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Identification and Characterization of a G Protein-Coupled Receptor Homolog Encoded by Murine Cytomegalovirus

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This report describes the identification of a murine cytomegalovirus (MCMV) G protein-coupled receptor (GCR) homolog. This open reading frame (M33) is most closely related to, and collinear with, human cytomegalovirus UL33, and homologs are also present in human herpesvirus 6 and 7 (U12 for both viruses). Conserved counterparts in the sequenced alpha- or gammaherpesviruses have not been identified to date, suggesting that these genes encode proteins which are important for the biological characteristics of betaherpesviruses. We have detected transcripts for both UL33 and M33 as early as 3 or 4 h postinfection, and these reappear at late times. In addition, we have identified N-terminal splicing for both the UL33 and M33 RNA transcripts. For both open reading frames, splicing results in the introduction of amino acids which are highly conserved among known GCRs. To characterise the function of the M33 in the natural host, two independent MCMV recombinant viruses were prepared, each of which possesses an M33 open reading frame which has been disrupted with the β-galactosidase gene. While the recombinant M33 null viruses showed no phenotypic differences in replication from wild-type MCMV in primary mouse embryo fibroblasts in vitro, they showed severely restricted growth in the salivary glands of infected mice. These data suggest that M33 plays an important role in vivo, in particular in the dissemination to or replication in the salivary gland, and provide the first evidence for the function of a viral GCR homolog in vivo.

Analysis of the complete genome of human cytomegalovirus (HCMV) several years ago revealed three homologs of cellular G protein-coupled receptors (GCRs) encoded by the UL33, US27, and US28 genes (9). Members of this large and diverse family of receptors function in signal transduction through cell membranes by activating G proteins and are triggered by small ligands such as hormones, neurotransmitters, and chemoattractant cytokines (23). It has recently become apparent that viral homologs of GCRs are not confined to HCMV. GCR homologs have been identified in poxviruses (6, 22) and in the following beta- and gammaherpesviruses: murine cytomegalovirus (MCMV) (28), human herpesvirus 6 (HHV-6) (15, 27), human herpesvirus 7 (HHV-7) (25), herpesvirus saimiri (HVS) (26), and equine herpesvirus 2 (31). Sequence analysis has indicated that the HVS ECRF3 gene and the HCMV US28 gene are most closely related to GCRs which bind leukocyte chemoattractant peptides, called chemokines (1, 24). Both gene products possess a disproportionately high homology with chemokine receptor N-terminal amino acid sequences, which have been shown to be involved in ligand binding and/or ligand localization (14). In agreement with these observations, in vitro studies demonstrated that cells transfected with either HVS ECRF3 or HCMV US28 specifically bound alpha or beta chemokines, respectively (1, 13, 24). Significantly, ligand binding to the transfected cells stimulated intracellular calcium mobilization, and while the subsequent activation of second-messenger pathways typical of G protein-mediated signal transduction responses has not yet been determined, it has been tempting to suggest that these viral GCR homologs play a role in cytokine-activated second-messenger systems.

In contrast to US28, analysis of the HCMV UL33 sequence did not suggest notable homology with chemokine receptors, due to the lack of conserved features at the N terminus. Subsequently, UL33 was shown to be nonessential in vitro (5), and the virulence of HCMV recombinant viruses deleted of UL33 was shown to be indistinguishable from that of wild-type HCMV in the SCID-Hu mouse model (4). Given the lack of potential candidate ligands for UL33 and the absence of a phenotype in vitro and in an immunodeficient in vivo model, there has thus far been no evidence for an important role of UL33 in the virus life cycle. If UL33 interacts with the host immune system, it is likely that the effects of deleting this gene will become apparent only in the context of an intact immune response.

MCMV has proved a useful model for HCMV disease, since its pathogenesis closely resembles that of HCMV (17). In addition, recent analysis of the complete nucleotide sequence of MCMV (Smith) has revealed a large number of open reading frames (ORFs) with significant homology to those of HCMV (28), thus supporting the observed biological similarities between these viruses. In this paper, we report the characterization of the MCMV ORF M33, which is conserved and collinear with HCMV UL33, HHV-6 U12, and HHV-7 U12. Initial alignment of the M33, UL33, and U12 ORFs showed a low level of conservation at the N terminus. However, we demonstrate that both UL33 and M33 are spliced at the N terminus and that the resulting authentic ORFs show an increased level of conservation with other mammalian GCRs. Finally, we present the growth kinetics of two independent MCMV recombinants for which the M33 ORF has been disrupted by insertion of a *lacZ* marker gene cassette. While dispensable for growth in fibroblasts in vitro, M33 is required for efficient

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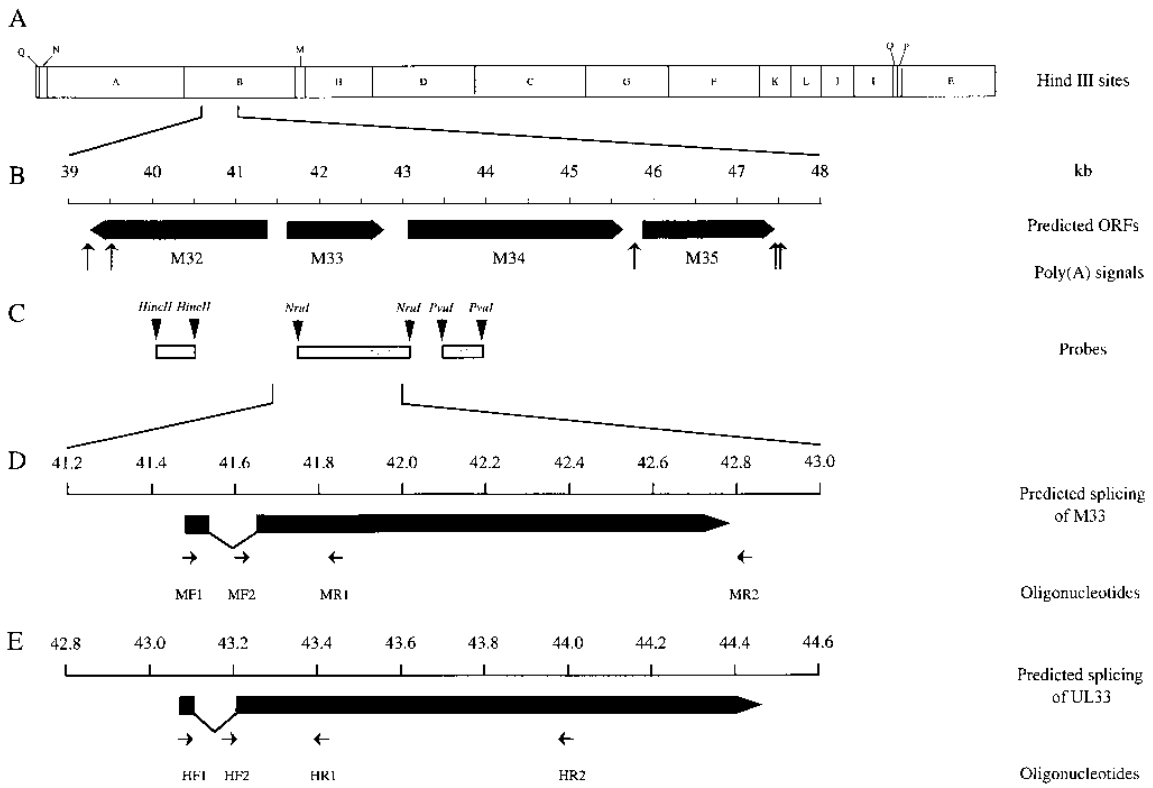

FIG. 1. Genetic organization of the region of the MCMV genome encoding the GCR homolog and the specificity of genomic fragments and oligonucleotides used in these studies. (A) Representation of the MCMV genome (Smith) as a *Hin*dIII map. (B) Expansion of the *Hin*dIII B region and arrangement of putative ORFs, M32 to M35, which are collinear and homologous with HCMV UL32 to UL35. All ORFs are trimmed to the first ATG. The locations of poly(A) signals are indicated. (C) Specificity of subgenomic MCMV fragments used as probes for Northern analysis. (D and E) Further expansion of the region encoding the predicted spliced M33 gene and the specificity of oligonucleotides used for RT-PCR assays (D) and for UL33 of HCMV (E).

replication in vivo, most notably in the salivary gland. Thus, this report provides the first direct evidence for the role of a viral GCR homolog during infection of the natural host.

MATERIALS AND METHODS

Cells. Primary mouse embryo fibroblasts (MEF) and human foreskin fibroblasts (HFF) were grown in minimal essential medium (MEM; Gibco) supplemented with 10% newborn calf serum (NCS).

Virus. The K181 strain of MCMV was used in this study. Wild-type and mutant viruses were propagated and subjected to titer determination on MEF as previously described (2). For in vivo studies, virus stocks were propagated in 3-week-old BALB/c mice (2). HCMV (AD169) (provided by Jane Allan, University of Western Australia) was propagated and subjected to titer determination on HFF.

Mice. Specific-pathogen-free BALB/c female mice (Animal Resource Centre, Murdoch, Western Australia) were maintained under minimal disease conditions for the duration of the studies.

Preparation of MCMV and HCMV DNA. MEF and HFF were infected with MCMV and HCMV, respectively, at a multiplicity of infection (MOI) of 0.01. When the cells exhibited an extensive cytopathic effect, infected-cell DNA was prepared by published methods (20).

Preparation of RNA. For the preparation of MCMV RNA, confluent monolayers of MEF were infected with MCMV at an MOI of 5. For immediate-early RNA, cells were maintained in growth medium containing 30 μg of cycloheximide per ml and RNA was prepared 4 h postinfection (p.i.). For early RNA, MEF were maintained in growth medium containing 250 μg of phosphonoacetic acid (PAA) per ml for 24 h prior to harvest. RNA was prepared from MEF maintained in the absence of drug treatment at 3 and 24 h p.i. For the preparation of HCMV RNA, HFF were infected with HCMV (AD169) at an MOI of 5. For early RNA, HFF treated with PAA were harvested at 60 h p.i. RNA was also harvested at 4, 8, and 60 h p.i. from HFF maintained in the absence of drug treatment. All RNA was prepared by the method of Chomczynski and Sacchi (11). Poly(A)$^+$ RNA was purified with oligo(dT)-coupled matrix (QIAGEN) as specified by the manufacturer.

Northern blot analysis. RNA samples (10 μg) were denatured at 65°C for 15 min in 20 mM morpholinepropanesulfonic acid (MOPS; pH 7)–1 mM EDTA–5 mM sodium acetate (MOPS buffer) containing 50% formamide and 2 M formaldehyde and then loaded onto 1.5% agarose gels containing 0.66 M formaldehyde prepared in MOPS buffer. After electrophoresis, the separated RNA was transferred to nylon membranes, UV irradiated, and prehybridized in 36% formamide–75 mM sodium citrate–0.75 M sodium chloride–0.1% bovine serum albumin–0.1% Ficoll–0.1% polyvinylpyrrolidone–0.1% sodium dodecyl sulfate (SDS)-50 mM sodium phosphate (pH 6.5)–200 μg of salmon sperm DNA per ml at 55°C overnight. [^{32}P]dCTP-labelled DNA probes were prepared by using a random prime kit (Bresatec). Radiolabelled probes were then incubated with prehybridized membranes in 10 ml of hybridization solution (36% formamide, 75 mM sodium citrate, 0.75 M sodium chloride, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% SDS, 200 μg of salmon sperm DNA per ml, 10% dextran sulfate) at 55°C overnight. The membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS solution (5 min per wash), twice with 0.2× SSC–0.1% SDS (15 min each wash), and twice with 0.02× SSC–0.1% SDS (15 min each wash). All washing steps were performed at 65°C. The membranes were then exposed to X-ray film at −80°C with an intensifying screen. Probes were stripped from the membranes by boiling in a solution of 1% SDS for 15 min. Removal of bound probe was confirmed by exposure of the membrane to X-ray film.

Synthesis, cloning, and sequencing of M33 and UL33 cDNA. For M33 cDNA, first-strand synthesis of poly(A)$^+$ selected RNA obtained from MCMV-infected MEF 2 h p.i. was performed with avian myeloblastosis virus (AMV) reverse transcriptase (RT) (Promega) and oligonucleotide MR2 (5′CCCTCATCCTGT CATTC3′) as the primer (Fig. 1D). Following the reverse transcription reaction, the cDNA products were diluted 10-fold and the M33 region was amplified by PCR with oligonucleotide MR1 (5′TAGATGCGGTTCGACAG3′) paired with either oligonucleotide MF1 (5′GGCTCGAGATGGACGTC3′, specific for the putative 5′ exon) or MF2 (5′ACATATCTCGCCCTCTC3′, specific for the putative intron). The RT-PCR product was cloned into pGEM-T (Promega), and four individually cloned products were sequenced by previously described methods (29). The same approach was used to generate, clone, and sequence HCMV UL33 cDNA, using RNA from HCMV-infected HFF at 4 h p.i. The cDNA was generated with oligonucleotide HR2 (5′TCATGAGAGAGACGTAGG3′) as

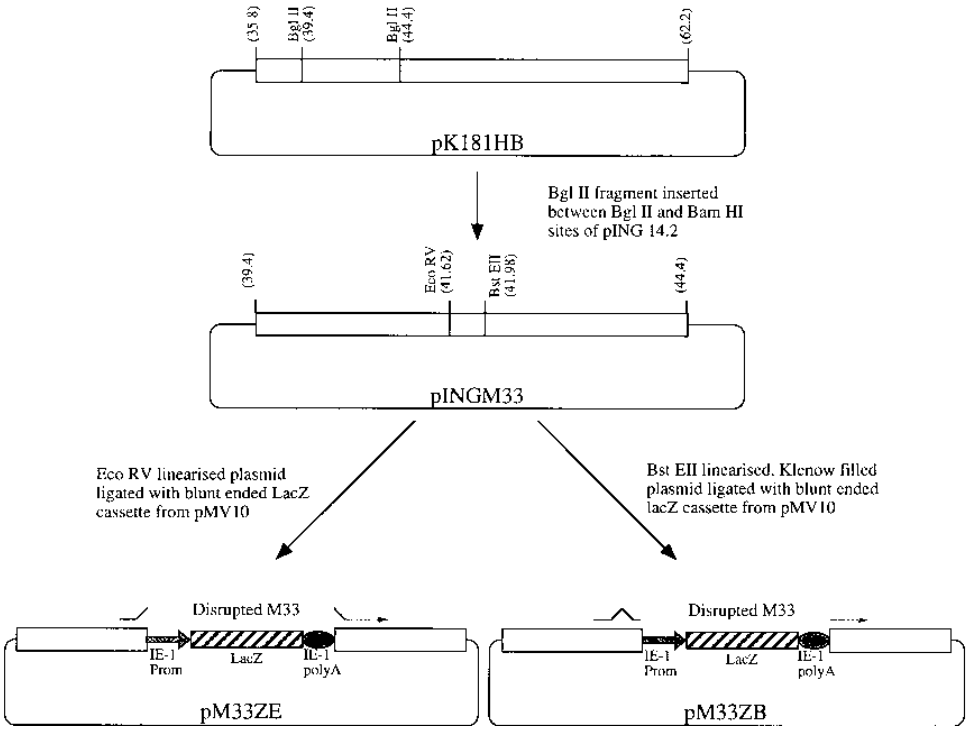

FIG. 2. Construction diagrams (not drawn to scale) for plasmids used in this study. Where appropriate, nucleotide positions are indicated according to the complete nucleotide sequence of MCMV (Smith). Additional details are given in the text.

the primer and was amplified with HR1 (5′GTAGAGTAGAGGTTGGTC3′) which was paired with HF1 (5′GACACCATCATCCACAAC3′, specific for the putative 5′ exon) or HF2 (5′CCGCTGACTTGTTTCTTC3′, specific for the putative intron) as shown in Fig. 1E.

Plasmid constructs. The derivation of plasmids used in this study is shown schematically in Fig. 2. Nucleotide positions are given according to the complete genomic sequence of MCMV (Smith) (28). Plasmid pK181HB (kindly provided by A. Scalzo, University of Western Australia), which contains the *Hin*dIII B fragment (nucleotides [nt] 35844 to 62239) of MCMV (K181) cloned into pGEM11Zf was used in this study. A 4.96-kb *Bgl*II fragment (nt 39402 to 44366) of pK181HB, which contains the M33 ORF (nt 41794 to 42777) together with flanking sequence, was subcloned between the *Bam*HI and *Bgl*II sites of pING14.2 (19) to generate plasmid pINGM33. A 3.8-kb blunt-ended β-galactosidase expression cassette, encoding the *lacZ* gene flanked by the HCMV IE1 promoter and poly(A) signals, was derived from pMV10 (12) by digestion with *Hin*dIII followed by a Klenow fill-in reaction. Two recombination plasmids were constructed by insertion of the β-galactosidase cassette within the M33 ORF at two different sites. For pM33ZE, the cassette was inserted into the *Eco*RV site (nt 41618, located in the putative intron), and for pM33ZB, insertion occurred at a *Bst*EII site (nt 41977, downstream of the putative splice acceptor site), which had been blunt ended by a Klenow fill-in reaction.

Transfection. Transfection of MEF was performed on subconfluent monolayers (35-mm dishes) by the modified calcium phosphate procedure of Chen and Okayama (10). Monolayers were transfected with 10 μg of MCMV (K181)-infected cell DNA plus 3 to 6 μg of the plasmid DNA of interest. At 16 to 20 h posttransfection, the monolayers were washed with MEM and fresh MEM–10% NCS was added. After a further 4 to 6 days of incubation, dishes were frozen (−80°C) and thawed and the contents were sonicated and stored as aliquots at −80°C. Transfection stocks were subjected to titer determination on MEF and incubated for 4 days under a MEM–2% NCS–0.7% carboxymethyl cellulose overlay, and the frequency of recombinant plaques was determined by fixing with glutaraldehyde and staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described previously (12). Transfection stocks with a recombination frequency of ≥0.05% were used to select cloned recombinant viruses as follows. Stocks were plated onto MEF at an appropriate density to allow picking of individual plaques. After 4 days of incubation, the overlay was removed and replaced with fresh overlay supplemented with 50 μg of X-Gal per ml. Blue plaques, visible after 1 to 3 h of incubation, were picked and stored in 1 ml of MEM–10% NCS at −80°C. Plaque samples were subsequently thawed, sonicated, and subjected to titer determination (using centrifugal enhancement), and recombinant plaques were identified and picked as described above. Each recombinant was selected by three rounds of plaque purification prior to confirmation of the recombination by Southern blotting.

Southern blot analysis. Infected-cell DNA was prepared from wild-type- or recombinant-infected MEF. The DNA was digested with *Eco*RI, and 2 μg of each sample was separated on a 0.8% agarose and transferred to nylon membranes by standard methods (20). The probes used, specific for the M33 region or *lacZ* cassette, were the 4.9-kb *Bgl*II fragment of pK181HB and the 3.8-kb *Hin*dIII fragment of pMV10 (described above), respectively. DNA fragments were gel purified prior to radiolabelling (Bresatec GigaPrime kit) and use in hybridization experiments as specified by the manufacturer.

In vitro growth of MCMV ΔM33 mutants. Multistep growth curves were determined for wild-type and mutant viruses in MEF. Cells were infected at an MOI of 0.01 for 1 h at 37°C. Following adsorption, the monolayers were washed with MEM, overlaid with MEM–2%NCS, and incubated for various times prior to harvest. Wells were harvested by freezing (−80°C) and thawed, and aliquots were stored at −80°C prior to titer determination on MEF monolayers.

In vivo growth of MCMV ΔM33 mutants. Mice were inoculated with wild-type MCMV or mutant viruses by the intraperitoneal route unless otherwise indicated. At the designated times p.i., the animals were sacrificed and salivary glands and/or livers and spleens were removed. All organs were individually weighed, homogenized in MEM–2% NCS, and centrifuged at 2,000 × *g*. The supernatant was stored at −80°C and subsequently subjected to titer determination on MEF. At least three to five mice per group per time point were assessed for each virus. Parental (wild-type) MCMV was included in each experiment as a control.

Nucleotide sequence accession number. The sequence of the M33 spliced gene of MCMV has GenBank accession number L41868.

RESULTS

Sequence analysis of MCMV M33. MCMV encodes a GCR homolog (designated M33) which is most closely related to, and collinear with, the HCMV UL33 (47% identity), HHV-6 U12 (28% identity), and HHV-7 U12 (29% identity) ORFs. The position and orientation of genes flanking M33 (all of which are conserved and collinear with HCMV) and the location of putative poly(A) sequences are shown in Fig. 1A and B. At the nucleotide level, M33 is similar to UL33 in that it lacks a consensus TATA promoter element and a proximal 3′ poly(A) signal. The M33 ORF encodes a polypeptide which possesses characteristic features of the GCR family, which

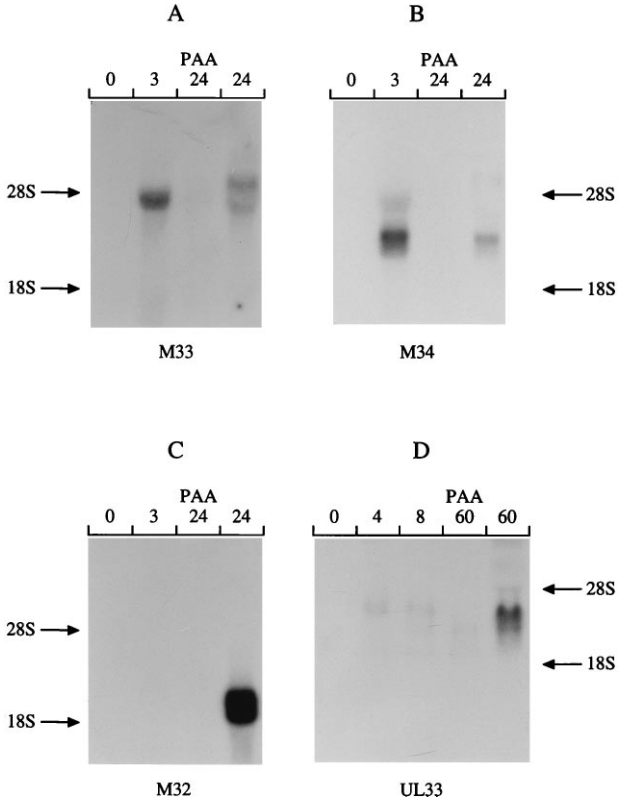

FIG. 3. Northern blot analysis of RNA isolated from MCMV-infected MEF (A to C) and HCMV-infected HFF (D). The time p.i. at which the RNA was harvested is shown above each lane; 0 h p.i. refers to uninfected cultures. Total RNA (10 μg) was separated in a denaturing agarose gel, blotted onto nylon, and hybridized with double-stranded DNA probes specific for either M33 (A), M34 (B), M32 (C), or UL33 (D) as described in the text. Transcript sizes were determined relative to RNA markers (Promega). The locations of 28S and 18S rRNA are indicated.

include the presence of multiple membrane-spanning domains, the conservation of specific motifs in transmembrane domains II and VII, the presence of two highly conserved cysteine residues in the second and third extracellular domains, and a serine/threonine-rich C terminus. However, the N-terminal amino acid sequence (spanning the predicted first extracellular domain) is missing in M33 and is truncated in UL33 and HHV-6 U12 in comparison with the protein sequences of the majority of mammalian and viral GCRs. Furthermore, the N termini predicted for the ORFs M33, UL33, and HHV-6 U12 are unusual in that they all lack N-linked glycosylation sites, unlike the majority of known GCRs. As described below, these anomalies can be resolved by splicing near the 5′ end of these transcripts, resulting in altered N-terminal sequences.

Transcriptional analysis of the M33 region. To characterize the expression of the M33 ORF during MCMV infection, the transcripts produced from the M33 region were identified by Northern blot analysis and the kinetic class of the transcripts was determined.

In the following description, nucleotide positions are listed according to the MCMV genomic sequence (28). Probe fragments were derived from pINGM33 (Fig. 2), and their positions are indicated in Fig. 1C. A 4.5-kb transcript was detected at 3 h p.i. with the M33-specific *Nru*I fragment (nt 41741 to 43176), and lower levels of this transcript were also detected at late times (Fig. 3A). This transcript could not be detected at 24 h p.i. in the presence of PAA, indicating that transcription of M33 is downregulated at late times in the absence of DNA replication. Moreover, the transcript could not be detected in cells infected in the presence of cycloheximide and harvested 3 h p.i. (data not shown), indicating that M33 is not expressed as an immediate-early gene. The 4.5-kb transcript is consistent in size with an RNA reading through both M33 and M34 ORFs and terminating at the first poly(A) signal 3′ of M34. An additional 5.5-kb transcript was detected at 24 h p.i. To confirm that the 4.5-kb transcript encodes sequences from both M33 and M34 and to determine whether the 5.5-kb transcript includes sequences upstream or downstream of M33, transcripts arising from the adjacent M34 and M32 ORFs were detected with the *Pvu*I (nt 43458 to 43901) and *Hinc*II (nt 40025 to 40624) fragments as probes, respectively (Fig. 1C). The *Pvu*I probe detected a 3.4-kb transcript (consistent with the expected size of the M34 transcript) at 3 and 24 h p.i. and an additional 4.5-kb transcript at 3 h p.i. (Fig. 3B). On a longer exposure than displayed in Fig. 3B, the 4.5- and 5.5-kb transcripts observed with the *Nru*I probe at 24 h p.i. were also detected with the *Pvu*I probe (data not shown). These data support the above proposal that the 4.5-kb transcript includes sequences from M33 and M34 and is therefore similar to HCMV UL33, which is expressed as a 3′ coterminal transcript with UL34 (22). The presence of transcripts initiating upstream of the M33 intron and continuing through the M34 ORF has been confirmed by RT-PCR analyses (data not shown). The *Hinc*II probe detected an abundant 2.1- to 2.4-kb late transcript, consistent with the size of M32 (Fig. 3C). Although the the M32 ORF possesses an internal poly(A) signal, this is unlikely to be utilized, given that it is a considerable distance (228 bp) upstream of the stop codon and is not followed by a GT-rich tract characteristic of transcription poly(A) signals (3). An alternative poly(A) signal (AGTAAA), which is 61 bp downstream of the M32 stop codon and is followed closely by a GT-rich tract, is more likely to be utilized. None of the probes reacted with RNA from uninfected MEF, indicating that the transcripts were specific for MCMV-infected RNA.

Temporal expression of the HCMV UL33 transcript. Previous studies of the three HCMV GCR transcripts (34) sug-

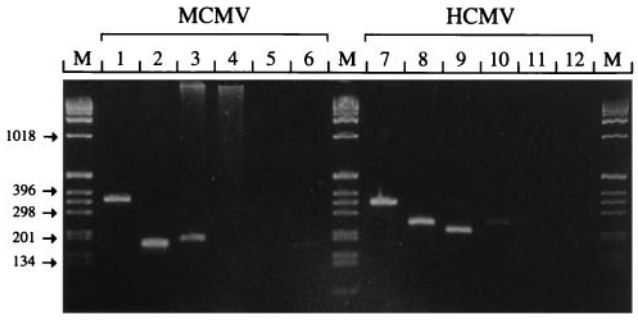

FIG. 4. Evidence for N-terminal splicing in MCMV M33 and HCMV UL33 genes. RT-PCRs of poly(A)$^+$ RNA from MCMV-infected MEF (3 h p.i.) or HCMV-infected HFF (4 h p.i.) were performed with oligonucleotides whose specificity is shown in Fig. 1D and E. First-strand synthesis with AMV-RT was performed by using oligonucleotide MR2 (for MCMV) or HR2 (for HCMV). Amplification of RT products by PCR was performed with the following oligonucleotide pairs: MF1/MR1 (lane 3) or MF2/MR1 (lane 4) for MCMV and HF1/HR1 (lane 9) or HF2/HR1 (lane 10) for HCMV. The same primer pairs were used to amplify viral genomic DNA by direct PCR, MF1-MR1 (lane 1), MF2-MR1 (lane 2), HF1-HR1 (lane 7), and HF2-HR2 (lane 8). To confirm that the amplified RT products were derived from RNA rather than from contaminating genomic DNA, control RT reactions were carried out in the absence of AMV RT (lanes 5, 6, 11, and 12). Products were separated on a 2.7% agarose gel. The relative migrations of molecular weight markers (in base pairs) are indicated in lanes M. No products were observed in uninfected cultures (not shown).

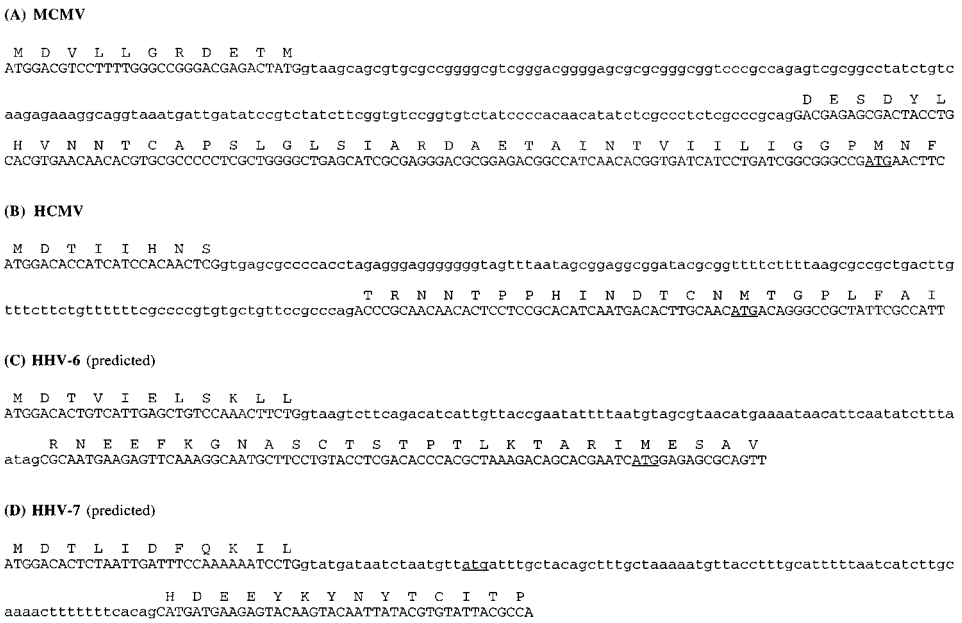

FIG. 5. Nucleotide sequence of the splice junction determined for MCMV M33 (A) and HCMV UL33 (B) or predicted for HHV-6 U12 and HHV-7 U12. The nucleotide sequences upstream and downstream of the splice sites are shown, with the predicted amino acid sequences shown above. The intron sequences are displayed in lowercase type. The initiating ATGs previously predicted for the unspliced ORFs are underlined.

gested that they were all expressed at late times p.i. and could not be detected from cells which had been maintained for 7 days p.i. in medium containing phosphonoformic acid, a viral DNA synthesis inhibitor. Following our observation that the M33 transcript could not be detected when DNA synthesis was blocked, despite being expressed from early times p.i., we investigated the expression of the UL33 transcript in HCMV-infected human HFF. Northern blot analysis was performed as described in Materials and Methods, using a double-stranded DNA probe specific for the complete UL33 ORF (a gift from Helena Browne, University of Cambridge). A 3.3-kb transcript (visible as a faint band in Fig. 3D) was detected at 4 and 8 h p.i. and again (more strongly) at late times p.i., consistent with an RNA reading through both UL33 and UL34 ORFs, terminating at the first poly(A) signal downstream of UL34 (Fig. 3D). These results are similar to those observed for M33, except that maximal detection of UL33 occurs at late rather than early times p.i. Similar to M33, no transcript was detected at 4 h p.i. in the presence of cycloheximide (data not shown). No transcript was detected at 60 h p.i. in the presence of PAA, which is consistent with the results of Welch et al. (34). Unlike the previous report, however, we conclude that UL33 expression does occur at early times prior to DNA replication. Additional evidence for expression of UL33 at early times p.i. was obtained by RT-PCR analysis as described below.

Both MCMV M33 and HCMV UL33 are expressed as spliced transcripts. Further inspection of the amino acid sequence alignment of M33 with UL33 showed that a significant level of conservation was maintained upstream of the first start codon in M33, suggesting that M33 may be expressed as a spliced transcript. Putative splice donor and acceptor sites were identified 286 and 114 bp upstream of the ATG, respectively. This arrangement results in a short 5′ exon, encoding an ATG followed by 10 codons, which is separated from the remainder of the ORF by a 161-bp intron. To test the existence of this splice, RT-PCR was performed with poly(A)$^+$ RNA from infected (3 h p.i.) MEF as the template and oligonucleotides specific for either the putative exon or intron sequences (Fig. 1D). For both primer pairs, PCR products of the predicted size were generated with MCMV-infected cell DNA as a template (Fig. 4, lanes 1 and 2). In contrast, an RT-PCR product was observed only in reactions amplified with the MF1-MR1 primer pair, and this product was approximately 160 bp shorter than the corresponding PCR product amplified from viral DNA, consistent with the predicted splice (lane 3). In control reactions performed as above but without the addition of AMV RT (lanes 5 and 6), no amplified products were observed, confirming that the RT-PCR products were derived from RNA transcripts rather than contaminating DNA. In addition, products of the same size were amplified from cDNA generated from oligo d(T)$_{15}$-primed RT reactions (data not shown). No products were observed with RNA from uninfected MEF (data not shown).

A similar splicing pattern for HCMV UL33 was also predicted on the basis of the identification of splice donor and acceptor sites 164 and 42 bp upstream of the published ATG, respectively. This arrangement results in a 5′ exon, encoding an ATG followed by seven codons, separated from rest of the ORF by a 121-bp intron. By using the same experimental approach as used to show the M33 splice, RT-PCR was performed with poly(A)$^+$ selected RNA from HCMV-infected (4 h p.i.) HFF as the template and oligonucleotides specific for the predicted exon and intron sequences (Fig. 1E). For both primer pairs, PCR products of the predicted size were generated with HCMV infected-cell DNA as the template (Fig. 4, lanes 7 and 8). By using the HF1-HR1 primer pair, an RT-PCR product approximately 120 bp shorter than the corresponding PCR product amplified from viral DNA was observed (lane 9), consistent with the predicted splice. By using the HF2-HR1 primer pair, a faint RT/PCR product can be observed, indicating that a proportion of the UL33 transcript is unspliced (lane 10). No products were amplified from reactions performed in the absence of AMV RT, confirming that the observed RT-PCR products were derived from RNA (lanes 11 and 12).

A

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          [ ---------------E I-------------] ___________TM I____________[ ===C I===] _________TM II_________[ ----E II-
MCMV M33  MDVLLGRDETMDESDYLHVNNTCAPSLGLSIARDAETAINTVIILIGGPMNFVVLTTQLLSNRIYRSSAPTLYMTNLYFANLLTVTMLPFLILSNRGQISSSPE
HCMV UL33 MDTIIHN--STRNNTPPHINDTCNMTGPLFAIRTTEAVLNTFIIFVGGPLNAIVLITQLLTNRVLGYSTPTIYMTNLYSTNFLTLTVLPFIVLSNQWLLPAGVA
HHV-6 U12 MDTVIELS-KLLRNEEFKGNASCTSTPTLKTARIMESAVTGITLTTSIPMIIIVVTTMILYHRVAKHNATSFYVITLFASDFVLMWCVFFMTVNRKQLFSFNRF
HHV-7 U12 MDTLIDFQ-KILLHDEEYKYNYTCI-TPTVRKAQRLESVINGIMLTLILPVSTVVICTLLIYYKWTKQTITSPYLITLFISDSLHSLTVLLLTLNREALTNLNQA
Conserved ** ..              * .*  .  .  .  *. .    .    *.  .*. * ..  .       . *.  *. ... .    . ... ..

          --] _______TM III_________[ ========C II========] ________TM IV______[ ---------------E III-----------------
MCMV M33  GCKLVAVTYYASCTAGFSTLALISVNRYRVIHQSTNKNAAGSKKKTYGVLALVWLTSLMCASPAPTYVTVLAH---DGDTPDSVHETCIIFFNYDQVKTVLATF
HCMV UL33 SCKFLSVIYYSSCTVGFATVALIAADRYRVLHKRT--YARQSYRSTYMILLLTWLAGLIFSVPAAVYTTVVMHHDANDTNNTNGHATCVLYFVAEEVHTVLLSW
HHV-6 U12 FCQLVYFIYHAVCSYSISMLAIIATIRYKTLHRRKKTESKTSSTGRNIGILL--LASSMCAIPTALFVKT--------NGMKKT-GKCVVYISSKKAYELFLAV
HHV-7 U12 LCQCVLFVYSASCTYSLCMLAVISTIRYRTLQRRTLNDKNNNHIKRNVGILF--LSSAMCAIPAVLYVQV--------EKKKGNYGKCNIHISTQKAYDLFIGI
Conserved  *. .   * . *. ..  .*.*.  **. ...        .       . .  *.. . . *.  .                    * . .       ..

          ] ________TM V________[ ======C III======] _________TM VI________[ -------E IV-------] __________TM VII______
MCMV M33  KILICIVWGVMPVVMMTWFYLFFYKRLKLTSYRRRSHTLAFVSTLILSFLVLQTPFVGIMIFDSYAVIEWDVTCESINSRDAVAMLARVVPNFHCMLNPVLYAF
HCMV UL33 KVLLTMVWGAAPVIMMTWFYAFFYSTVQRTSQKQRSRTLTFVSVLLISFVALQTPYVSLMIFNSYATTAWPMQCEHLTLRRTIGTLARVVPHLHCLINPILYAL
HHV-6 U12 KIVFSFIWGVLPTMVFSFFYVIFCKALHDVTEKKYKKTLFFIRILLLSFLLIQIPYIAILICEIAFLYMPQNTCFWLARVEILQLIIRLMPQVHCFSNPLVYAF
HHV-7 U12 KIVYCFLWGIFPTVIFSYFYVIFGKTLRALTQSKHNKTLSFISLLILSFLCIQIPNLLVMSVEIFFLYIANTSCLGTIQREIVQIISRLMPEIHCLSNPLVYAF
Conserved *..   .**  * .. . ** .*   .   .  .   ** *.  *..**. .* * . ..  .          *        .  . *..*. **  **..**.

          __[ ================================================C IV================================================]
MCMV M33  LGRDFNKRFMQCITGKLFSRRRMLQERAGVRSPSTP----------------------HRAARQLA-KIGTLTRSCSRSELQRSASAPPPQ------------
HCMV UL33 LGHDFLQRMRQCFRGQLLDRRAFLRSQQNQRATAETNLAAGNNSQSVATSLDTNSKNYNQHAKRSVSFNFPSGTWKGGQKTASNDTSTKIPHRLSQSHHNLSGV
HHV-6 U12 TGGELRNRFTACFQS--FFPKTLCSTQKRKDSDASEHDQNSKSKASV----------------------------------------------------EKNQPL---
HHV-7 U12 TRTDFRLRF---YD---FIKCNLCNSSLKRKRNPLTIKN-------------------------------------------------------------------
Conserved    ..  *         .              .
```

B

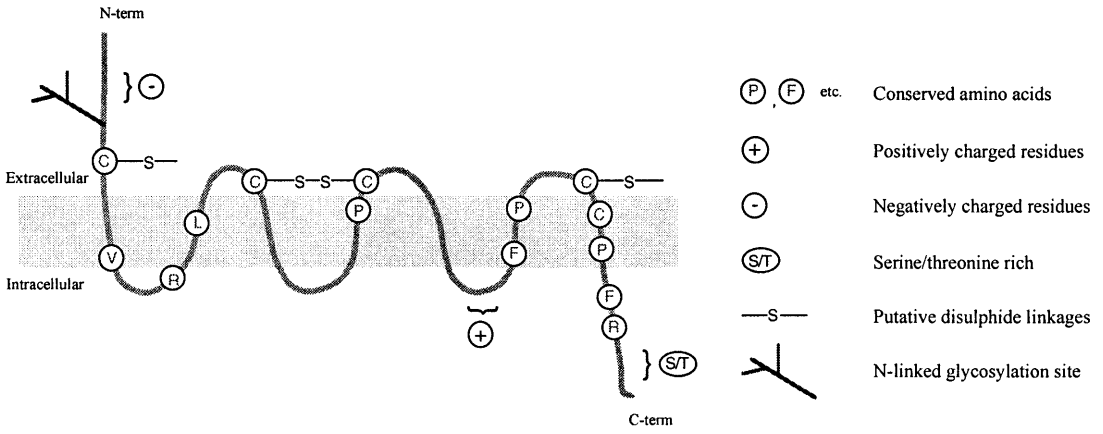

FIG. 6. (A) Multiple alignment of the betaherpesvirus GCR homologs compiled with Clustalw (33). Amino acids conserved among the four sequences are indicated by * (identical) and . (similar). The predicted extracellular (E), transmembrane (TM), and cytoplasmic (C) regions are indicated above the aligned sequences and numbered sequentially from the N terminus with Roman numerals. The positions of amino acids flanking the splice junctions identified (MCMV and HCMV) or predicted (HHV-6 and HHV-7) near the N terminus of each of the sequences are singly underlined. Features conserved with chemokine receptors are indicated by double underlining (potential N-linked glycosylation site) or in boldface type (specific amino acid residues). (B) Diagrammatic representation of the predicted topology for the GCR receptor homologs, highlighting features characteristic of chemokine receptors (adapted from reference 23).

The M33 and UL33 RT-PCR products were cloned into pGEM-T (Promega) and sequenced on both strands by using forward and reverse universal primers and fluorescent dye terminators on an automated sequencer (Applied Biosystems). At least four individual clones were sequenced for each RT-PCR product. Sequencing of these products confirmed the existence of the spliced mRNA, and the identified splice junctions and intron sequences are shown in Fig. 5A and B. Inspection of the HHV-6 U12 sequence identified similar splice donor and acceptor sites upstream of the previously published ATG (15), and putative splice donor/acceptor sites are similarly present in the HHV-7 U12 sequence (25); the predicted exon and intron sequences should these sites be utilized are shown in Fig. 5C and D. Figure 6A shows an alignment of the authentic M33 and UL33 amino acid sequences with the predicted spliced U12 genes of HHV-6 and HHV-7. The predicted topology and conserved features characteristic of chemokine receptors noted from this alignment are displayed in Fig. 6B and are further described in Discussion.

Construction of MCMV recombinants with disrupted M33. To investigate the biological significance of M33, MCMV recombinants in which the M33 coding region had been disrupted by insertion of a *lacZ* expression cassette were produced. Two separate insertion sites were used, as shown in Fig. 2, and the resulting recombinants were designated KΔ33E (disrupted at an *Eco*RV site) and KΔ33B (disrupted at a *Bst*EII site), with subscripts indicating independent recombinants (e.g., $K\Delta33B_{T2}$, $K\Delta33B_{T8}$). Recombinant plaques were

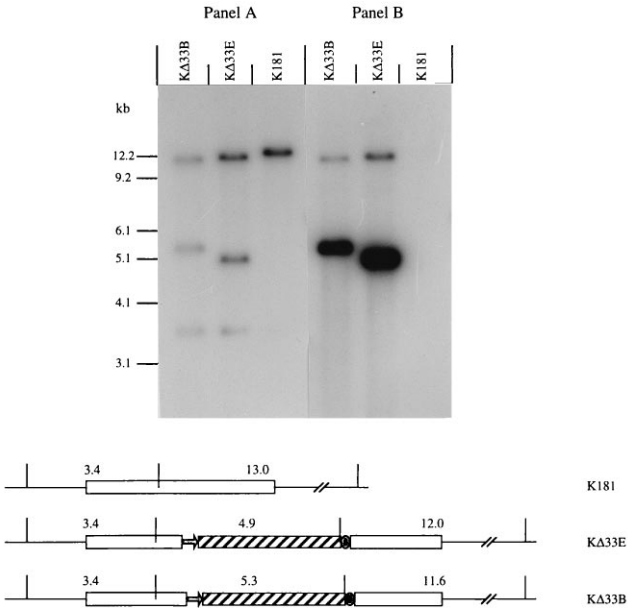

FIG. 7. Southern blot analysis of MCMV recombinants. DNA samples were prepared from cells infected with either wild-type K181 or recombinant virus (KΔ33B$_{T2}$ or KΔ33E). Samples were digested with *Eco*RI, separated on a 0.8% agarose gel, and blotted onto nylon membranes prior to hybridization to radioactively labelled probes. (A) The 5-kb *Bgl*II fragment of pINGM33 was used as the probe; (B) the 3.8-kb *lacZ* cassette from pMV10 was used as the probe. The position of DNA markers is indicated in kilobase pairs. The predicted positions of *Eco*RI sites for K181 and the two recombinants are shown schematically below the blot. Open boxes represent the 5-kb *Bgl*II region, and the hatched box indicates the *lacZ* ORF, with fragment sizes indicated in kilobases.

identified following cotransfection of viral and plasmid DNA as blue plaques in the presence of X-Gal (see Materials and Methods). Following three rounds of plaque purification, stocks of each recombinant were prepared and virus-infected cell DNA was analyzed by restriction enzyme digestion followed by Southern blot hybridization. Figure 7 shows the result of this analysis for KΔ33B$_{T2}$ and KΔ33E, in comparison with wild-type K181. All samples were digested with *Eco*RI and hybridized to probes specific for either the M33 region (Fig. 7A) or the *lacZ* cassette (Fig. 7B). The bands observed for both recombinants are as predicted following correct insertion of the *lacZ* cassette, and there is no evidence of contaminating wild-type virus, confirming that the stocks are clonally pure. (In Fig. 7A, the 3.4-kb band present in the K181 track is faint but could be clearly detected following a longer exposure of the blot.) The recombination for KΔ33B$_{T8}$ was confirmed in a similar manner (data not shown), and analysis of ethidium-stained digests (*Eco*RI and *Sac*I) for the three recombinants did not reveal any adventitious deletions elsewhere in the genome.

M33 is still transcribed in fibroblasts infected with KΔ33E. To determine whether insertion of *lacZ* within the M33 intron disrupted the M33 transcript for KΔ33E, RT-PCR was performed with poly(A)$^+$ selected RNA extracted from fibroblasts infected 3 h previously with either wild-type or recombinant viruses. The RT reaction was primed with MR2, and PCR was subsequently performed across the splice junction with MF1 and MR1. As shown in Fig. 8, an RT-PCR product corresponding to the spliced M33 transcript was detected in fibroblasts infected with both K181 and KΔ33E, demonstrating that the insertion of the *lacZ* cassette within the intron sequences of M33 did not prevent splicing to produce the authentic M33 transcript, presumably because the transcription termination signal of the cassette was inefficiently utilized. The intron of M33 may therefore provide a suitable site for the expression of heterologous genes without disrupting viral gene expression. The KΔ33E recombinant was subsequently used as a control virus recombinant to determine the effect, if any, of the expression of *lacZ* from the M33 region on the resulting phenotype in vitro and in vivo.

M33 is not required for replication in fibroblasts in vitro. Multistep growth curves were generated to determine whether the mutations in M33 affected the growth of the recombinant viruses in cell culture. Primary MEF were infected at an MOI of 0.01, and virus titers were determined from these cultures at various times p.i. and compared with the growth of wild-type MCMV (K181) (Fig. 9). No significant difference in replication between the mutants and wild-type MCMV was observed, indicating that M33, like UL33 (5), does not encode a function that is important for growth in fibroblasts in vitro.

M33 is critical for growth in salivary glands in vivo. Tissue culture-passaged MCMV (K181) is severely attenuated for growth in vivo during the acute phase, and thus a quantitative comparison of the replicative abilities of the recombinant viruses with wild-type MCMV is difficult. However, following a single passage in the salivary glands of mice, wild-type MCMV replicates to high titers in a number of target organs, particularly the spleen and liver. In an attempt to generate virulent salivary gland virus stocks of the recombinant viruses for in vivo growth studies, 3-week-old BALB/c mice were inoculated intraperitoneally with $10^{4.7}$ PFU of either KΔ33B$_{T2}$ or KΔ33B$_{T8}$ recombinant viruses. Control mice received the same dose of tissue culture-derived wild-type K181 virus or KΔ33E. During the acute stage of infection, virus was recovered from the spleens and livers of all wild-type- and KΔ33E-infected mice, although, as expected, the titers were low (10^2- $10^{2.7}$ PFU/organ [Table 1]). In comparison, MCMV could not be recovered from a number of KΔ33B$_{T2}$- and KΔ33B$_{T8}$-infected mice, although in those which were positive, the titers were not

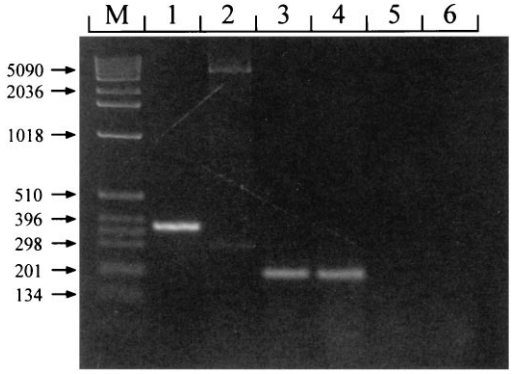

FIG. 8. Demonstration of transcription of correctly spliced M33 transcripts for recombinant KΔ33E. PCRs of DNA and RT-PCRs of poly(A)$^+$ RNA from MCMV-infected MEF cultures (24 h p.i.) were performed with oligonucleotides whose specificity is shown in Fig. 1D. Cultures were infected with either K181 (lanes 1, 3, and 5) or KΔ33E (lanes 2, 4, and 6). Amplification of infected cellular DNA by PCR was performed with oligonucleotide pair MF1-MR1 (lanes 1 and 2). First-strand synthesis from poly(A)$^+$ RNA with AMV RT was performed with oligonucleotide MR2 (lanes 3 and 4). Amplification of RT products by PCR was performed with oligonucleotide pair MF1-MR1 (lanes 3 to 6). To confirm that the amplified RT products were derived from RNA rather than from contaminating genomic DNA, control RT reactions were carried out with oligonucleotide MR2 in the absence of AMV RT (lanes 5 and 6). Products were separated on a 1.8% agarose gel. The relative migration of molecular mass markers (in base pairs) are indicated in lanes M. No products were observed in uninfected cultures (not shown).

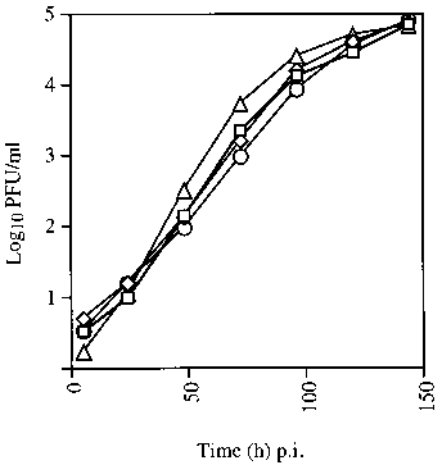

FIG. 9. Multistep growth curves of wild-type MCMV, KΔ33E, KΔ33B$_{T2}$, and KΔ33B$_{T8}$ in MEF. Confluent monolayers (in 24-well trays) were infected with 0.01 PFU per cell of either K181 MCMV (△), KΔ33E (□), KΔ33B$_{T2}$ (◇), or KΔ33B$_{T8}$ (○). Following virus adsorption (1 h at 37°C), monolayers were washed, fresh medium was added (2 ml per well), and the mixture was incubated for various times prior to harvesting (cells and supernatant). Samples were stored at −80°C prior to titer determination on MEF. Each point represents the mean of triplicate samples; standard errors were ≤10% of the mean value and are not shown.

significantly different from those in wild-type infected mice. However, while mice inoculated with either wild-type MCMV or KΔ33E yielded high levels of virus in the salivary glands on day 17 p.i., MCMV was not detected in the salivary glands of mice inoculated with the KΔ33B recombinants. Subsequent experiments with higher doses of inoculum and/or harvesting at different times p.i. also failed to elicit detectable levels of MCMV from the salivary glands of weanling mice. In addition, MCMV was not recovered from mice inoculated with $10^{3.7}$ PFU of either KΔ33B$_{T2}$ or KΔ33B$_{T8}$ recombinants directly in the salivary gland (Table 1, i.g.). These experiments indicate that M33 is critical for growth in vivo, particularly in the dissemination to and replication of virus in the salivary gland.

DISCUSSION

It is now apparent that a number of different viruses from within the herpesvirus and poxvirus families encode GCR homologs. Since cellular GCRs show considerable diversity, both in ligand specificity and in effects upon cellular behavior following ligand binding, many different roles can be envisaged for these viral GCRs. Of the GCR homologs encoded by HCMV, US28 has attracted particular attention as a potential chemokine receptor. In agreement with hypotheses based on the high level of sequence homology at the N terminus of US28 to known beta-chemokine receptors, US28 has been shown to bind beta-chemokines and to mediate cellular signalling as measured by intracellular calcium flux (13, 24). Similarly, the HSV GCR homolog ECRF3 has been shown to interact with alpha-chemokines (1).

TABLE 1. Growth of wild-type MCMV and M33 recombinant viruses in vivo

Virus[a]	Virus titer[b] in expt 1 ($10^{4.7}$ PFU i.p.)			Virus titer[b] in expt 2 ($10^{3.7}$ PFU i.p./i.g.)	
	Spleen	Liver	Salivary gland	Salivary gland (i.p.)	Salivary gland (i.g.)
Wild-type K181	2.0 ± 0.2	2.7 ± 0.1	7.30 ± 0.2	4.9 ± 0.1	4.6 ± 0.4
KΔ33B$_{T2}$	1.6	2.0	<1.0	<1.0	<1.0
KΔ33B$_{T8}$	1.3	1.8	<1.0	<1.0	<1.0
KΔ33E	1.7 ± 0.3	2.0 ± 0.2	5.81 ± 0.3	3.4 ± 0.2	2.3 ± 0.3

[a] In each experiment groups of three or four mice were infected with each virus.

[b] In experiment 1, virus titers were determined on day 3 p.i. (for the spleen and liver) or day 17 p.i. (for the salivary gland). In experiment 2, virus titers were determined on day 7 p.i., following i.p. or intraglandular (i.g.) inoculation of virus. Virus titers are expressed as the $\log_{10}$ ± standard error. Values without standard errors represent the titers of a single positive sample; the titers of the remaining samples in each group were <1.0.

The conservation of HCMV UL33 in MCMV, HHV-6, and HHV-7, of which homologs in the gamma- and alphaherpesviruses have not been identified, suggests that these gene products are important for the biological characteristics of betaherpesviruses. While preliminary analysis of these ORFs did not show notable conservation with GCRs at their N termini, this study has identified splicing in both the M33 and UL33 transcripts which, in contrast to the unspliced products of M33 and UL33, display an increased level of conservation with other mammalian GCRs and known viral homologs. Predicted splicing for the U12 genes of HHV-6 and HHV-7 likewise results in a higher degree of conservation at the N termini. When the above four sequences are aligned, it is apparent that a number of features characteristic of chemokine receptors are conserved, as illustrated in Fig. 6. These features include a conserved N-terminal glycosylation site and cysteine residue, the latter of which is invariant among the chemokine receptor subfamily of GCRs (23). In addition, the authentic M33 N terminus possesses two pairs of acidic residues which are also common to chemokine receptors. Although there are no signature motifs common to all chemokine receptors, they possess, in addition to an acidic glycosylated N terminus, a short third intracellular domain, which is enriched for basic residues. Furthermore, in contrast to many other GCRs, the first and fourth cysteines of the chemokine receptors, which are predicted to be disulfide linked, are highly conserved.

By analogy with US28 and ECRF3, it is tempting to speculate that the UL33 homologs may similarly display properties associated with chemokine receptors which lead to activation of the infected cell. It should be noted, however, that while US28 and ECRF3 were both found to have a high degree of amino acid identity at the N terminus in comparison with known chemokine receptors (MIP-1α/RANTES and IL-8 receptors, respectively), such a high level of conservation is not apparent for the UL33 homologs. Since the N terminus of GCRs is significant in determining the ligand-binding capacity, it is possible either that the UL33 homologs are not involved in chemokine binding or that they may interact with chemokines for which cellular receptors have yet to be determined.

Despite proving to be nonessential for growth in primary MEF in vitro, tissue culture stocks of the M33 null MCMV recombinants were defective in replication in vivo, in particular in the salivary gland, which is the major site of replication and persistence. Previous histological studies of MCMV-infected mice have shown that serious acinar cells of the salivary gland are the principal cells that support MCMV replication in vivo (16, 30). As cell lines derived from the salivary gland are not yet available, we are unable to make a direct, quantitative comparison of the replicative ability of wild-type and KΔ33B recombinants in these cells. Nevertheless, we were unable to detect virus replication following intraglandular inoculations with cell-free virus stocks of the KΔ33B recombinants. While these results suggest that M33 directly affects MCMV replication in salivary gland cells, it is also possible, given that MCMV infection in vivo is highly cell associated, that M33 plays a role

in the trafficking of infected leukocytes or the transmission of virus from infected leukocytes to target organs. Indeed, although a quantitative assessment of the M33 null mutants during acute infection was not possible due to the low levels of virus replication, it is possible that there is a global in vivo attenuation exhibited by the M33 null mutants, given that virus was not recovered from the spleens and livers of the majority of mice during acute infection. It is unlikely that the reduction in MCMV replication was due to the expression of β-galactosidase per se, as the KΔ33E recombinant, which contained *lacZ* within the M33 intron was shown to be replicate to levels comparable with wild-type MCMV.

Previous studies have identified another MCMV gene, *sgg-1*, which is also important for high virus growth in the salivary gland (18, 21). Unlike M33, homologs of *sgg-1* have not been identified in any other betaherpesviruses, suggesting that it may encode a function of particular importance to MCMV rather than of general significance for betaherpesviruses.

While the strict species specificity of the betaherpesviruses prevents studies of the HCMV and HHV-6/7 GCR homologs in the immunocompetent host, the murine model of CMV infection provides an excellent vehicle for the expression and characterization of betaherpesvirus genes in the context of a natural infection. The identification of several MCMV genes nonessential for growth in vitro have been recently reported (7, 8, 18, 21, 32) and their role in vivo characterised (7, 8, 18). The availability of the complete nucleotide sequence for MCMV will provide the basis for further, systematic analyses. The high level of sequence conservation between UL33 and M33 implies a conserved biological function which may allow the UL33 gene of HCMV to substitute for the MCMV counterpart. Toward this end, we are developing an in vivo system for the characterization HCMV UL33 and other betaherpesvirus GCR homologs.

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