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Cytomegalovirus Determinant of Replication in Salivary Glands

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Murine cytomegalovirus carrying a deletion mutation disrupting the expression of a gene dispensable for growth in cultured cells was found to disseminate poorly in the mouse. The mutation resulted in a dramatic decrease in the expression of a 1.5-kb major and a 1.8-kb minor β transcript from a region adjacent to the *ie2* gene in the viral genome. Nucleotide sequence determination indicated that 323 bp, including a predicted polyadenylation signal, was deleted from this β gene. In cultured cells, the plaque morphology and growth characteristics of the mutant were similar to those of parental or rescued wild-type viruses. Following intraperitoneal inoculation of BALB/c mice, growth of the mutant in the salivary gland was dramatically reduced 10,000-fold, while growth in the liver and spleen was not dramatically affected. The β gene was thus denoted *sgg*1 (salivary gland growth gene 1). Neither intranasal infection nor direct inoculation into the salivary glands completely overcame the restriction of growth in this organ, suggesting that the *sgg*1 gene encoded a determinant of tissue tropism. To investigate the impact of the *sgg*1 mutation on virus dissemination via the blood, the virus titer in peripheral blood leukocytes was determined. No difference was found between the *sgg*1 mutant and rescued wild-type virus. Thus, murine cytomegalovirus *sgg*1 gene products appear to be involved in entry or replication of virus in salivary gland cells.

Murine cytomegalovirus (CMV) is a betaherpesvirus that has been widely used as a model for human CMV infections. This virus has a 230-kbp linear DNA genome, a highly regulated replication cycle, and similarity to human CMV in many aspects of its biology, replication, and pathogenesis (13). Murine CMV establishes acute, persistent, and latent infections in the mouse (reviewed in references 13, 14, 29, and 30). The map position of a limited number of genes with homology between human and murine CMV suggests that these two viruses may have a colinear genome arrangement (3, 18, 19, 24, 26, 43).

Different strains of mice exhibit different susceptibilities to infection by murine CMV. Major histocompatibility complex-linked genes (1, 4, 10) as well as non-major histocompatibility complex-linked genes (10, 33, 40) have been identified as determinants of strain susceptibility. Virus strains also demonstrate different virulence characteristics (28). In many strains of mice, murine CMV replication in the salivary gland continues for prolonged periods of time. In all strains of mice, murine CMV grows to highest peak titers in the salivary gland, reaching peak levels of 10^8 to 10^9 PFU/g of tissue within 7 to 14 days postinoculation. Salivary gland virus is more virulent than that produced in other organs or in cell culture and can be attenuated by a single passage in cell culture (16, 31). This attenuation, however, is readily reversed by passage again in the mouse. The mechanism of this presumed epigenetic modification is not known but may be mediated through differences in adsorption and penetration (15, 34).

Tropism for the salivary gland is central to the biology and epidemiology of CMVs. Persistent and recurrent shedding from the salivary gland is believed to be the principal means by which these viruses spread in the population. Given the lack of an animal model for human CMV, murine CMV provides a useful setting in which to assess the viral genes that are dispensable for growth in cultured cells but that influence tissue tropism, pathogenesis, or latency. There have been reports of temperature-sensitive virus mutants exhibiting differences in both tissue tropism and virulence (37, 38, 47). Several of these mutants exhibit reduced ability to grow in salivary glands; however, the mutations responsible for this behavior have not been characterized.

A leukocyte-associated viremia is a hallmark of acute human CMV infection (7, 36) and has been demonstrated after intraperitoneal (i.p.) inoculation of murine CMV (2). This viremia is believed to enable virus dissemination from initial sites of viral replication in the peritoneum and spleen to secondary sites such as the salivary gland and lung. Experiments have shown that highly susceptible mouse strains such as BALB/c experience a more significant level of viremia than more resistant strains of mice (2), although the detection of viremia is difficult even in these susceptible strains. The role of viral gene products as determinants in the interaction of the virus with leukocytes has not been determined.

We previously described a method to engineer site-specific mutations into the murine CMV genome and showed that the α gene *ie2* was dispensable for viral replication in cell culture (23). We have characterized a spontaneous deletion mutant that arose in the course of using this methodology and have shown that the deletion lies within a β gene, called salivary gland growth gene 1 (*sgg*1), that influences replication in the salivary gland.

(A preliminary presentation of this work was made at the 15th International Herpesvirus Workshop in August, 1990.)

MATERIALS AND METHODS

Cells and viruses. Wild-type (strain Smith-K181, from M. Colin Jordan) and mutant viruses were grown and plaque assayed in NIH 3T3 fibroblast cells (ATCC CRL 1658) that

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were grown in Dulbecco's modified Eagle's medium (GIBCO-Life Technologies, Bethesda, Md.) supplemented with 10% NuSerum (Collaborative Research, Waltham, Mass.). Infections for the preparation of virus stocks and viral DNA were performed at a multiplicity of infection (MOI) of 0.01 PFU per cell, and infections for the preparation of viral RNA were performed at an MOI of 10 PFU per cell. Virus stocks were prepared from infected cells by sonicating the cells in a mixture of 50% medium and 50% autoclaved skim milk. Because of the marked defect in growth in the salivary gland exhibited by the mutant viruses, tissue culture-propagated (rather than more virulent salivary gland-propagated) virus stocks were prepared and used throughout these studies.

Animals. Three-week-old male BALB/c.ByJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were inoculated i.p. with 10^6 PFU of virus. For intraglandular inoculation, 6-week-old male BALB/c.ByJ mice were anesthetized by i.p. administration of tribromoethanol and inoculated with 10^6 PFU of virus (in 25 μl) inoculated directly into each submaxillary gland through an incision in the ventral midline of the neck. Animals received food and water ad libitum and were sacrificed by CO_2 asphyxiation. Organs were harvested and sonicated as a 10% (wt/vol) suspension in medium-skim milk. Sonicates were centrifuged at 200 × *g* for 5 min to pellet debris and were stored at −80°C until plaque assays were performed. The limit of virus detection in organ homogenates was 100 PFU/ml of sonicate. Those samples that were negative at a 10^{-2} dilution were assigned values of 1.5 log PFU for determination of geometric mean titers. Peripheral blood leukocytes (PBLs) were prepared from whole blood collected by cardiac puncture and added to 1.5 ml of phosphate-buffered saline (PBS)–8 mM EDTA, and after erythrocytes were lysed in 0.15 M NH_4Cl, the PBLs were collected and resuspended in 2.5 ml of medium. Half of the cells were sonicated for plaque assay and half were left intact for infectious center assays on subconfluent NIH 3T3 cell monolayers in 6-well cluster dishes (Becton Dickinson). After 14 h, these cultures were overlaid with 0.75% carboxymethylcellulose, and plaques or infectious centers were stained with Giemsa 4 days after inoculation.

Virulence assay. Groups of five 3-week-old male mice were inoculated i.p. with dilutions of virus (1×10^6, 5×10^6, 1×10^7, and 5×10^7 PFU). Animals were observed twice daily until 7 days after inoculation. No further deaths occurred after this time. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (35).

Recombinant plasmid and virus construction. Plasmid cloning was by standard methods (20). pON401 (Fig. 1) carries the *Hin*dIII L fragment of murine CMV DNA (25) cloned into the pBR322-based plasmid vector pMT11 (41). To construct pON427, the 4.0-kbp *Xho*I-*Xba*I fragment of pON283 (5), containing the human CMV *ie*1 promoter-enhancer (positions −219 to −19 relative to the start site of transcription) fused to the amino terminus of a chimeric *lacZ* gene (41), was made blunt ended by using Klenow DNA polymerase. The resulting fragment was then inserted between two *Hpa*I sites in pON401, replacing 79 bp that contained the TATA box and transcription start site of the *ie*2 gene (27), a strategy previously used to disrupt expression of this gene (23). pON480 was constructed by first digesting pON401 with *Hpa*I and then ligating to introduce a deletion of the 79-bp *Hpa*I fragment. pON400 (Fig. 1) carries murine CMV *Eco*RI-E (25) in pMT11. pON458 carries a 1.5-kbp *Eco*RI-*Hin*dIII fragment with the deletion mutation from RM427 DNA in the plasmid vector pUC9 (48), and pON446 carries the analogous 1.8-kbp wild-type fragment from parental virus in pGEM-2 (Promega). pON455 carries the 99-bp *Bal*I-*Cla*I fragment from the 3′ end of *sgg*1 cloned into *Hin*cII-*Acc*I-digested Bluescript KS− (Stratagene). Additional fragments from pON446, a 422-bp *Hin*dIII-*Msc*I fragment (pON477 and pON481) and a 187-bp *Cla*I-*Xho*I fragment (pON456 and pON484), were cloned in both orientations by using pGEM-3Zf(+) and (−) (Promega) and subjected to nucleotide sequencing (39). Other fragments were cloned in only one orientation by using pGEM-3Zf(+) [a 254-bp *Cla*I-*Nae*I fraction (pON4002) and a 524-bp *Hin*dIII-*Cla*I fragment (pON4004)] or by using pGEM-3Zf(−) [a 285-bp *Msc*I-*Xho*I fragment (pON486)]. *Msc*I is an isoschizomer of *Bal*I which is used to designate this site on the figures.

To construct virus recombinants, linearized plasmid and intact viral DNA were cotransfected into NIH 3T3 cells by using calcium phosphate precipitation as described previously (23, 42). Optimal recombination was obtained when approximately 1.0 μg of linearized plasmid DNA was cotransfected with 0.5 to 5.0 μg of viral DNA in a 25-cm^2 tissue culture flask. $lacZ^+$ virus was isolated as described previously (23) by blue plaque phenotype after being overlaid with agarose-containing medium supplemented with 300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and recombinant viruses in which mutations were rescued were either isolated as nonstaining plaques after being overlaid with X-Gal or were isolated by plaque hybridization (12). For each cotransfection, several candidate recombinant viruses were characterized and plaque purified four times prior to the preparation of virus stocks and DNA. Recombinant virus DNA structure was analyzed by restriction digestion and blot hybridization using ^{32}P-labeled probes prepared by nick translation (20) or random primer synthesis (8). For RNA blot hybridization, the *ie*2-specific probe was a *Pst*I-*Bam*HI fragment (27) isolated from pON401 and the *sgg*1-specific probe was an *Eco*RI-*Hin*dIII fragment in the plasmid pON446.

DNA and RNA analysis. Protocols for harvesting viral RNA, for DNA and RNA blot hybridization, and for RNase protection analysis have been described previously (9, 12). To prepare genomic viral DNA, 850-cm^2 roller bottles (Becton Dickinson) of infected cells were rinsed twice with PBS, overlaid with 10 ml of 100 mM NaCl–10 mM Tris base–10 mM EDTA–0.6% sodium dodecyl sulfate (SDS)–0.1 mg of proteinase K per ml (pH 8) and incubated overnight at 37°C. Lysates were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then extracted twice with an equal volume of water-saturated ether. The DNA was spooled on a glass rod after the addition of 2 volumes of isopropanol, dried, and resuspended in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. Although this preparation contained host cell DNA, it was optimal for transfection.

RESULTS

Construction of recombinant viruses. RM427 was isolated following the cotransfection of intact murine CMV DNA and plasmid pON427 linearized at a unique *Bam*HI site within the polylinker portion of the plasmid vector sequences. Recombinant viruses were identified by blue plaque phenotype after being overlaid with X-Gal. Blue plaques were picked and plaque purified four times, after which RM427 DNA was prepared and its structure was analyzed. In addition to the expected changes in *Hin*dIII-L due to the

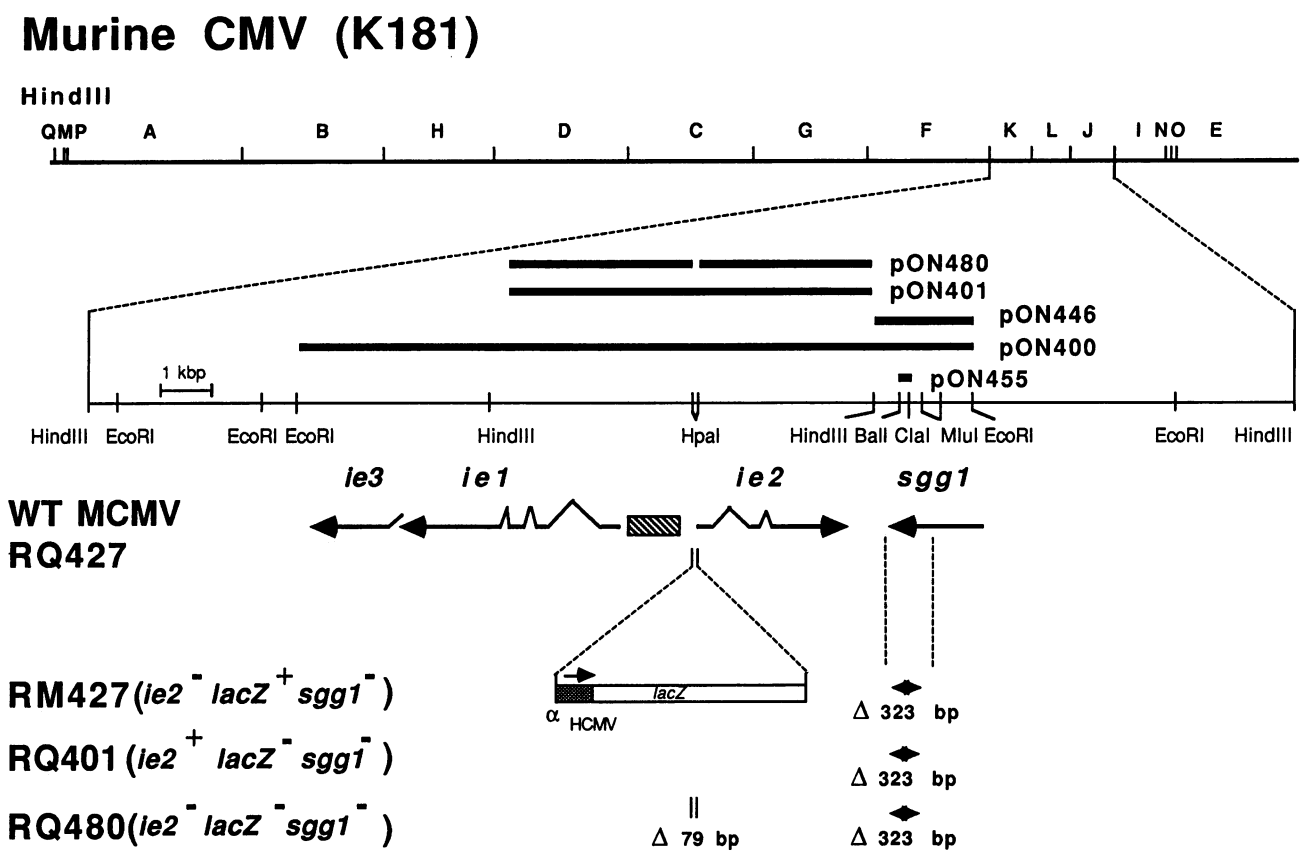

FIG. 1. Structure of recombinant plasmids and viruses. The top line depicts a *Hin*dIII map of the murine CMV genome (25) with the *Hin*dIII K, L, and J regions expanded. Complete maps for *Eco*RI and *Hin*dIII sites and only selected *Hpa*I, *Cla*I, *Mlu*I, and *Bal*I sites are shown in this region. The sequences carried in plasmid clones pON400, pON401, pON480, pON446, and pON455 are indicated by solid lines above the region, and the α genes *ie*1, *ie*2, and *ie*3 are shown by arrows below the region. The directions of transcription and splicing patterns of these α genes (18, 26, 27) are depicted along with the position of the murine CMV enhancer (hatched box) between *ie*1 and *ie*2. The *sgg*1 gene is depicted along with its direction of transcription. This arrangement of genes is found in parental wild-type (WT MCMV) murine CMV and RQ427. Below the expanded region, the genotypes of mutant viruses RM427, RQ401, and RQ480 are given in parentheses, along with a depiction of the presence of a *lacZ* insertion in *ie*2 (in RM427), a 79-bp deletion in *ie*2 (in RQ480), and a 323-bp deletion in *sgg*1 (in all three mutants). The human CMV *ie*1 promoter-enhancer containing sequences from −219 to −19 (shaded rectangle), used to drive the expression of *lacZ*, is indicated (α_{HCMV}), and a short arrow shows the direction of transcription. The double arrowheads indicate the position of the 323-bp deletion in *sgg*1, and the double lines indicate the position of the 79-bp deletion in *ie*2.

insertion of *lacZ* (22, 23), DNA blot analysis revealed the deletion of about 300 bp in the 1,800-bp *Hin*dIII-*Eco*RI fragment adjacent to the *ie*2 gene (Fig. 2). This was the only deletion observed for this viral DNA when restriction digests were compared with the parental wild-type virus. To map the deletion more precisely, the 1,500-bp *Hin*dIII-*Eco*RI fragment was cloned from the RM427 genome in pON458, and its restriction enzyme cleavage map was compared with that of the wild-type *Hin*dIII-*Eco*RI fragment. The location of the deletion was confirmed by the lack of any hybridization with pON455, a plasmid carrying a 99-bp *Bal*I-*Cla*I DNA fragment from within the deleted region, as shown diagrammatically in Fig. 1.

Three *lacZ* negative viruses were then derived from RM427. The first, RQ401, rescued the *ie*2 mutation; the second, RQ480, replaced the *lacZ* insertion mutation in *ie*2 with a 79-bp deletion; and the third, RQ427, rescued both the *ie*2 mutation and the adjacent 300-bp deletion. RQ401, RQ480, and RQ427 were isolated following cotransfection of RM427 viral DNA with pON401 (linearized with *Hin*dIII), pON480 (linearized with *Hin*dIII), and pON400 (linearized with *Eco*RI), respectively. Rescued viruses RQ401 and RQ480 were identified by their nonstaining phenotype after being overlaid with X-Gal. The completely rescued virus, RQ427, was identified by plaque hybridization with the 99-bp *Bal*I-*Cla*I fragment from the region deleted in RM427. RQ401, RQ480, and RQ427 were plaque purified four times, and their genomic structures were verified by restriction enzyme digestion analysis and DNA blot hybridization (data not shown). Figure 2 shows the presence of the 1.5-kbp *Hin*dIII-*Eco*RI fragment in RM427 and RQ401 and the 1.8-kbp fragment in RQ427. The structures of all recombinant virus genomes are depicted in Fig. 1.

Recombinant virus gene expression and structure. To confirm the expected disruption of *ie*2 and to determine whether the deletion altered the expression of any genes in the region, RNA from cells infected with the parental wild type, RM427, RQ401, and RQ427 was prepared and subjected to analysis. First, the impact of the *lacZ* insertion on *ie*2 expression was investigated, and expression was found to be disrupted in a manner similar to what was observed with a previous *ie*2-deficient virus (23). Figure 3A shows the levels

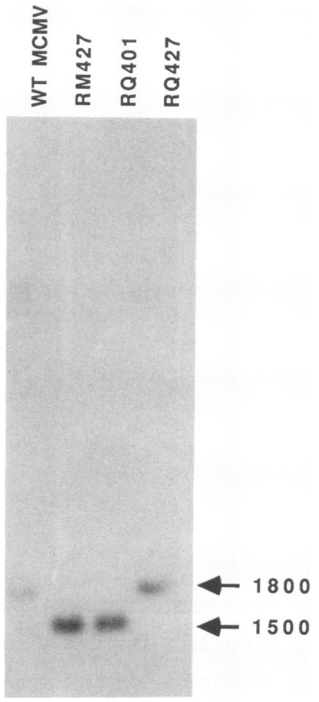

FIG. 2. DNA blot hybridization analysis of recombinant virus genome structure. Parental murine CMV (WT MCMV), RM427, RQ401, and RQ427 DNA was digested with *Hin*dIII and *Eco*RI, and the resulting fragments were separated on a 0.5% agarose gel. After transfer to nitrocellulose, the blot was hybridized with ^{32}P-labeled pON446 carrying the 1,800-bp *Eco*RI-*Hin*dIII fragment from the region adjacent to the *ie*2 gene (Fig. 1). The smaller, 1,500-bp fragments of RM427 and RQ401 as well as the larger, 1,800-bp fragments of parental murine CMV and RQ427 are indicated.

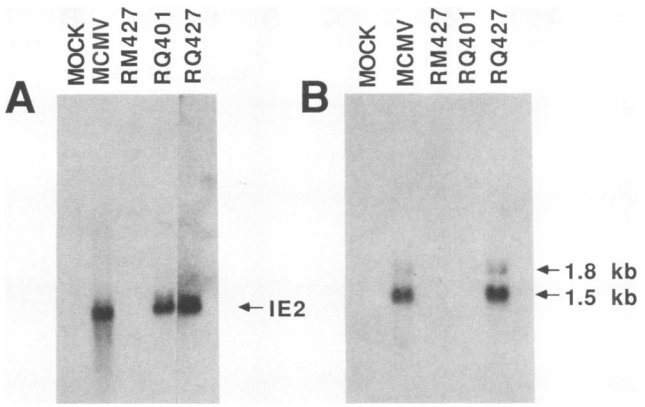

FIG. 3. Analysis of transcripts from *ie*2 and adjacent regions. (A) RNA blot to detect *ie*2 transcripts. Two micrograms (per lane) of whole cell RNA extracted from NIH 3T3 cells infected with wild-type murine CMV, RM427, RQ401, or RQ427 in the presence of cycloheximide (50 μg/ml) from 1 h before to 8 h after infection was separated on a 1% agarose–6% formaldehyde gel, transferred to nitrocellulose, and hybridized with an *ie*2-specific ^{32}P-labeled probe. (B) RNA blot to detect transcripts from the region adjacent to *ie*2. Whole cell RNA (2 μg) extracted from NIH 3T3 cells 8 h after infection with wild-type murine CMV, RM427, RQ401, or RQ427 was separated on a 1% agarose–6% formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pON446. MOCK refers to mock-infected cell RNA. MCMV, murine CMV.

of the 1.75-kb *ie*2 transcript expressed during a cycloheximide (50 μg/ml) block from 1 h before to 8 h after infection with parental wild-type and mutant viruses. Expression of this transcript by RM427 was reduced by at least 100-fold, whereas expression by RQ401 and RQ427 was at the same level as that of the wild type. RM480, which carried a deletion in place of the *lacZ* insertion, showed a disruption in *ie*2 expression similar to that of RM427 (data not shown). Thus, the lack of expression of *ie*2 transcripts correlated with the genome structure of the recombinant viruses.

The deletion mutation was found to disrupt a β gene adjacent to *ie*2. Analysis of RNA harvested at 8 h postinfection is shown in Fig. 3B. RM427 and RQ401, which both carried the 300-bp deletion, failed to express detectable levels of one abundant 1.5-kb transcript and one less abundant 1.8-kb transcript. The rescued virus RQ427 expressed both of these transcripts to wild-type levels, thus suggesting that the loss of both transcripts in RM427 was due to the deletion. These two transcripts were not expressed in the presence of cycloheximide (data not shown). Further experiments also revealed that, like some other CMV β genes, the 1.8- and 1.5-kb transcripts continued to be expressed at late times postinfection (24 h).

Hybridization to RNA blots with DNA probes generated from within the 1.8-kbp fragment of pON446 consistently detected both the 1.5- and 1.8-kb transcripts, indicating that the coding sequences for both transcripts were largely or completely contained within the *Hin*dIII-*Eco*RI region (data not shown). The transcripts arising from this region were shown to be transcribed from right to left by using RNase protection analysis with RNA probes generated from either the T7 or SP6 promoter flanking the *Hin*dIII-*Eco*RI insert in pON446. As shown in Fig. 4A, a probe generated from the T7 promoter and extending from the *Hin*dIII to the *Mlu*I site (676 bp) into this region protected a 337-nucleotide species. Thus, a 3′ terminus mapped downstream of the *Mlu*I site. No other protected species were generated from this region, suggesting that both the 1.5- and 1.8-kb RNA species terminated at the same position. The nucleotide sequence of this region, from an *Xho*I site adjacent to the *Mlu*I site extending to a *Pvu*II site adjacent to the *Hin*dIII site, was determined by using the following series of plasmid clones (described in Materials and Methods): pON455, pON477, pON481, pON456, pON484, pON4002, pON4004, and pON486. The sequence revealed that the 337-nucleotide protected region was likely to be the most 3′ proximal exon of this gene; this finding was confirmed by sequencing a cDNA clone that carried the 3′ end of this RNA (data not shown). When this sequence was compared with the analogous region from the mutant virus by sequencing pON458, a 323-bp region was found to be deleted (Fig. 4B). The deletion removed most of this exon as well as a consensus polyadenylation signal adjacent to the position of the mapped 3′ end (Fig. 4C).

RQ401 grows well in cultured cells and exhibits virulence. The size and morphology of plaques made by RQ401 and RQ427 were comparable to those of wild-type murine CMV. The growth rate of the mutant RQ401 was indistinguishable from that of the parental wild-type virus, and the growth of the rescued virus RQ427 was similar (Fig. 5A). Stock titers for all of these viruses were between 8.3 and 8.9 $\log_{10}$ PFU/ml, indicating that the β gene, like *ie*2 (23), was dispensable for viral growth in cell culture.

An LD_{50} experiment was performed with the virus mutants. The LD_{50} of the mutant virus RQ401 was virtually identical to that of the parental wild-type virus, and the

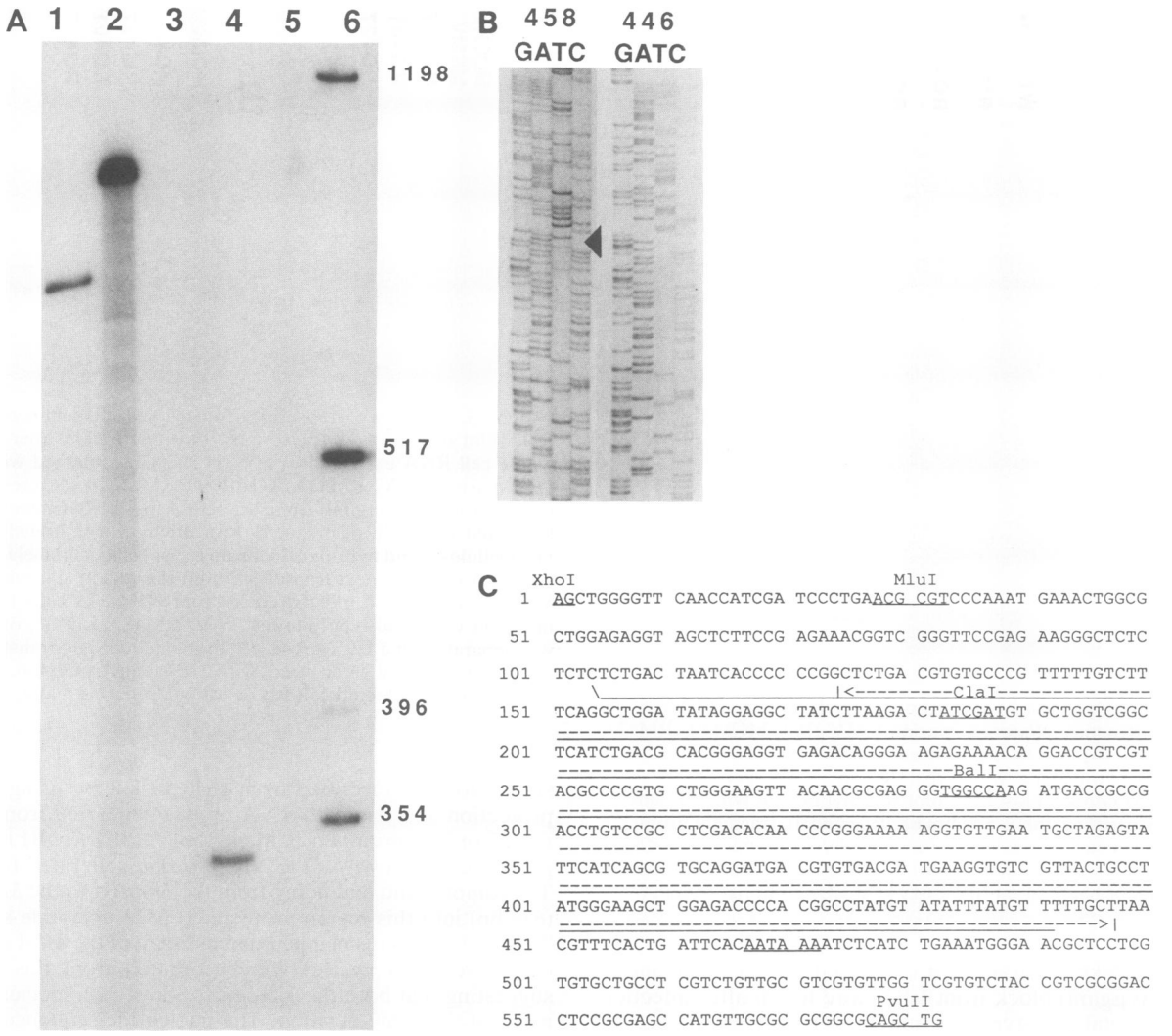

FIG. 4. Direction of transcription and structure of the 3′ exon of *sgg*1. (A) RNase protection assay. Strand-specific RNA probes were generated from pON446 after digestion with *Mlu*I (see Fig. 1) by using either SP6 or T7 RNA polymerase. The SP6 probe represented sequences adjacent to the *Eco*RI site, and the T7 probe represented sequences adjacent to the *Hin*dIII site. Lanes: 1, SP6-generated probe; 2, T7-generated probe; 3, T7 probe hybridized with uninfected cell RNA; 4, T7 probe with murine CMV-infected cell RNA (8 h postinfection); 5, SP6 probe with murine CMV-infected cell RNA (8 h postinfection); 6, size markers, with sizes in nucleotides indicated to the right. (B) Nucleotide sequence of the noncoding strand across the deletion carried by pON458 (the deletion point is indicated by an arrowhead) compared with that of wild-type murine CMV (pON446). (C) Summary of nucleotide sequence from the last two nucleotides of an *Xho*I site to a *Pvu*II site in wild-type viral DNA. The overlined sequence from 155 to 491 depicts the 3′ exon and likely polyadenylation sequence (AATAAA) from this gene. The position of the 323-bp deletion found in RM427 and RQ401 DNA is indicated by broken arrow (<- - ->). Restriction enzyme sites and the AATAAA sequence are underlined.

rescued virus RQ427 appeared to be slightly more virulent than wild-type virus (Table 1). Further, there was no difference in the day of death following inoculation with any of these viruses, and all deaths were observed within a similar 7-day time period. Animals inoculated with a sublethal dose (10^6 PFU i.p.) of virus exhibited different levels of disease, as indicated by measuring weight loss at 5 days postinoculation. All animals infected with parental wild-type virus lost weight and on average were only 86% of their initial weight, whereas all animals inoculated with either RQ401 or RQ427 continued to gain weight and were on average about 15 to 20% heavier at 5 days postinoculation. Thus, the mutation in the β gene did not correlate with altered virulence, although both RQ401 and RQ427 induce less disease than wild-type virus at this sublethal inoculation.

Replication of recombinant viruses in mouse spleen and liver was examined. Groups of 3-week-old BALB/c.ByJ mice were inoculated i.p. with 10^6 PFU of RQ401, RQ427, or parental wild-type virus, and the virus titer in these organs was determined at various days postinoculation. In the liver, the mutant (RQ401) replicated as well as the wild-type did (Table 1), with peak titers occurring at 3 days postinoculation. In spleen sonicates, however, the titer of the mutant virus was more than 1 order of magnitude less than that of the parental wild-type virus. To determine whether the peak growth occurred at a different time in mutant-inoculated animals, we measured viral growth at 1, 3, 5, and 7 days postinoculation. Compared with the parental wild type, both RQ401 and RQ427 exhibited a slightly lower peak growth in the spleen (Fig. 5B), but these data suggested that the

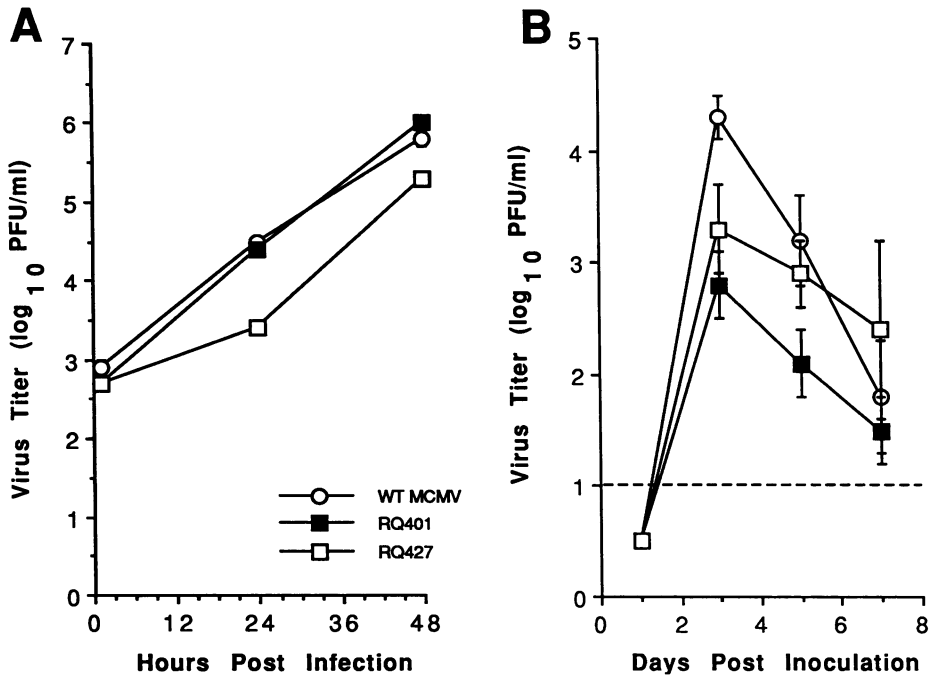

FIG. 5. Virus growth in cell culture and in the spleen after i.p. inoculation. (A) Cell cultures were infected at a multiplicity of infection of 5 PFU per cell, collected at 1, 24, and 48 h postinfection, and sonicated, and the titer was determined by plaque assay. All titers are expressed as the geometric mean (± the standard error of the mean) of the virus titer determined on three parallel cultures of 10^5 cells. (B) The spleens were collected from groups of three to five mice at 1, 3, 5, and 7 days after i.p. inoculation with 10^5 PFU; the geometric mean (± the standard error of the mean) is shown. The data from day 3 were from animals inoculated on a different day than the others, and the limit of sensitivity was 10 PFU/ml (indicated by the dashed line). WT MCMV, parental murine CMV.

characteristic was not rescued when the mutation in the β gene was repaired. Taken together with the LD_{50} data, the growth characteristics suggest that the β gene mutation carried by RQ401 was not responsible for any growth deficiency or attenuation.

The deletion mutation affects ability to grow in the salivary gland. Even though these viruses all replicated relatively well in the spleen and liver following i.p. inoculation, the mutant virus replicated very poorly in the salivary gland (Fig. 6). The peak titer of RQ401 replication in the salivary gland was reduced on average 10,000-fold relative to that of the parental wild type or the rescued virus (RQ427). Because the rescued virus, RQ427, expressed wild-type levels of *sgg*1 transcripts and exhibited normal growth characteristics in the salivary gland, the mutation disrupting this β gene in RQ401 was the most likely cause of poor replication. These results suggested that the gene product(s) encoded by the β gene played an important role in influencing the level of growth in salivary glands, and so the gene was designated salivary gland growth gene 1 (*sgg*1).

TABLE 1. Growth and virulence characteristics of mutant viruses

Virus	LD_{50}[a]	Wt loss[b]	Titer[c] in:	
			Spleen	Liver
WT MCMV[d]	7.4	5/5 (0.86)	4.3 ± 0.2	3.0 ± 0.5
RQ401	7.3	0/5 (1.20)	2.8 ± 0.3	3.3 ± 0.4
RQ427	6.6	0/5 (1.15)	3.3 ± 0.4	ND

[a] Expressed as $\log_{10}$ PFU.
[b] Number of animals exhibiting weight loss 5 days after i.p. inoculation with 10^6 PFU per total number of animals; the average weight at 5 days divided by the average weight of animals at the time of inoculation is shown in parentheses.
[c] Peak titer (3 days after i.p. inoculation) expressed as the geometric mean titer ± the standard error of the mean ($\log_{10}$ PFU/ml of 10% tissue sonicate); three to six animals per group. ND, not determined.
[d] WT MCMV, parental murine CMV.

In preliminary experiments, replicaton of the *sgg*1 mutant in the salivary gland was found to be as poor after intranasal (i.n.) inoculation as after i.p. inoculation. Peak titers in the salivary gland were 1,000- to 10,000-fold lower than those of the parental wild-type virus when compared at 7 or 15 days postinoculation (data not shown). To establish whether the mutant was deficient in its ability to grow in the salivary gland, we used direct inoculation of the salivary gland. Groups of six mice were inoculated directly into the submaxillary salivary glands with 10^6 PFU of the *sgg*1 mutant RQ401 per gland and were compared with groups inoculated with parental and rescued wild-type viruses. Seven days after intraglandular inoculation, the growth of the *sgg*1 mutant was detectable but was reduced 30- to 100-fold compared with growth of the parental wild-type virus or rescued virus RQ427 (Table 2). While the differences in these titers were lower than observed after either i.p. or i.n. inoculation, *sgg*1 mutants were clearly unable to replicate as efficiently as wild-type viruses even when directly introduced into this target organ. Thus, the mutant virus's poor growth was related to a defect in the ability to replicate in cells located in the salivary gland. Importantly, this defect was rescued with the repair of the *sgg*1 gene. The less dramatic difference between the *sgg*1 mutant and wild-type viruses following direct inoculation of the gland, as opposed

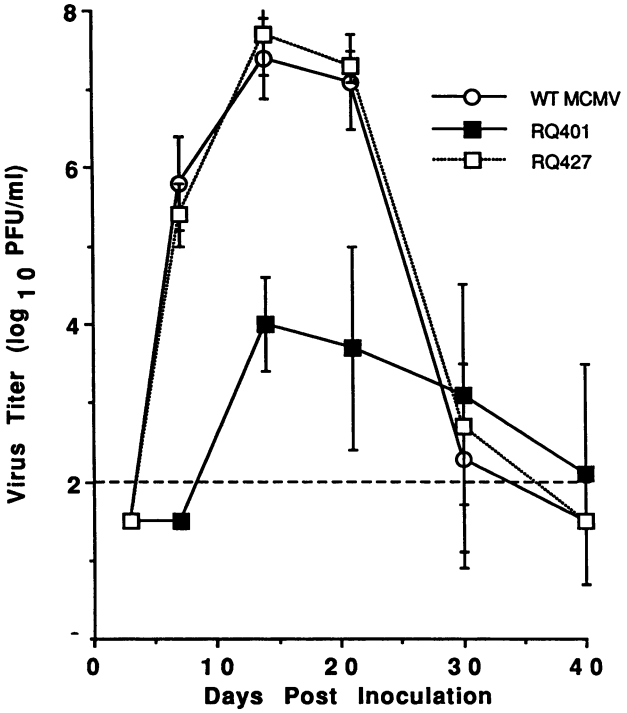

FIG. 6. Growth in the salivary gland following i.p. inoculation. Groups of 4 to 10 3-week-old mice were inoculated i.p. with 10^6 PFU of virus. Salivary gland stocks were prepared from groups of animals at 3, 7, 14, 21, 30, and 40 days after inoculation. The virus titer was determined by plaque assay on NIH 3T3 cells, and results are depicted as the geometric mean (± the standard error of the mean). Data were combined from four separate experiments, and all recombinant viruses were assayed at least three times. The limit of sensitivity was 10^2 (2.0 $\log_{10}$ units) PFU/ml (indicated by the dashed line). WT MCMV, parental murine CMV.

to i.p. or i.n. inoculation, suggested that dissemination to the salivary gland may also be influenced by the mutation.

The deletion does not affect cell-associated viremia. In order to determine the nature of the interaction of wild-type and *sgg*1 mutants with cells in the blood that might be involved in viral dissemination, we inoculated animals i.p. with 10^6 PFU and prepared PBLs at the peak of viremia (2), at 5 days postinoculation. The titers of virus recovered from sonicated cells and the numbers of cells yielding infectious centers were determined (Table 3). While there were marked differences between the parental wild type and the *sgg*1 mutant, the rescued virus RQ427 was found in levels comparable to those of the *sgg*1 mutant. Thus, the differences we had observed between the abilities of the *sgg* mutant RQ401 and the rescued virus RQ427 to replicate in the salivary gland did not result from any differences in their respective levels of viremia. While all recombinant viruses exhibited a reduced viremia relative to that of the parental wild-type virus, the *sgg*1 mutation did not contribute to this phenotype. Furthermore, the reduced viremia by the rescued wild-type virus RQ427 did not alter the ability of this virus to replicate in the salivary gland after either i.p. or i.n. inoculation. Taken together with the results of direct inoculation of the salivary gland, these data suggest that the *sgg*1 mutation influences the ability of virus to interact with or replicate in cells in the salivary gland and, although virus associated with leukocytes, dissemination from these cells may be compromised in some way.

TABLE 2. Growth in salivary gland after intraglandular (i.g.) inoculation

Virus	Titer at 7 days postinoculation ($\log_{10}$ PFU/ml)[a]	
	i.p.[b]	i.g.[c]
WT MCMV	6.5 ± 0.5	4.5 ± 0.1
RQ401	<2	3.0 ± 0.4
RQ427	5.2 ± 0.4	5.0 ± 0.2

[a] Geometric mean ± standard error of the mean. The limit of sensitivity was 2 $\log_{10}$ PFU/ml.
[b] Inoculum of 10^6 PFU, with two animals per group.
[c] Inoculum of 10^6 PFU per salivary gland, with six animals per group.

TABLE 3. Virus detection in leukocytes

Virus	Titer at 5 days postinoculation[a]	
	Sonicated[b]	Infectious centers[c]
WT MCMV	3.3 ± 0.2	4.1 ± 0.1
RQ401	1.4 ± 0.6	2.7 ± 0.5
RQ427	0.6 ± 0.2	2.2 ± 0.4

[a] Geometric mean ± standard error of the mean (either $\log_{10}$ PFU per milliliter or $\log_{10}$ infectious centers per milliliter of blood). There were five mice per group.
[b] PFU per milliliter of blood after sonication.
[c] Infectious centers per milliliter of blood after cocultivating cells on NIH 3T3 cell monolayers.

Influence of *ie*2 on viral growth characteristics in the mouse. We previously reported that the *ie*2 gene could be disrupted without altering viral growth characteristics in cell culture (23). The availability of mutations disrupting *ie*2 in addition to *sgg*1 made it possible to assess whether this α gene influenced viral growth in the mouse. We tested RM427, which carries a *lacZ* insertion in *ie*2, and RQ480, which carries a 79-bp deletion in *ie*2 (Fig. 1), and found both of these to exhibit growth qualities in cell culture and in the spleen, liver, and salivary gland analogous to those of RQ401 (data not shown), suggesting that the disruption of *ie*2 expression did not contribute dramatically to the ability to grow in these organs.

DISCUSSION

We have identified a murine CMV β gene, *sgg*1, that influences dissemination in the host to a specific target organ, the salivary gland. Our results suggest that products of this gene play a role in the ability of virus to efficiently interact with or replicate in salivary gland cells. Studies following murine CMV cytopathology have indicated that acinar cells are the most prominent target cells in the salivary gland (11, 14, 30). Thus, the function of the *sgg*1 gene products might be predicted to be important for entry or replication in this cell type. The role of the *sgg*1 gene will be better understood once *sgg*1 mutants are compared with the wild type for the ability to infect salivary gland acinar cells or similar cell epithelial types and once the gene structure and nucleotide sequencing studies of *sgg*1 are complete.

The spontaneously arising mutation carried by RQ401 is located at the 3′ end of the gene and apparently disrupts the polyadenylation signal, AAUAAA, of two transcripts encoded in this region. The two transcripts that are disrupted in

*sgg*1 mutants are large enough to encode proteins in the range of 30 to 70 kDa, and preliminary analyses have indicated that β proteins of 37, 50, and 70 kDa are affected by the mutation (22). Whether any or all of these proteins are the products of the *sgg*1 gene requires additional work. The deletion lowers steady-state RNA levels of the predominant 1.5-kb transcript at least 100-fold. This effect is most likely due to the instability of the transcripts arising as a result of the mutation rather than to any direct effect on the transcription rate of this gene (21).

Virus carrying the *sgg*1 mutation was as virulent as the wild type as determined by LD_{50} analysis, but it did not cause as much weight loss as did wild-type virus given at the same input dose. Taken together with the growth defect of *sgg*1 mutants, this observation indicates that replication of virus in the salivary gland is probably not a significant factor in a lethal infection of BALB/c mice. Furthermore, the *sgg*1 gene products did not it appear to be responsible for weight loss during infection, because the rescued virus RQ427 exhibited the same phenotype as RQ401. This characteristic appeared to be better correlated with the slightly lower growth in the spleen of both RQ401 and RQ427 than of the parental virus. These observations are consistent with previous suggestions indicating that aggressive viral growth in the spleen or liver might lead to weight loss, necrosis, and death (17), and they provide evidence that both recombinant viruses carried another mutation unrelated to *sgg*1 that influenced weight loss as well as levels of growth in peripheral blood cells and organs such as the spleen.

While RQ401 exhibited less replication in PBLs than did the parental wild-type virus, this defect was not rescued along with the *sgg*1 mutation in RQ427. Thus, poor dissemination may have contributed to, but was not the primary reason for, poor growth in the salivary gland by the *sgg*1 mutant. In support of this conclusion, poor growth in leukocytes did not prevent RQ427 from reaching levels in the salivary gland comparable to those of the parental wild-type virus. Thus, a poor titer in leukocytes per se did not result in poor dissemination to the salivary gland in these studies.

In the herpesvirus family, there are other examples of genes that are needed for efficient virus growth in specific tissues. In herpes simplex virus type 1 (HSV-1), the thymidine kinase gene, a β gene, is essential for replication in the neuron but is dispensable for replication in tissue culture or in many other tissue types (32, 44–46). Other HSV-1 genes (6) have also been implicated in HSV-1 neuronal cell tropism and neuroinvasiveness. Thus, as with the much more extensively studied example of HSV-1, CMV genes play critical roles as determinants of tissue tropism in the infected animal host. Our work opens the way to a better understanding of the role of CMV gene products in the important interaction of this virus with blood leukocytes and the salivary gland. These interactions are critical to the survival and spread of CMV.

Specific tropism for host tissues, particularly for the salivary gland, is a critical determinant of CMV biology. Tropism for the salivary gland and resultant persistent and recurrent shedding in saliva appears to define one major route of human CMV transmission in normally healthy human populations. Tropism for a blood leukocyte population and the risk of transmission by blood transfusion defines a second major route of CMV transmission in human populations. The work we have reported here demonstrates that a gene which is dispensable for viral replication in cell culture has an impact on organ tropism. Although the mutation we have characterized arose spontaneously, we have expanded the techniques available for manipulating the murine CMV genome and for studying their phenotypes in the infected mouse host. Targeted mutagenesis is sure to yield information on the function of other genes and to lend new insight into the complex biology of this group of viruses.

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