HUMAN CYTOMEGALOVIRUS UL21.5 GENE IS EXPRESSED AS AN "EARLY-LATE" GENE IN CULTURED HUMAN FIBROBLASTS

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Summary. – The human cytomegalovirus (CMV) UL21 5 gene encodes a secreted glycoprotein of unknown function. Both the UL21.5 protein and mRNA accumulate in abundance at late stages of infection making the RNA an attractive target for diagnosis of active CMV infection. The UL21.5 was originally described as a 'spliced late' gene (SLG) (Rawlinson and Barrell, *J Virol* **67**, 5502 (1993)). However, we found that the the UL21.5 mRNA was detectable in CMV-infected patients before the onset of CMV DNA replication (Boriskin *et al.*, *J Clin Virol.*, in press). Here, we re-examined the UL21.5 mRNA kinetic class in CMV-infected human fibroblast culture using a RNAse protection assay and RT-PCR. The UL21.5 mRNA was detectable before the "true late" UL75 RNA, was resistant to a CMV DNA replication inhibitor but moderately sensitive to inhibitors of protein synthesis. In the presence of protein synthesis inhibitors the UL21.5 mRNA was detectable only by a nested reverse transcription – PCR (RT-PCR) with the bulk of it in unspliced form. This suggests that splicing factors for UL21.5 mRNA are encoded by the virus rather than by the cell Our results indicate that UL21.5 should be defined as an "early-late" rather than a "late" (L) CMV gene

Key words: human cytomegalovirus; UL21.5 gene, early-late gene

Human CMV infects over 60% of adults worldwide and, like other herpesviruses, probably remains latent for life, with the host undergoing episodes of reactivation and superinfection depending on immunological status. The outcome of infection is governed by an interplay between cellular and humoral immunity, but in immunocompetent individuals it is mostly subclinical. In those who are immunosuppressed, like patients with AIDS or organ transplantation, especially bone marrow transplant recipients, there may be a range of illnesses collectively termed "CMV disease". Due to the introduction of prophylactic or preemptive CMV therapy with ganciclovir, the incidence of CMV disease in high risk patients has been significantly reduced (Singh, 2001). However, the success of preemptive antiviral treatment depends on early detection of ongoing CMV infection by laboratory tests, among which those based on nucleic acid amplification are the most sensitive. Furthermore, detection of viral RNA transcripts rather than viral DNA genome, presents a better indication of active CMV infection.

The CMV genome has a complex organization and is functionally divided into immediate-early (IE), early (E) and late (L) groups of genes with respect to their temporal activation during the infection cycle in cell culture (Wathen and Stinski, 1982). For CMV diagnosis, it is important which group of genes is targeted. The IE genes may be transcribed without concomitant viral DNA replication. Hence, their mRNA detection cannot discriminate latency from active infection, whereas CMV L RNA transcripts are the best indicators of active CMV infection.

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Abbreviations: BMT = bone marrow transplant; CHX = cycloheximide; CMV = cytomegalovirus; E = early, FITC = fluorescein isothiocyanate; gH = glucoprotein H; HEL = human embryonic lung, HRS = histidyl-tRNA synthetase, IE = immediate early; L = late, MIE = major immediate early, MOI = multiplicity of infection; PAA = phosphonoacetic acid; p.i. = post infection; RNAse = ribonuclease; RT-PCR = reverse transcription – PCR

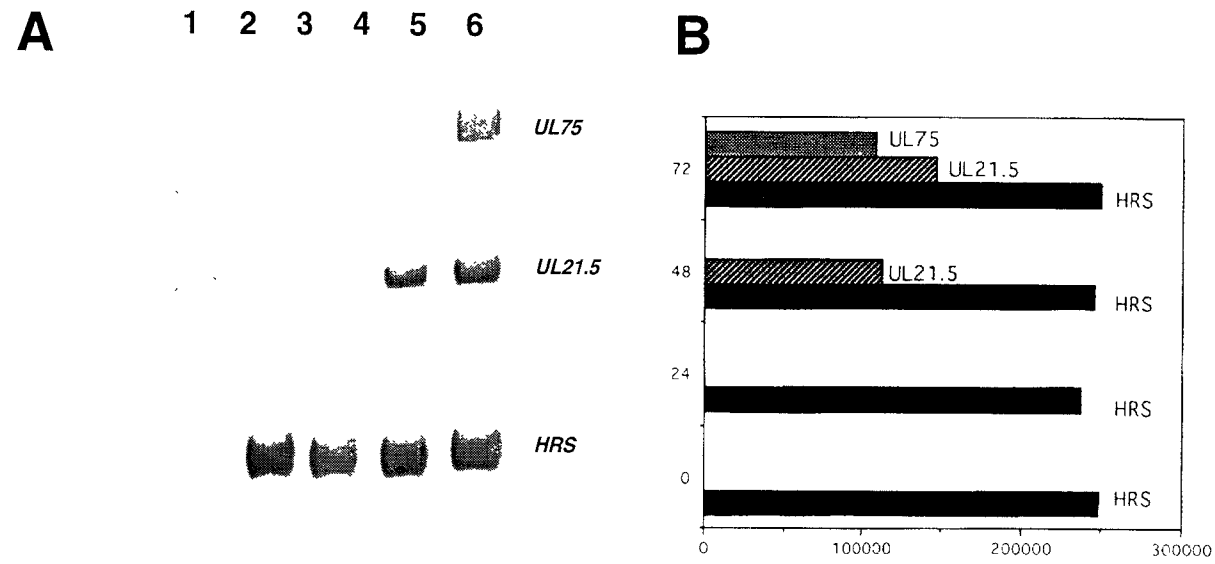

Fig. 1
Time-course analysis of transcription of CMV UL21.5 and UL75 genes

A Image of mRNAs, detected as protected probe fragments, for CMV UL21 5 (175 nt) UL75 (257 nt) genes and the housekeeping (cellular) HRS RNA gene (111 nt) used as internal standard RNAse-treated probes (lane 1), RNAse-untreated probes (lane 2), RNAs isolated at 0 hr p i (lane 3), 24 hrs p i (lane 4), 48 hrs p i. (lane 5), and 72 hrs p i (lane 6) The signals from the RNAse-untreated probe (control) are substantially weaker because only one-tenth of the sample was loaded on the gel

B Comparative intensity of bands in (A) as measured with the aid of the NucleoScan GelExpert software. Ordinate hrs p i Abscissa band absorbance

Rawlinson and Barrell (1993) have described a group of spliced late genes (SLG) of CMV which on detection were found to provide stronger evidence of CMV replication compared to the expression of IE genes (Nelson *et al.*, 1996). In a follow-up study we have shown the clinical utility of the RT-PCR for one of CMV SLG glycoproteins, the UL21.5, when applied to CMV diagnosis in BMT patients (Boriskin *et al.*, in press). However, in the majority of our BMT patients with CMV infection this, late by definition, viral RNA was detectable one week before first positive CMV DNA PCR although CMV L genes are supposed to be expressed after the onset of DNA replication. Therefore, we re-examined the kinetic class of UL21.5 RNA in CMV-infected human fibroblast culture.

Temporal pattern of UL21.5 mRNA expression was compared to that of a true late CMV UL75 transcript (Chambers *et al.*, 1999) using the ribonuclease (RNAse) protection assay. To this end, primary human embryonic lung (HEL) cell cultures were infected with a high titer stock of laboratory CMV AD169 strain at a multiplicity of infection (MOI) of 1 FFU per cell (Milne *et al.*, 1998). Total RNA was isolated by Trizol reagent (Life Technologies) and treated with 2 U of RNAse-free DNAse I (Ambion Inc., USA) per 1 µl of RNA solution. The templates for RNA probe preparation comprised gel-purified PCR products for a housekeeping gene, histidyl-tRNA synthetase (HRS, spliced RNA transcripts of 110 bp), spliced CMV UL21.5 RNA transcripts (SLG, 175 bp) and unspliced CMV UL75 RNA transcripts (257 bp) encoding glycoprotein H (gH). Details of RT-PCR and DNA PCR assays for these genes are given in Nelson *et al.* (1996) and. Milne *et al.* (1998). The sensitivities of the nested and single round RT-PCR assays for SLG were equal to 1 and 1000 copies of synthetic SLG RNA transcripts, respectively (Nelson *et al.*, 1996). The templates for the antisense and sense transcripts incorporated the T7 or SP6 polymerase promoters, respectively, using the Lig'n'Scribe kit (Ambion). One µl (20 ng) of gel-purified template was used in an *in vitro* transcription reaction using the BioProbe RNA transcript labeling kit with fluorescein isothiocyanate (FITC)-12-UTP (Enzo-Sigma, USA). The RNAse protection assay was carried out using the RPAIII kit (Ambion) as directed by the manufacturer. FITC-labeled RNA probes were precipitated by LiCl and digested with 2U of RNAse-free DNAse I per µg of DNA template. The DNAse pretreatment of isolated RNA preparations or DNAse treatment of transcription reaction products was sufficient to remove the residual DNA as verified by lack of signal in DNA PCR for respective templates (results not shown).

Ten ng of each probe was hybridized separately to 10 µg of total RNA isolated from CMV-infected cells at 24, 48 and 72 hrs post infection (p.i.). The hybrids were digested

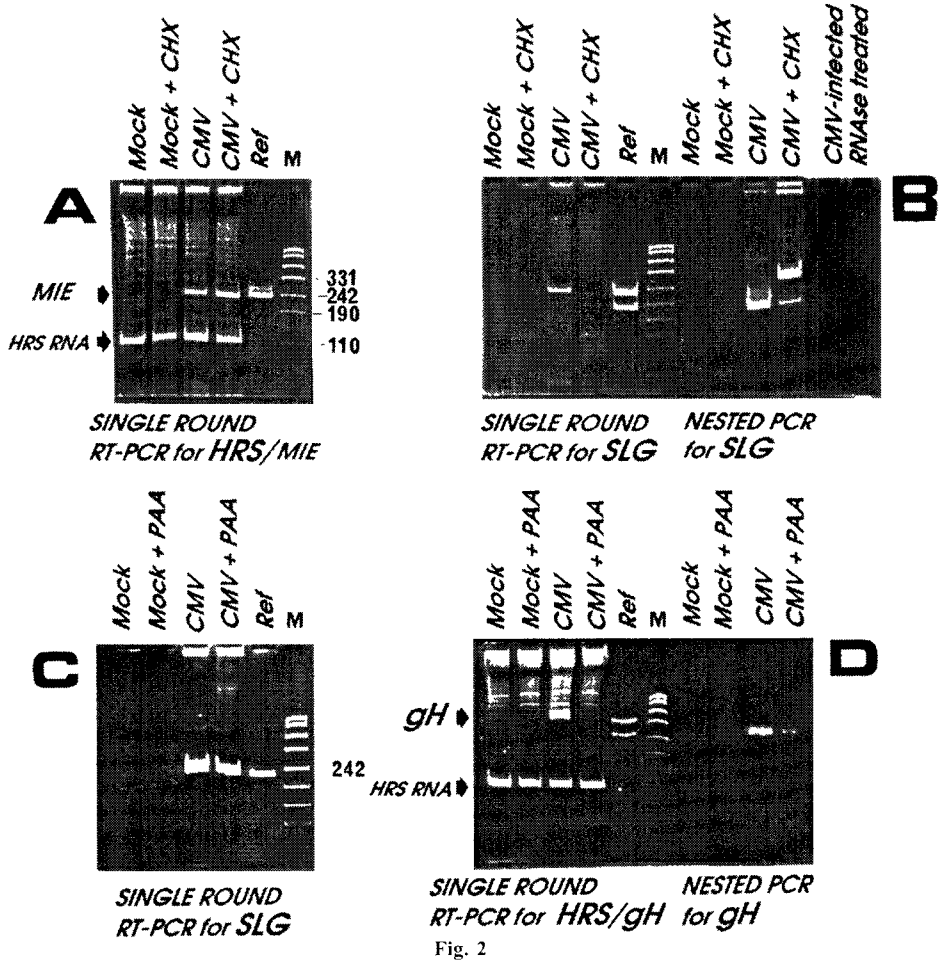

Fig. 2

RT-PCR analysis of expression of UL21.5 RNA transcription in the presence of CMV protein synthesis inhibitors (A, B) or CMV DNA replication inhibitor (C, D)

Ref lanes show positions of purified PCR products of both single and nested PCR rounds for each of the three targeted CMV genes MIE mRNA, unspliced (256 bp) (A), SLG (UL21.5) spliced mRNA, first round PCR (227 bp) and nested round PCR (175 bp) (B, C), SLG (UL21.5) unspliced mRNA, nested round PCR (258 bp) (B), unspliced gH (UL75) mRNA, first round PCR (357 bp) and nested round PCR (257 bp) (D) The spliced host housekeeping HRS mRNA (internal control) is 111 bp (A, D)

with a mixture of 0.25 U/ml RNAse A and 10 U/ml RNAse T1 which completely degraded unprotected probes (Fig. 1). For each time interval p.i. the individual digests of HRS, SLG and gH duplexes were pooled, co-precipitated, dissolved in a gel loading buffer (95% formamide, 18 mmol/l EDTA, 0.025% blue dextran, and 0.025% SDS), denatured at 95°C for 3 mins and loaded (2 µl) on 0.2 mm thin 6% denaturing polyacrylamide gel. Individual components were separated electrophoretically at 600 V in a NucleoScan 2001 Unit (NucleoTech Corp., USA) equipped with laser beam and GelExpert software which allowed gel image processing, editing and analysis.

Fig. 1 demonstrates that UL21.5 (SLG) RNA transcripts were first detected 48 hrs p.i. and before UL75 (late gH gene) RNA which was expressed no sooner than 72 hrs p.i. Uninfected cell RNA hybridized only with the HRS probe resembling the pattern of CMV-infected cells at 24 hrs p.i. No signal resulted from total cell RNA hybridization to sense HRS, SLG or gH probes (negative controls, results not shown).

To ascertain the correct kinetic class of SLG transcripts we analyzed their pattern of expression in the presence of inhibitors of CMV protein or DNA synthesis. In this set of experiments we used RT-PCR which allowed simultaneous processing of multiple samples. Unlike viral E or L RNA, the IE RNA is transcribed from input CMV genome and, as their transcription is not dependent on *de novo* cellular protein synthesis, it accumulates in the presence of protein synthesis inhibitors. HEL cells grown in 25 cm^2 culture

flasks were pretreated with a combination of cycloheximide (CHX) and anisomycin (final concentrations 40 μmol/l and 10 μmol/l, respectively) for 1 hr or left untreated (negative control). Half of inhibitor-treated or untreated cells were infected with CMV at MOI of 1 and total RNA was extracted at 18 hrs p.i. from mock-infected untreated, mock-infected treated, CMV-infected untreated and CMV-infected treated cells. The RNAs were digested with RNAse-free DNAse I and subjected to RT-PCR for HRS, CMV major IE (MIE) and CMV SLG transcripts using the First Strand cDNA synthesis kit (Amersham Pharmacia Biotech) and all three gene-specific reverse primers in the same RT reaction followed by separate nested PCR for each of the three individual genes. The primers and reaction conditions for MIE PCR were as described by Vinogradskaya *et al.* (1995). The whole PCR reaction mixtures (50 μl) were loaded on 6% polyacrylamide gel and run in a Mini Protean electrophoresis cell (BioRad, USA) followed by ethidium bromide staining. All PCR product DNAs were validated by direct dideoxy sequencing using the Sequenase 2.0 kit (Amersham Pharmacia Biotech).

A single round RT-PCR for a non-spliced MIE transcript yielded a 256 bp band indistinguishable by size from its DNA-derived PCR product. However, a simultaneously transcribed and co-amplified HRS RNA yielded only a spliced 111 bp RNA band with no sign of its unspliced 360 bp template DNA-derived product (Fig. 2A). The HRS RNA was present in CMV-infected or CMV-uninfected cells regardless of whether they were treated or untreated with CHX. As expected, the MIE RNA was not inhibited by CHX (Fig. 2A). In contrast, the 227 bp band characteristic for SLG RNA and visible in CMV-infected CHX-untreated cells, did not appear in CMV-infected CHX-treated cells after a single round PCR (Fig. 2B), suggesting its transcriptional sensitivity to protein synthesis inhibitors. However, after a nested round PCR two bands were seen in CMV-infected CHX-treated cells (Fig. 2B): a 175 bp spliced RNA band which was only a fraction of the intensity of its counterpart in CHX-untreated cells, and a major 258 bp non-spliced size band equally attributable to SLG RNA or DNA-derived PCR product. Direct dideoxy sequencing confirmed its SLG origin and presence of an intron. The absence of PCR signals in RNA preparations from matched CMV-infected, CHX-treated or untreated cells argue that the 258 bp band was derived from contaminating CMV DNA. Rawlinson and Barrell (1993) have predicted that this gene is likely to encode both spliced and unspliced messages. The results suggest that, although not as CHX-resistant as MIE RNA, SLG RNA is synthesized in the presence of protein synthesis inhibitors in smaller amounts detectable only by nested PCR. The bulk of SLG RNA synthesized in the presence of protein synthesis inhibitors comprise a non-spliced form with only the minority of transcripts being processed to a mature spliced RNA. This, in turn, suggests that SLG splicing factors are encoded by the virus rather than by the host cell.

Next, we studied SLG RNA expression in the presence of a CMV DNA replication inhibitor, phosphonoacetic acid (PAA). HEL cells were pretreated with 200 μg/ml PAA for 1 hr and both PAA-treated and untreated cells were infected with CMV at MOI of 1. Total cellular RNA was harvested at 72 hrs p.i., DNAse-treated and subjected to RT-PCR for HRS, SLG and gH (true late) genes. As in experiments with protein synthesis inhibitors, only the spliced 111 bp HRS RNA band was seen with no trace of contaminating 360 bp HRS DNA band (Fig. 2D). The SLG RNA expression was unaffected by PAA (Fig. 2C) whereas the 357 bp gH RNA band could not be seen after the single round PCR on PAA-treated cells (Fig. 2D) but appeared as a 257 bp band after the nested PCR in both PAA-treated and untreated cells (Fig. 2D).

From these results we conclude that CMV UL21.5 gene (SLG) cannot be defined as late, because its RNA is expressed sooner than that of the true late UL75 gene and, unlike UL75, is resistant to inhibition of CMV DNA synthesis. On the other hand, its expression is more sensitive to protein synthesis inhibition than that of an IE gene. Therefore, the UL21.5 could be rather classified as an "early-late" CMV gene.

The function of UL21.5 protein is unknown. It has been described as a predominant CMV protein species secreted by CMV-infected fibroblasts (Mullberg *et al.*, 1999) and UL21.5 RNA has been considered one of a few abundant CMV RNA species being sequestered non-specifically into new virus particles during virion assembly at the late stages of infection (Bresnahan and Shenk, 2000). The UL21.5 protein was proposed to function as a virokine interacting with host cellular receptors or cytokines (Mullberg *et al.*, 1999). The fact that the UL21.5 mRNA is synthesized in vast excess compared with its DNA template made it an attractive target for early diagnosis of active CMV infection.

The early-late nature of UL21.5 RNA transcripts helps in explaining its detection before the onset of CMV DNA replication in CMV-infected patients (Boriskin *et al.*, in press).

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