Human cytomegalovirus late-phase maturation is blocked by stably expressed UL32 antisense mRNA in astrocytoma cells

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Human cytomegalovirus (HCMV) open reading frame UL32 codes for the basic phosphoprotein pp150 (ppUL32), an abundant constituent of the virion tegument. In order to study its potential role in the assembly and/or transport of progeny particles, astrocytoma cell lines (U373MG) were generated, stably expressing a 2.1 kb 5′ fragment of UL32 in antisense orientation under the control of the HCMV major immediate early promoter. The steady-state level of the UL32 sense mRNA and pp150 synthesis were strongly reduced in infected antisense cell lines. Neither immediate early and early gene expression, nor viral DNA replication, was inhibited; the expression of the late gene product gB (gpUL55) was also reduced, but mainly at the level of translation. Control experiments indicated that this differential effect of UL32 antisense expression on the synthesis of viral products was specific. As a consequence of the inhibitory effect, virus yield was significantly reduced in antisense mRNA cell lines. Ultrastructural comparison of control and antisense cells revealed no difference in nucleocapsid forms in the nucleus. However, in the cytoplasm of antisense cells, DNA-containing C capsids and virions were absent and abnormal forms of non-infectious enveloped particles were observed. The data suggest the involvement of pp150 either in the transport of DNA-containing particles through the nuclear envelope or in the stabilization of capsids in the cytoplasm. Thus, UL32 antisense mRNA appears to interfere strongly with virus maturation during the late phase of the infectious cycle.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus which acts as a severe human pathogen in immunocompromised individuals (Alford & Britt, 1993). The HCMV genome contains over 200 open reading frames (ORFs), the products of most of which have not been characterized (Mocarski, 1996). Like other herpesviruses, HCMV consists of a double-stranded DNA enclosed within an icosahedral nucleocapsid. The nucleocapsid is surrounded by the envelope in which several glycoproteins are inserted. Between envelope and nucleocapsid lies an amorphous phosphoprotein matrix, referred to as the tegument, which forms a structural link between the nucleocapsid and the envelope. In addition to the infectious virions, two species of deficient particles can be observed: (i) non-infectious enveloped particles (NIEPs) which are similar in structure and composition to virions but contain no DNA; and (ii) dense bodies, which contain no nucleocapsid, but consist of spherical tegument protein aggregates. Both types of particle are enveloped and exocytosed (Irmiere & Gibson, 1983).

The morphogenesis of HCMV begins with the assembly of nucleocapsids in the nucleus. It is widely accepted that assembled HCMV nucleocapsids leave the nucleus by budding at characteristic patches through the inner nuclear membrane, resulting in enveloped intermediates in the perinuclear space (Radsak et al., 1991). The subsequent steps are still controversial. One proposal is that fusion with the outer nuclear membrane delivers de-enveloped progeny virions into the cytosol (Rixon, 1993). In a second step, naked virions are enveloped by cytoplasmic cisternae (Tooze et al., 1992), a mechanism by which the virion obtains its final envelope and can be released to the extracellular space (for review, see Griffith & Rottier, 1992).

For both budding at the inner nuclear membrane and envelopment at cytoplasmic cisternae, a specific interaction between components of the respective membrane and progeny...
capsids needs to be postulated. As for the membrane component, the viral glycoprotein gB (gpUL55) can be detected in the inner nuclear membrane (Radsak et al., 1990) as well as in the final envelope and is thus a potential participant in these interactions.

Given its structural localization in the virion, the tegument needs to mediate this mechanism, although it is not yet clear whether the nucleocapsids acquire the tegument only in the cytoplasm or, at least partially, in the nucleus. In other viral systems it is well established that tegument proteins play a crucial role in budding processes. The M protein of paramyxovirus is considered as the central organizer of assembly and budding (Peeples, 1991); the matrix protein of human immunodeficiency virus type 1 can induce budding even in the absence of other viral components (Wills & Crawen, 1991).

Little is known about the organization and function of the HCMV tegument, although a large number of proteins have been assigned to it (Sapa et al., 1994). There is evidence that the tegument proteins of the input virus play an important role in viral gene regulation and modification of host cell metabolism during the initial stages of infection, but no experimental data are available for a possible function in the maturation or transport of virions. For example, pp65 (ppUL83) is transported in the nucleus immediately after infection (Schmolke et al., 1995b). pp65 has been successfully applied for pp65 (Schmolke et al., 1995) and is thought to be a protein kinase (Somogyi et al., 1990). However, it has been shown to be non-essential for replication in cell culture (Schmolke et al., 1995b). Tegument protein pp71 (ppUL82) has been identified as a trans-activator of gene expression (Liu & Stinski, 1992) and is also transported to the nucleus (Hensel et al., 1996).

The basic phosphoprotein pp150 (ppUL32) is a very abundant component of the virion tegument. pp150 is the 1048 amino acid product of the ORF UL32 (Jahn et al., 1987). It is highly immunogenic, multiply phosphorylated and modified by O-linked N-acetylglucosamine at two residues (Greis et al., 1994). It has no apparent counterpart in either herpes simplex virus type 1 or 2, but shares a 25% identity to p100 of human herpesvirus 6 (Neipel et al., 1992). pp150 is estimated to represent 20% of the virion mass (Gibson, 1983; Baldick & Shenk, 1996), but is under-represented in infected cells (Weiner & Gibson, 1983) compared with its relative abundance in virions, supports the hypothesis that pp150 might contribute to assembly and transport processes of progeny particles. The aim of this work was to reveal the possible involvement of pp150 in virion morphogenesis by inhibiting its expression during infection.

Genetic analysis of HCMV genes with deletion mutants has been successfully applied for pp65 (Schmolke et al., 1995b). However, this approach can be used only for non-essential genes, because systems for the efficient propagation and rescue of mutants are not available. On the other hand, stable antisense mRNA-expressing cell lines have been shown to be a reliable tool for the specific inhibition of HCMV gene expression (Dal Monte et al., 1996; Ripalti et al., 1995). In this report, we describe the effect of expression of UL32 antisense mRNA under the control of the major immediate early promoter (MIEP) on the replication of HCMV AD169 in the permissive astrocytoma cell line U373MG.

**Methods**

- **Cells and virus.** Human astrocytoma cells (U373MG) and human primary foreskin fibroblasts (HFF) were grown as described earlier (Ripalti et al., 1995). HCMV strain AD169 was used for all experiments. Infections were carried out at an m.o.i. of 3. All infections were tested for comparable efficiency by immunofluorescence staining of IE-antigen 24 h post-infection (p.i.) in cells grown on glass coverslips in the same dish. Titres in virus stocks and cell culture supernatants were determined by end-point dilution as follows: logarithmic dilutions were used to infect HFF in four parallel series; infected cells were identified by immunostaining of IE-antigen 24 h p.i. and counted in the last positive culture.

- **Plasmids.** The UL32 antisense expression vector pRClUL32AS was constructed as follows. The 2.1 kb SstI fragment (bp 83 to +2020) of UL32 was excised from pCM1017 (Fleckenstein et al., 1982) and blunt-ligated in pbBluescript KS(−) (Stratagene) after removal of the 3' protruding termini. The 2.1 kb Apal fragment representing bp 83 to +1933 of UL32 was excised and ligated in antisense orientation in the Apal site of the expression vector pRC/CMV (Invitrogen).

- **Stable transfection of U373MG.** Linearized pRC/CMV and pRC/UL32AS were used in parallel to transfect U373MG by electroporation. DNA was cleaved by ScaI, precipitated and dissolved in sterile H2O. Cells were trypsinized, washed and resuspended in DMEM without supplements. Plasmid DNA (30 µg) was added to 2 × 106 cells in 800 µl DMEM in an electroporation cuvette (4 mm gap) and incubated on ice for 10 min. Transfection was performed with the Gene-Pulser electroporation apparatus (Bio-Rad) at 270 V and 960 µF. Cells were diluted in DMEM–10% FCS and seeded in 96-well plates. G418 (Gibco–BRL) was added 2 days after transfection at a concentration of 400 µg/ml and changed every 3 days for 4 weeks. Neomycin-resistant colonies were transferred to individual dishes and screened for UL32 antisense mRNA expression by Northern blot analysis.

- **Preparation of RNA and Northern blot analysis.** Total RNA was extracted from mock-infected and HCMV-infected cells at different times after infection using the RNaseasy Kit (Qiagen) according to the manufacturer’s instructions. Purified RNA (10–20 µg) was separated in a denaturing agarose gel, partially hydrolysed in 50 mM NaOH for 10 min, and blotted on uncharged nylon membranes (Qiabran, Qiagen) as described elsewhere (Sambrook et al., 1989).

  The UL32 Apal DNA fragment (bp 83 to +1933) was radiolabelled with [α-32P]dCTP by random priming using the Megaprime Labelling Kit (Amersham Buchler). The same DNA fragment cloned in the Bluescript KS(−) vector (Stratagene) was used to generate radiolabelled strand-specific RNA probes by T7 and T3 transcription with [α-32P]UTP. Hybridization was performed in QuickHyb hybridization buffer (Stratagene) according to the manufacturer’s instructions. A 500 bases DNA fragment of UL55 (bp 1451–2348) was used to detect UL55 mRNA.

- **DNA dot blot hybridization.** Total genomic DNA was extracted from mock-infected and HCMV-infected cells at 12 and 72 h p.i. with the QIAamp Blood Kit (Qiagen) according to the manufacturer’s instructions.
Residual RNA was digested with DNase-free RNAse (Boehringer Mannheim). Serial dilutions (ranging from 2–0·0032 µg) were transferred to uncharged nylon membranes. Hybridization was performed as above with the XhoI–HindIII DNA fragment of the HCMV AD169 genome HindIII fragment I, representing bp 15147–17243 (UL5–9) according to the GenBank HCMV genome sequence (Bankier et al., 1991). It was repeated with the UL32 Apal DNA fragment leading to the same result.

**Antibodies.** The polyclonal antiserum against pp150 (PAb XP1) was produced against a recombinant β-galactosidase–pp150 (aa 555–705) protein (Scholl et al., 1988). Precipitation of gB was carried out with the monoclonal antibody 27–156 (Spaete et al., 1988). The polyclonal antiserum BGE1 was raised against a glutathione S-transferase–pp65 (aa 325–511) protein (Hensel et al., 1995). BS510 (Plachter et al., 1992) is a monoclonal antibody to a ppUL44 (p52)–fusion protein. The monoclonal anti-CMV early nuclear protein antibody (Du Pont), which recognizes IE1 and IE2, was used for titration, infection control and Western blot analysis.

**Immunoprecipitation.** This was performed as described previously (Hensel et al., 1995). Briefly, uninfected and infected cells were radiolabelled 1 or 3 days p.i. for 3 h with [35S]methionine and [35S]cysteine (50 µCi/ml), followed by a chase of 1 h. For immunoprecipitation with different antibodies, three types of extract were prepared in parallel from one cell preparation using the following lysis buffers. For precipitation of gB, cells were lysed in 500 µl NP40 buffer (150 mM NaCl, 1%, v/v, NP40, 0·05 M Tris–HCl pH 8, 100 units/ml aprotinin, 1 mM PMSF) and for pp52 precipitation in 500 µl RIPA buffer (1%, v/v, NP40, 0·5% w/v sodium deoxycholate, 0·1% SDS, 150 mM NaCl, 0·05 M Tris–HCl pH 8, 100 units/ml aprotinin, 1 mM PMSF). For pp150, lysis was performed in 100 µl 2% SDS–0·05 M Tris–HCl at 95°C for 5 min followed by dilution to 2 ml with NP40 buffer–1% BSA. Aliquots of cell extracts of identical trichloroacetic acid-insoluble radioactivity were used. The immunocomplexes were washed five times in RIPA buffer. After SDS–PAGE, protein bands were quantified using a phosphor-imager (Molecular Dynamics) in three individual experiments.

**Electron microscopy.** U373MG (1 x 10⁶ cells) was grown in 60 mm plastic dishes and were infected with HCMV. At 3 days p.i., the monolayer was prepared for electron microscopy as described elsewhere (Schmolke et al., 1995 b). Briefly, cells were preincubated with horseradish peroxidase (HRP) at a concentration of 10 mg (Schmolke b), followed by dilution to 2 ml with NP40 buffer–1% BSA. Aliquots of cell extracts of identical trichloroacetic acid-insoluble radioactivity were used. The immunocomplexes were washed five times in RIPA buffer. After SDS–PAGE, protein bands were quantified using a phosphor-imager (Molecular Dynamics) in three individual experiments.

**Test for the presence of soluble antiviral factors.** HCMV- and mock-infected cell lines were cultivated for 2 days in MDME−2% FCS. Supernatants were collected, clarified by centrifugation at 3000 g for 10 min and irradiated at 254 nm for 30 min. U373MG and HFF were treated either with these supernatants or with fresh MDME−2% FCS for 24 h prior to HCMV infection. At 4 days p.i. the effect of pretreatment was examined by virus titration.

**Results**

**Establishment of astrocytoma cell lines stably expressing UL32 antisense mRNA**

The SstII–ApaI gene fragment of the ORF UL32 which codes for pp150 was isolated from the cosmid pCM1017 (Fleckenstein et al., 1982) and cloned in antisense (AS) orientation in the eukaryotic expression vector pRC/CMV (Fig. 1). This fragment (bp −83 to +1993 according to Jahn et al., 1987) represents 60% of the ORF and 30% of the target sense mRNA, including the start codon and most of the 5′ untranslated region. In the resulting construct, pRC/UL32AS, the fragment is expressed under the control of the CMV MIEP, which guarantees high expression upon HCMV infection. The construct, as well as the vector pRC/CMV, was transfected into U373MG, and neomycin-resistant cell clones were isolated by selection with the neomycin analogue G418. Antisense UL32 mRNA-expressing cell lines were identifed by Northern blot analysis with a UL32-specific DNA probe. This expression was stable for at least 20 passages.

**Viral UL32 mRNA expression is reduced in antisense mRNA-expressing cell lines**

Two representative antisense cell lines, U3/AA35 with a high level of expression and U3/AA32 with a relatively low level of expression of AS mRNA, were selected for further experiments and were compared to the vector control cell line, U3/RC1. In order to study the expression kinetics of the AS mRNA, cells were HCMV-infected at an m.o.i. of 3. In this and all subsequent experiments, the efficiency of infection was compared by measuring the degree of immunostaining of IE-antigen 24 h p.i. At different times p.i., the total RNA was isolated and submitted to Northern blot analysis with a UL32-specific DNA probe (Fig. 2).

The control cell line U3/RC1 showed the typical late gene expression pattern of the dominant 6·2 kb UL32 mRNA and weaker major and minor mRNA (Jahn et al., 1987) starting at 48 h p.i. In mock-infected AS cells the 2·1 kb AS mRNA was detectable in U3/AA35 at a low level, but in U3/AA32 was detected only after long exposure. Upon infection the AS mRNA steady-state level was strongly upregulated, increased in both cell lines up to 48 h p.i. and stayed at that level when the viral sense UL32 mRNA reached its maximum at 72 h p.i. The correct orientation of both mRNA species was verified with strand-specific RNA probes (data not shown). In both
Table 1. Reduction of viral mRNA levels and protein synthesis in UL32 antisense mRNA cell lines

Data were quantified by phosphor-imaging of Northern blots and of immunoprecipitations, and collected from the three individual experiments. See Figs 2 and 3 and text for details. The reduction is presented as a percentage of the value measured for the control cell line U3/RC1 in each experiment. The measurements of mRNA were normalized to G3PDH detected in each lane. Immunoprecipitations were normalized using lysates of identical protein-bound radioactivity.

<table>
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<tr>
<th>mRNA</th>
<th>U3/AA32</th>
<th>U3/AA35</th>
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<tr>
<td>UL32</td>
<td>73% (±14)</td>
<td>85% (±2)</td>
</tr>
<tr>
<td>UL55</td>
<td>17% (±5)</td>
<td>37% (±5)</td>
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<tr>
<td>pp150 (ppUL32)</td>
<td>79% (±12)</td>
<td>90% (±5)</td>
</tr>
<tr>
<td>gB (gpUL55)</td>
<td>67% (±14)</td>
<td>80% (±8)</td>
</tr>
<tr>
<td>pp65 (ppUL83)</td>
<td>0% (±15)</td>
<td>23% (±6)</td>
</tr>
<tr>
<td>pp52 (ppUL44)</td>
<td>1% (±7)</td>
<td>3% (±11)</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>72 h p.i.</td>
<td>24 h p.i.</td>
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<tr>
<td>UL32</td>
<td>72 h p.i.</td>
<td>24 h p.i.</td>
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<tr>
<td>UL55</td>
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<td>pp150 (ppUL32)</td>
<td>79%</td>
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<td>gB (gpUL55)</td>
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<td>pp65 (ppUL83)</td>
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In antisense cell lines, the UL32 sense mRNA was reduced compared to the control cells. In order to quantify this observation, the amount of viral UL32 sense mRNA at 72 h p.i. was measured by phosphor-imaging in three independent experiments. The results were normalized to the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA detected in each lane and summarized as a percentage of reduction compared to U3/RC1 (Table 1). The reduction in
UL32 AS mRNA blocks HCMV late-phase maturation

Fig. 3. Representative immunoprecipitation of pp150 and other viral antigens in the early and late phase. UL32 antisense cell lines (U3/AA32 and U3/AA35) and the vector transfectant (U3/RC1) were radiolabelled and lysed at 24 and 72 h p.i. Using the indicated antibodies, pp150 (ppUL32), the late protein gB (gpUL55) and the delayed early protein pp65 (ppUL83) were precipitated from the same sample at 72 h p.i. In each experiment the lysates were normalized to identical protein-bound radioactivity. The early protein pp52 (ppUL44) was precipitated 24 h p.i. (See Table 1 for quantification of protein bands.) Note that only the 130 kD gB precursor is shown; its cleavage was not impaired. Mock-infected U3/RC1 (Mo) was used to control the specificity of precipitations.

In order to study the selectivity of the effect of UL32 AS mRNA, the amount of the major 3–7 kb transcript of UL55 (the ORF of the late glycoprotein gB) was quantified under the same conditions after hybridization with a UL55-specific DNA probe. As shown in Fig. 2 and Table 1, the UL55 mRNA was also reduced in U3/AA32 and U3/AA35, but to a lesser extent (17 and 37%, respectively).

Analysis of pp150 biosynthesis and other viral proteins

The analysis of mRNA levels does not always reflect the full extent of AS action because sense mRNA inactivation can also be due to stable formation of RNA:RNA duplexes (Kim & Wold, 1985). Thus, the effect of AS mRNA on viral protein synthesis was visualized by immunoprecipitation and subsequent quantification. Different antigens were precipitated from the same preparation 72 h p.i. to guarantee identical conditions (Fig. 3). In each experiment, the lysates of different cell lines were normalized to identical protein-bound radioactivity. Quantification of protein bands was carried out by phosphor-imaging in three independent experiments (Table 1). As expected, the synthesis of pp150 (ppUL32) was significantly inhibited in AS cell lines. The degree of reduction compared to control cells was dependent on the level of AS mRNA expression, ranging from 79% in the low expressor U3/AA32, to 90% in the high expressor U3/AA35.

Precipitation of the late gene product glycoprotein gB (gpUL55) revealed that the inhibitory effect was not restricted to pp150, although gB was reduced to a lower extent (67 and 80%, respectively). The delayed early gene product pp65 (ppUL83) was less affected than gB (0 and 23%, respectively). To address the question of whether protein synthesis in the early phase was also influenced, the true early protein pp52 (ppUL44) was precipitated from cells extracted 24 h p.i., at a time when the AS mRNA is upregulated, and was not reduced. Furthermore, Western blot analysis showed that the immediate early IE1 expression was not diminished at 72 h p.i. (data not shown). Therefore, UL32 AS mRNA clearly reduced the synthesis of pp150. The inhibitory effect on other viral proteins was restricted to the late phase.

Replication of viral genomic DNA is not inhibited

There is no evidence that the late structural gene product pp150 is involved in viral DNA replication. To rule out a nonspecific effect of AS mRNA transcription on viral DNA synthesis, AS and control cells were infected and total DNA was extracted at 72 h p.i. The amount of replicated viral DNA in the cell lines was compared by dot-blot hybridization. Neither hybridization with a UL32 nor that with a UL5–9 specific probe (Fig. 4) revealed a significant difference. Thus,
UL32 antisense mRNA had no non-specific effect on mechanisms involved in viral DNA replication. Furthermore, neither pp150 nor any of the other potentially affected gene products appeared to have an influence on these processes.

Production of infectious virus is reduced in AS mRNA cell lines

The production of infectious virus was compared in order to address the question whether the UL32 AS mRNA has an effect on virion maturation or release. Cells were infected at an m.o.i. of 3. At 1 day p.i., comparable infection was verified by immunostaining of IE-antigen. The background viral titre in the supernatant at that time was found to be 10^1 p.f.u./ml (data not shown). In view of the fact that virion release in to the medium of U373MG cells is generally delayed and accumulation is relatively low, the supernatants of control and AS cell lines were titrated at 6 days p.i. These experiments show that the production of infectious virus was significantly reduced in AS cell lines (Fig. 5). The reduction was 2–3 orders of magnitude greater than for the control cell lines U373MG and U3/RCl1, indicating a severe block in maturation or release of progeny virions.

Maturation of cytoplasmic progeny virions is blocked in UL32 AS mRNA cell lines

Ultrastructural analysis was performed to reveal potential alterations in HCMV morphogenesis which could be the cause of a low production of infectious particles in AS cells. At 3 days p.i., infected control and AS cells (m.o.i. = 3) were prepared for electron microscopy.

Figs 6 and 7 demonstrate that obvious alterations of progeny morphogenesis in AS mRNA cell lines were observed, whereas most morphological aspects appeared to be unchanged. HCMV morphogenesis begins in the nucleus with the assembly of immature and mature capsids, referred to as A, B and DNA-containing capsids (Rixon, 1993). The A capsid appears as a round shell structure with an electron-lucent core; the B capsid contains an inner scaffold in addition to the outer shell, which is thought to be removed to package the viral genome, giving rise to DNA-containing capsids. The latter are characterized by the electron-dense DNA core. Fig. 6(a, c) demonstrates comparable amounts of B and A capsids in the nuclei of control and AS cells.

The late cytopathic effect in the cytoplasm is characterized by a rearrangement of the Golgi complex and accumulation of membrane cisternae and viral particles in the vicinity of the centrosome, referred to as the viroplasm (Soveri et al., 1988). At that stage, envelopment of progeny is seen to occur in association with the tubular endosome which was visualized by uptake of fluid phase marker HRP (Fig. 6), as described previously (Tooze et al., 1992). These characteristic alterations of the morphology of the infected cell were observed in all cell lines to the same extent (Fig. 6b, d). However, no or very few DNA-containing particles could be seen in the cytoplasm of AS cell lines, whereas in control cells a large number of mature cytoplasmic progeny were found (Figs 6b; 7a–d, arrows). In the AS cells, the overall number of particles and enveloping events was not reduced (Fig. 6d, arrowheads). However, NIEPs were predominant along with a subset of particles with irregularly shaped cores (Fig. 7e–h), very rarely seen in control cells. At the ultrastructural level the tegument of the NIEPs seemed not to be reduced. The immunofluorescence pattern of pp150 (Hensel et al., 1995) was unchanged except for the weaker signal (data not shown), which suggests that no general morphological alteration is caused by UL32 AS mRNA. In summary, the only morphological difference between control and antisense cells was the absence (or a significant reduction) of DNA-containing particles in the cytoplasm, together with the appearance of irregularly shaped particles.

Inhibition is not due to indigenous clonal heterogeneity or soluble antiviral factors

To exclude the possibility that the observed effects were due to the indigenous heterogeneity of clones isolated by the selection procedures, the inhibition of pp150 was compared by immunoprecipitation and subsequent quantification in four vector-control and four UL32 AS cell lines which were not identical with those described above. The mean inhibition of pp150 synthesis in AS cell lines was of 57% (SD 17%) compared to the mean control value (SD 24%).

As double-stranded RNA can induce production of interferon (IFN), it was necessary to exclude the possibility that inhibition was due to soluble antiviral factors secreted.
UL32 AS mRNA blocks HCMV late-phase maturation

Fig. 6. Ultrastructural analysis of infected antisense and control cells. HCMV-infected cells were incubated with HRP and prepared for electron microscopy 3 days p.i. HRP (black precipitate) labels the endosomal compartment with an electron-dense precipitate. The micrographs of the vector control U3/RC1 (a, b) reflect the observations from both U3/RC1 and the parental U373MG; those of the antisense cell line U3/AA35 (c, d) are representative for both U3/AA32 and U3/AA35. In the nuclei of infected control and antisense cells, no difference could be detected (a, c): many B capsids, several DNA-containing capsids (C) and few A capsids were embedded in the HCMV-induced skein, as is usually seen (Severi et al., 1992). The cytoplasm of all cells (b, d) shows the virus-induced characteristics to the same extent, including the accumulation of viral particles and HRP-labelled membrane cisternae around the centriole (ce). In contrast to control cells (b), no or very few electron-dense capsids or virions (arrows) were found in the cytoplasm of antisense cells (d), but NIEPs (arrowheads) were equally represented. Scale bars: (a, c) 200 nm; (b, d) 1 µm.
Fig. 7. Fine structural appearance of cytoplasmic progeny in control and antisense cells. Representative cytoplasmic particles from control cells (a–d) and antisense cells (e–h) 3 days p.i. Mature viral particles with dense DNA-containing core (a, b) and NIEPs (c, d) are frequently found in control cells. Together with NIEPs, deficient particles (e–h) are usually found in antisense cells. These particles are characterized by an irregularly shaped, often lucent core. Note the double membrane of the particles, indicating correct envelopment. Only particles enveloped during the HRP incubation are labelled by the black precipitate in the intermembrane space (e). Scale bar, 200 nm.

preferentially by AS cell lines. As IFN can act synergistically with tumour necrosis factor (Lucin et al., 1994) and potentially with other factors, the effect of AS cell culture supernatant on HCMV replication was tested directly, in preference to measurement of individual cytokines. The supernatants of HCMV-infected and mock-infected AS and control cell lines, harvested at 48 h p.i., were used to pre-incubate U373MG and HFF cells for 24 h prior to HCMV infection at an m.o.i. of 3. In none of the samples did the pre-incubation cause a reduction of viral titres in U373MG or HFF as compared to control supernatants, thus indicating that the inhibition in UL32 AS cell lines was not due to soluble antiviral factors.

Discussion

Direct genetic analysis of essential HCMV genes is hampered by difficulties in obtaining virus mutants and by the lack of complementing cell systems. Mutational analysis has been applied successfully, but so far only to non-essential genes (Schmolke et al., 1995 b; Jones & Muzithras, 1992). In contrast, the AS mRNA approach has been shown to serve efficiently as an alternative methodology for the study of HCMV gene function (Bryant & Sinclair, 1993; Ripalti et al., 1995; Dal Monte et al., 1996). In these studies, the expression of immediate early and early genes has been inhibited, resulting in a block of replication in the immediate early or early phase, and revealing the essential role of these gene products in early events. The present study addresses the question whether the AS approach is appropriate to investigation of the function of the true late gene product pp150 (ppUL32). If the gene is essential, its inhibition should not impair virus replication until the late phase, thus causing an accumulation of maturational intermediates.

For this purpose we generated astrocytoma cell lines (U373MG) stably expressing the 5′ portion of the pp150 ORF UL32 in antisense orientation. A low and a high UL32 AS mRNA expressor cell line were selected for further investigation. Both cell lines exhibited reduced levels of viral UL32 sense mRNA compared to the control. On the basis of several observations, we conclude that the observed effect was caused directly by UL32 AS mRNA. Firstly, the extent of inhibition reflects the different levels of AS mRNA in the two cell lines.
Secondly, the inhibition of ppUL32 protein biosynthesis correlates with the reduction of UL32 mRNA levels, whereas the inhibition of gB takes place mainly at the level of translation, as the reduction of UL55 transcription is lower than that of its protein synthesis. Thirdly, a possible nonspecific effect due to the variability between different cell clones or due to soluble antiviral factors could be ruled out in control experiments.

As a consequence of UL32 sense mRNA neutralization, the synthesis of pp150 was significantly reduced as determined by immunoprecipitation, and the extent of reduction corresponded to the AS mRNA level in the two cell lines. The expression of the late gene gB was also inhibited, whereas no influence could be detected on immediate early and early gene expression. The effect of UL32 AS mRNA appears therefore to be restricted to the late phase. This notion is supported by the finding that viral DNA replication was not impaired. The restriction of the AS mRNA inhibition to the late phase is a novel observation and presents the possibility of studying the effect of late gene inhibition on advanced maturational events. This restriction reflects the specificity of the approach, as the UL32 AS mRNA level is already upregulated at 24 h p.i., but has no apparent influence on early gene expression at that time. It affects gene expression exclusively in the late phase when the target sense mRNA is transcribed. Furthermore, the expression of gB (gpUL55), which has no sequence homology to pp150, is mainly inhibited at the level of translation. We therefore favour a mechanism in which the AS mRNA acts directly via RNA:RNA interaction only with the UL32 sense mRNA leading to degradation of both RNA species (Nellen & Lichtenstein, 1993), and that gB is presumably reduced indirectly as a consequence of the inhibition of pp150. This would suggest a feedback mechanism in late gene expression. The concomitant (although lower) reduction of a late gene such as gB makes it difficult to ascribe the observed effects on HCMV maturation to pp150 alone.

The reduced virus titre, together with the fact that viral biosynthesis was not impaired until the late phase, indicated a block of viral morphogenesis. Electron microscopy studies were therefore performed to search for possible maturational intermediates. Morphological analysis and quantification must be considered carefully, especially when virus production is relatively low, as in astrocyoma cells. Nevertheless, obvious and reproducible similarities and differences were observed between AS cell lines and control cells. In the nucleus of all HCMV-infected cells, B, A and also DNA-containing capsids were observed in comparable numbers, embedded in the amorphous skein. These are considered as the final maturational stage in the nucleus (Rixon, 1993). This indicates that the assembly of capsids and packaging of the genomic DNA is not impaired by AS mRNA, an observation which corresponds to the finding that DNA replication is not reduced.

The cytoplasm also showed the typical reorganization in all cell lines, including the accumulation of viral particles and endosomal cisternae labelled by HRP, appearing as viroplasm around the centrosome. But in contrast to control cells, DNA-containing virions were almost completely absent in AS cells. Instead, NIEPs appeared to have increased in number, and a subset showed an irregular core structure.

The morphological data clearly differ from those observed in the study of Dal Monte et al. (1996). The application of UL38 AS mRNA, presumably by interference with UL82 expression, caused a severe block of maturation in the nucleus, resulting in the absence of C capsids and no profound alteration in the cell morphology. The different effects of the two studies underline the phase specificity of the AS mRNA approach.

The discrepancy in UL32 AS mRNA cells between the normal maturation in the nucleus and the low number of DNA-containing particles in the cytoplasm may be explained either by defective transport of DNA-containing capsids through the nuclear envelope, or by reduced stability of C capsids in the cytoplasm.

The first assumption implies a role for pp150 in transport processes. It has been proposed that tegumentation of capsids could take place in the nucleus to mediate budding through the nuclear envelope (Roizman & Sears, 1996). The recent localization of pp150 in the nucleus is consistent with this proposal (Hensel et al., 1995). This would explain the absence of C capsids in the cytoplasm, but not the unimpaired transport of NIEPs, as the tegument composition of NIEPs and virions has been shown to be similar (Irmiere & Gibson, 1983), suggesting that they are transported by the same mechanism.

The second explanation is based on pp150 acting to stabilize the nucleocapsid. B capsids are stabilized by a second proteinaceous layer outside the outer capsid shell. This structure is degraded to form the A capsids and the latter are transformed to electron-dense capsids by the packaging of the genomic DNA. It is known that pp150 is very tightly associated with capsids (Benko & Gibson, 1989). This might help to stabilize the capsids and prevent disintegration in the cytoplasm. Consequently, only NIEPs which keep the inner ring remain stable, and the irregular particles described above might be the result of capsid instability. Disintegration might also occur in the nucleus, which would prevent accumulation of C capsids; there is no accumulation in AS cells. Further investigations are necessary to determine which of these interpretations is correct.

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References


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