Induction of cellular polyadenylation factors in HCMV-infected permissive hTERT-HFFs

Similar patterns of reduction and induction of CstF-64, PTB and hypo- and hyper-phosphorylated SF2 were observed following HCMV infection of hTERT-HFFs (Fig. 3), although the timing differed slightly from HFFs (Fig. 2). CstF-64 was induced at 1 h p.i. and transiently reduced at 4 and 6 h p.i. in HCMV-infected hTERT-HFFs (Fig. 3, top panel). Again, CstF-64 was induced by 8 h and more dramatically at 48 h p.i. Full-length PTB was detected throughout HCMV infection, although of noticeably lower abundance at 4 and 6 h p.i. When the full-length PTB isoform was reduced, smaller PTB isoforms, of ~25 and 36 kDa, were detected using a monoclonal antibody against the PTB carboxy terminus at 4, 8, and 48 h p.i. (Fig. 3, second panel). In contrast to primary HFFs, hypo- and hyper-phosphorylated SF2/ASF was readily detected throughout IE times (1–8 h p.i.) in immortalized fibroblasts. However, hypo-phosphorylated SF2 levels were reduced at 24 and 48 h p.i. (Fig. 3, third panel). Loading of similar amounts of cellular protein was monitored using an anti-actin antibody (bottom panel).

### Induction of cellular polyadenylation factors in HCMV-infected hTERT-RPEs

To determine the cell specificity of HCMV induction of polyadenylation factors, we examined the effects of HCMV infection in epithelial cells. To that end, hTERT-RPEs, which can be differentiated in vitro and are permissive for HCMV (Detrick et al., 1996; Coats et al., 2000; Rambhatla et al., 2002), were infected with HCMV, harvested at various times p.i. and examined for the presence of critical cellular polyadenylation factors (Fig. 4). Unlike primary HFFs (Fig. 2), CstF-64 was readily detectable in nuclear extracts from uninfected, life-extended hTERT-RPEs (Fig. 4a and b, top panel). CstF-64 was present at 4 and 8 h p.i., but was greatly reduced in abundance at 24, 48, 96, 120 and 144 h p.i. CstF-64 levels were increased at 72 h p.i. in HCMV-infected hTERT-RPE nuclear extracts, similar to the second induction observed a day earlier in HCMV-infected HFFs (Fig. 2, 48 h p.i.), albeit at a later time of infection. Full-length PTB was detected in relatively high abundance in uninfected hTERT-RPEs and also in HCMV-infected cells at 4, 8 and 72 h p.i. (Fig. 4a and b, second panel). Interestingly, smaller PTB species were detected at 24 and 48 h p.i. and at a lesser degree at 96, 120 and 144 h p.i. Similar truncated PTB products have been described in uninfected cells (Bothwell et al., 1991; Gooding et al., 2003) and during poliovirus infection (Back et al., 2002a, b) and hepatitis A virus infection (Venkatramana et al., 2003).

SF2/ASF was detected throughout HCMV infection with induction of hypo-phosphorylated SF2/ASF at 72 h p.i. (Fig. 4a and b, third panel). Even gel loading was monitored by probing with an anti-actin antibody (Fig. 4a and b, bottom panel). Similar to the results with HFFs, there was no noticeable difference in the levels of U1 or Sp-100 during HCMV infection of hTERT-RPE cells (R. Adair and A. M. Colberg-Poley, unpublished results).

### UL37x1 unspliced RNA increases in abundance compared with UL37 spliced RNAs during HCMV infection of hTERT-HFF cells

To determine the effects of regulation of the polyadenylation factors during permissive HCMV infection on UL37 pre-mRNA processing, we examined the relative abundance of UL37x1 unspliced RNA and UL37 spliced RNAs in HCMV-infected hTERT-HFFs (Fig. 5). At 4 h p.i., the relative abundance of UL37x1 unspliced RNA was approximately ninefold higher than UL37 spliced RNAs (Fig. 5a and e). Interestingly, the most abundant spliced UL37 RNA was the UL37 RNA, as other UL37 spliced RNAs were barely detectable by RT-PCR. The abundance of the UL37x1 unspliced RNA was lower than the levels of the major IE, IE1 and IE2 RNAs at 4 h p.i. in HCMV-infected hTERT-HFFs.
After induction of CstF-64 and PTB (8 h p.i.) in hTERT-HFFs (Fig. 3), the abundance of UL37x1 and UL37 spliced RNAs both increased dramatically (Fig. 5b and e). Nonetheless, UL37x1 unspliced RNA was even more abundant (approx. 27-fold) than UL37 spliced RNA. The complexity of UL37 pre-mRNA alternative splicing was increased at 8 h p.i. as another UL37 spliced RNA, UL37_d, was readily detected. The abundance of the UL37x1 RNA was comparable to that detected for IE1 and IE2 transcripts in HCMV-infected hTERT-HFFs at 8 h p.i.
The abundance of the UL37x1 unspliced RNA remained elevated in HCMV-infected hTERT-HFFs at 24 h p.i. (Fig. 5c). Moreover, the UL37x1 unspliced RNA was greatly favoured as its level was approximately 81-fold higher than the UL37 spliced RNAs in HCMV-infected hTERT-HFFs at 24 h p.i. (Fig. 5e). At this time, the levels of CstF-64 were increased above uninfected cells, and at 4 and 6 h p.i., while hypo- and hyper-phosphorylated SF2 levels were reduced (Fig. 3). Multiple UL37 spliced RNAs were detected and the most abundant UL37 spliced RNAs detected were UL37di and UL37 spliced RNA observed earlier (Fig. 5b). UL37x1 RNA levels were comparable to other HCMV IE RNAs. IE1, IE2 and pp65 RNAs in HCMV-infected hTERT-HFFs were increased in abundance at 24 h p.i. (Fig. 5c).

At 48 h p.i., when the abundance of CstF-64 was dramatically increased and hypo-phosphorylated SF2 was reduced in these infected cells (Fig. 3), the relative abundance of UL37x1 unspliced RNA was even higher (approx. 243-fold) than that of UL37 spliced RNAs (Fig. 5d and e). The abundance of UL37x1 unspliced RNA continued to be comparable to that of IE2 and slightly more abundant than IE1 RNA in HCMV-infected hTERT-HFFs at 24 h p.i. (Fig. 5c). The abundance of pp65 RNA increased above UL37x1, IE1 and IE2 RNAs at 48 h p.i. Multiple UL37 spliced RNAs were detected and the most abundant of these was, once again, UL37 spliced RNA. Taken together, these results showed that the relative predominance of UL37x1 unspliced RNA over UL37 spliced RNAs increased dramatically with increasing time of HCMV infection, following the increased abundance of CstF-64. In contrast, the complexity of UL37 RNA splicing increased as hypo-phosphorylated SF2 decreased in abundance.

**DISCUSSION**

This study strived towards our long-term goal of understanding how HCMV alters post-transcriptional processing of its transcripts. Here, we examined the influence of a DSE in UL37x2 and of consensus PTB binding sequences on the mutually exclusive processing events of HCMV UL37 premRNA: polyadenylation at the UL37x1 site and UL37x1–x2 splicing. We also correlated the induction of important cellular RNA processing factors, including CstF-64 and PTB, which bind UL37 premRNA cis elements, with functional consequences, i.e. changes in the processing of UL37 premRNA during HCMV infection in different cell types.

Of the mutants tested (Su et al., 2003a, and this paper), the DSE mutant resulted in the largest decrease in the efficiency of polyadenylation at the UL37x1 site. This mutation was predicted to prevent binding of CstF-64 and therefore affect
only polyadenylation at the UL37x1 site, and indeed the DSE mutant selectively decreased UL37x1 polyadenylation but not UL37x1–x2 splicing. These findings suggested that competition between the splicing and polyadenylation machineries observed at the juxtaposed UL37x2 acceptor does not occur at the UL37x2 DSE.

Conversely, the UL37 PTB mutant sharply reduced UL37x1–x2 splicing and, to a lesser degree, polyadenylation at the UL37x1 site. These results suggested that the PTB/polypyrimidine tract sequences may play roles both in UL37x1 RNA cleavage and in UL37x1–x2 RNA splicing.

PTB can act as a regulator of mRNA expression through both its positive and negative effects on mRNA 3' processing (Castelo-Branco et al., 2004). PTB may enhance cleavage at the UL37x1 site, as with C2 complement pre-mRNA (Moreira et al., 1998). Consistent with this suggestion was the finding that mutation of the UL37x1 polyadenylation signal did not abolish the in vitro production of the UL37 cleavage product (R. Adair and A. M. Colberg-Poley, unpublished results).

High-affinity PTB binding sites act as splicing silencers when present within or adjacent to regulated exons (Wagner & Garcia-Blanco, 2002; Shen et al., 2004). The consensus PTB binding site is immediately upstream of the UL37x2 acceptor. The target 1 PTB mutant was predicted dramatically to affect usage of the UL37x2 acceptor, reducing its prediction score as an acceptor from 0·99 to 0·52 (http://www.fruitfly.org/seq-tools/splice.html), even though it retained a short polypyrimidine sequence proximal to the UL37x2 acceptor and another further upstream. It should be noted that the mutant splicing phenotypes observed (Su et al., 2003b, and this paper) were consistent with these computer model predictions.

Consistent with its predicted score, the PTB mutant sharply reduced UL37x1–x2 RNA splicing. The reduction in UL37x1–x2 splicing most likely resulted from the loss of pyrimidines required for the UL37x2 acceptor. The reduction in UL37x1 polyadenylated RNA suggested that the PTB core binding site also has a role in cleavage and polyadenylation of UL37 pre-mRNA. We are currently dissecting these two roles of PTB sequences in UL37 pre-mRNA processing by generating a PTB mutant lacking the consensus site but retaining an elevated splice acceptor score (R. Adair and A. M. Colberg-Poley, unpublished results).

We have previously shown that our target minigenes accurately reproduced splice site and polyadenylation site selection (Su et al., 2003a). However, the ratio of polyadenylated to spliced messages in the transfected cells did not replicate that seen during permissive HCMV infection.

**Fig. 4.** Regulation of cellular factors favouring polyadenylation at IE (a) and early and late (b) times of HCMV infection of hTERT-RPEs. hTERT-RPEs were infected with HCMV (m.o.i. of 3) and nuclear extracts prepared at 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 and 144 h p.i. Nuclear extracts were also prepared from uninfected (Un) hTERT-RPEs. Ten micrograms of nuclear proteins was resolved by SDS-PAGE as in Fig. 2, blotted and reacted with antibodies against CstF-64 (diluted 1 : 50), PTB (1 : 250), SF2/ASF (1 : 250) or actin (1 : 100). Arrows indicate CstF-64 (top panel), full-length and shorter PTB products (second panel), hyperphosphorylated (upper band) and hypophosphorylated (lower band) SF2/ASF (third panel).

**Fig. 5.** Production of UL37x1 unspliced and UL37 spliced RNAs in HCMV-infected hTERT-HFFs. hTERT-HFFs were infected with HCMV at an m.o.i. of 3 and harvested at 4 h (a), 8 h (b), 24 h (c) or 48 h (d) p.i. Poly(A)+ RNA (750 ng) was reverse transcribed and 200 ng serially diluted in threefold steps. Each dilution was PCR amplified using primers specific for UL37x1 (top panel, primers 264/140), UL37 (second panel, primers 264/196), IE1 (third panel, primers 300/301), IE2 (fourth panel, primers 321/322) and pp65 (bottom panel, primers 298/2990. The identities of the various UL37 RNA species were based on previous work (Adair et al., 2003) with * marking a PCR heteroduplex product. For negative controls, RNA or reverse transcriptase was omitted from the reaction (H2O and "RT," respectively). Migration of molecular size markers is indicated on the left of each gel. (e) Summary of relative abundances of UL37x1 unspliced and UL37x1–x2 spliced RNA in HCMV-infected hTERT-HFFs. The end-point dilution of each UL37 cDNA was used as a measure of relative abundance at each time point of infection and the ratios between unspliced and spliced UL37 RNAs were generated by dividing the relative abundance of UL37x1 by that of UL37x1–x2 RNA.
HCMV alters cellular RNA processing factors.
HCMV infection of target 1-transfected cells increased the UL37x1 polyadenylated species (Y. Su and A. M. Colberg-Poley, unpublished results). This finding further argued that the alteration of polyadenylation factors observed herein during HCMV infection favoured polyadenylation at the UL37x1 site. In spite of its limitations, the minigene system thus provides a powerful genetic tool with which to study UL37 pre-mRNA processing.

The effect of mutations on their predicted binding sites and their regulation during HCMV infection suggested that CstF-64, PTB and SF2 factors have a significant impact on the processing of UL37 pre-mRNA. HCMV infection induced CstF-64 at IE and early times of infection in HFFs, which coincided with the induction of PTB and hypo- and hyper-phosphorylated SF2. The induction of hypo-phosphorylated SF2, PTB and CstF-64 at 48 h p.i. in HFFs corresponded well with a reduction in splicing of UL37 phosphorylated SF2, PTB and CstF-64 at IE and early times of infection in hTERT-HFFs. The induction of hypo-phosphorylated SF2, PTB and CstF-64 at 48 h p.i. in HFFs, which coincided with the induction of PTB and hypo- and hyper-phosphorylated SF2. The induction of hypo-phosphorylated SF2, PTB and CstF-64 at IE and early times of infection in HCMV-infected hTERT-HFFs and hyper-phosphorylated SF2. The induction of hypo-phosphorylated SF2, PTB and CstF-64 at 48 h p.i. in HFFs corresponded well with a reduction in splicing of UL37 pre-mRNA (Adair et al., 2003).

To reduce the effect of the inherent variability in transfection efficiency, passage number and abundance of cellular factors in primary HFFs, we repeated the experiments using hTERT-HFFs (Bresnahan et al., 2000) and then under stringent conditions of primary HFF cell synchronization. Following the initial induction of CstF-64 and PTB (8 h p.i.), the levels of UL37x1 unspliced RNA increased dramatically in HCMV-infected hTERT-HFFs. Conversely, UL37 spliced RNA increased both in abundance and diversity, correlating with decreasing levels of hypo-phosphorylated SF2 (8 and 24 h p.i.). However, at later times of infection when CstF-64 levels increased dramatically (48 h p.i.), the UL37x1 unspliced RNA became the overwhelmingly predominant product of UL37 pre-mRNA processing. In synchronized G0 HFFs, the induction of hypo-phosphorylated SF2 occurred within IE times, while induction of CstF-64 and PTB increased progressively from 24 h p.i. to late times of infection. These results establish the reproducibility of induction of polyadenylation factors during HCMV infection of human fibroblasts under different physiological conditions.

Induction of polyadenylation factors during HCMV infection was not cell-type specific as it was observed upon infection of both permissive fibroblasts and epithelial cells. Nonetheless, the timing of the induction differed among cell types, most likely reflecting inherent cellular physiology, their immortalization and the timing of HCMV gene expression within each cell type. Consistent with this suggestion, the CstF-64 levels in uninfected HFFs, G0 HFFs and hTERT-HFFs detected was low, whereas CstF-64 levels in uninfected hTERT-RPEs were higher. In addition, induction of CstF-64 during HCMV infection of G0 HFFs was slower than in HFFs, hTERT-HFFs and hTERT-RPEs.

Induction of CstF-64 was observed dramatically in cell types (HFFs and hTERT-HFFs) in which the abundance in uninfected cells was lower, but not in others (hTERT-RPEs) in which its abundance was already high. These results suggested that HCMV infection induced the critical CstF-64, if needed, for processing of its transcripts. In support of this possibility was the finding that, when CstF-64 levels were still low, the relative abundance of the essential UL37x1 RNA was low, and it increased following the induction of CstF-64. It should also be noted that the induction of cellular PA and splicing factors observed herein predictably resulted in the patterns of UL37 pre-mRNA splicing described previously (Adair et al., 2003).

Induction of PTB mostly paralleled induction of CstF-64. Our finding of PTB in HFFs, G0 HFFs, hTERT-HFFs and hTERT-RPEs was consistent with its widespread expression in multiple cell types (Gil et al., 1991). Intriguingly, the induction of PTB was preceded by the appearance of smaller PTB isoforms, which reacted with a monoclonal antibody against the PTB carboxy terminus. They may represent either cleavage products (Bothwell et al., 1991; Back et al., 2002a, b) or products of alternatively spliced PTB RNAs (Gooding et al., 2003).

PTB-mediated repression can be relieved by tissue-restricted paralogues such as nPTB/brPTB (Chou et al., 2000; Markovtsov et al., 2000). The smaller PTB forms, which appear during HCMV infection, may contribute to partial relief and allow increased UL37x1–x2 RNA splicing. Induction of the smaller PTB forms during HCMV infection correlated inversely with the ability of full-length PTB to bind UL37 pre-mRNA sequences during HCMV infection, as monitored by gel-shift assays (R. Adair and A. M. Colberg-Poley, unpublished results). Nonetheless, PTB binding to UL37 pre-mRNA sequences may span more than the core site, as a PTB core mutant still weakly bound PTB in vitro. Interestingly, the splicing complexity of UL37 pre-mRNA increased in hTERT-RPEs following the decrease in full-length PTB (G. W. Liebisch and A. M. Colberg-Poley, unpublished results), similar to the results obtained with hTERT-HFFs.

Both hypo- and hyper-phosphorylated SF2/ASF were induced during HCMV infection of HFFs and G0 HFFs in which the uninfected cell levels were low. Conversely, in hTERT-HFFs and RPEs, hyper-phosphorylated SF2 was detected and not dramatically increased by HCMV infection. This selective induction of hypo- and hyper-phosphorylated SF2 suggested that both forms play key roles in the processing of HCMV RNAs. SF2 is an essential splicing factor and phosphorylation regulates its role during splicing (Xiao & Manley, 1998). The decrease in hypo-phosphorylated SF2 at 24 and 48 h p.i. of hTERT-HFFs correlated with an increase in the complexity of UL37 spliced RNAs. The putative importance for SF2 in processing of HCMV-spliced RNAs is further suggested by a potential SF2 binding site (nt 51120–51129) within target 1 but not within target 1S, which is cryptically spliced (Su et al., 2003a). Alternatively spliced IE1 and IE2 RNAs appear earlier than UL37 spliced RNAs during HCMV infection of hTERT-RPEs. Whether the complexity of IE1/2
The rapidity with which dramatic changes in the abundance of cellular polyadenylation factors occurs during HCMV infection suggests that the HCMV product responsible for this alteration is either a virion component or an IE protein. Moreover, induction at late times of infection is consistent with the possibility that a virion component, then synthesized, may be responsible for the induction. It is known that activation of the phosphatidylinositol-3-OH kinase/Akt pathway through anti-IgM–B-cell receptor cross-linking promotes expression of phosphorylated SR proteins and confers efficient splicing of bovine papilloma virus pre-mRNAs in SF2-deficient cells (Liu et al., 2003). Binding of HCMV virions to the epidermal growth factor receptor prior to entry into permissive HFFs has recently been implicated in the induction of the phosphatidylinositol-3-OH kinase/Akt pathway (Wang et al., 2003). Thus, induction of signaling upon HCMV binding and entry may play a role in altering the balance of polyadenylation and splicing factors necessary for processing of its RNAs.

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