Characterization of the murine gammaherpesvirus 68 ORF74 product: a novel oncogenic G protein-coupled receptor

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Murine gammaherpesvirus (MHV-68) is well established as a small animal model for the study of gammaherpesviruses. The MHV-68 genome contains an open reading frame (ORF74) that has significant sequence homology with mammalian G-protein coupled receptors (GPCRs) and the GPCR from the related Kaposi’s sarcoma-associated herpesvirus (KSHV). Here we show that the MHV-68 ORF74 is predicted to encode a GPCR since it has seven potential transmembrane helices and that it has other sequence motifs in common with GPCRs. Of interest is the observation that the sequence around a conserved arginine at the start of the second intracellular loop suggests that the ORF74 product may signal constitutively (agonist independent). Given that the ORF74 product is predicted to encode a GPCR we named it MHV-GPCR. In studies on the transcription of the MHV-GPCR, we determined that it was encoded on multiple early transcripts of 3–4, 4–4, 6–6 and 8–7 kb in size. At least one of these transcripts was bicistronic, containing the ORF encoding the Bcl-2 homologue also. In vivo, we found that MHV GPCR was expressed during acute infection but also during persistence, particularly in the lungs of infected mice. Immunofluorescence studies indicated that the MHV GPCR protein was expressed on the surface of cells in patches. Finally, like the KSHV GPCR, expression of the MHV GPCR resulted in transformation of NIH 3T3 cells. We surmise, therefore, that the MHV GPCR may act in concert with genes with which it is expressed such as vBcl-2 to enhance the growth and survival of MHV-68-infected cells.

Introduction

Murine gammaherpesvirus (MHV-68) is a lymphotropic herpesvirus isolated from wild rodents (Blaskovic et al., 1980). Sequence and biological analyses have shown that it is closely related to Epstein–Barr virus (EBV) and Kaposi’s sarcom-associated herpesvirus (KSHV) (Sunil-Chandra et al., 1992a, b; Virgin et al., 1997). However, unlike these human gammaherpesviruses, MHV-68 can infect a wide range of cells in tissue culture (Svobodova et al., 1982). It also infects laboratory mice (Blaskovic et al., 1984) thus making it an excellent small animal model for the study of gammaherpesvirus pathogenesis (Stewart, 1999). An acute interstitial pneumonia ensues after intranasal infection with MHV-68. Peak titres of virus occur at 5–7 days post infection (p.i.) and the productive infection at this site is resolved by the immune response by 14 days p.i. (Ehtisham et al., 1993; Sunil-Chandra et al., 1992a), although a lifelong latent infection is then established at this site (Stewart et al., 1998). The virus then spreads to the spleen where it establishes latency and persists throughout the lifetime of the host in B cells, macrophages and dendritic cells (Flano et al., 2000; Sunil-Chandra et al., 1992b; Usherwood et al., 1996; Weck et al., 1996, 1999).

The G protein-coupled receptor (GPCR) super-family of seven transmembrane spanning receptors comprises over 250 members. They all share characteristic structural features that most likely reflect their common mechanism of action (Strader et al., 1995). Mutations in GPCRs resulting in constitutive signalling activity have been associated with human disease (Parma et al., 1993; Schipani et al., 1995). In addition, a number of cellular GPCRs including the muscarinic acetylcholine receptor (Gutkind et al., 1991) and the serotonin 1c receptor have been shown to be oncogenic when expressed ectopically (Julius et al., 1989).

Several gamma-2 herpesviruses including herpesvirus saimiri (HVS) and KSHV contain an open reading frame (ORF74) encoding a functional GPCR that has significant
sequence identity (32 and 30% respectively) with CXCR2, the high-affinity interleukin 8 receptor (Ahuja & Murphy, 1993; Arvanitakis et al., 1997). In the case of KSHV, the GPCR binds a wide range of chemokines and has constitutive (agonist-independent) activity. It also exhibits transforming activity in 3T3 cells and is tumorigenic in nude mice (Arvanitakis et al., 1997). Thus, it has been proposed that the KSHV GPCR is a viral oncogene that can exploit cell-signalling pathways to induce cell proliferation that may be important in viral pathogenesis and oncogenesis.

MHV-68 encodes a number of cellular homologues that are postulated to be involved in immune evasion and virus persistence (Virgin et al., 1997). A cluster of such genes is encoded at the right-hand end of the genome. The products of two of these ORFs [homologues of cyclin D (ORF72), Bcl-2 (M11)] have been shown to be functional (Roy et al., 1993; van Dyk et al., 1999; Wang et al., 1999). A third gene in this cluster is ORF74. Like the analogous genes in HVS and KSHV, MHV-68 ORF74 shares significant (28%) sequence similarity with CXCR2.

The aim of this study was to characterize the expression and activity of the MHV-68 ORF74 gene product. We describe the transcription pattern of the gene and the expression pattern of the protein in cells. We also show that the ORF74 gene product is a viral oncogene with transforming activity in vitro.

Methods

Cell lines and virus. Murine gammaherpesvirus-68 (MHV-68) strain g2.4 was propagated and quantified by plaque assay using BHK-21 + Cells and virus.

Table 1. Primers used for PCR analysis

<table>
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<th>Gene</th>
<th>Primer</th>
<th>Genome coordinate</th>
<th>Sequence</th>
<th>Product size (bp)</th>
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</tbody>
</table>

Methods

Cell lines and virus. Murine gammaherpesvirus-68 (MHV-68) strain g2.4 was propagated and quantified by plaque assay using BHK-21 + Cells and virus.

RNA preparation from MHV-68 infection in vitro. BHK cells (8 × 10^5) were infected at 5 p.f.u. per cell. RNA specific for the immediate early stage of infection was harvested at 8 h p.i. from cells that were incubated with 100 µg/ml cycloheximide (CHX) for 30 min prior to adsorption of the virus and for the rest of the infection. Early RNA was isolated at 24 h p.i. from cells that were pre-treated for 2 h with 100 µg/ml of phosphonoacetic acid (PAA) prior to and during infection. Late RNA was derived from infected cells maintained without treatment for 24 h. At appropriate time-points, monolayers were washed and harvested by scraping. Cells were pelleted and total RNA purified as described previously (Stewart et al., 1994). Poly(A)^+ RNA was prepared from total RNA using an mRNA Purification kit (Amersham Pharmacia).

Northern analysis. Poly(A)^+ RNA (10 µg/lane) was electrophoresed through formaldehyde gels, transferred to nylon membranes and probed with [α-32P]dCTP-labelled DNA probes as described previously (Stewart et al., 1994). Molecular mass determinations were made using an RNA ladder (Life Technologies).

Preparation of RNA from MHV-68-infected mouse tissues. BALB/c mice were infected intranasally with 4 × 10^5 p.f.u. of MHV-68 as previously described (Sunil-Chandra et al., 1992a). Three mice were sacrificed at 2 weeks and 10 months p.i. and lung and spleens were removed and snap-frozen at −70 °C. A portion of tissue from each
organ was removed and total RNA prepared using an RNeasy kit (Qiagen).

**RT–PCR analysis.** Total RNA (2 µg) was treated with 10 U of RNase-free DNase I (Amersham Pharmacia) at 37 °C for 30 min. cDNA was synthesized at 42 °C with 200 U of reverse transcriptase (Superscript II, Life Technologies) in the presence of 100 nM dNTPs (Amersham Pharmacia). A portion (1–5 µl) of cDNA was subject to PCR with primer sets specific for the MHV-68 ORFs vBcl-2 (M11), ORF74, ORF50 and ORF57. Separate PCR reactions for murine β-actin were done for each cDNA sample. The sequences of primer sets used and the predicted sizes of PCR products are given in Table 1. Primers for ORF50 and ORF57 were as described by Virgin et al. (1999). PCR reactions were carried out with 1 U per reaction of Taq DNA polymerase (Life Technologies) in the presence of 100 nM dNTP, 2.0 mM MgCl₂ and 50 pmol of each primer in a total reaction volume of 50 µl. Cycling parameters were the same for each primer set used and were: 96 °C, 45 s; 45 °C, 60 s; 72 °C, 120 s for 35 cycles with a final extension at 72 °C for 7 min. Electrophoresed PCR products were transferred to Hybond N⁺ (Amersham Pharmacia) in 0.4 M NaOH. Blots were hybridized using standard techniques with DNA probes random prime-labelled with [α-32P] dCTP. Viral gene-specific DNA probes for labelling were obtained from cloned viral DNA fragments that were cleaved from plasmid vectors and purified prior to labelling. Murine β-actin blots were hybridized with a full-length chick β-actin probe (Cleveland et al., 1980). A series of PCR controls was incorporated. To control for the carry over of viral DNA, PCR was performed on cDNA synthesized in the absence of reverse transcriptase. Controls for false positive signals included PCR analysis of cDNA from spleen and lung tissue from uninfected mice. Contamination was also controlled for with PCR performed in the absence of cDNA. These control reactions were negative in all cases.

**Plasmid vectors and molecular cloning.** Molecular cloning was carried out essentially as described by Sambrook et al. (1989). pVR1255 was obtained under license from Vical Inc. (Norman et al., 1997). This vector contains the CMV IE enhancer/promoter, CMV intron A, cloning sites and a rabbit β-globin polyadenylation site. HA-tagged Jκ contained

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**Fig. 1.** Diagrammatic representation of the structural motifs present in the putative MHV-68 ORF74 gene product. (A) Amino acid sequence of the MHV-68 ORF74 product with the predicted structural motifs highlighted as follows; transmembrane helices (black shading, white text), N-linked glycosylation sites (boxed), intracellular domains (overlined), conserved motifs (asterisk below), targets for phosphorylation (underlined). (B) Comparison of the sequence around the start of the second intracellular domain. GPCR sequences from mammalian CXCR2, f-Met–Leu–Phe receptor (fMLPR), KSHV and MHV-68 were aligned using the program ClustalW. Residues that are identical in two or more sequences are highlighted in black. The sequence surrounding the conserved arginine at the start of the second intracellular domain is boxed.
in the expression vector pSG5 (Stratagene, pSG5-3a) was obtained from Clare Sample (Zhao et al., 1996). The retroviral expression vector pBabe/puro (Morgenstern & Land, 1990) contains the Moloney murine leukemia virus LTR, which drives expression of the inserted gene, and a puromycin-resistance cassette. The EBV LMP cDNA was obtained as a BamHI fragment in pAT153 (Stewart et al., 1989). The SV40 early region was contained in pBR328 (pPVU-0) (Kalderon et al., 1982).

**DNA electroporation.** Cells (2 × 10⁶) were mixed with 20 µg of linearized plasmid DNA and electroporated using a double-pulse setting (HV = 600 V, 25 µF, 99 Ω; LV = 260 V, 1500 µF, 329 Ω; 0-1 s interpulse delay) on an EasyJet electroporator (EquiBio). The efficiency of transfection was estimated using a control plasmid expressing GFP. Transiently transfected cells were generally harvested 24 h p.i.

**Immunofluorescence.** Indirect immunofluorescence was carried out as described by Stewart et al. (1994). The primary antibody was rat anti-haemagglutinin (clone 3F10, Roche) used at a dilution of 1:250. After staining the cells were incubated with PBS containing 1 µg/ml propidium iodide and 10 µg/ml RNaseA for 30 min at 37 ºC in darkness. Cells were then washed twice in PBS and mounted in Citifluor (UKC). Slides were examined using a Leica TCSNT confocal microscope.

**Focus formation assay.** Stably transfected cell lines were grown to confluence in six-well plates. The medium (DMEM + 10% foetal calf serum) was replaced on alternate days for 3-4 weeks until cellular foci were observed under the light microscope.

**Growth in soft agar.** Soft agar assays were performed as described by Baichwal & Sugden (1989). Cellular foci (diameter > 50 µm) were counted with the aid of a calibrated graticule and a light microscope.

**Prediction of protein structure.** Structural motifs were predicted from protein sequence using the following programs (which can be found at the listed web sites). The position of transmembrane helices and the orientation of the molecule in the membrane was predicted using ‘TMpred’ (Hofmann & Stoffel, 1993) which is found at http://www.ch.embnet.org/software/TMPRED_form.htm. The presence of common motifs was determined using ‘Scan Prosite’, which is found at http://expasy.ch.embnet.org/tools/sprosite.html. The alignment of multiple proteins was performed using the Blosum scoring matrix with the ‘ClustalW’ program (Thompson et al., 1994), which is found at http://www.ch.embnet.org/software/Clustalw.htm. The prediction of phosphorylation site was made using the program ‘NetPhos’ (Blom et al., 1999), which is found at http://www.cbs.dtu.dk/services/NetPhos/.

**Results**

### Structural features of the MHV-68 ORF 74 product

The predicted MHV-68 ORF74 product showed significant but low sequence identity at the amino acid level with mammalian CXCR2 (28%) and KSHV GPCR (25%). To determine if this similarity was relevant we analysed whether the MHV-68 ORF74 product contained conserved sequence motifs that are characteristic of GPCRs and relate to function. The results are shown in Fig. 1. Analysis of potential transmembrane helices using the program ‘TMpred’ revealed the presence of seven transmembrane spanning domains that are a defining characteristic of GPCRs (highlighted black). The predicted orientation of the molecule is that the hydrophilic N-terminal domain is extracellular and that the C-terminal domain is intracellular (shown overlined). There are three predicted intracellular (overlined) and three extracellular loops. GPCRs contain a highly conserved aspartic acid–arginine–tyrosine (DRY) motif at the start of the second intracellular loop (Dohman et al., 1991; Probst et al., 1992). As seen in Fig. 1(B), the corresponding sequence in the MHV-68 ORF74 product is histidine–arginine–cysteine (HRC). A Y to C difference has been seen for some native GPCRs such as the f-Met–Leu–Phe receptor (fMLPR) and is thus conservative (Boulay et al., 1990). The D to H change, however, is non-conservative. Mutation at this residue is found in other gammaherpesvirus GPCRs (Ahuja & Murphy, 1993; Arvanitakis et al., 1993) and associated with constitutive GPCR signalling (Burger et al., 1999). Like other GPCRs, there are two potential N-linked glycosylation sites in the N-terminal domain. Two other residues that are highly conserved in GPCRs, a cysteine in the second extracellular loop and a tryptophan in transmembrane domain IV (Probst et al., 1992), are also conserved in the ORF74 gene product (Fig. 1A, asterisk). In addition, it contains a highly cationic third intracellular loop that is a consistent feature of mammalian chemokine receptors (Gao et al., 1993; Murphy et al., 1992). Using the program ‘NetPhos’ there were predicted to be two serine residues in the C-terminal tail that are potential targets for phosphorylation (Fig. 1A, underlined). Taken together, all these predicted features indicated that the ORF74 gene product was likely to be a functional GPCR that had
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MHV-GPCR from an ancestral mammalian chemoattractant receptor. We have therefore named the product MHV-GPCR.

Transcription of the MHV-68 GPCR in vitro

We analysed expression during lytic infection in vitro by Northern analysis. Using 10 µg of total RNA we were unable to detect GPCR message (not shown). Poly(A)+ RNA was thus extracted from cells and used at 10 µg per track. To determine the temporal expression of GPCR mRNA we blocked all but immediate early gene expression with cycloheximide (CHX) and blocked late gene expression with phosphonoacetic acid (PAA). A Northern blot of the gel was hybridized sequentially with probes specific for MHV-GPCR, MHV-68 glycoprotein gp150 and β-actin. The results are shown in Fig. 2.

The β-actin probe detected a 2-4 kb message of similar intensity in all tracks confirming mRNA integrity and loading. The gp150 probe detected a 1-7 kb transcript that was inhibited by the addition of PAA. This confirms lytic viral gene expression and shows that PAA blocked late gene expression. The GPCR-specific probe detected several transcripts at 12 and 24 h.p.i. The major messages were 3-4 and 4-4 kb in size. Two minor transcripts of 6-6 and 8-7 kb were also present. Expression was reduced but not inhibited by PAA. This indicates that the GPCR is a delayed early gene whose expression continues through into late infection. The blot

Fig. 3. Co-transcription of MHV GPCR and vBcl-2. (A) RT–PCR was carried out on random-primed cDNA produced from C127 cells infected with MHV-68 using an upper primer specific for vBcl-2 and a lower primer corresponding to the GPCR. The reaction was performed either in the presence (+ RT) or the absence (– RT) of reverse transcriptase. Molecular mass determinations were made with a 1 kb DNA ladder (Mr). The sizes (kbp) of relevant bands are shown at the side. (B) 3'-RACE was performed using one primer at the 5' end of the GPCR ORF (GPCR sense) and oligo(dT)18 as the other primer. (C) Diagrammatic representation of positions of transcripts mapped in this work. The relative sizes and positions of Bcl-2, ORF73, GPCR and ORF75 open reading frames are shown by open arrows. The absolute position in the genome of relevant points is indicated above by the nucleotide number. The relative sizes and positions of products and transcripts described herein are shown below this.
Fig. 4. *In vivo* transcription of GPCR and vBcl-2. RNA was extracted from the spleens and lungs of BALB/c mice at 2 weeks and 10 months p.i. with MHV-68 as indicated above. RT–PCR was then performed using sets of primers to viral genes and cellular \( \beta \)-actin as indicated at the left. In one case, reverse transcriptase was omitted to control for carry-over DNA (no RT). Products were then analysed by Southern blotting. The individual mice used are indicated at the bottom.

probed with GPCR was exposed to film for 10 times longer than when probed with \( \beta \)-actin and gp150. Thus, the GPCR-specific messages were much lower in abundance.

The size of all transcripts exceeded that of the 1-0 kb GPCR coding region. It was therefore possible that they represented polycistronic messages. The closest ORF on the same strand of the MHV-68 genome is M11, which encodes the viral Bcl-2 homologue and lies upstream (see Fig. 3C). A Northern blot containing mRNA from cells 24 h p.i. was therefore screened using a probe specific for vBcl-2. As shown in Fig. 2, messages of 8-7, 6-6, 4-4, 3-4, 2-4 and 1-4 kb were revealed. The blot had to be exposed to film for a similar length of time as that probed with GPCR and therefore the vBcl-2-specific transcripts were also low abundance. Thus, a subset comprising the larger four of these mRNAs were of a similar size to the GPCR-specific mRNAs. It was possible, therefore, that these mRNAs were bicistronic and contained vBcl-2 and GPCR.

To study further the possible co-transcription of vBcl-2 and GPCR, RT–PCR was performed using primers (shown in Table 1) at the 5’ end of vBcl-2 (vBcl-2 sense) and at the 3’ end of the GPCR ORF (GPCR antisense). The results (Fig. 3A) showed a 2.7 kb product. No product was seen when the same reaction was performed in the absence of reverse transcriptase. This size of product corresponds to an unspliced message that contains both vBcl-2 and the GPCR genes (see Fig. 3C). This was confirmed by sequencing of the product. This PCR product could be generated from either the 4-4 or 3-4 kb GPCR and vBcl-2-specific mRNAs (Fig. 2). To analyse the 3’ end of the mRNAs, 3’-RACE was performed using one primer at the 5’ end of the GPCR ORF (GPCR sense) and oligo(dT)\(_{18}\) (Fig. 3B). A product of approximately 1-6 kb was seen that when sequenced was found to terminate at 106637 in the genome. This could potentially be the termination point for all the GPCR-specific mRNAs.

Thus, as shown in Fig. 3(C), at least one of the forms of GPCR mRNA is unspliced, bicistronic, and also contains the vBcl-2 gene.

**Expression of GPCR in vivo**

To confirm previous data on MHV GPCR transcription (Virgin *et al*., 1999) and to study the expression during persistent infection of lungs, we performed RT–PCR on lung and spleen tissue from mice intranasally infected with MHV-68. We analysed expression at 2 weeks p.i. where acutely productive infection is resolving in the lung, persistence is establishing and latently infected cells are present (Sunil-Chandra *et al*., 1992a, b). Analysis was also performed at 10 months p.i. where a steady state of persistence and latency exists. We used primers specific for ORF50, ORF57, GPCR, vBcl-2 and \( \beta \)-actin. ORFs 50 and 57 are only transcribed during the productive cycle (Liu *et al*., 2000; Mackett *et al*., 1997; Wu...
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et al., 2000). The sensitivity of the PCRs was checked by limiting dilution of cloned templates and was one copy in all cases. Control amplifications performed without reverse transcriptase (no RT) were negative. As shown in Fig. 4, β-actin transcription was detected in all samples. Expression of ORF50 and ORF57 was detected in both the spleen and the lung 2 weeks p.i. in all three mice, but by 10 months expression was negative. This indicated that productive viral gene expression was still occurring at 2 weeks p.i. but by 10 months p.i. was undetectable. GPCR and vBcl-2 were expressed in the spleen and lung of all mice at 2 weeks p.i. By 10 months p.i., GPCR and vBcl-2 were expressed in the spleen of one mouse but were found in all lungs tested.

Therefore, GPCR and vBcl-2 were both expressed during acute and persistent infection in vivo. Since no concomitant lytic cycle gene expression was seen during persistence, this was indicative of expression in latently infected cells.

Expression pattern of the GPCR protein in cells

No antibodies to the MHV GPCR are available. To study GPCR protein we utilized a transient transfection system with a gene tagged at the 3′ end with a sequence encoding three copies of an influenza virus haemagglutinin (HA) epitope. The tagged GPCR gene was inserted into the expression vector, pVR1255 [pVR1255/GPCR-(HA)3]. Human 293 cells were transfected with expression constructs and protein expression was detected with rat monoclonal anti-HA; the cell nuclei were counterstained with propidium iodide (PI) and fluorescence then visualized by confocal microscopy. Cells transfected with empty vector alone showed no positive staining with anti-HA and cells transfected with a construct known to express an HA-tagged version of Jκ exhibited characteristic positive nuclear staining (not shown). As shown in Fig. 5, cells transfected with pVR1255/GPCR-(HA)3 showed positive staining in patches with anti-HA. This staining was quite separate from the intranuclear PI staining and, when the two images were overlaid, it could be seen that the pattern of anti-HA staining was consistent with expression at the plasma membrane. Thus, the pattern of MHV GPCR expression is in keeping with the fact that it is homologous to growth factor receptors that are found at the cell surface.

Transforming activity of the GPCR

The effects of expression of MHV GPCR on cell growth were assessed in NIH 3T3 cells. Focus formation by these cells represents a morphological manifestation of transformation associated with a loss of contact inhibition that normally limits the density of these cells. Transformation was assessed in two ways, by focus formation and colony formation in soft agar. The GPCR coding sequence was inserted into the vector pBabe/puro (pBabe/GPCR). As positive controls we used the EBV latent membrane protein-1 (LMP) gene (pBabe/puro-LMP) and the SV40 early region (pPVU-0). NIH 3T3 transfectants were selected with puromycin and cell clones expressing the GPCR were grown until focus formation was observed (3 weeks). The results are shown in Fig. 6. Cells transfected with empty pBabe/puro plasmid did not exhibit loss of contact inhibition or focus formation. However, cells transfected with pBabe/puro GPCR (as well as pBabe/puro-LMP and pPVU-0; not shown) readily showed loss of contact inhibition (middle panel) and formed foci (lowest panel). To assess the anchorage independence of the clones, we grew lines from individual transformed foci and tested their ability to
form foci in soft agar. As shown in Fig. 7, cell clones expressing the GPCR and the positive control cell clones produced significantly more foci than the negative control clones that contained the empty pBabe/puro vector.

These results therefore demonstrate that, like KSHV-GPCR, the MHV GPCR exhibits transforming activity \textit{in vitro}.

\section*{Discussion}

We have shown low levels of mRNA encoding the MHV GPCR at early and late time-points during lytic infection \textit{in vitro} and during virus persistence \textit{in vivo}. Further, we demonstrated co-transcription of the GPCR with the gene encoding vBcl-2. GPCR protein was expressed at the cell surface in patches and its expression was associated with growth transformation.

In contrast to a previous report (Virgin et al., 1999), we were able to detect GPCR and vBcl-2 mRNAs by Northern analysis. However, the level of expression was low, bordering on the level of detection (Fig. 2). This indicates a high degree of transcriptional control. There were four GPCR-specific messages (3-4, 4-4, 6-6 and 8-7 kb). Similar sized messages were detected by a vBcl-2 probe suggesting co-transcription of the two ORFs. This interpretation is strengthened by the fact that RT–PCR using primers specific for the 5' end of vBcl-2 and the 3' end of GPCR demonstrated an unspliced bicistronic transcript. 3'-RACE data showed that GPCR-containing messages terminate at 106637. The combined length of the overlapping RT–PCR and 3'-RACE products is approximately 3-2 kb. Thus, it is probable that the 3-4 kb transcript corresponds to this with additional 5' sequences and a poly(A) tail (Fig. 3 C). Analysis of potential promoter elements using the program ‘Promoter 2.0’ (Knudsen, 1999) produced strong predictions upstream of the vBcl-2 gene at around positions 102000 and 103000. No such elements were predicted directly upstream of the GPCR. Put together, these data are consistent with the hypothesis that the 4-4 and 3-4 kb mRNAs are bicistronic, originate upstream of vBcl-2 at around nucleotides 102000 and 103000 respectively and terminate downstream of the GPCR.

The vBcl-2 probe hybridized to two additional unshared transcripts (1-4 and 2-4 kb). There is a non-canonical polyadenylation signal (AGTAAA) at position 104216, downstream of the vBcl-2 but upstream of the GPCR gene. It has been shown experimentally that an identical signal sequence is used by the MHV-68 latency-associated M2 gene as a polyadenylation signal (Husain et al., 1999). It seems likely, therefore, that the two smaller vBcl-2 specific mRNAs terminate just downstream of this. This signal is clearly ignored in the production of the longer bicistronic transcripts. The size of the vBcl-2-specific transcripts and the distance between the two poly(A) signal sequences (2-4 kb) makes it possible for them to have the same starting points as the 3-4 and 4-4 kb transcripts. To date, we have been unable to locate the precise 5’ end of the mRNAs by using 5'-RACE. Due to the complex nature of the transcription across this region, precise definition of the composition of these mRNA demands the cloning and sequencing of their cDNAs. However, as yet no cDNAs corresponding to the GPCR or vBcl-2 have been found in a library from which both lytic and latent genes have been isolated (J. Sample, personal communication).

The observation that the vBcl-2 ORF is contained on the GPCR mRNAs is interesting. This is therefore similar to KSHV where the homologous GPCR gene is expressed as the 3’ ORF in a bicistronic transcript, although in this case the upstream gene is K14 (v-Ox-2) (Kirshner et al., 1999; Talbot et al., 1999). The co-expression of MHV-68 vBcl-2 and GPCR backs up the finding of transcription of both ORFs during persistence in the mouse. This confirms the results of previous work by Virgin et al. (1999), who described expression of both in the lymphoid compartment of B cell-deficient mice. Our results therefore extend this analysis to intact mice and show that co-expression of these genes occurs most consistently in the lung, an organ that we have previously shown to be important for MHV-68 persistence (Stewart et al., 1998). The fact that both mRNAs are found during persistence in the absence of lytic cycle genes (ORF50 and ORF57) strongly implies that they are expressed and function in latently infected cells. Definitive confirmation of this and the definition of the cell type involved (epithelial or lymphoid) will rely upon the direct visualization of both transcripts in the same cells. However, to date we have been unable to detect either GCPR or vBcl-2 by \textit{in situ} hybridization \textit{in vitro} or \textit{in vivo} due to the low abundance of the transcripts. The fact that MHV-68 vBcl-2 and GPCR are both expressed during lytic infection and during latency suggests either that the promoter elements are active during both phases of the lifecycle or that there are separate promoters. There is a precedent for the former in that both EBV EBNA 1 and LMP transcripts
are produced during latency but are also induced during lytic replication (Tori et al., 1998; Zetterberg et al., 1999).

Previous work has shown that the KSHV GPCR is oncogenic in the sense that it is able to induce transformation of mouse 3T3 cells in vitro (Arvanitakis et al., 1997; Bais et al., 1998). Our data therefore show that the MHV GPCR is not only similar in sequence but also in transforming activity to the KSHV homologue. The transforming ability of KSHV GPCR is thought to be a function of constitutive signalling in the absence of ligand (Arvanitakis et al., 1997). Light has recently been shed on the molecular basis of this by the finding that mutation of the aspartate (D) in the highly conserved DRY motif in the second intracellular loop of CXCR2 and histamine H(2) receptor (mimicking KSHV GPCR, see Fig. 1B) confers constitutive signalling and transforming ability (Alewijnse et al., 2000; Burger et al., 1999). The equivalent motif in the MHV-68 GPCR is HRC (Fig. 1B). Although the DRY motif is highly conserved, C replacing the Y is seen in certain functional GPCRs such as the FMLPR receptor (Boulay et al., 1990). Thus MHV-68, like KSHV has a mutated form of this motif that may underlie the ability of the protein to confer growth-inducing properties on cells.

The fact that vBcl-2 and GPCR are encoded on the same mRNA suggests that these ORFs may be co-translated and may have coordinated functions in the virus life-cycle. We have shown here that the GPCR can enhance the growth properties of cells and it has been shown that vBcl-2 can protect cells from apoptotic death (Bellows et al., 2000; Wang et al., 1999). Thus it may be envisaged the GPCR functions to activate virus infected cells and that the vBcl-2 acts at the same time to prolong the life of the same cells not only during acute infection but also during persistence in the lung. Considerable sub-pleural lymphoid accumulations are seen after MHV-68 infection (Sunil-Chandra et al., 1992a). Thus, it is possible that the vBcl-2 and GPCR expression seen in latency by RT–PCR is derived from either lymphoid or epithelial cells. Both genes might therefore play a role in the development of the lymphoproliferative disease seen in long-term infected mice (Sunil-Chandra et al., 1994). The exact function of the GPCR in the virus life-cycle, however, awaits the characterization of a virus with this ORF mutated. Further, the functional similarities between the MHV-68 GPCR and the KSHV homologue make this and future studies particularly pertinent with respect to the modelling the pathogenesis of KSHV-associated disease.

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