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CHARACTERIZATION OF THE ORF TRL12 OF HUMAN CYTOMEGALOVIRUS

Presentata da: Dott.ssa **Giada Rossini**

Relatore	**Coordinatore**
Chiar.ma Prof.ssa	Chiar.mo Prof.
Lucia Manzoli	**Lucio Cocco**

Settore scientifico-disciplinare: BIO/11 Biologia Molecolare

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INTRODUCTION

Human Cytomegalovirus

1. CHARACTERISTIC FEATURES OF THE HERPESVIRUSES

Human cytomegalovirus (HCMV) is a highly species specific herpes virus that infects and is carried by the majority of the human population. Herpesviruses are highly prevalent, affecting an estimated 70-100% of the world's population. To this date, the human herpesvirus family consists of eight different members: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV) and human herpesviruses 6A, 6B, 7 and 8 (HHV6A, HHV6B, HHV7 and HHV8). The criteria for including a virus in the herpesvirus family is based on the architecture of the virion. A herpesvirus particle is composed of a core containing a linear double-stranded DNA molecule, which is enclosed within an icosadeltahedral capsid of 100 nm, containing 162 capsomers. The capsid is surrounded by a tegument layer which consists of amorphous protein material. In the mature virion, these structures are enclosed in a lipid bilayer envelope, which contains a number of viral glycoproteins on its surface. The mature virions range in size from 150-200 nm.

All herpesviruses share four significant common properties: 1) they encode a large array of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins such as thymidine kinase, DNA polymerase, helicase, primase and protein kinases; 2) the synthesis of viral DNA and the capsid assembly occurs in the nucleus of infected cells. Capsids are enveloped as the transit through the nuclear membrane and mature within the cytoplasm and intracellular compartments; 3) production of infectious progeny virus is mainly accompanied by the destruction of the infected cells; 4) after primary infection, all herpesviruses persist in a latent state in their host and may be reactivated later in life. In cells harbouring latent virus, the viral genome appears in the form of a closed circular structure, and few if any viral proteins are produced.

Herpesvirus latency is established in different cell types; HSV-1, HSV-2 and VZV remain in a latent state in neurons of dorsal root ganglia, whereas latent EBV has been found primarily in memory B cells. HCMV establishes latency mainly in the myeloid cell lineage. For a herpesvirus to persist in its host, some general conditions must be fulfilled:

the virus must be able to infect cells without producing infectious progeny and therefore the virus infection cannot be cytopathic, host mechanisms must permit long-term maintenance of the viral genome, and the virus must be able to avoid elimination by the immune system. A delicate balance must therefore exist between the virus and its host to avoid virus elimination by the immune system or host destruction by the infection. Persistence in an immunocompetent host has put an enormous evolutionary pressure on these viruses to develop strategies to avoid immune recognition.

2. VIRION STRUCTURE

The virion of HCMV consists of a 100-nm diameter icosahedral nucleocapsid containing a 230-kbp, double-stranded linear DNA genome surrounded by proteinaceous layer defined as the tegument or matrix, which, in turn, is enclosed by a lipid bilayer containing a large number of viral glycoproteins (**Fig. 1**). The mature virion particle is 150-200 nm in diameter. The viral genome in the characterized cytomegaloviruses is a linear DNA ranging in size from 200-240 kbp which is significantly larger than other herpesviruses and the human CMV genome exhibits a pattern of terminal and inverted repeats that vary in size in various strains.

HCMV-infected cell cultures produce the infectious virions and another two types of morphological particles: non-infectious enveloped particles (NIEP) and dense bodies (DB).

NIEPs are defective viral particles composed of enveloped immature capsides (type B) that lack DNA, but contain the viral scaffolding/assembly protein (AP) normally absent from fully mature nucleocapsids (C-capsids). DBs are enveloped particles that lacks an assembled n ucleocapsid and viral DNA, but contain several tegument proteins of which ppUL83 (pp65 or lower matrix protein) is the most abundant. The relative amounts of the three forms depends on the number of passages in cell culture and the viral strain. .

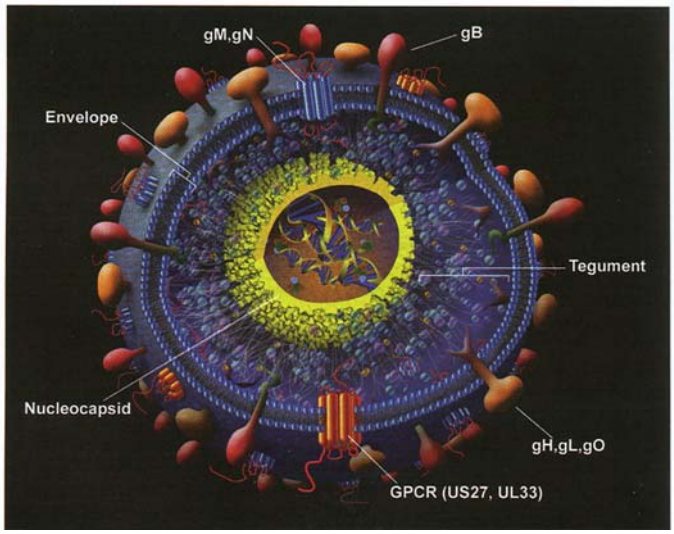


FIGURE 1. HCMV structure. HCMV virions comprise three major layers. The first layer is the nucleocapsid containing the double stranded viral DNA genome, which is surrounded bt a proteinaceous tegument layer. The tegumented capsids are enveloped by a host-derived lipid bi-layer that is studded with viral glycoproteins

2.1 Structural proteins of the virion

The proteome of the HCMV is extraordinarily complex and encodes a spectrum of proteins that include regulatory proteins , virion structural proteins, proteins that facilitate evasion of the host innate and adaptive immune responses, and proteins that modulate host cell transcription.

The HCMV virion has been structurally divided into three regions: the capsid , tegument, and the envelope.

Capsid protein. The capsid is the innermost structure of the virion; is a icosahedral structure consisting of 12 pentons, 150 hexons, and 320 triplexes[**1, 2**]. The protein components of the capsid include at least five proteins: UL86 (the major capsid protein, MCP), UL48-49 (the smallest capsid protein, SCP), UL85 (minor capsid protein, mCP), UL46 (minor capsid binding protein), and fragment of UL80 (assembly protein) [**1, 3, 4, 5, 2, 6**]. Through protein interactions and the scaffolding activity of the uncleaved UL80, these proteins self- assemble into an icosahedral structure that incorporates unit length viral DNA [**1, 7, 2, 6**]. The major capsid protein, pUL86, is the most abundant protein component of the capsid (960 copies) and forms the penton and hexons of the icosahedral capsid [**1, 2**]. The minor capsid proteins, pUL85 (two copies) and the pUL46 (one copy), form the triplexes that are located between the pentons and hexons [**1, 7, 2**].

Tegument proteins. The virion tegument has been described as an amorphous coating of the capsid that maintains the association between the virion envelope and the capsid. The tegument appears to be formed by the sequential addition of proteins, first in the nucleus and then in the cytoplasm. The tegument proteins may be involved in the maturation of progeny virions, or may influence viral and cellular events in the early stages of infection, such as release of viral DNA from disassembling virus particles or the regulation of viral and cellular promoters. Most tegument proteins are phosphorylated and are highly immunogenic. Some tegument proteins are expressed in the cytoplasm (ppUL32, pp150 or ppUL99, pp28) or in the nucleus (ppUL69) throughout the replicative cycle of HCMV [**8**]. Others such as the tegument proteins ppUL53 and ppUL83 (pp65) are expressed in the nucleus of cells early after infection but late in infection are localized predominantly in the cytoplasm [**8**]. Thus it is unclear whether nuclear tegument proteins associate with the capsid in the nucleus or at a later step in the cytoplasmic assembly compartment [**1, 2**]. Electron microscopic studies of virion morphogenesis suggested that at least some of the tegument layer is acquired in the nucleus.

The role of individual proteins in the maintenance of the structure of the virion remains to be determined. For example, deletion of one of the most abundant tegument proteins, ppUL83 (pp65), resulted in a growth modified viral phenotype that replicated in tissue culture [**9**]. Thus it is likely that many of the tegument proteins have functions unrelated to a structural role of the virion tegument.

Tegument proteins that have been shown to regulate viral gene expression or modify host cell response to HCMV infection include ppUL82 (pp71), ppUL84, ppUL47, ppUL69, ppUL26 and possibly ppUL83 (pp65) [**10, 11, 5, 12, 13, 14, 15**]. As examples, pp71 has been shown to inhibit the expression of genes associated with induction of interferon responses.

In addition, at least two tegument proteins are thought to be essential for the nuclear egress of the tegumented capsids presumably through modification of the nuclear cytoskeleton and nuclear membranes [**16**].

The tegument protein, ppUL99 (pp28) appears to be essential for virus replication, perhaps by providing an essential function for virion envelopment [**17, 18**].

2.2 Envelope glycoproteins.

The virion envelopment is exceedingly complex and to date the composition of this virion structure is incompletely defined. Although homologs of others herpesviruses including gB, gH, gL, gM and gN have been identified, functional or structural homologs of α-herpesvirus gC, gD, gE, gI or gK have not been defined in HCMV. Several of the more abundant HCMV glycoproteins have been shown to exist as disulfide-linked complexes within the virion, gCI (gB), gCII (gM/gN), and gCIII (gH, gL, gO) [**19, 20, 21, 22, 23**]. The relative abundance of the various glycoproteins is incompletely defined but recent studies using mass spectroscopy has suggested that glycoproteins in envelope of HCMV are present in the following order of abundance: gM/gN>gB>gH/gL/gO [**24**]. To date, gB, gM/gN, and gH have been shown to be essential for the production of infectious virus [**25**].

Glycoprotein B. Several structural and antigenic features if HCMV glycoprotein B (gB) are conserved in other CMVs [**26, 27, 28**]. The mature gB is a type I membrane glycoprotein consisting of 110-116 kDa ectodomain or surface component linked by disulfide bonds to a 55 kDa transmembrane component. Together these are linked through interchain disulfide bonds to form the mature homodimer [**29, 30**]. The gB is extensively glycosylated and contains approximately 50-60 kDa of N-linked sugars and at least one O-linked carbohydrate modification [**31**]. Others posttranslational modifications of gB include the phosphorylation of serine at amino acid position 900, a modification that has been shown to be responsible for the interaction of this glycoprotein with components of the endosomal recycling system, including PACS-1 (phosphofurin acidic cluster sorting protein-1) [**32, 33, 34, 35, 36**]. gB is expressed on the membrane of infected cells and is incorporated in the virions during the envelopment process in different subcellular compartments.

gB is highly immunogenic and is the major target of neutralizing immune response. gB specific neutralizing antibodies are able to inhibit the attachment of virions to the cellular surface.

Glycoprotein H, L, O. The gCIII complex of glycoproteins was originally thought to consist of glycoprotein H (gH) and glycoprotein L (gL) based on studies of α-herpesviruses. Studies from at least two laboratories definied a third component of the complex, a viral glycoprotein that was subsequently shown to be encoded by the ORF UL74 (gO) [**37, 38, 39**]. Homologs of gO have been described in Epstein-Barr virus and

HHV-6, although in this later case this glycoprotein has been termed gQ. The HCMV gO is highly glycosylated membrane glycoprotein with a cleavable signal sequence [**40, 41**]. Recent studies have indicated that deletion of the gO gene does not prevent the assembly and release of infectious virus, although the mutant virus appears growth impaired [**25**]. One of the most striking results from studies of gO is the variation of the nucleotide sequence in the 5' end of the UL74 gene [**42, 43**]. It is of interest that the gO gene is adjacent to UL73 ORF which encodes gN, a viral glycoprotein that also exhibits significant amino acid and nucleotide variation between viral strains.

Similar to the gH of others herpesviruses, HCMV gH requires coexpression of gL for intracellular transport and terminal carbohydrate modifications [**44**]. HCMV gL remains localized in the endoplasmic reticulum (ER) when expressed in the absence of gH. Because gO is not required for the production of infectious virus *in vitro*, it also unclear whether it is required for transport of the gH/gL complex to the assembly compartment or if authentic glycosylation of gH and gL require the expression of gO. The gH of HCMV is the target of virus-neutralizing antibodies and these antibodies appear to function by blocking a post attachment event such as membrane fusion or virus penetration [**45, 46**]. Although anti-gH monoclonal antibodies appear broadly reactive, HCMV can modulate gH expression to evade virus neutralization, a phenomena similar to that reported for pseudorabies virus [**39, 47**]. The incorporation of gH into the envelope of infectious virus has been shown to be variable and, under antibody selection, infectious virion containing limiting amounts of gH could be selected [**39**]. This finding suggested that the stoicheiometry of the envelope glycoproteins that are required for production of infectious particles is variable and assembly of the virion envelope is considerably less structurally constrained as compared with the more highly ordered capsid.

Glycoprotein M, N. The predicted structure of HCMV glycoprotein M (gM) includes seven membrane spanning domains, structure that appears relatively conserved among gMs of α-, β-, γ-herpesviruses [**48**]. The HCMV gM contains a single N-linked carbohydrate modification [**23**]. Although the structure of gM remains undefined, an obvious structural requirement for gM is complex formation with the product of the UL73 orf, gN [**23**]. This complex forms in the ER and is dependent on both covalent disulfide bonds and noncovalent interactions [**23**]. Native folding and intracellular transport of both gM and gN require complex formation [**23**]. Infectious virus cannot be recovered from viral genomes with deletion in either the UL100 or UL73 gene [**25**]. This in contrast to findings in the α-herpesvirus and pseudorabies virus in which deletion of gM was not

associated with loss of infectivity unless additional genes encoding glycoprotein E and glycoprotein I were also deleted [**49**]. This suggests that requirements for glycoprotein function may vary considerably between different herpesviruses.

The HCMV gN is unique in its structure and its sequence variability [**50, 23, 51**]. The UL73 orf encodes a 129 amino acid polypeptide with a signal sequence and a single C-terminal hydrophobic domain typical of a type I glycoprotein. The mature gN (complexed with gM) has an estimated molecular mass of nearly 60 kDa and glycosidase treatment indicates that approximately 40 kDa of the mass of the mature gN is carbohydrate [**23**]. These modifications are almost exclusively O-linked sugars, a finding consistent with the predicted serine/threonine content of the primary sequence. The role this posttranslational modification of gN plays in the biology of the HCMV is unknown, but it is of interest that the total number of sites for O-linked carbohydrate modification is relatively conserved regardless of the amino acid sequence variation in the ectodomain of gN. The second unique characteristic of gN is the variability in the predicted amino acid sequence of the gNs from different clinical viral isolates [**50, 51**]. In some cases, gNs from clinical isolates have up to 20% amino acid variability in the approximate 100 amino acid ectodomain, whereas the primary sequence of the short cytoplasmic tail of the gN molecule is conserved. Pignatelli and coworkers have analyzed the sequence from more than 200 HCMV isolates and grouped gNs into four major genotypes, with two genotypes further segregating into additional subgroups [**52**]. Sequence analysis suggested that variations in the ectodomain of gN resulted from positive selective pressure such as would be observed with a virus neutralizing antibody response [**52**]. The finding that gN in virions is possibly under positive immune selective pressure could argue that extensive carbohydrate modifications serve to shield the gN from antibody recognition similar to that which has been observed for HIV gp120 [**53**].

HCMV-specific glycoproteins. In addition to these well-described glycoproteins, the envelope of HCMV has also been shown to contain several glycoproteins that do not have obvious homologous proteins in other herpesviruses. These include gpTRL10, gpTRL11 and gpUL132 [**54, 55, 56**]. Deletion of gpTRL10 and gpUL132 have no effect on assembly of infectious virus, although the phenotype of these viruses remains incompletely characterized [**57, 56**].

the product of the TRL11 orf has been shown to have Fc binding activity and may responsible for the Fcγ receptor activity observed in infected cells [**54**].

Glycoproteins encoded by genes within the US region (US2-11) have been shown to have dramatic effects on the expression of major histocompatibilily complex class I and II antigens and thus have been most well studied as immune evasion gene products expressed in infected cells [**58**]. Convincing evidence of their presence in the envelope of infectious virus has not been presented. Recent studies have suggested that the chemokine receptor homologs encoded by US28, US27, and UL33 may be incorporated into the particle; however, it is unknown whether they are in the envelope [**59, 60**].

Additional glycoproteins such as that encoded by the UL4 orf (gp48) has been suggested to be components of the infectious virus, but they localization to the envelope has not been confirmed [**61**].

It is almost assured that additional HCMV specific glycoproteins will be found in the envelope of the infectious virion. Even if these proteins are shown to be nonessential for *in vitro* replication of HCMV, it is presumed that they have a role in the *in vivo* replication and spread of HCMV.

3. VIRUS GENOME

The HCMV genome is the largest of all herpesviruses and has a high G+C content. Like that of herpes simplex virus-1, it contains an arrangement of unique long (UL), unique short (US), and repeat regions (**Fig. 2**). Since each long and short region can be oriented in either direction, four genomes isomers are produced in viral progeny (Class E structure). In contrast, the genome of animal CMV, as well as those of other betaherpesviruses, are linear without repeat regions (Class F genomes). Inversion of UL and US regions is mediated by direct repeat sequences (*a*, *b*, *c*) at the genome termini and by inverted repeat elements at the UL-US junction (*a'*, *b'*, *c'*). the repeated *a* sequence that occurs as a direct element at the termini and in the inverted orientation at the UL/US junction promotes genome isomerisation, since it contains the *cis*-acting *pac* (packaging) elements needed for DNA cleavage.

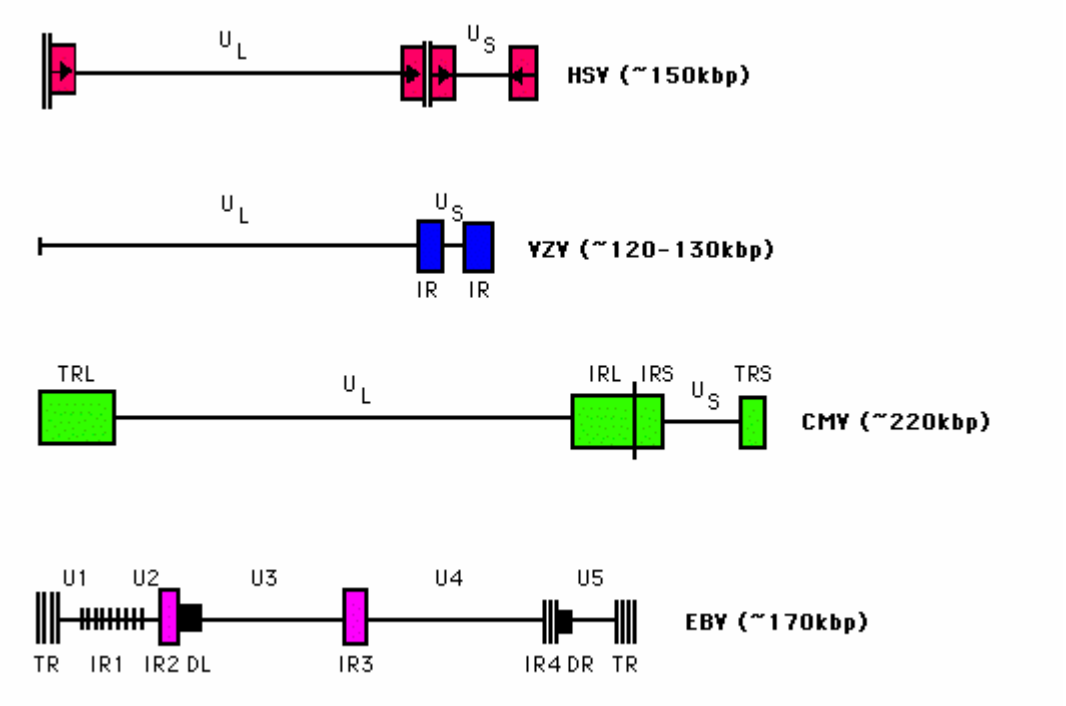


FIGURE 2. Schematic rapresentation of herpesviruses genomes.
HSV: herpes simplex virus; VZV: varicella zoster virus; CMV: cytomegalovirus; EBV: Epstein Barr virus

The AD169 laboratory strain is the only completely sequenced HCMV. Analysis of its 230-kbp genome has revealed that it encodes 255 ORF of ~ 100 or more amino acids [**62, 63**]. These ORFs are designated sequentially according to their location within the unique and repeated regions. Additional ORFs have been identified in the Towne and Toledo laboratory strains. In latter, as well as in clinical isolates, the inverted *b'* repeat is deleted and replaced by additional UL region of ~ 15 Kbp, containing 19 additional ORFs that are absent in the Ad169 genome [**62**]. The unique ORF UL1-154 and US1-36 blocks are separated by duplicated IRL1-14 and J1I genes and the partially repeated IRS1 gene. The UL region is flanked at the 5' end by the duplicated TRL1-14 and J1L (identical to IRL1-14 and J1I), whereas the US gene block is flanked at its 3' end by the TRS1 gene and by the third copy of a J1 gene (J1S).

Sequence homology searches and experimental biochemical and/or genetic studies have assigned functional roles to only some of the more then 200 HCMV ORFs [**63**]. However, analysis of the phenotypes of spontaneous deletion mutants of the AD169 strain, as well as those of virus-bearing deletions or inactivation at specific loci, has indicated the products of more the 50 HCMV ORFs are dispensable for productive replication in

fibroblast cultures. These findings, along with observations that the proteins responsible for functions common to all herpesviruses, such as basic DNA replication, virion organization, and maturation, do not account for all the ORFs, indicate likely, therefore, that much of the coding capability has evolved to optimize infection by influencing dissemination, growth in target tissues and pathogenesis, and in counteracting host immune reactivity.

4. VIRUS GROWTH CYCLE AND VIRAL GENE EXPRESSION

4.1 Initial steps of HCMV infection: virus binding and penetration

HCMV causes systemic infection and exhibits a tropism for fibroblasts, endothelial cells, epithelial cells, monocyte/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils and hepatocytes [**64, 65, 66**]. This exceptionally broad cellular tropism is the root of HCMV disease manifestation of most organ systems and tissue types in the immunocompromised host. Although HCMV is considered to have a very restricted cell tropism in vitro, entry into target cells is very promiscuous, as HCMV is able to bind, fuse and initiate replication in all tested vertebrate cell types. Productive in vitro replication is only supported by primary fibroblasts, endothelial cells and differentiated myeloid cells as well as certain astrocyte cell lines [**64, 67**].

Virus attachment and penetration are rapid and efficient in both permissive and non permissive cell types. However, since productive replication is observed in a very restricted range of human cells, a post-penetration block to viral gene expression is thought to restrict replication in non permissive cells [**66**].

The ability of HCMV to enter such a wide range of cells is highly indicative of multiple cell specific receptors, broadly expressed receptors or a complex entry pathway in which a combination of both cell specific and broadly expressed cellular receptors are utilized.

Viral entry is the result of a cascade of interactions between viral and cellular proteins that culminate in fusion of the virion envelope with the cellular plasma membrane by a PH-inde pendent mechanism.

The initial events in the replication cycle are only partially understood.

It has been known for some time that HCMV initiates infection via a tethering interaction of virions and cell-surface heparan sulphate proteoglycans (HSPGs) [**68**]. At least in cell

culture system, HCMV engagement of HSPGs is thought to play a crucial role in recruiting virions to the cell surface and enhancing the engagement of others receptors. HCMV virions were initially shown to bind β2 microglobulin (β2m) [**69**]. This observation led to numerous binding studies concluding that HCMV tegument binds β2m as it is released from cells. This β2m-HCMV complex was then thought to associate with alpha chain of HLA class I antigens [**70, 71, 60**] However, it was later determined that β2m expression had no correlation with in vitro entry or in vivo spread of infectivity [**71, 72, 73**]. Virus-cell overlay blots were used to globally analyze cellular proteins that could bind to HCMV virions. These studies identified a cell-surface protein of approximately 30 kDa, whose expression correlated with cells permissive for entry [**67, 74**]. This protein was later identified as annexin II [**75**]. Annexin II was also shown to bind HCMV virions [**75, 76**]. Upon further study it was found that gB was able to directly interact with annexin II and that this protein was able to enhance HCMV binding and fusion to phospholipid membranes [**77, 78**]. However, cells devoid of annexin II are fully permissive for entry and initiation of infection [**79**]. What role, if any, annexin II plays in the life cycle of HCMV is unknown but, given its membrane-bridging activity, it remains formally possible that this enhances entry, cell-cell spread and/or maturation and egress.

CD13, or human aminopeptidase N, has also been implicated as a HCMV receptor. This hypothesis was based on that fact that only human peripheral blood mononuclear cells (PBMCs) that were CD13 positive supported productive infection [**80, 34**].

A consideration of HCMV-induced signalling cascades led Wang et al.(2003) to hypothesize a role for epidermal growth factor receptor (EGFR) as an HCMV receptor. A limitation of the study, however, was that there was no experimental evidence that EGFR functioned in a entry event per se. Nor was it shown if EGFR was required for the delivery of virion components across the plasma membrane. Lastly, is important to note that EGFR is not expressed on all HCMV permissive cells, including those of haematopoietic lineage.

Cellular integrins may serve as co-receptors for cytomegalovirus. The first and foremost observation about HCMV biology was its namesake characteristic, cytomegaly, or cell enlargement. In vitro studies initially demonstrated a unique cytopathogenic effect (CPE) of infected cells, with HCMV-infected cells appearing round and enlarged with intracellular viral inclusion bodies [**81**]. The cause of this phenomenon was widely speculated upon; theories for HCMV-induced cell rounding included cation influx,

suppression of fibronectin synthesis and integrin down-regulation [**82, 81, 83, 84, 85**] have tested the hypothesis that integrins were involved in the HCMV entry pathway. (**Fig. 3**)

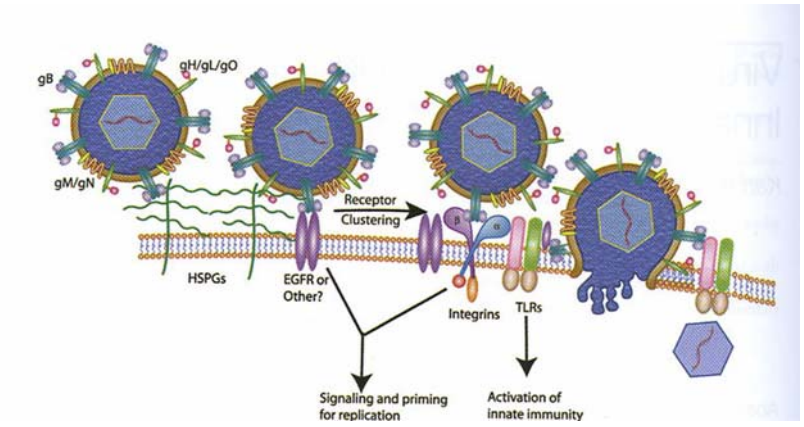


FIGURE 3. Working model for HCMV entry into cells.

Attachment to the cell surface is followed by penetration, mediated by fusion of the virion envelope and the cell surface in a pH-independent manner.

However, final fusion of the viral envelope with the cell membrane to allow viral penetration is thought to require a further priming event mediated by the heteroligomeric gH-gL-gO complex with as yet unidentified receptors. Fusion of the virus and cell membranes is followed by entry into the host cytoplasm of the nucleocapsid and tegument proteins, and their rapid translocation into the nucleus, where pp65 is detected < 1 hr p.i. and expression of IE1/IE2 products follows shortly thereafter. Several studies have shown Interaction of HCMV glycoproteins with their receptors is enough to generate an intracellular signal transduction pathway, leading to the alteration of cellular gene expression. Viral attachment may therefore stimulate specific cellular processes that facilitate viral replication.

4.2 Regulation of viral gene expression

During productive infection, the HCMV genome is expressed in a temporally coordinated and regulated cascade of transcriptional events that lead to the synthesis of three categories of viral proteins described as immediate-early (IE or α), early (E or β), and late (L or γ). Failure in the expression of early gene and subsequent viral DNA replication rather than attachment and/or penetration may be the
restricting event in non permissive cells. HCMV genes are transcribed in the infected cell nucleus by RNA polymerase II and the associated basal transcription machinery, with the intervention of host-encoded transcription factors whose activity may be stimulated by viral transactivators.

4.2.1 Characteristics and functions of the immediate-early proteins

HCMV gene expression initiates from a few IE proteins within l hr p.i. without de novo protein synthesis. The IE genes include the major IE (MIE) UL122/123 genes (IE1 and IE2) and auxiliary genes, such as UL36–UL38, UL115–UL119, IRS1/TRS1, and US3. The MIE proteins, alone or in synergism, are required for subsequent expression by acting as transactivators and autostimulators of viral genes. In addition, these proteins have a deep impact on host cell physiology since they regulate the expression of
a large number of host cell genes. MIE proteins are encoded by the ie1/ie2 genes (UL122/UL123), whose expression is regulated by a complex enhancer-modulator element that functions in a tissue- and cell-type-specific manner, and exerts its strong transcriptional activity through interactions with several host transcription factors whose binding sites are closely distributed within the regulatory element.

4.2.2 Characteristics and functions of the early proteins

Expression of E or β genes depends on the presence of functional IE proteins and is unaffected by inhibitors of viral DNA replication. They are divided into two subclasses: β1 (E) and β2 (E-L) according to their time of expression. β1 genes are transcribed within 4–8 hr p.i., β2 transcription 8–24 hr p.i.. The functional data indicate that E genes encode mostly non-structural proteins, including viral DNA replication factors, repair enzymes, and proteins involved in immune evasion. The expression profiles of microarrays of viral

DNA recently have provided a temporal map of IE, E, and L genes in the entire viral genome. Hybridization of such microarrays to cDNAs prepared from
HCMV-infected cells treated with ganciclovir (GCV) to block viral DNA replication has revealed that 36% of the more than 150 ORFs scored positive for expression were unaffected by GCV and, therefore, classified as E. These E genes are dispersed throughout the HCMV genome.

4.2.3 Characteristics and functions of the late proteins

The L proteins are the last class of gene products expressed during HCMV replication. Their transcription begins more than 24 hr p.i. and requires prior viral DNA replication. Late or γ gene expression leads to the synthesis of two subclasses of L proteins (γ1 and γ2) in accordance with their time of expression and sensitivity to viral DNA replication inhibitors. γ1 (leaky late) transcription occurs 24–36 hr p.i., and is reduced by such inhibitors. γ2 (true late) transcription occurs 24–48 hr p.i., and is strictly dependent on DNA replication. The L proteins have mainly structural roles and primarily contribute to the assembly and morphogenesis of the virion.

4.3 Virion Morphogenesis

Formation of HCMV capsids and packaging of viral DNA occur in the nucleus.
Assembled cytomegalovirus nucleocapsids are exported from the nuclear compartment by sequential events including (i) *primary envelopment* at the inner nuclear membrane and (ii) de-envelopment at the outer nuclear membrane prior to (iii) *secondary envelopment* of naked cytoplasmic nucleocapsids at cytoplasmic cisternae, and (iv) release of mature enveloped particles by exocytosis. [**86**] and later Severi et al. (1979) deduced from electron microscopic studies that acquisition of an envelope derived from the inner nuclear membrane (INM) (*primary envelopment*) was only temporary.
Regarding viral exit, budding of nucleocapsids at the INM leading to *primary envelopment* produces vesicular (temporarily enveloped) structures in the perinuclear space that are normally not seen in uninfected cells. The temporary envelope is lost by *de-enevelopment* at the outer nuclear membrane (ONM) which is contiguous with the rough endoplasmic reticulum (RER).

Final maturation occurs by a secondary budding process (*secondary envelopment*) of naked unenveloped nucleocapsids at cytoplasmic vesicles thereby acquiring an envelope with processed membrane proteins. These transport vesicles are targeted to the plasma membrane which fuses with the vesicle membrane and enveloped mature viral particles are exocytosed into the extracellular space.

There are still controversial views regarding the definition of the cellular compartment of secondary envelopmnent. Clearly, the cellular membranes are enriched by viral envelope proteins whose cytoplasmic domains evidently interact with cellular factors [**87**] and may modify the compartmental properties.

It generally accepted that viral transmembrane glycoproteins are subject not only to anterograde transport along the cellular exocytosis pathway to plasma membrane from where they are retrieved to the cytoplasmic compartment of final viral maturation but also to a retrograde transport from the site of biosynthesis, namely the rough endoplasmic reticulum (RER), into the inner nuclear membrane presumably by lateral diffusion [**5, 88, 89**]. Presence in the INM has been demonstrated for the conserved glycoproteins B and H (gB and gH; [**55, 90**].

There is genetic and biochemical evidence from the herpes simplex virus (HSV) that two conserved viral proteins, UL31 and UL34, are essential for viral exit from the nucleus. Transient coexpression of these viral gene products results in colocalizaton at the nuclear envelope [**91, 92**]. There evidence from recent reports that the recombinant UL34 product not only induces morphological alterations of the nuclear envelope similar to those in infected cells [**34**] but that the UL34/UL31 complex also triggers modifications of the nuclear lamina, possibly by direct interaction with lamin A/C [**93**]. Only limited information is presently available for the counterparts of HCMV, UL50 and UL53. Interestingly, pUL50 was found in the INM following solitary expression and morphological changes were comparable to those in cells cotransfected with M50/p35 and M53/p38 [**16**]. On the other hand, when transfected alone pUL53 exhibited diffuse nuclear distribution but colocalize with lamin B in punctuate patches along the nuclear periphery in HCMV-infected cells [**94**].

5. PATHOGENESIS

The pathogenesis of infections with human cytomegalovirus have modelled in small animals and primates utilizing the respective CMVs. In most cases, acute infection is associated with significant levels of virus replication and dissemination to multiple organs. In the immunocompetent animal, such infections are rapidly controlled by a number of effectors functions of the innate and adaptive immune response. The pathogenesis of acute HCMV infections can be readily explained by the control of virus replication and the resolution of virus-induced cytopathology. There appears to be a linkage between levels of virus replication, organ dysfunction, and disease in patients as well as in experimental models with acute CMV infections. In contrast, chronic infections with CMV have as a major component of their pathogenesis a bidirectional relationship between viral gene expression and the host inflammatory response such that viral persistence is facilitated by the host inflammatory response and the host inflammatory response is fuelled by the presence of the virus. In these cases, diseases can be attributed to both viral and host functions. The viral gene products that appear to play a role in chronic inflammation have evolved with CMVs and are likely unimportant for replication in vitro. As such, defining the role of these viral functions in disease associated with chronic HCMV infections almost certainly will require relevant animal models.

5.1 Immunity

HCMV infections are kept under control by the immune system. Histopathologic and immunohistochemical examination of necropsy tissues has indicated that the virus initially enters via the epithelium of the upper alimentary, respiratory, or genitourinary tracts. However, since infection is readily established by transfusion and transplantation, initial infection of epithelial cells does not seem essential [**95**]. Cytotrophoblasts form a barrier between the maternal and fetal circulation, but readily allow HCMV replication in vitro, suggesting that the fetus is infected hematogenously [**2, 96**]. Leukocytes and vascular endothelial cells aid the spread of HCMV, however, is rarely achieved, and the viral genome remains at selected sites in a latent state. Initiation of productive replication results in transient virus shedding and recrudescent disease [**97**]. Since severe infection is usually restricted to individuals with impaired cell-mediated immunity, it is evident that this arm of the immune response provides the most protection. Even so, the supportive

role of the humoral system in keeping CMV loads below critical thresholds must not be overlooked.

5.1.1 Cell-Mediated Immunity

Mouse models have been employed to determine the role of specific components of the immune response and their recognition of viral proteins [**98**]. Early studies demonstrated that a CMV-specific cytotoxic T lymphocyte (CTL) response was required for recovery from CMV infection. Suppression of CTLs caused reactivation and dissemination of natural infection. Further studies showed that both natural killer (NK) cells and CD8+ cytotoxic T-cells are of primary importance in the prevention of recurrence [**99, 100, 72**]. Adoptive transfer of CD8+ cells protected mice from lethal challenge independent of CD4+ T helper cells. CD8+ T-cell clones specific for both structural and non structural proteins were identified. However, CD8+ depletion with abs or in mouse mutants deficient in MHC Class I expression revealed additional antiviral activities mediated by CD4+-derived cytokines not seen when CD8+ cells are present. Lastly, experiments with mice deficient for perforin and granzyme- or Fas-mediated cytotoxicity have shown that replication is controlled by NK and CD8+ cells via the perforin and granzyme-mediated pathway,

whereas the Fas-Fas ligand system is not critical [**101, 102**]. Since infection (apart from congenital forms) is most severe in patients with dramatically impaired cell-mediated immunity, such as BMT recipients, and those with AIDS, it is evident that this arm of the immune response provides the most protection [**103**]. Its investigation in studies of lymphocyte proliferation in response to CMV antigens has shown that in most seropositive immunocompetent adults, this usually takes the form of CD4+ cell proliferation in response to envelope glycoproteins gB and gH, the lower matrix protein pp65, and IE proteins, in addition to other proteins [**26, 10, 104**]. The function of proliferating CD4+ cells in resistance to HCMV infection is not clear. Studies in mice point to several antiviral effector functions, such as the production of cytokines and CD4+-

mediated cytotoxicity. The virus-encoded proteins that are targets of CTLs include a collection of structural and non-structural forms [**105, 106**]. Comparison of CTL precursor frequencies for envelope gB or the 72-kDa IE1 protein revealed a low frequency of gB specific CTLs (< 6%), whereas 18–58% of CTLs recognized IE1-expressing target cells.

The high frequencies for pp65 and IE1 proteins found in subsequent studies show that they are the chief targets of the CTL-mediated immune
response. Although a direct role for virus-specific CTL responses in the resolution of infection has yet to be defined, in vivo evidence of the control of HCMV infection by CTLs has been obtained through the adoptive transfer of virus-specific clones. Passive administration of autologous HCMVspecific CD8+ CTLs at set intervals after BMT to seronegative recipients of marrow from seropositive donors generated a vigorous CMV-specific CTL response without
onset of viremia or CMV disease [**107**]. These studies provided the first evidence for the role of CTLs in controlling human CMV infection, and opened new therapeutic perspectives for CMV treatment in organ transplantation.

5.1.2 Humoral Immunity

During primary infection, immunocompetent individuals produce anti-HCMV immunoglobulin (Ig)M class abs that persist for 3–4 months, followed a few weeks later by IgG class abs that persist for life. Experimental and clinical findings show that the humoral response is beneficial.
Mice immunized against murine CMV gB were protected against a lethal challenge , and immunization of pregnant guinea pigs against guinea pig CMV envelope glycoprotein protected their fetuses in the same way [**108**]. Intrauterine infection is less severe when transmitted by means of recurrent rather than primary maternal infection . During primary infection, women who transmit the virus in utero have high levels of total IgG characterized by low avidity and neutralizing activity. Correlation of an enhanced antibody response with a poor fetal prognosis suggests that fetal antibody is responsible for immunopathology. Primary infection is more frequent and more severe when the renal transplant recipient is seronegative and the donor seropositive. Alleviation of this severity by preimmunization with the attenuated Towne strain [**109**] or administration of high-titer anti-CMV Igs [**110**] also shows that humoral immunity is beneficial. Many CMV proteins are recognized by the humoral immune system [**111, 112**]. The envelope glycoproteins (mainly gB and gH) are the targets of virus-neutralizing abs in both human and mouse models. The predominance of gB as a target is best explained by its dominant immunogenicity and abundance compared with other components of the envelope. Anti-gH abs have a potent, complement-independent, but minor neutralizing activity. Viral

tegument components, including pp28 (UL99), pp65 (UL83), and pp150 (UL32), trigger an intense and long-lasting ab response that provides an indirect measure of viral replication and correlates with the clinical outcome. However, these abs are
unable to react with the surface of virions and infected cells, and are of limited importance in a protective response.

5.2 Immune evasion by Human Cytomegalovirus

A characteristic feature of infection in the normal host is persistence of the viral genome in a nonproductive form at specific anatomical sites for months or even years [**99**]. This ability to avoid elimination by the immune system is the result of (i) induction of a latent state of infection, (ii) exploitation of immunologically privileged tissues for replication (i.e., epithelial cells of the salivary glands expressing an insufficient number of MHC Class I molecules to trigger clearance by CD8+ cell, and (iii) expression of genes that interfere with the immune response [**113**]. Escape from CD8+ cells is mediated by several mechanisms, all of which block the expression of MHC Class I molecules complexed with a potentially important CTL target, the 72-kDa IE1 phosphoprotein, or which degrade MHC Class II molecule proteins, and, thus, prevent presentation of viral antigen to CD4+ lymphocytes.

HCMV deletion mutants have been used to map two gene regions whose products are associated with the downregulation of MHC Class I complex formation; namely, US11 and that spanning US2 and US5 [**114**]. Both US2- and US11-encoded proteins lead to the accumulation of MHC Class I heavy chains in the cytosol of infected cells, where they are degraded by the proteosome, thus shortening their half-life and preventing their expression on the cell surface. Binding of peptides to MHC Class I molecules depends on transport across the ER membrane by a specific transporter complex designated TAP 1/2 (transporter associated with antigen processing). Only after their introduction into the ER can peptides form heterodimers with MHC Class I molecules and b2-microglobulin and be recognized by CD8+ CTLs. Despite a significant increase in the expression of TAP molecules in infected cells, the HCMV glycoprotein encoded by the US6 gene inactivates the TAP system by interfering with formation of the TAP1- TAP2-MHC Class I-b2-microglobulin complex. The finding that lysis of HCMV-infected fibroblasts by IE-specific CTL is usually low, but increases in fibroblasts infected with a mutant HCMV lacking the UL83 encoding the matrix pp65, suggests that phosphorylation of the 72-kDa

IE1 protein by pp65 interferes with its antigenic processing and presentation to CD8+ cells [**5, 115, 116**]. Experiments have demonstrated that NK cells are involved in the control of CMV infection. Beige mice (genetically deficient in functional NK cells) are more susceptible to murine CMV [**117**]. In humans, MHC Class I HLA–E molecules protect target cells from NK cells by binding to inhibitory receptors on their surface. A convincing mechanism for escape from NK cell attack stems from the observation that the HCMV UL40 gene encodes for a glycoprotein containing a nine amino acid sequence homologous to the MHC Class I sequence that up-regulates the expression of Class I HLA-E molecules [**118**]. Lastly, mechanisms interfering with chemokine-driven inflammation are exploited by HCMV to evade the immune response. HCMV-infected cells express viral G-proteincoupled

receptor (GPCR) homologues encoded by US27, US28, UL33, and UL78, which are needed for virus replication in vitro [**119, 62**]. The US28-encoded GPCR binds to and sequesters several b-chemokines and, thus, enables the virus to evade the immune response.

5.3 Persistence and release from the host

Cells in bone marrow and peripheral blood are the chief reservoirs for latent CMV infection. CMV DNA is found in a small percentage of peripheral blood monocytes, and gene expression is limited to the E genes. These findings and the demonstration that tissue macrophages differentiated from circulating monocytes also express E and L genes have led to the formulation of the following model: bone marrow precursors of blood monocytes are the site of

CMV latency and provide a means of dissemination upon differentiation into circulating monocytes. Differentiation of latently infected monocytes into macrophages leads to reactivation and productive infection. Support for this model is provided by the reactivation of CMV by allogeneic stimulation in vitro of peripheral blood mononuclear cells from seropositive healthy donors [**34**]. Moreover, it has been found that a small percentage (close to 0.01%) of CD33+/CD14+ or CD33+/CD15+ bone marrow mononuclear cells from seropositive human donors express transcripts associated with latency. In contrast, T lymphocytes, B lymphocytes, and CD33- mature granulocytes and macrophages are negative [**120**].

Recurrent infection can be defined as indefinite, but intermittent, excretion of the virus from single or multiple sites. It should be distinguished from the prolonged excretion typical of primary infection and also infection in the immunocompromised host, where productive infection, as measured by viral excretion, is markedly increased. In allograft recipients and AIDS patients, the incidence of virus excretion from multiple sites approaches 100%. During primary, recurrent, or persistent infection, CMV is shed in multiple body fluids, such as saliva, urine, tears, cervicovaginal fluid, breast milk, and semen, probably due to virus replication in glandular epithelial cells, accompanied by virus release into excretions.

6. EPIDEMIOLOGY OF HUMAN CYTOMEGALOVIRUS INFECTION

Epidemiology of human cytomegalovirus infection HCMV is one of the most successful parasites. It is found in both the developed industrial societies and in isolated aboriginal groups. Following infection, it is excreted in body fluids (urine, saliva, tears, semen, milk, and cervical secretions) for months to years. Infection is usually mild and subclinical. The unsuspecting host is thus able to spread the virus both vertically and horizontally. Virus can appear following primary infection, reinfection, or reactivation.

Infection is life long. Latent CMV may reactivate and produce infectious virions that are shed in the saliva, urine, and other body fluids. Reactivation is usually asymptomatic, and enables the virus to spread horizontally and vertically.

Congenital infections

About 10% of infants are infected by the age of 6 months, following transmission from their mothers via the placenta, during delivery, and by breast feeding [**121, 122**]. Transplacental infection can occur both in women infected for the first time during pregnancy and those infected long before conception (recurrent infection). Primary infection during pregnancy as a source of fetal infection was suggested by the observation that transplacental transmission ranges from 20% to 40% when primary infection varies from 0.7% to 4.1% [**123**]. In the case of recurrent infections, high rates of congenital infections are observed in populations with higher rates of maternal seropositivity, suggesting that most congenital infections are caused by reactivation of latent virus [**124, 125**]. Breast-feeding is the most common route of transmission. Polymerase chain reaction

(PCR) studies have also demonstrated a strong relationship between the presence of viral DNA in milk and transmission to the infant. The precise route of infection throughout childhood is not known, although close contact is required. Infection increases when people live in crowded, unhygienic conditions, and hence is most common in socially disadvantaged countries. The incidence of congenital infection is highest in poor communities, since most women are infected before puberty. Reactivation is thus a more frequent cause of congenital infection than primary maternal infection, although the latter presents a greater risk to the fetus than recurrent infection.

Primary infection does not usually result in a clinical illness, and cannot be identified in pregnant women. Its status as a risk factor for fetus infection is thus uncertain. Intrauterine infection occurs in only one-third of pregnant women with primary infection. The ways in which the fetus escapes infection are unknown, although macrophages in the placenta may constitute a barrier.

Transfusion

Primary infection with HCMV via blood transfusions was noted in the mid-1960s, although epidemiologic studies have demonstrated that this is an uncommon route. Attempts to culture HCMV from fresh donor blood have rarely been successful. It is thus assumed that the virus is latent in the blood cells of healthy donors and is reactivated following transfusion when they encounter an allogenic stimulus. The nature of the leukocytes carrying latent virus is unknown, although attention is being increasingly focused on the monocytes/macrophages [**126, 34**].

Solid organ transplantation

HCMV is a significant post-allograft pathogen. Several studies have shown that seronegative recipients of an organ from a seropositive donor are at risk of acquiring a primary infection and that 60%–80% develop a more severe disease than seropositive recipients of seropositive organs, suggesting that acquired immunity modulates infection [**127, 128**]. The contribution of immunity to resistance to HCMV disease, however, decreases as the level of immunosuppression increases. Variables that contribute to post-transplant infection include donor and recipient serological status, type of immunosuppression, source of allograft, HLA matching of donor and recipient, and type and amount of blood products used [**129, 130**]. Since the main determinant of transmission via blood is the presence of leukocytes, reduction of the cell contamination

of blood products has dramatically decreased the incidence of transfusion-acquired infection in allograft recipients [**129**]. The effects of infection extend well beyond the immediate post-transplant period. It appears that HCMV contributes to graft loss independently of graft rejection. Following kidney transplantation, a histologically distinct glomerulopathy may result from infection of the graft and may significantly alter its survival [**131, 132**]. The role of HCMV infection in rejection is most convincing in the case of cardiac allografts. Accelerated development of coronary atherosclerosis associated with post-transplant HCMV infection has been reported and found to be independent of reports of age, lipid levels, and immunosuppression regimen [**133**].

BMT

The incidence of HCMV infection following allogenic bone marrow transplant (BMT) ranges from 32% to 70%, with an average of 50%, regardless of the prior serological status of the recipient and donor. Typing of strains has shown that infection is due to a virus derived from the recipient [**134, 135, 136**]. Its incidence in seronegative recipients receiving a seropositive marrow is lower than in seropositive recipients receiving a seropositive marrow, suggesting a transfer of adoptive immunity from donor to recipient. Thus, the most critical event is reactivation of a latent virus in seropositive BMT recipients, whereas in seronegative recipients, transmission mostly occurs through the larger quantities of blood products that they receive compared with solid organ transplant recipients. Increasing evidence indicates that apart from the serological status of donor and recipient, administration of blood products carries a significant risk of exposure to HCMV infection following BMT.

Another major risk factor is the immunosuppressive regimen.

AIDS patients

HCMV is a leading opportunistic agent in acquired immunodeficiency syndrome (AIDS). The increased survival of HIV-infected individuals following more effective prophylaxis and treatment of bacterial and protozoal infections, accompanied by virtual inactivity of their immune system, has augmented the importance of HCMV infection in these patients. Three major organ systems are clinically affected: the CNS, the lungs, and the gastrointestinal tract. Retinitis is the most frequent CNS infection directly attributable to HCMV replication, and the most sight-threatening [**137, 138, 139, 140**]. Autoptic immunohistochemical analysis has shown HCMV presence in all layers of the retina,

without underlying choroidal involvement. Absence of virus in the endothelium of the retinal microvasculature indicates that its hematogenous spread does not follow replication in these cells, but that perhaps the virus breaks through the capillary endothelium.

7. MANIPULATING CYTOMEGALOVIRUS GENOMES BY BAC MUTAGENESIS

The generation of CMV mutants has been a difficult task especially because of the large genome size and the slow replication cycle of the CMVs. The recent cloning of CMV genomes as infectious bacterial artificial chromosome (BAC) in *E. Coli* opened new horizons for the construction of mutant CMVs by utilizing the methods of bacterial genetics.

The genetic content of the AD169 strain was estimated at 145 genes and that of wild-type HCMV at between 164 and 167 genes. The majority of the proteins encoded by HCMV has not yet been characterized. Even for those proteins about which we have some functional knowledge, e.g. the envelope glycoproteins, a clear understanding of the mode of action is usually missing. A number of ORFs has been subcloned in plasmid expression vectors and their functions studied independently of the viral infection and separately from other viral genes, for istance by transient transfection experiments. However, many viral proteins have to interact with other viral or cellular proteins in order to exert their effects and many genes display a distinct temporal expression pattern during infection cycle. Thus, it is desirable to study the role of gens in the context of the viral infection. For many years the size of the CMV genomes represented an insumontable barrier, which prevented their cloning as a single entity. Only the development for new cloning vectors, e.g. the *E.Coli* F-factor-derived bacterial artificial chromosome (BAC) that offered a cloning capacity of up to 300 kbp, made this vision become real.

The BAC vector sequences were introduced into the viral genome by homologous recombination utilizing the cellular recombination machinery (**Fig. 4A**). to this end, recombination plasmid was constructed that contained the BAC vector flanked by viral DNA sequences homologous to the intended insertion site in the viral genome. The linearized recombination plasmid was transfected into fibroblasts cells followed by infection with the respective CMV strain [**120**]. recombinant viruses carrying the BAC

vector integrated into their genomes were amplified. Circular replication intermediates of the viral genomes were isolated from infected cells and electroporated into *E. Coli.* Bacterial clones harbouring CMV BACs were selected by means of the antibiotic resistance encoded on the vector backbone. The CMV BACs could be propagated stably in E. Coli [**120**]. Upon transfection into permissive cells, the CMV BACs gave rise to formation of plaques and infectious virus (**Fig. 4B**). the CMV BACs displayed a remarkable stability in *E. Coli*: after days or even weeks of propagation in suitable *E.Coli* hosts the BACs did not show an altered restriction profile and remained infectious. This property formed the basis for the targeted manipulation of the BAC-cloned CMV genomes in *E.Coli.*

Mutagenesis of BAC-cloned CMV genome: ET Recombination

The establishment of methods to manipulate the CMV genomes in *E.Coli* was the next step after successful BAC cloning. A mutagenesis technique uses a linear DNA fragment carrying a selectable marker (e.g. kanamycin resistance gene) flanked by DNA sequences homologous to the viral target site and to introduce the marker by two simultaneous recombination events, i.e. by double cross-over. (**Fig. 4C**). Some recombination systems of bacteriophages such as the *recE* and *recT* gene functions of the E.Coli prophage Rac or the redα and redβ functons of phage lambda utilize linear DNAsubstrates. These recombination proteins are highly efficient and need only 30 to 50 nucleotides of homologous sequences to mediate recombination. Thus, the linear recombination substrates can easily be generated by PCR with the homologous sequences being incorporated into the synthetic oligonucleotides used as primers for the amplification of the selectable marker .

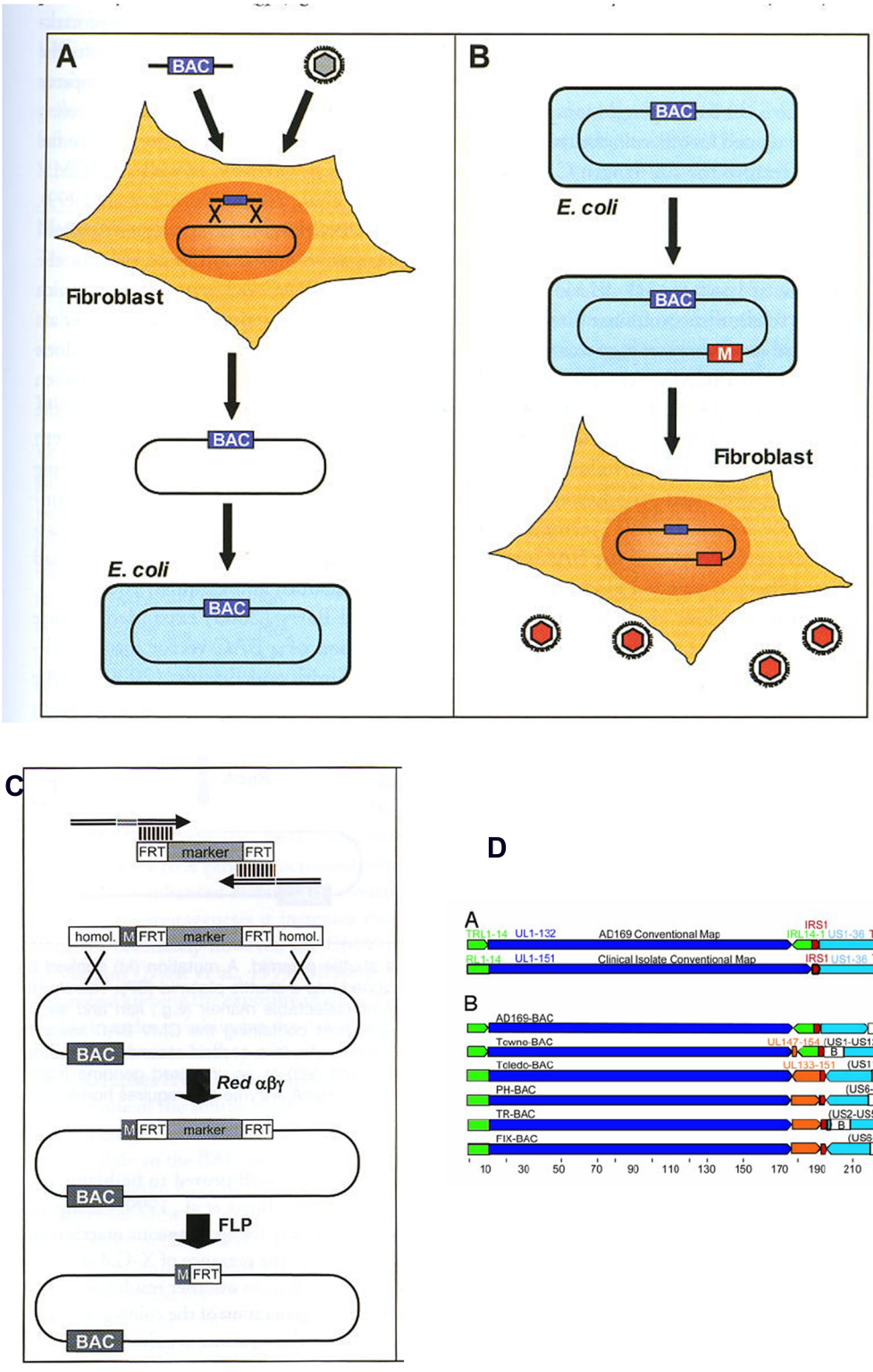


FIGURE 4. A) Cloning of a CMV genome as BAC. **B**) The principle of generating mutant CMVs using BAC technology. **C**) BAC mutagenesis using linear DNA fragments. **D**) Cytomegaloviruses cloned as BACs in *E.Coli*.

MATERIALS AND METHODS

Cells and Viruses. HCMV strain TR12 and recombinant viruses were propagated in primary human foreskin fibroblasts (HFF) grown in minimal essential medium (Invitrogen, Karlsruhe, Germany) supplemented with 5% fetal calf serum, glutamine (100 mg/liter), and gentamicin (350 mg/liter).

To prepare intracellular particles, cells from infected cultures were trypsinized and collected via low speed centrifugation. The cell pellet was subjected to three freeze-thaw cycles and cellular debries was removed by centrifugation at 1000 x g. supernatants were subjected to high speed centrifugation (23 000 rpm in a Beckmann SW27 rotor) for 70 min, the pellet was resuspended in medium and stored at -80°C.

Virus titer were determined by indirect immunofluorescence using a monoclonal antibody (MAb) against the immediate early protein 1 (IE1) of HCMV as described. (Andreoni M. et al 1989).

COS7 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, glutamine (100 mg/liter), and gentamicin (350 mg/liter).

Preparation of eukaryotic expression constructs and DNA transfection

pcTRL12myc

The entire TRL12 reading frame was amplified from HCMV strain TR12 with the following primers that contain HindIII and EcoRI restriction sites, **TRL12-TR-5' (44)** and **TRL12-TR-3'**(45). The PCR product was cleaved with the appropriate restriction enzymes and inserted into the expression vector pcDNA3.1myc/His (Invitrogen, Carlsbad, Calif.). The resulting plasmid, pcTRL12myc/his, encoded full-length pTRL12 fused to a C-terminal Myc/His tag. The integrity of pcUL132myc/his was confirmed by DNA sequencing.

pcTRL12/sig-HA

Two different TRL12 DNA insert were PCR amplified HCMV strain TR12. The first insert (fragment A), encoding amino acids 33-190 of pTRL12 protein, was amplified with primers that contain EcoRI and XhoI restriction sites, *TRL12-33-EcoRI s (82)* and *TRL12-570-XhoI as (32)***.**

The second insert (fragment B), encoding amino acids 191-410 of pTRL12 protein, was amplified with primers that contain XhoI and XbaI restriction sites, *TRL12-570-XhoI s (33)* and *TRL12-stop-XbaI as (34)***.** The PCR products were cleaved with appropriate restriction enzymes and inserted into the expression vector pcDNA3-sig-HA containing the signal

sequence of TRL12 and in frame the epitope HA. The resulting plasmid, pcTRL12-sig-HA, encoded full-length fused to a N-terminal HA tag. The integrity of pcUL132myc/his was confirmed by DNA sequencing.

Transient transfection of eukaryotic cells. COS7 cells were transfected with the respective DNAs using *Lipofectamine 2000* reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's suggestion except that the transfection mixture consisted of 1 µg of DNA, 95 µl of Dulbecco's modified Eagle's medium, and 6 µl of Lipofectamine Plus reagent. The mixture was added to a cell culture dish (3.5-cm diameter, seeded with 2×10^5 cells 1 day before). After 48 h, cells were harvested, washed three times with phosphate-buffered saline (PBS), and stored at −20°C until used.

Image analysis. COS7 cells grown on glass coverslips in 24-well plates were transfected with 1 to 2 µg of plasmid DNA using *Lipofectamine 2000*. Fibroblasts, also grown on glass coverslips in 24-well plates, were infected with virus at a multiplicity of infection of 0.5. 24 hours to 48 hours later, the coverslips were washed and fixed in 2.0% paraformaldehyde in PBS. The fixed cells were permeabilized with Triton X-100 containing buffer (0,1%) and then blocked with PBS-1% bovine serum albumin. Primary antibodies, were then added. Following washing, antibody binding was detected with the appropriate secondary antibody conjugated with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (Dianova). Images were collected using a Zeiss Axioplan 2 fluorescence microscope fitted with a Visitron Systems charge-coupled device camera (Puchheim, Germany). Images were processed using MetaView software and Adobe Photoshop.

Monoclonal antibodies utilized: mouse anti-MCP; mouse anti-IE-1 (p63-27); mouse anti-gB (27-287); mouse anti-gH (14-4B); mouse anti-pp150 (XP-1); Myc-specific monoclonal antibody; rabbit and mouse anti-HA; rabbit anti-calreticulin (endoplasmic reticulum marker; Dianova, Hamburg, Germany); rabbit anti-GM130 (Golgi marker; Dianova); sheep anti-TGN46 (trans-Golgi network marker; Serotec)

Preparation of GST-TRL12 fusion protein and antiserum. The TRL12 carboxyl-terminal region was amplified from TR DNA by PCR with the following primers containing either BamHI or XhoI: *TRL12-TR BamHI 5 (42)* and *TRL12-TR XhoI 3 (43)*. The PCR product was digested with both endonucleases and inserted into the expression vector pGEX-1P (Pharmacia Biotech, Freiburg, Germany). The resulting plasmid, GST/TRL12c, contained nucleotides (nt) 1123 to 1230 of TRL12-TR fused to the glutathione *S*-transferase (GST) gene. Correct insertion was confirmed by DNA sequencing. Plasmid DNA was used to transform *Escherichia coli* BL21 for expression of GST fusion proteins. The fusion protein was induced and purified from *E. coli* lysates according to the manufacturer's instructions. The 34-kDa fusion, was used to raise an antiserum in mice.

Immunoprecipitation. Immunoprecipitation of Myc-containing proteins was carried out with the μMACS c-Myc-tagged protein isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions.

SDS-PAGE and immunoblotting. Lysates of infected cells were incubated in sample buffer containing 15 mM Tris-Cl (pH 6.8), SDS, 2% (vol/vol), b-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% bromophenol blue for 20 minites at room temperature. The samples were boiled at 95°C for 5 min and the centrifugated at 10 000 g. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 15% polyacrylamide gels under standard conditions. Proteins were transferred to nitrocellulose membranes, and membranes were blocked with PBS containing 0.1% Tween 20 and 5% powdered milk. Antibodies and sera were diluted in PBS containing 0.1% Tween 20. Antibody binding was detected with horseradish peroxidase-coupled anti-murine immunoglobulin and the ECL detection system (Pharmacia Biotech, Freiburg, Germany).

BAC mutagenesis and reconstitution of TRL12 recombinat viruses. Mutagenesis of TR12 was performed using linear DNA fragments for homologous recombination. Recombinant viruses on the genetic background of strain TR12 were constructed using TR-BAC (GenBank AC146906).

Deletion mutant Del-TRL12 was created by site-directed mutagenesis using a PCR-generated fragment electroporated into *Escherichia coli* strain DH10B harbouring the TR bacterial artificial chromosome (BAC) and expressing bacteriophage λ functions *red*αβγ from plasmid pkD46 (Datsenko, K.A. et al. 2000 – GenBank AY048746). The primers that were used to generate the recombination fragments represented hybrids in which the 5′ end was homologous to sequences flanking the HCMV region to be deleted (50 nucleotides) and 3′ ends were homologous to the kanamycin resistance gene as present in plasmid pcpo15-Link2 (30 nucleotides). PCR amplification of the recombination fragment was performed with primers *TRL12-TR Kan5(44)* and *TRL12-TR Kan3(45)*. Thus, the resulting PCR product consisted of a kanamycin resistance gene flanked by short stretches of HCMV-specific sequences.

The PCR-fragment was electroprorated into *E.Coli* DH10B carrying the TR-BAC and the plasmid pkD46 for recE/T mediated recombination. Following homologous recombination in *Escherichia coli*, colonies carrying kanamycin and chloramphenicol resistance were selected on agar plates containing kanamycin (30µg/ml) and chloramphenicol (30µg/ml).

To confirm the integrity of the recombined BAC, digestion of DNA with the appropriate restriction enzymes was carried out and analyzed via agarose gel electrophoresis in comparison to the parental TR-BAC. To confirm recombination at the predicted site PCR analysis as well as DNA sequence analysis of the TRL11-TRL13 region was performed.

The resulting BAC-Del-TRL12 was used for transfection of fibroblasts and reconstitution of infectious virus Del-TRL12.

*Recombinant myc-tagged virus***.** To create BAC-TRL12myc a PCR fragment was generated using HCMV/kanamycin hybrid primers as described above. PCR amplification was performed with primers *BAC-TRL12myc-s(80)* and *BAC-TRL12myc-as(81)*. The 5′ primer contained an additional sequence coding for the *myc* epitope (AEEQKLISEEDLL). This PCR fragment was used for recombination in BAC-TR12 as described above.

Reconstitution BAC-derived virus RV-delTRL12/TR12 . MRC-5 cells (2,5 x 10^5 cells per well) were seeded into six-well dishes. Two days later, 1 µg of HCMV BAC DNA, prepared using Nucleobond PC 100 columns (Macherey-Nagel, Düren, Germany) together with 1 µg of pcDNApp71tag plasmid DNA was cotransfected with the Superfect reagent (QIAGEN, Hilden, Germany). Serum-free Dulbecco's modified Eagle's medium was added to the

transfection mixture to a total volume of 100 µl and incubated for 30 min at room temperature. MRC-5 cells were serum starved for 30 min and subsequently 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum was added together with the transfection mixture for 4 h at 37°C. After removal of the transfection mixture the cells were incubated for 7 days in cell culture medium. The cells were split and cultured until a cytopathic effect was observed. Propagation of the infectious virus was through cocultivation of infected and uninfected cells. To test for reversion of the recombinant viruses, primers X and Y were used.

Viral DNA Accumulation analysis by Real-Time PCR. For analysis of viral DNA accumulation during infection, intracellular particles from TR and Del-TRL12 were used to infect 6 x 10^4 fibroblast in 24 well-dishes at MOI 0,1. DNA copy numbers were analyzed on days 1, 3, 6, 9 and 12 after infection by quantitative real time PCR.

Multistep growth curves for TR-based viruses. Intracellular particles were directly used to assay the growth kinetics of the viral mutants. To this end, 1 day after seeding 2,5 × 10^5 HFF per well in 6-well plates, cells were infected with wild type virus (TR) and TRL12 deletion mutant (Del-TRL12) at MOI of 0,5 in a volume of 1 ml. After incubation at 37°C for 4 h the infectious mixture was removed, cells were washed twice with PBS, and fresh medium was added. At days 3, 6, 9 and 12 after infection, the cells were harvested and intracellular particles were isolated as described above. Different dilutions of these virus preparations were used directly to determine the virus titre by indirect immunofluorescence using a monoclonal antibody (MAb) against the immediate early protein 1 (IE1) of HCMV as described.

DNA sequence analysis. To establish the coding sequences for TRL12 in clinical isolates, DNA was extracted from lysates of infected fibroblasts and amplified by PCR using primers *TRL11 EcoRI(59)* and *TRL13-TR BamHI(62)*. Nucleotide sequences were determined on an ABI 377 using the dye terminator sequencing kit according to the manufacturer's instructions (PE Applied Biosystems, Foster City, Calif.).

Sequence and phylogenetic analysis. The nucleotide and amino acid sequence described in this study were aligned using the ClustalW software and displayed as a printable output by BOXSHADE Server (http//www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed by Hein method and the lasergene'99 System package (DNASTAR), choosing the MegAlign option. Phylogenetic analysis was confirmed by ClustalW software and MEGA software alignment and tree display methods.

Nucleotide accession numbers. TR-BAC (GenBank AC146906);

PRIMERS

TRL12-TR Kan5 (44)
5'ACCGTCAAAGACGCAACAACCTTACGTACCGACAAACGGTATATGTAATTAGT
CACGACGTTGTAAAACGACGGCCAGTG – 3'

TRL12-TR Kan3 (45)
5'TAGTGTGCGTGCCATGTGAGATGTCATAGTGACCTGTTTTGCGCATAAGAAGCT
TCAAAAGCGCTCTGAAGTTCCTATAC - 3'

BAC-TRL12myc-s (80)
5'AGATGATATTAGTGAATCTAGCCTTGTTGTGCAATATCATCCTGAACATGAAGA
CGAACAAAAACTCATCTCAGAAGAGGATCTGTAAAGTCACGACGTTGTAAAACG
ACGGCCAGTG - 3'

BAC-TRL12myc-as (81)
5'ATTCCGAAAACGTGGATATGATTACCGTCCATGTAATCGCAAGATGCCAGTGCA
TAGCTTCAAAAGCGCTCTGAAGTTCCTATAC - 3'

TRL12-TR 5 (39)
5'- CACCAAGCTTATGCGCGTCAACCGTCAAAG -3'

TRL12-TR 3 (40)
5'- GGTAGAATTCGTCTTCATGTTCAGGATG -3'

RecTRL12myc-as (85)
5'- ATTCCGAAAACGTGGATATGATTACCG -3'

TRL12-TR BamHI (42)
5' – CTCAGGATCCATGCTTCTTTTTTTTGTTC -3'

TRL11 EcoRI (59)
5' – TTATGAATTCTGGTTACGGATTCCCAAGAGG -3'

TRL13-TR BamHI (62)
5' – CACTGGATCCGTATTTGTCGTAGCAGTTGG – 3'

TRL12-TR rec3 (53)
5’ – TAGTGTGCGTGCCATGTG – 3’

Kan 2,5 (63)
5’ – CTCATGCTGGAGTTCTTC – 5’

TRL12-570-XhoI as (32)
5’ – ATGGCTCGAGTGGAGTTGATTTCACAGG – 3’

TRL12-33-EcoRI s (82)
5’ – ATGGGAATTCAGCACCGATACCAACAAT – 3’

TRL12-570-XhoI s (33)
5’ – ATGACTCGAGCTAACGGCAGAAGAAGG – 3’

TRL12-stop-XbaI as (34)
5’ – ATTGTCTAGATTAGTCTTCATGTTCAGG – 3’

TRL12-TR XhoI 3’ (43)
5’ – ACTTCTCGAGGTCTTCATGTTCAGGATG – 3’

ORF TRL12: GENERAL FEATURES

Human cytomegalovirus has a glycoprotein coding capacity which far exceeds that of other herpesviruses. The genome of the laboratory-adapted strain AD169 encodes at least 57 open reading frames (ORFs) with predicted characteristics of glycoproteins or glycoproteins exons. Remarkably, fewer than 10 glycoprotein gene products have been characterized with respect to biosynthesis, function, and incorporation into the virion. The envelope glycoproteins of herpesviruse have multiple role in the viral replication cycle, including essential functions such as attachmen, penetration, cell-to-cell spread, envelopment and maturation of nascent virus particles. To understand these processes, it necessary to have detailed knowledge of the structure and function of envelope glycoproteins.

The reading frame TRL12 of cytomegalovirus codes for a protein of approximately 417 amino acids which has all characteristics of a type I glycoprotein.

The only information available about TRL12 gene concerns its transcription kinetic. Preliminary results (unpublished data) indicate that TRL12 protein is encoded by a 2,4 or 2,7 kb mRNA with a early-late kinetic.

Chee et al. [**62**] identified nine multigene families in AD169 strain and an additional three were recognized in low passage isolates [**141**]. One, the RL11 family consists of 12 genes (RL11, **RL12**, RL13, UL1, UL4, UL5, UL6, UL7, UL8, UL9, UL10 and UL11) oriented left to right near the left terminus of the genome and arranged contiguously but for the presence on the opposing strand of two unrelated genes (UL2 and UL3). RL11, RL12 and the 5'-portion of RL13 are present twice in the AD169 genome by virtue of their location in an inverted repeat. This repeat is much smaller in low passage strains, which consequently contain single copies of these genes [**44**]. All members of the RL11 family (generically termed "RL11 genes") are also present in CCMV, which has counterparts of all but UL1 [**142**]. Cytomegalovirus of mouse, rat and tupaia lack RL11 genes [**143, 39, 144**]. These proteins that are expressed only by individual genera of a herpesvirus subfamily are termed "private proteins" and could contribute to the specific properties of individual herpesviruses, such as cell tropism or pathogenicity.

RESULTS

1. GENOMIC LOCATION OF TRL12 IN HCMV GENOME AND PREDICTED FEATURES OF THE PROTEIN.

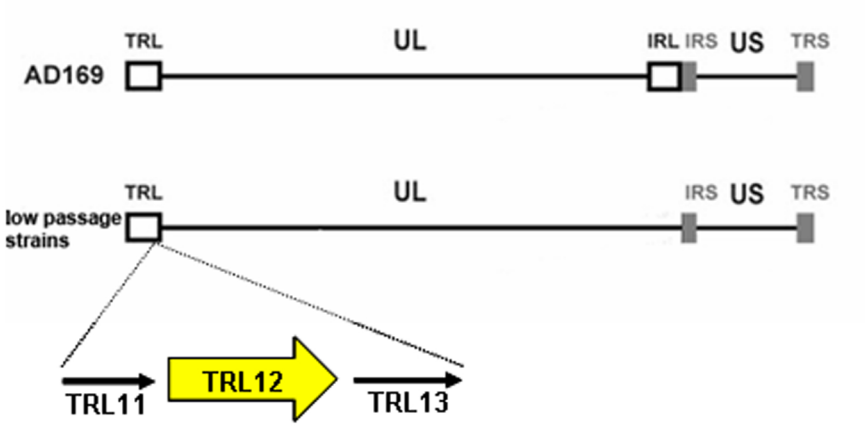
HCMV AD169 and low-passage isolates differ in two major aspects. First, as originally reported for Toledo, low-passage isolates contain a DNA segment that is absent from AD169 (UL133 to UL151). Second, low-passage isolates lack an internal long repeat (IRL). A deletion and duplication produced these changes in AD169.

The coding region for gpTRL12 is located within the repeat sequences flanking the long unique segment of the viral genome (terminal repeat long (TRL) and internal repeat long (IRL)). In the laboratory adapted HCMV strain AD169 the reading frame is present twice and termed TRL12 and IRL12, respectively. In contrast, all low passage clinical isolates examined so far contain only the TRL12 reading frame since large parts of IRL including IRL12 are replaced by unique coding sequences. Thus, besides the fact that extensively passages HCMV strains have lost a substantial part of the coding sequence, low passage isolates have the advantage that only a single copy of the TRL12 gene needs to be deleted. The integrity of TRL12 was conserved in all HCMV strains during these deletion/recombination/inversion events, suggesting an important function of TRL12 for replication.

The ORF TRL12 of AD169 codes for a protein of 416 amino acids with a theoretical molecular mass of 47,4 kDa. Computer predictions classify the TRL12 polypeptide as a typical type I glycoprotein with a signal sequence between amino acids 1 and 30 and a membrane anchor between residues 370 and 390 **(Fig. 5)**. The predicted primary amino acid sequence of TRL12 AD169 contains 17 motifs for addition of N-linked carbohydrate modification and several motifs for O-linked glycosylation in the extraluminal domain of TRL12.

The short cytoplasmatic tail contains the motifs, YXXΦ that is known to be implicated in endosomal/lysosomal targeting.

The extracellular domain contains a region encompassing ~ 120 amino acids, containing several cysteins residues, which may be involved in disulfide bonding. This region has been found to be similar to the CR domain of adenovirus E3 proteins which has an immunoglobulin-like (Ig-like) fold (INTERPRO IPR013783) (**Fig. 6B**). Domains with an Ig-like fold consists of a beta-sandwich of seven or more strands arranged into two sheets



*MRVACRRPHHLTYRHTAYTIIIFYILHRVT*CNSTTTNTASITSPNTASTTFVTSVFSTPN
NNTSTTPHTSVTSQASTIGNITNVTSDLSTFTTVYSTFNTSYANISNTAATTELI
STNTNTILSFTNVTANATSSYNTTITVTITSDETSHNVSTNTALISTPWLTNCSA
TTYTTYNRTNSSNACHTETTIIRFKETNTTGIEGSNVTIKGNSTWDCLSVAWIR
HYNRSTHGHHLGHRKNAHTQSWYWLRILTSHTVCHSQHERPSLYHDLCRSC
NNTELHLYDLNITNSGRYSRRCFKENYFTGHHEDENFYLLVTPKNHTEAINAT
FVCPRYNTDIENEDREKGSQHTNNTHHHKRNLYHSSQRSRTVWTIVLVCMA
CIVLFFARRAFNKKYHMLQDTVSESEFIVRYHTEHED

FIGURE 5. Genomic localization of ORF TRL12 and structural prediction of the protein product. **A)** Localization of TRL12 within the genomes of HCMV strains. **B)** Computer prediction of the hydrophobicity. The potential signal and anchor membrane sequences are indicated by boxes. **C)** Amino acid sequence of AD169 TRL12. The putative signal peptide is shown in italic; the putative transmembrane region is underlined; the boxed sequence indicates the putative Ig-domain of TRL12.

usually linked by conserved Cys residues (**Fig. 6C**). One feature of Ig-like domains is that they contain relatively few highly conserved residues. These domains are often involved in interactions.

2. ANALYSIS OF TRL12 POLYMORPHISM

Reading frame TRL12 from 30 cytomegalovirus strains has been amplified and sequenced. Comparison of sequences showed that this locus is highly variable between different HCMV isolates. Phylogenetic analysis, performed on nucleotide and amino acid sequences, revealed that the identified variants are not randomly distributed, but are clearly clustered in 4 distinct groups (**Fig. 6A**) The four main groups diverged on the amino acid level in the rage of 60-70% and showed a high level of amino acid identity (80-100%) among strains within each group.

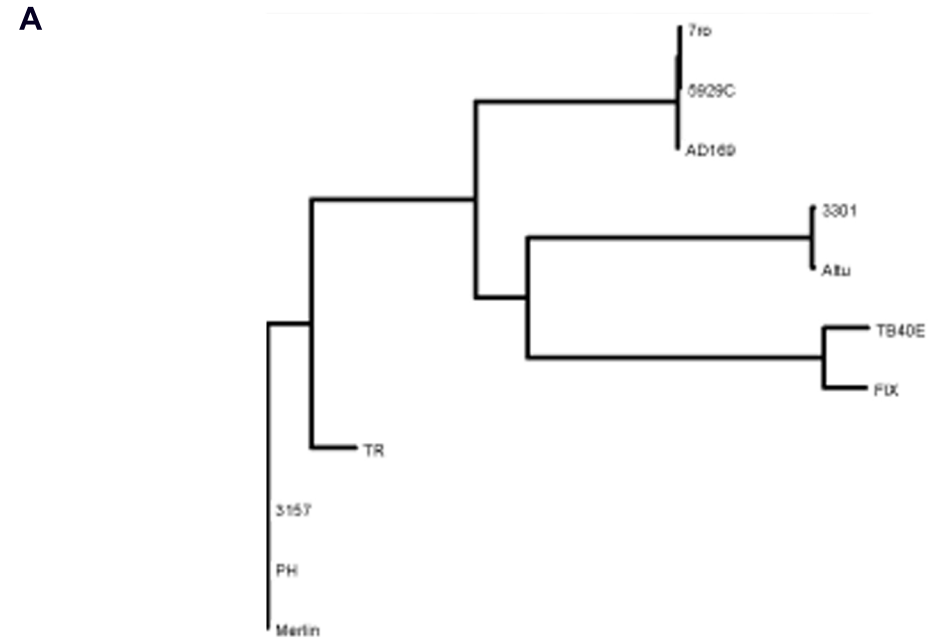
Alignment of TRL12 amino acid sequences of clinical strains and comparison using the AD169 sequence as the arbitrary reference sequence, showed that amino acid variation is dispersed over all the protein sequence (**Fig. 7**). In particular, high variability is evident in the N-terminal region, predicted to be the extracellular domain of the protein in which are concentrated most of post-translational modification sites (N- and O-glycosylation sytes).

Despite the impressive variability of the amino acid sequence, the Ig-like domain is conserved in its structure as well as the number and the position of cysteine residues confirming that there is a very tight constrain upon this domain (**Fig. 6B**).

3. CLONING, RECOMBINAT EXPRESSION OF pTRL12 AND INTRACELLULAR DISTRIBUTIN OF THE PROTEIN

To analyze the protein product of ORF TRL12 in the absence of viral infection, the coding sequence from strain TR was inserted into the vector pcDNA3myc/his, allowing the synthesis of a pTRL12 protein containing an epitope-tagged carboxyl terminus. Furthermore, the TRL12 coding sequence was inserted into the vector pcDNA-sig-HA, containing the TRL12 signal sequence cloned in frame with the HA epitope. The resulting plasmid, pcTRL12-sig-HA, encoded full-length TRL12 fused to a N-terminal HA tag (**Fig. 8A**).

Following transient transfection in COS7 cells, the protein was analyzed 24h and 48h after transfection by indirect immunofluorescence (IIF) using a Myc-specific monoclonal antibody and/or a HA-specific monoclonal antibody.



B

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(193) 193       200       210       220       230       240       250       260       270       280       290       300       310       320       338
AD169 (190) TIIRFKETNTTGIEGSNVTIKGNSTW-DCLSVAWIRHYNRSTHGHHLGHRKNAHTQSWYWLRILTSHTVCHSQHERPSLYHDLCRSCNN-TELHLYDLWITNSGRYSRRCFKENYF-TGHHEDENFYLLVTPKNHTEAINA-TFVC
3301 (172) KPIKVNKTDIKAEEWTNVTIQSNFTIPHCHKVVWIRQYNLTTHGDYFPTRYKRPFVKGA---LYSREICGHTYTHNLLHSYDLCISCDN-GTLHLYGVNTTHSGRYTARCHIYEHNNHGTHEDKNFNLIIYPRNNTNNTNG-IWIC
TB40E (181) TISQESETLLKAIQGDNITIIHNLTTTSCYKTAWLRHFNISTHRKYTHPNIKSGKFSNHSLKILHSRVLCEWQTHYLKHHYDLCFTCDQNLSLSLYGLNFTHSGKYSFRCYKSGHP---SEQNQNFNLQVHPRNNTNETHVNPWIC
TR (182) ETIPVKSTPLTAEEGTNITIHGNDTW-DCPDVVWYRHYNWSTHGHHIYPNTHYKTLIHR-RKILTSHPICYSDRSSPTAYHDLCRSCNK-TELRLYDLNTTNSGRYSRRCYKQYHH-QGPHEDENFGLTVNPRNNTDNYTI-P-VC
Consensus (193) TIIKVKET LTAIEGTNITI GN TW DCHKVVWIRHYNISTHGHHIHPR KA TLS H LKILTSHIICHSQTH PSHYHDLCRSCNN TELHLYGLNTTNSGRYSRRCYK H GSHEDENFNLIVHPRNNTNNTNI PWIC
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C

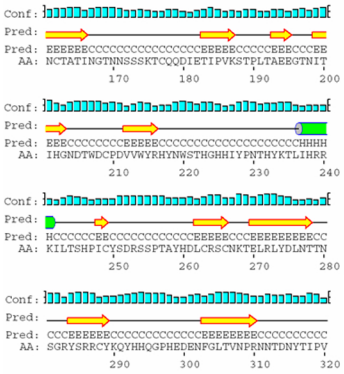


FIGURE 6 **A**) Results of phylogenetic analysis of 11 TRL12 protein sequences representative of 30 sequences. The tree was generated using MegAlign software. **B**) Alignment of the Ig-like domain in 4 TRL12 sequences representative of each genotype. **C**) Schematic rapresentation of secondary structure of TRL12 (strain TR) Ig-like domain. Prediction at http://bioinf.cs.ud.ac.uk/psiform.html

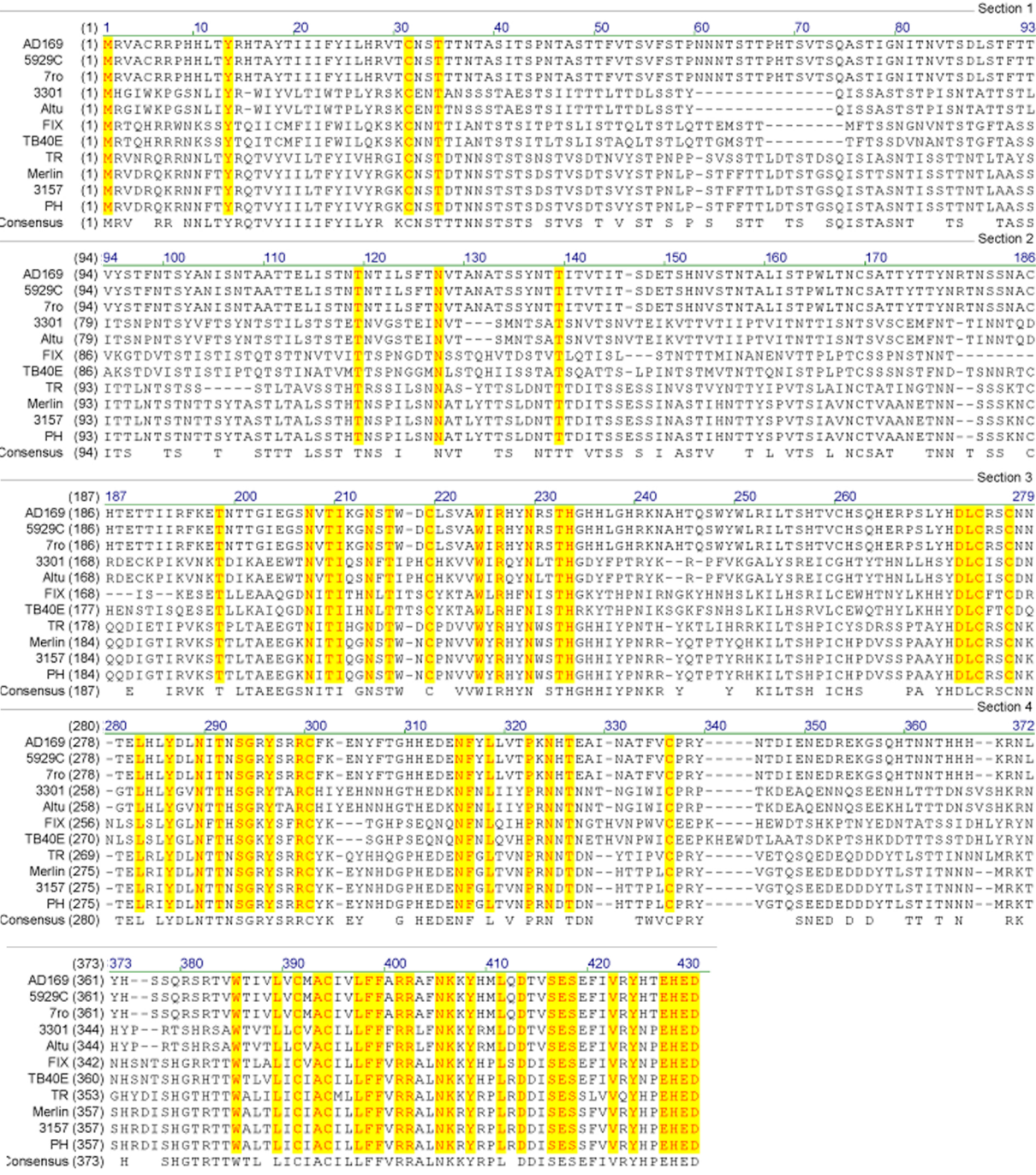
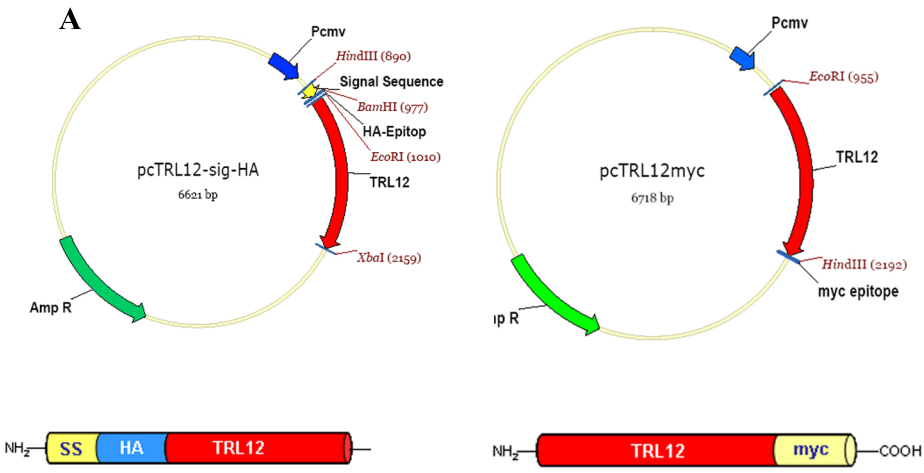


FIGURE 7. Alignment of TRL12 amino acid sequence of HCMV strains representative of different genotypes and comparison using the AD169 sequence as the arbitrary reference sequence.Identical residues are highlighted in yellow. The amino acid sequences were aligned using the ClustalW method.



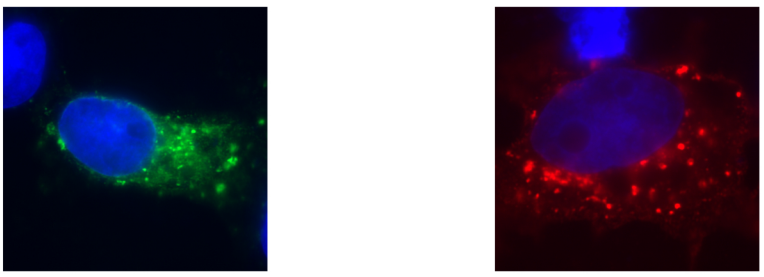


Figure 8. COS7 cells were transfected with pcTRL12-sig-HA and/or pcTRL12myc vectors (**A**). **B**) 24 h post transfection cells were fixed and processed for immunofluorescence with monoclonal Ab anti-HA. **C**) Surface staining of TRL12-HA.

The protein pTRL12 shows an exclusively cytoplasmatic localization. The intracellular staining patter of pTRL12 detected with anti-HA and -Myc antibodies were indistinguishable. With both antibodies a perinuclear vesicles and tubular compartment and numerous vesicles in the periphery of the cell were stained. The staining was mainly associated with very irregular granules or vesicle-like structures that were especially condensed near the nucleus (**Fig. 8B**). In cells expressing very high amounts of pTRL12-HA, cell surface expression of pTRL12 was detected in immunofluorescence by using monoclonal antibody anti-HA (**Fig. 8C**).

To better define the intracellular distribution of pTRL12, has been used transient expression of pHA-TRL12 followed by fluorescence imaging with antibodies directed against the HA-tagged viral protein and against cellular markers of the secretory pathway (**Fig. 9**)

Hardly any colocalization with endoplasmic reticulum (ER) marker calreticulin was found. Recombinant pTRL12 had a vesicular staining patter that largely colocalize with TGN46, a marker of the *trans*-Golgi network, that shows an altered morphology comparable to that observed for several HCMV glycoproteins during the infection. Minimal overlap of the signal with markers for the Golgi (GM130) was found. The ability of recombinantly expressed pTRL12 to mediate its own localization to the TGN demonstrates that HCMV pTRL12 contains all of the *cis*-acting elements necessary for TGN localization. Inspection of the pTRL12 cytoplasmic domain for the presence of trafficking motifs that are known to function in others viral proteins reveals a number of motifs that may be involved in the intracellular trafficking of HCMV pTRL12.

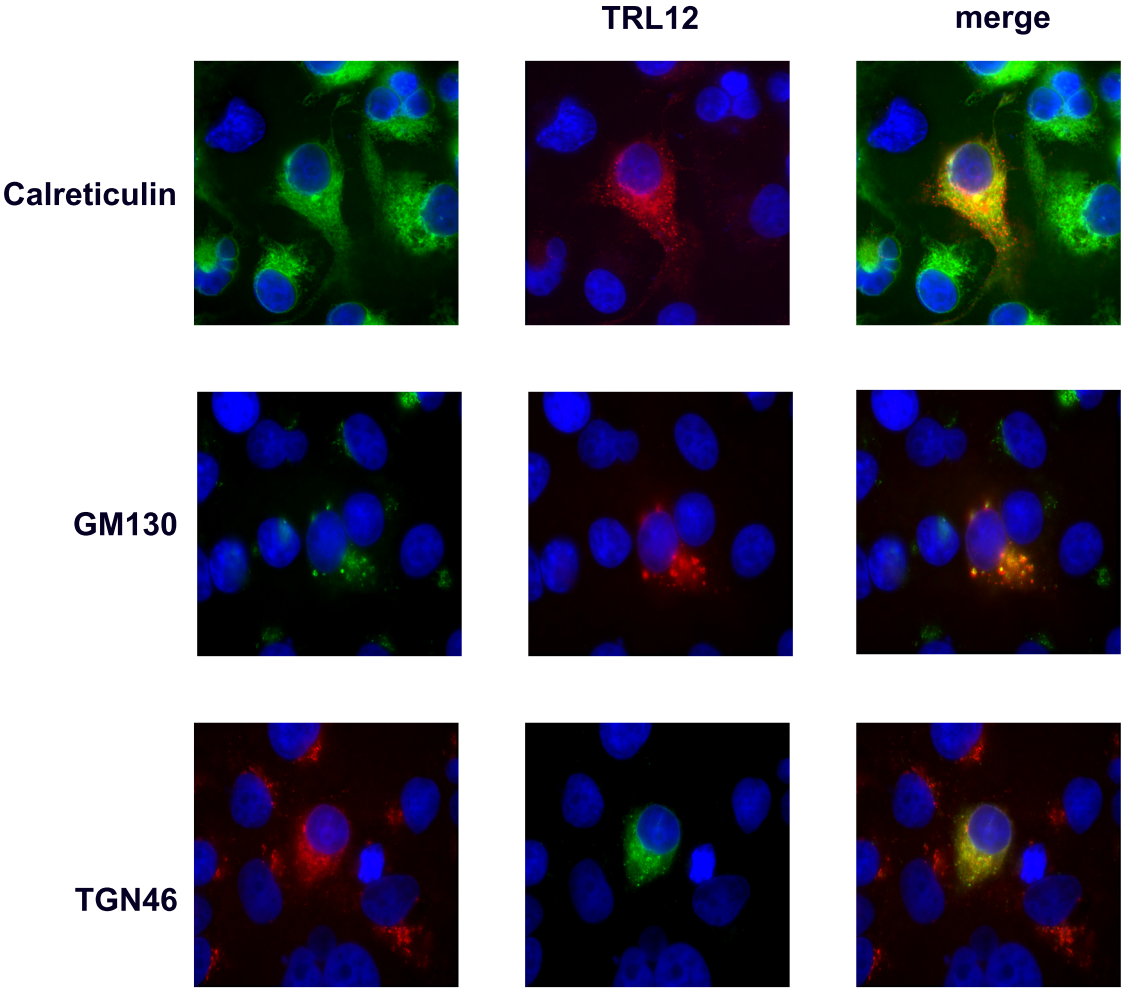


FIGURE 9. Intracellular localization of pTRL12 in transfected cells. COS7 cells were transfected with pcTRL12-sig-HA. Intracellular localization was determined by comparing the signal from HA-specific Ab (pTRL12) with those of antibodies for components of the secretory pathway (endoplasmic reticulum, calreticulin; Golgi, GM130; *trans*-Golgi network, TGN46). The appearance of yellow in the merged pictures indicates colocalization of signals. Cell nuclei are stained with DAPI:

4. TRL12 PROTEIN EXPRESSION IN INFECTED CELLS

Immunoblot analyses were carried out to characterize the expression of pTRL12 in infected cells. Fibroblasts were infected with wild-type strain TR and mutant virus del-TRL12 and cell lysates were prepared 96 h post infection. In infected cells, one major signal at 97-150 kDa was observed using a mouse anti-TRL12 serum raised against the carboxyl terminus of the protein (see Materials and Methods). Control analysis using a monoclonal antibody specific for gB (p27-287) showed the expected molecular-weight. (**Fig. 10A**)

The theoretical molecular mass of TR-TRL12 is 47,4 kDa. Thus, the 97- to 150-kDa protein most probably represents fully modified gpTRL12, carrying N-linked and O-linked carbohydrates. The diffuse migration as well as the presence of multiple bands, however, indicated that the protein carried some modification.

To obtain more information about the nature of the 97- to 150-kDa protein it was performed an immunoprecipitation analysis using lysates of cells infected with a recombinant virus TR-TRL12-Myc expressing gpTRL12 containing a Myc epitope tag at the carboxyl terminus (the construction of recombinant viruses is described collectively below). As shown in (**Fig. 10B**), the protein recognized by an anti-Myc monoclonal antibody were identical to the signals obtained with the polyclonal anti-TRL12 mouse serum.

A hallmark of many HCMV glycoproteins that have been identified thus far is the formation of disulfide-linked homomeric or heteromeric protein complexes. Therefore, complex formation of gpTRL12 was analyzed in cells infected with the recombinant virus TRL12myc. Immunoprecipitation analysis of viral lysates carried out in the absence of reducing agents showed a migration pattern of gpTRL12 (**Fig. 10B**) that was unchanged compared to that under reducing conditions observed in western blot (**Fig. 10A**), indicating that gpTRL12 is not part of a disulfide-linked complex.

4.1 Intracellular localization of gpTRL12 in infected cells.

To define the intracellular localization of gpTRL12 in infected cells, fibroblasts infected with recombinant virus TR-TRL12-Myc. Since the anti-pTRL12 antiserum was less sensitive and gave a higher background than the monoclonal anti-Myc antibody; therefore it was not possible to detect the untagged protein. The cells were infected for 72 h and used for fluorescence imaging with antibodies directed against the Myc epitope. In infected cells

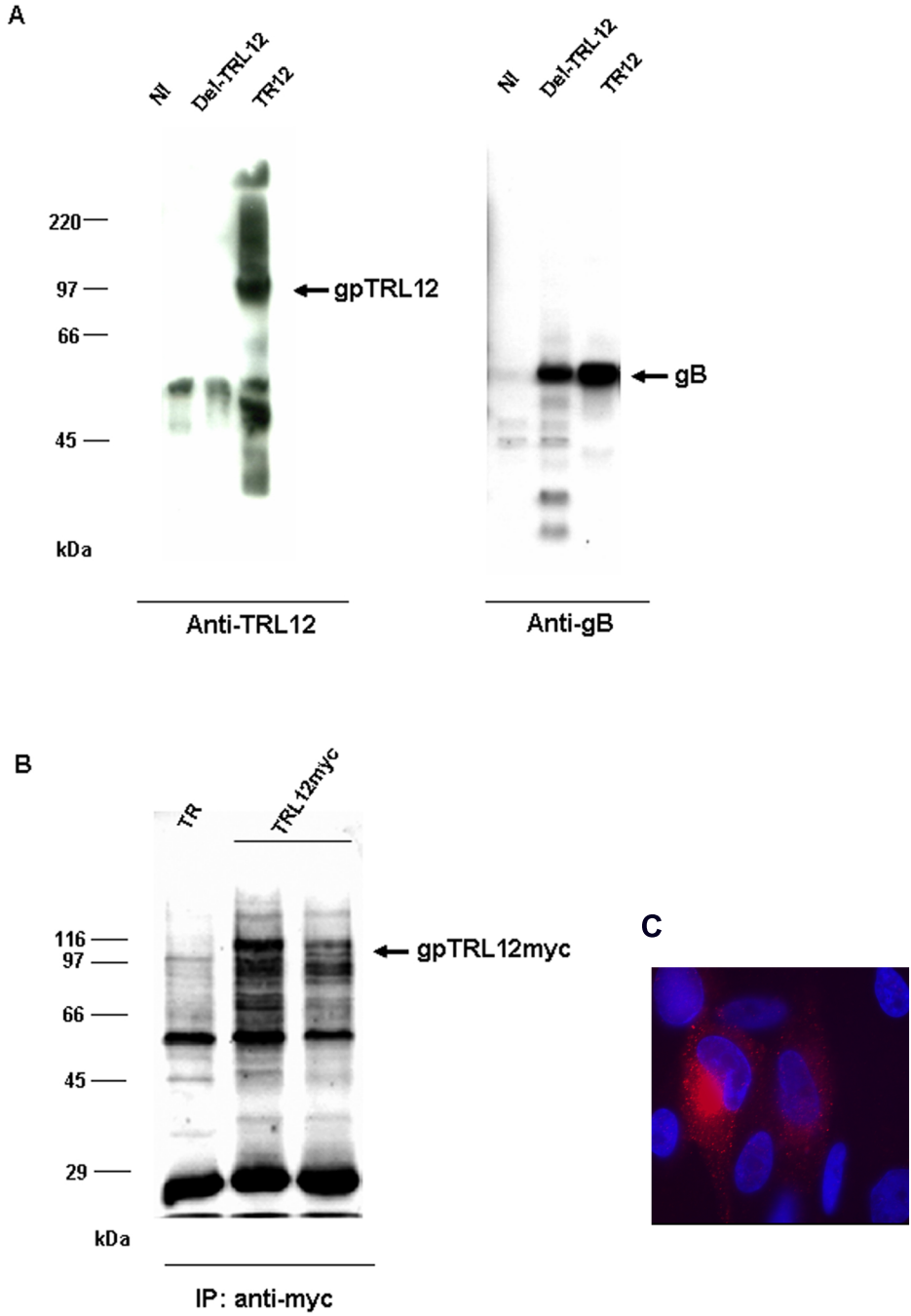


FIGURE 10. gpTRL12 is present in lysates of infected cells. **A**) Lysates of non infected cells (NI), infected cell with TR12 (TR12 lane) and Del-TRL12 (Del-TRL12 lane) viruses were analyzed by western blot using Abs directed against gpTRL12 (anti-TRL12) and gB (anti-gB; p27-287). **B**) Lysates of cells infected with TR12 virus and TRL12myc recombinant virus were analysed by immunoprecipitation using monoclonal Ab anti-myc. **C**) Intracellular localization of gpTRL12myc in infected cells. Fibroblasts were infected with the recombinant virus TRL12myc for 72 h and processed for immunofluorescence with monoclonal Ab anti-myc.

gpTRL12myc accumulated in a juxtanuclear location, corresponding to the *trans*-Golgi network, which has been argued to constitute the virus assembly compartment [**8**]. (**Fig. 10C**)

5. CONSTRUCTION OF TRL12 RECOMBINANT VIRUSES.

Aim of these experiments was to characterize the importance of pTRL12 in the viral replication cycle. To this we generated a deletion mutant in a low passage HCMV isolate (TR) for two main reasons: (i) AD169, the laboratory adapted HCMV strain does not contain the full set of genes characteristic for HCMV; (ii) the ULb' region, which is missing in AD169, encodes a number of glycoproteins which potentially could interact with pTRL12.

In order to investigate the role of pTRL12 in the replication of HCMV, a series of recombinant viruses were generated, using a strategy that includes the use of an infectious clone of HCMV maintained as a bacterial artificial chromosome in *E. Coli*. All manipulations were carried out in bacteria and mutations are introduced into the HCMV BAC using linear PCR fragments. This methodology takes advantage of the lambda phage recombination system (*RED* locus) expressed from an inducible promoter that enables the use of a linear single-stranded DNA to target genes of interest carried on BACs maintained in *E Coli*.

5.1 Generation of BAC-delTRL12-TR

To elucidate the role of pTRL12 in HCMV replication, a deletion mutant lacking the entire TRL12 open reading frame was generated.

To generate a TRL12 null mutant, BAC technology has been employed [**145**]. The basic strategy is to replace the TRL12 locus in the HCMV genome (TR-BAC) with a selectable antibiotic resistance gene that is generated by PCR using primers with homology extensions. The PCR fragment was electropored into bacteria harboring the HCMV TR genome as BAC as well as the pKD46 plasmid encoding the recombination functions of phage λ (for a detailed description, see Materials and Methods). The predicted recombination product would have the ORF TRL12 deleted and replaced by the kanamycin resistance gene.

The TR-BAC was preparated from the TR clinical isolate. The BAC clone was generated by substituting BAC cassette for the US2-US5 region of the TR genome by using the method of Borst et al.[**120**]. TR-BAC carries the chloramphenicol resistance cassette.

The PCR fragment was generated by using primers with 50-nt extensions that are homologous to regions adjacent to the gene to be deleted and 30-nt priming for the template plasmid, pCP-o15, carrying the kanamycin resistance gene that are flanked by FRT (FLP recognition target) sites (**Fig. 11**)

PCR products were gel-purified, treated with *Dpn*I and then transformed into bacteria carrying the TR-BAC and the Red helper plasmid (pKD46). Insertion of the cassette resulted in the deletion of the entire TRL12 ORF except for 60 bp of the 5'end and 190 bp of the 3'. Colonies carrying BACs with the ORF TRL12 deleted were isolated after overnight incubation at 37°C on Cm/Km selective plates. Two BAC cloneS, designed *BAC-delTRL12-TR* were further characterized.

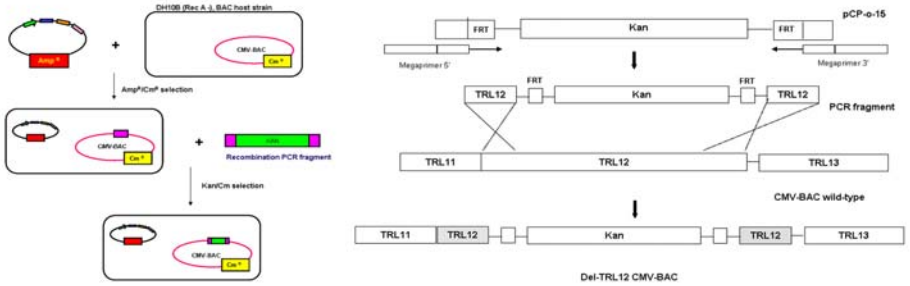


FIGURE 11. Schematic overview of BAC mutagenesis using the helper plasmid (left). Generation of PCR recombination fragment using megaprimers with 50-nt extention that are homologous to regions adjacent to the gene to be deleted and 30-nt priming for the template plasmid, pCP-o15.

5.2 Characterization of BAC-delTRL12-TR

The DNA of the resulting BAC was analyzed for overall integrity by restriction analysis, PCR analysis and nucleotide sequencing in the interested genomic region.
BAC-DNA from both the wild-type TR and the recombinant BAC-delTRL12-TR was isolated, digested with *Bgl*II and *Eco*RI and analyzed on a 0,6% agarose gel and restriction patterns were compared in order to verify that no spurious rearrangements had occurred during the recombination process (**Fig. 12A**).
In BAC-delTRL12-TR, replacement of TRL12 with the kanamycin cassette caused an additional *Bgl*II restriction fragment since the kanamycin gene from pCP-o-15 contains a single *Bgl*II site (**Fig. 12C**).

PCR analysis over the BAC-DNA from both the wild-type and the recombinant BAC-delTRL12-TR showed the correct replacement of the ORF TRL12 by the kanamycin gene. PCR analysis was performed using the pair primers: *TRL11EcoRIs(59)*/*TRL13BamHI as (62)*, *TRL11EcoRIs(59)*/*TRL12TRrec3(53)*, *TRL11EcoRIs(59)*/*Kan2,5(63)*. With the first set of primers, a 2,2 Kbp fragment was amplified from the recombinant BAC-delTRL12-TR as predicted due to the presence of the kanamycin cassette and a 1,7 Kbp PCR product was obtained from the parental BAC-TR. With the second set of primers, a 1,9 Kbp fragment was predicted for the recombinant BAC-delTRL12-TR and a 1,3 Kbp fragment for the wild type BAC-TR. With the third set of primers, a 0,5 Kbp fragment was predicted for the recombinant BAC-delTRL12-TR; no amplification product for the wild-type BAC-TR was predicted since the primer Kan2,5 anneals specifically to the kanamycin cassette sequence (**Fig. 12B**).

Taken together, the results of restriction, PCR and sequencing analyses showed that recombination events took place at the correct sites of the HCMV genome.

5.3 Generation of recombinant myc-tagged virus: BAC-TRL12myc-TR

To create BAC-TRL12myc a PCR fragment was generated using HCMV/Kanamycin hybrid primers as described above. The 5′ primer contained an additional sequence coding for the *Myc* epitope (AEEQKLISEEDLL). This PCR fragment was used for recombination in BAC-TR12 as described above. PCR products were gel-purified, treated with *Dpn*I and then transformed into bacteria carrying the TR-BAC and the Red helper plasmid (pKD46).

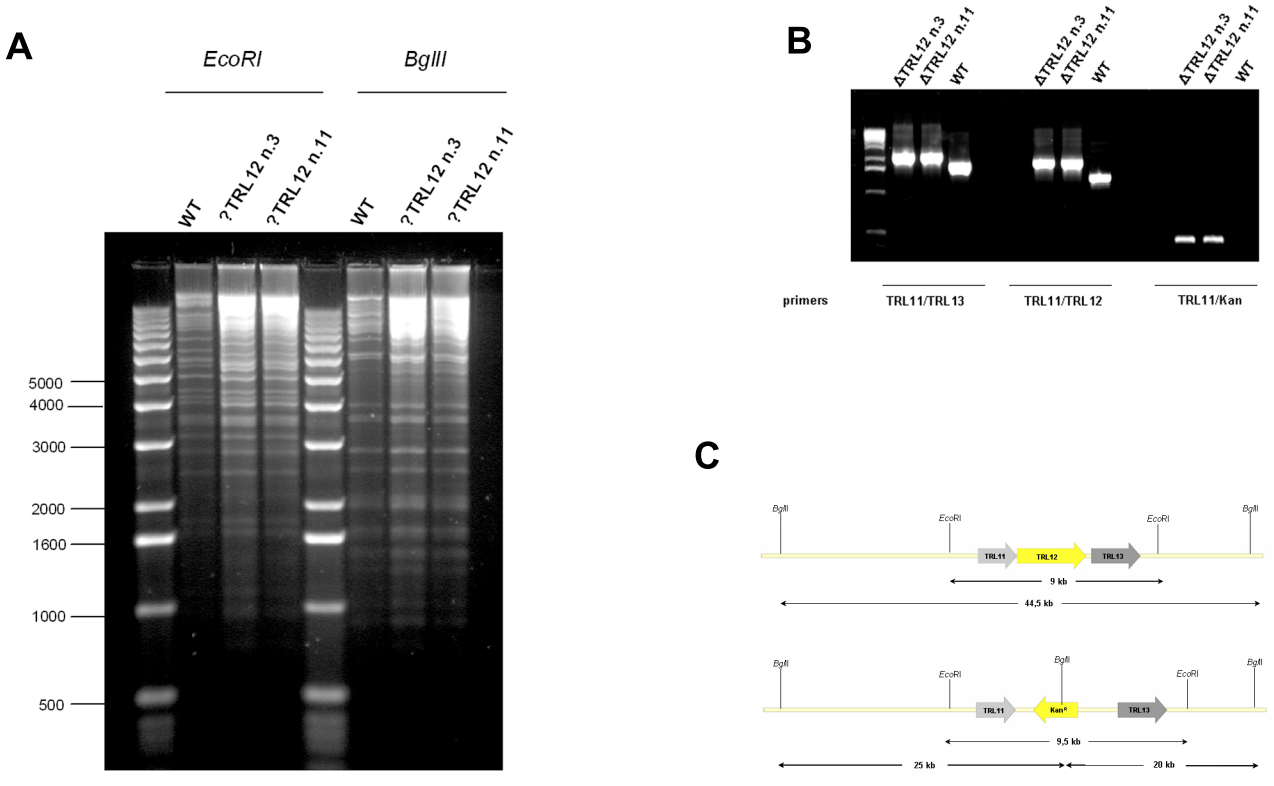


FIGURE 12. Structural analysis of HCMV BAC plasmids. **A**) *Eco*RI and *Bgl*II restriction of wild-type TR (WT) and deletion mutants (Del-TRL12 clone n.3 and Del-TRL12 clone n11) BACs. The scheme in **C** shows the *Eco*RI and *Bgl*II restriction sites in relation to the relevant genomic segments of HCMV. **B)** For verification of correct recombination sites within the HCMV genome, PCR analysis of bacterial clones harbouring the indicated BACs were performed using pair of primers TRL11/TRL13, TRL11/TRL12 and TRL11/Kan.

Insertion of the cassette resulted full-length TRL12 fused to a C-terminal Myc tag. Colonies carrying BACs with the ORF TRL12 fused with Myc tag were isolated after overnight incubation at 37°C on Cm/Km selective plates. Two BAC clones, designed *BAC-TRL12myc-TR* were further characterized.

5.4 Characterization of BAC-TRL12myc-TR

The DNA of the resulting BACs were analyzed for overall integrity by restriction analysis, PCR analysis and nucleotide sequencing in the interested genomic region.
BAC-DNA from both the wild-type TR and the recombinant BAC-TRL12myc-TR was isolated, digested with *Bgl*II and *Eco*RI and analyzed on a 0,6% agarose gel and restriction patterns were compared as described above (**Fig. 13A**).
In BAC-TRL12myc-TR, insertion of the kanamycin cassette caused an additional *Bgl*II restriction fragment since the kanamycin gene from pCP-o-15 contains a single *Bgl*II site.
Insertion of the Myc epitope sequence at the C-terminal of TRL12 caused an additional *Eco*RI restriction fragment (**Fig. 13B**).

PCR analysis over the BAC-DNA from both the wild-type and the recombinant BAC-TRL12myc-TR showed the correct insertion of the Kanamycin cassette. PCR analysis was performed using the primers: *RecTRL12myc-as (42)* and *TRL12-TR-BamHI (85)*. A 1,8 Kbp fragment was amplified from the recombinant BAC-TRL12my-TR as predicted due to the presence of the kanamycin cassette. As predicted, a 350 bp PCR product was obtained from the parental BAC-TR. Sequencing analysis showed the in frame insertion of the Myc epitope at the C-terminal of TRL12 (**Fig. 13C**).
Taken together, the results of restriction analysis, PCR and sequencing showed that recombination events took place at the correct sites of the HCMV genome.

5.5 Reconstitution of infectious TRL12 recombinant viruses

To test whether the recombinant BACs could be reconstituted to infectious viruses, BAC DNA was purified from BAC-containing *E.Coli* and transfected into MRC-5 cells to reconstitute recombinant virus. Cotransfection of a pp71 expression plasmid led to a 30-fold enhancement in plaque formation as described [**10**]. One week after transfection, the cells

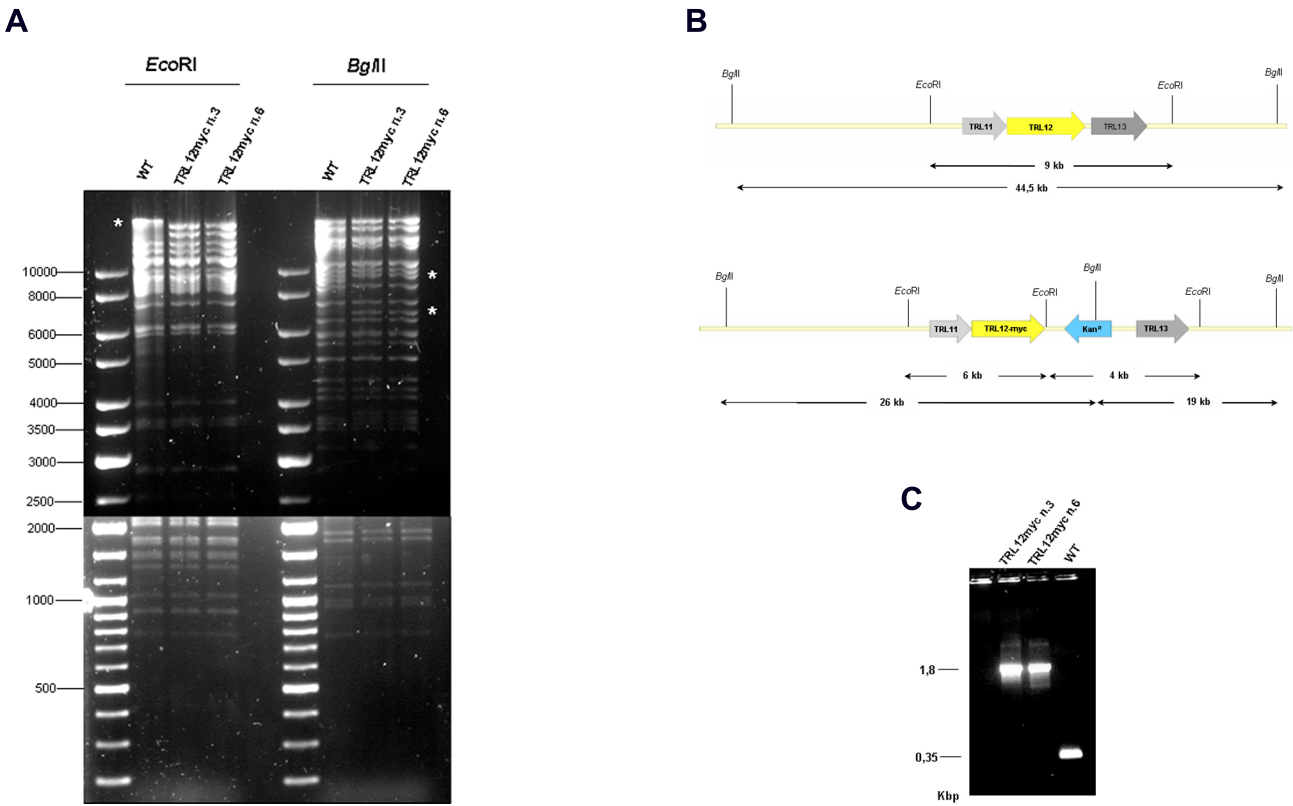


FIGURE 13. Structural analysis of HCMV plasmids. **A**) *Eco*RI and *Bgl*II restriction of wild-type TR (WT) and recombinant (TRL12myc clone n.3 and TRL12myc clone n.6) BACs. The scheme in **B** shows the *Eco*RI and *Bgl*II restriction sites in relation to the relevant genomic segments of HCMV. **C)** For verification of correct recombination sites within the HCMV genome, PCR analysis of bacterial clones harbouring the indicated BACs were performed using an opportune pair of primers.

were transferred to 2-cm^2 flasks and cultured for about 2 weeks until plaques appeared. Particles could be reconstituted from the recombinant BACs, indicating that the TRL12 ORF is not absolutely necessary for viral growth.

6. GROWTH PROPERTIES OF TRL12 MUTANT VIRUS

6.1 Analysis o f plaque formation and virus spread

Concomitant with productive infection, HCMV induces cytopathogenic effects (CPE) in tissue cultures of various origins, which include cell rounding, cell enlargement, formation of inclusion bodied and lysis of infected cells.

Most HCMV strains require adaptation to HF cells in culture before they replicate efficiently in these cells. Recent clinical isolates are distinguished from cell culture adapted strains by lack of release of cell-free infectious progeny from infected cells. The progeny virus is highly cell-associated and development of CPE or plaques can take as long as several weeks, requiring subculture of infected cells. In contrast, both AD169 and Towne form distinctive plaques in less than 7 days and a single round of replication resulting in cell free virus in the culture fluid requires 96h or less to complete.

In order to study the morphology and the size of plaque formation, human fibroblasts (HF) were infected with Del-TRL12 and TR viruses at multiplicity of 0,5 PFU/cell.. After 4 h the viral inoculum was removed, the cells were washed and subsequently incubated 10 days at 37°C until approximately 80% of the cells showed CPE.

In **Fig. 14A** a comparison of representative plaques in culture infected with Del-TRL12 and TR is shown. The morphology of the plaque induced by TR is characterized by the formation of small foci, a typical plaque morphology associated to a cell-associated spread; whereas, infection with Del-TRL12 leads to an even distribution of the infection through the cell culture.

In order to further investigate the plaque formation properties, fibroblasts were infected with Del-TRL12 and TR (multiplicity of infection of 0,5 PFU/ml) and assayed 3, 10 and 14 days post infection for expression of HCMV IE-1 by immunofluorescence. (**Fig. 14B**). At day 10 post infection, the TRL12 deletion mutant Del-TRL12 induced plaques significantly larger than those induced by the wild-type virus TR. At day 14 post infection the Del-TRL12 plaque extension might be consistent with the spread of infection via supernatant.

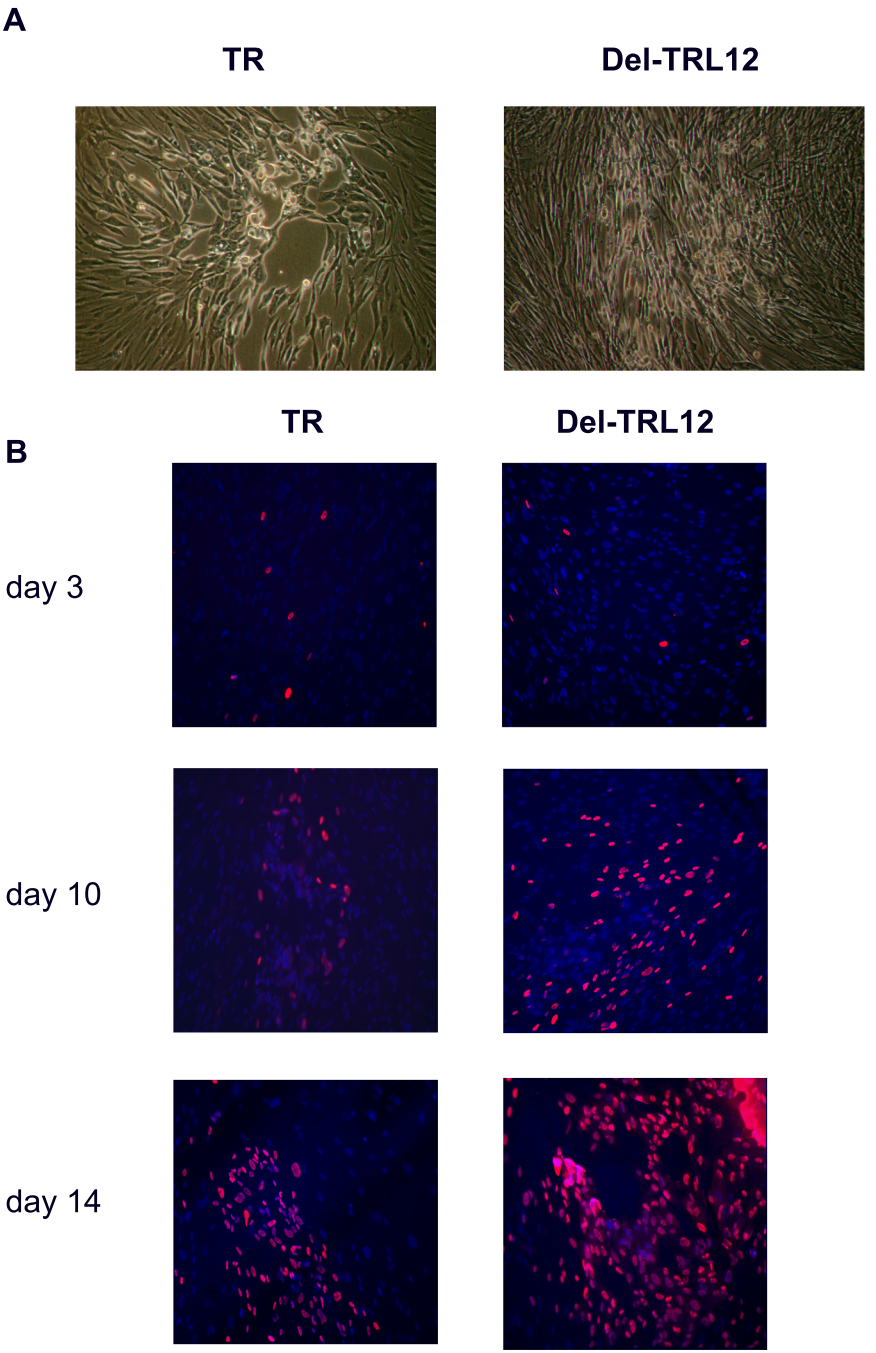


FIGURE 14. Analysis of plaque formation of deletion mutant Del-TRL12. **A**) Comparison of representative plaques in culture infected with wild type TR and deletion mutant Del-TRL12 viruses at 10 days post infection. **B**) Fibroblasts were infected with TR and Del-TRL12 viruses at multiplicity of infection 0,5. Cells were fixed and processed for immunofluorescence by using antibodies specific for the HCMV IE-1. Cell nuclei were stained with DAPI (blue). The purple color of nuclei indicates IE-1 staining.

6.2 Growth kinetic of Del-TRL12 virus

To investigate possible effects of TRL12 deletion on the kinetic of virus replication and to extend the findings of the plaque size determinations, multi-step growth kinetic of the engineered mutant virus, Del-TRl12, were performed. The extremely low to non-existent infectivity present in the

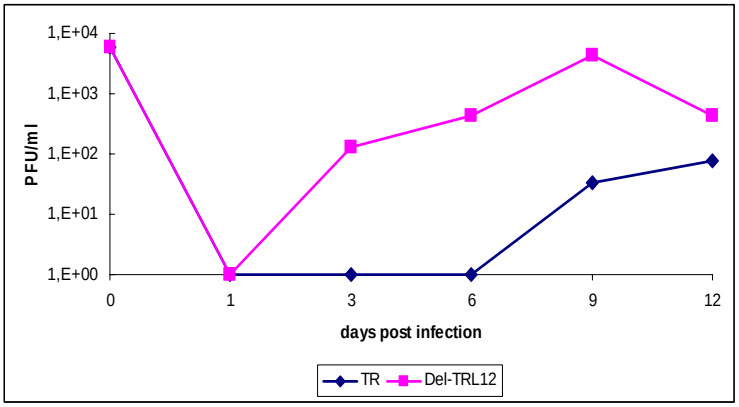


Figure 15: Replication of TRL12 mutant virus. Fibroblasts were seeded in six-well dishes and infected with TR and Del-TRL12 viruses (multiplicity of infection, 0.5). At the indicated days post infection, cells were harvested and intracellular particles were isolated and titrated using indirect immunofluorescence with an antibody directed against the immediate-early antigen 1. Each data point represents the mean of three independent infected cultures.
TR: wild type strain; Del-TRL12: recombinant virus lacking ORF TRL12.

tissue culture supernatant of TR strain made it impossible to analyze the replication kinetics using extracellular virus. Therefore, intracellular particles were isolated for this assay (see Material and Methods). Preparations from both TR and Del-TRL12 contained sufficient infectious virus to perform multistep growth curves. Fibroblasts were infected with identical amounts of infectious virus (MOI.= 0,5) from either the parental virus TR or the mutant Del-TRL12. The virus titers from cells infected with TR and Del-TRL12 were determined over a period of 12 days. Experiments were done in triplicate and the results are summarized in **Fig. 15**.

Strikingly, the final virus yield from cells infected with Del-TRL12, obtained after 12 days of infection, was increased approximately 10-100 fold as compared to wild type parental virus TR. At day 9 post infection, the deletion mutant Del-TRL12 reached a titer of 4,5 x 10^3

PFU/ml and declined thereafter, whereas the parental strain TR reached titers of approximately 50 PFU/ml that slightly increased at the day 12 post infection.

The number of HCMV immediate early protein-positive nuclei was comparable 24h after infection indicating that the infection rate of fibroblasts was not affected by TRL12 gene. These results indicated that the deletion of ORF TRL12 from the genome of low-passage HCMV strain accelerated and increased virus production in fibroblasts. Then, the locus TRL12 is detrimental to cytomegalovirus growth in fibroblasts.

6.3 DNA accumulation after infection with Del-TRL12

Viral DNA accumulation during infection has been analyzed by real-time PCR.
Intracellular particles from TR and Del-TRL12 corresponding to 6 x 10^3 PFU/ml were used to infect fibroblasts. Viral DNA copy numbers were analyzed on days 1, 3, 6, 9, 12 after infection by quantitative real-time PCR, both in the supernatant and in the cellular pellet.

As can be observed in **Fig. 16** the ratio of viral DNA copies to infectious intracellular particles did not differ considerably between the two preparations (6 x 10^6 for TR and 10^7 for Del-TRL12). The relative ratio of viral DNA to infectivity suggests that the viral inoculum of Del-TRL12 contained the same number of infectious particles as the wild-type virus TR.
Consistent with the results observed for the replication kinetic of Del-TRL12, the increase of Del-TRL12 infectious titers observed in **Fig. 15** is reflected by an increase in intracellular viral DNA accumulation. As shown in **Fig. 16A**, at days 9 at 12 post infection, the number of viral genome copies from cells infected with Del-TRL12, was increased approximately 100 fold as compared to wild type parental virus TR.
Strikingly, as can been observed in **Fig. 16B**, disruption of TRL12 resulted in release of viral progeny to the medium, in contrast to the wild-type virus TR that, being a recent clinical isolates, is characterized by more cell-associated spread and by lack of release of cell-free infectious progeny from infected cells. It is further interesting to note that, at day 12 p.i., the slight drop in cell-associated viral yield (**Fig. 15**) and viral DNA copies (**Fig. 16A**) for Del-TRL12 virus is associated to an increased release of viral genome (**Fig. 16B**) and virus progeny (data not shown) to the medium.
Deletion of TRL12 resulted in a 10-100 fold increase in intracellular particles and a concomitant enhanced release of virus to the medium in HF culture. The results of growth

kinetics and DNA accumulation are largely a reflection of virus-induced plaque size because the number of infected cells are dependent on the size of the plaque.

A) **B)**

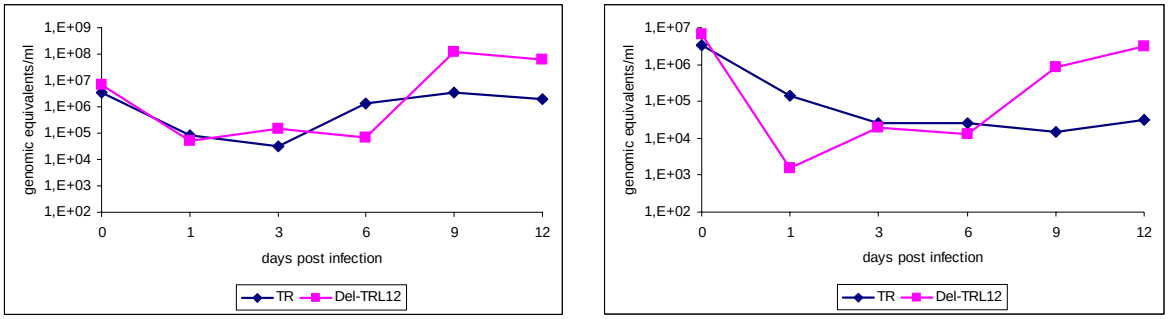


Figure 16. Analysis of viral DNA accumulation in cellular pellet (**A**) and in the supernatant (**B**) during infection with TR and Del-TRL12 viruses. Intracellular particles from TR and Del-TRL12 corresponding to 6 x 10^3 PFU were used to infect fibroblasts. Viral DNA copy numbers were analyzed on days 1, 3, 6, 9, 12 after infection by quantitative real-time PCR, both in the supernatant and in the cellular pellet.

6.4 Analysis of MCP, pp150, gB and gH expression in cells infected with Del-TRL12

Fibroblasts cells were infected with wild-type virus TR and deletion mutant Del-TRL12 at multiplicity of infection 0,5 PFU/cell and were processed at 72 h post infection with antibodies specific for capsid protein (MCP), tegument protein (pp150) and envelope proteins (gB and gH). All these proteins appeared to localize in the same region in wild type and deletion mutant infected cells. Therefore, the expression and localization of capsid, tegument and envelope proteins is not dependent on gpTRL12 accumulation. (**Fig. 17**)

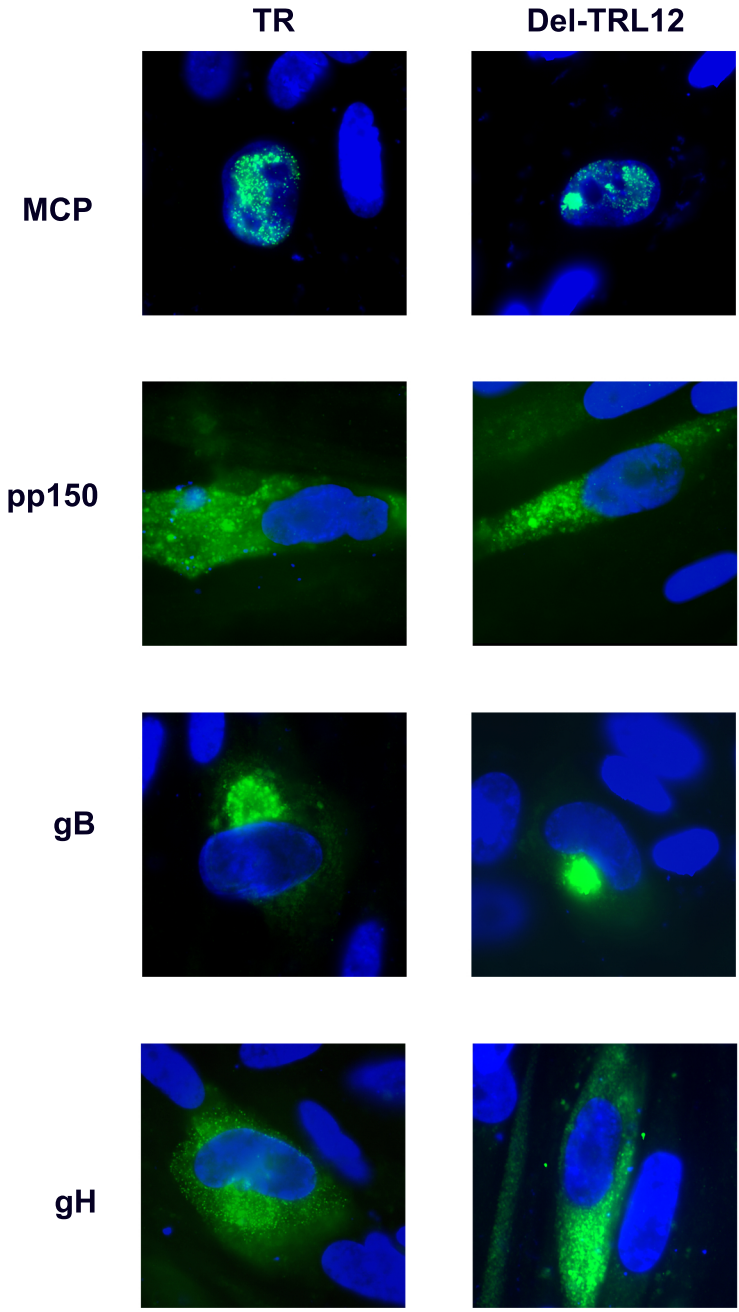


FIGURE 17. Localization of viral proteins expressed in fibroblasts infected with wild-type TR and deletion mutant Del-TRL12 viruses. Cells were infected with TR and Del-TRL12 at multiplicity of infection 0,5 PFU/cell and processed for immunofluorescence at 72 h post infection with antibodies specific for capsid protein (MCP), tegument protein (pp150) and envelope proteins (gB and gH).

DISCUSSION

The coding capacity of human cytomegalovirus (HCMV) for glycoproteins by far exceeds that of other herpesviruses. Few of these proteins have been characterized so far. The gene product of reading frame TRL12 has been investigated.

The coding region for gpTRL12 is located within the repeat sequences flanking the long unique segment of the viral genome (terminal repeat long (TRL) and internal repeat long (IRL)). In the laboratory adapted HCMV strain AD169 the reading frame is present twice and termed TRL12 and IRL12, respectively. In contrast, all low passage clinical isolates examined so far contain only the TRL12 reading frame since large parts of IRL including IRL12 are replaced by unique coding sequences. Thus, besides the fact that extensively passages HCMV strains have lost a substantial part of the coding sequence, low passage isolates have the advantage that only a single copy of the TRL12 gene needs to be deleted. The integrity of TRL12 was conserved in all HCMV strains during these deletion/recombination/inversion events, suggesting an important function of TRL12 for replication. TRL12 does not have homologs in others herpesviruses. These proteins that are expressed only by individual genera of a herpesvirus subfamily are termed “private proteins”. These proteins likely play very critical roles in the in vivo replication and pathogenic behaviour of the respective viruses.

The putative protein product of TRL12 is a glycoprotein with a theoretical mass of 47,4 kDa. Transcription analysis (unpublished data) revealed that the gene is transcribed with a early-late kinetic from the laboratory-adapted strain AD169 and low-passage isolates.

Immunoblot analyses were carried out to characterize the expression of pTRL12 in infected cells. One major signal at 97-150 kDa was observed using a mouse anti-TRL12 serum raised against the carboxyl terminus of the protein.

Since, the theoretical molecular mass of TR-TRL12 is 47,4 kDa, the 97- to 150-kDa protein most probably represents fully modified gpTRL12, carrying N-linked and O-linked carbohydrates. The diffuse migration as well as the presence of multiple bands, however, indicated that the protein carried some modification. According to computer-based predictions, gpTRL12 (AD169 strain) carries 19 sites for addition of N-linked glycosylation (NetNGlyc 3.1 server; http://www.cbs.dtu.dk) and several motifs for O-linked glycosylation (NetOGlyc 3.1 server; http://www.cbs.dtu.dk). Additional modifications such as phophorylation, which has also been detected on other HCMV structural glycoproteins such as gB, could further add to the diffuse migration patter see in PAGE.

In infected cells, gpTRL12myc accumulated in a juxtanuclear location, similar to others HCMV glycoproteins such as gB and gpUL132. This compartment has been shown to contain proteins found in the *trans*-Golgi network and late endosomesand has been argued to constitute the virus assembly compartment. The structural glycoproteins must be targeted to this compartment and, late in infection, become concentrated within organelle. This proposed assembly pathway appears to be a default assembly pathway for all herpesviruses.

Interestingly, the integrity of the Golgi/TGN was disturbed by the cytomegalovirus infection. In uninfected cells the Golgi/TGN was represented by a number of tubules, whereas in infected cells the Golgi/TGN exhibited a rather vesicular appearance. During HCMV infection was observed an expansion of the TGN from the normal ribbon-like staining patter of the TGN46 in uninfected cells to a more expanded mesh-like morphology after infection [**34**].

The finding that recombinant gpTRL12 can be localized to the *trans*-Golgi network in the absence of other viral function demonstrates that, similar to other structural glycoproteins of HCMV such as gB and gpUL132, gpTRL12 contains all of the *cis*-acting elements necessary for *trans*-Golgi network localization. This is in contrast to a number of others HCMV structural glycoproteins, including gH, gM, gN and gpTRL10, which require complex formation with other viral proteins in order to reach the more distal parts of the secretory system [**23, 55**]. Inspection of the gpTRL12 cytoplasmic domain for the presence of trafficking motifs that are known to function in these processes reveals a number of motifs that may be involved in this intracellular trafficking. A tyrosine-based motifs YXXΦ (where Y is tyrosine, X is any amino acid and Φ is any bulky hydrophobic amino acid) was found. YXXΦ motifs mediate the incorporation of membrane proteins into transport vesicles due to interaction with cellular adaptor proteins. In addition, gpTRL12 contains acidic cluster motifs which potentially interact with the connector protein PACS-1 (phosphofurin acidic cluster sorting protein 1), which redirects proteins from the endosomes to the trans-Golgi network, a proposed site of tegument assembly and virion envelopment. Thus, it seems likely that trafficking of gpTRL12 involves transport to the plasma membrane and recycling from there to the endosomal compartment involved in virus envelopment. Future experiments will test this hypothesis.

In order to investigate the role of pTRL12 in the replication of HCMV, a series of recombinant viruses were generated, using a strategy that includes the use of an infectious clone of HCMV maintained as a bacterial artificial chromosome in *E. Coli*.

A deletion mutant lacking the entire TRL12 open reading frame was generated in a low passage HCMV isolate (TR) was generated.

Concomitant with productive infection, HCMV induces cytopathogenic effects (CPE) in tissue cultures of various origins, which include cell rounding, cell enlargement, formation of inclusion bodied and lysis of infected cells.

Recent clinical isolates are distinguished from cell culture adapted strains by lack of release of cell-free infectious progeny from infected cells. The progeny virus is highly cell-associated and development of CPE or plaques can take as long as several weeks, requiring subculture of infected cells. In contrast, both AD169 and Towne form distinctive plaques in less than 7 days and a single round of replication resulting in cell free virus in the culture fluid requires 96h or less to complete.

The morphology of the plaques induced by wild-type strain TR is characterized by the formation of small foci, a typical plaque morphology associated to a cell-associated spread; whereas, infection with deletion mutant Del-TRL12 leads to an even distribution of the infection through the cell culture.

At days 10 and 14 post infection, the TRL12 deletion mutant Del-TRL12 induced plaques significantly larger than those induced by the wild-type virus TR. The plaque extension might be consistent with the spread of infection via supernatant.

To investigate possible effects of TRL12 deletion on the kinetic of virus replication and to extend the findings of the plaque size determinations, multi-step growth kinetic of the engineered mutant virus, Del-TRl12, were performed.

Strikingly, the final virus yield from cells infected with Del-TRL12, obtained after 12 days of infection, was increased approximately 10-100 fold as compared to wild type parental virus TR. These results indicated that the deletion of ORF TRL12 from the genome of low-passage HCMV strain accelerated and increased virus production in fibroblasts. Then, the locus TRL12 is detrimental to cytomegalovirus growth in fibroblasts.

Consistent with the results observed for the replication kinetic of Del-TRL12, the increase of Del-TRL12 infectious titers is reflected by an increase in intracellular viral DNA accumulation. At days 9 at 12 post infection, the number of viral genome copies from cells infected with Del-TRL12, was increased approximately 100 fold as compared to wild type parental virus TR.

Strikingly, disruption of TRL12 resulted in release of viral progeny to the medium, in contrast to the wild-type virus TR that, being a recent clinical isolates, is characterized by more cell-associated spread and by lack of release of cell-free infectious progeny from infected cells.
Deletion of TRL12 resulted in a 10-100 fold increase in intracellular particles and a concomitant enhanced release of virus to the medium in HF culture. The data are most compatible with an increased cell-to-cell spread of the delta-TRL12 recombinant virus. The results of growth kinetics and DNA accumulation are largely a reflection of virus-induced plaque size because the number of infected cells are dependent on the size of the plaque. This increase results in larger plaques which, in turn, lead to increased virus yield in the growth curve experiment since virus from the entire culture was assayed.. The increased number of infected cells will probably also produce a higher number of infectious particles in the supernatant. The increased number of infected cells will probably also produce a higher number of infectious particles in the supernatant. It is believable to explain the higher titres of cell-associated virus of Del-TRL12 compared to the wild-type virus TR, by a faster spread via the supernatant. The PCR also indicates that the rate of viral DNA synthesis is not different between wt and delTRL12 in the early phases after infection. The higher yield at later times might reflect the increased number of infected cells due to increased spread.
Expression of pTRL12 (wild-type virus, TR) delayed and reduced virus production in fibroblasts and supported cell-associated virus spread leading to the formation of small foci rather than to an even distribution of the infection through the cell culture.
These data, clearly indicates that there is a negative effect of the TRL12 gene product on growth properties of limited passaged cytomegalovirus strains in fibroblast cell culture.
It could be speculated that the reduced virus production was due to a change in the virus-release phenotype. The TRL12 protein apparently seems to be involved in redirecting the virions to more cell-associated, and therefore slower, spread. This is the reverse of what is selected for when clinical isolates are adapted for productively in fibroblasts cultures.
Further experiments, where cell-cell-spread is inhibited by agarose overlay or by addition of neutralizing antibodies will clarify the point. It will be interesting to find out whether the increased plaque size has something to do with the virion envelope or an altered plasma membrane or both.

A number of glycoproteins have been described as polymorphic [**50, 51, 52**]. A consequence of variations in structural glycoproteins could be a strain specific humoral immune response that incompletely protects against infection from a genetically divergent HCMV strain. However, although it is known that the humoral immune response against HCMV carries a

strain specific component, little is known about the antigens involved. Since, due to the exquisite species-specificity of HCMV, no animal model exists for the analysis of potential vaccines, as much knowledge as possible must be gained from detailed analysis of HCMV glycoproteins with respect to strain variations, function during the viral replication cycle and induction of a protective humoral immune response. A rational approach towards the development of an effective vaccine can only be made after this knowledge has been gained.

Analysis of gpTRL12 coding sequences from clinical isolates revealed high sequence variability over the entire protein sequence. Phylogenetic analysis, performed on nucleotide and amino acid sequences, revealed that the identified variants are not randomly distributed, but are clearly clustered in 4 distinct groups. The four main groups diverged on the amino acid level in the range of 60-70%. Although the four groups showed a high degree of divergence, the identity within each group approaches 80-100%. Despite the impressive variability of the amino acid sequence, the Ig-like domain is conserved as well as the number and the position of cysteine residues confirming that there is a very tight constrain upon this domain. High variability is particularly evident in the N-terminal part of the molecule, predicted to be exposed, where are concentrated most of the post-translational modifications sites (N- and O-glycosylation sites.). High variability associated to variation in number and precise location of glycosylation sites might be indicative of strong immune pressure. Altered patterns of glycosylation in viral proteins can be a mechanism for evasion of T cell and neutralizing antibody responses since it changes the phenotypic and antigenic properties of the virus. Further studies will be focus to find out if gpTRL12 induces neutralizing antibodies during natural infection and if the neutralizing ability of these antibodies is strain-specific.

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