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The Equine Herpesvirus 2 E1 Open Reading Frame Encodes a Functional Chemokine Receptor

GRAZIA CAMARDA,[1] GAIA SPINETTI,[1] GIOVANNI BERNARDINI,[1] CATHERINE MAIR,[2] NICK DAVIS-POYNTER,[2] MAURIZIO C. CAPOGROSSI,[1] AND MONICA NAPOLITANO[1]*

Laboratorio di Patologia Vascolare, Istituto Dermopatico dell'Immacolata-Istituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy,[1] and Laboratory of Virology, Animal Health Trust, Newmarket, England[2]

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Several herpesviruses contain open reading frames (ORFs) that encode potential homologs of eucaryotic genes. Equine herpesvirus 2 (EHV-2) is a gammaherpesvirus related to other lymphotropic herpesviruses such as herpesvirus saimiri and Epstein-Barr virus. The E1 ORF of EHV-2, a G protein-coupled receptor homolog, shows 31 to 47% amino acid identity with known CC chemokine receptors. To investigate whether E1 may encode a functional receptor, we cloned the E1 ORF and expressed it in stably transfected cell lines. We report here the identification of the CC chemokine eotaxin as a functional ligand for the EHV-2 E1 receptor. Chemokines are likely to play a role in the regulation of immune functions in equine hosts during EHV-2 infection and, via interaction with E1, may affect viral replication and/or escape from immune responses.

Viruses often integrate in their own genomes several cellular genes involved in the control of cell growth and differentiation and/or in the regulation of immune functions. These "pirated" genes may confer a replicative advantage to infecting viruses by interfering with cellular functions and by subverting the host immune system through molecular mimicry (2, 16, 22). Herpesviruses and poxviruses frequently contain in their genome host-derived genes homologous to immunoregulatory genes, such as cytokines and cytokine receptors (4, 28), or to cell cycle regulatory molecules such as bcl-2 and cyclins (4, 40, 49).

The gamma subfamily of herpesviruses generally replicates in lymphoblastoid, epithelial, and fibroblastic cells, with the former group of cells being the preferred site of latency. This subfamily is further divided into the γ1 genus (typified by the Epstein-Barr virus [EBV]) and the γ2 genus (e.g., herpesvirus saimiri [HVS] that infects nonhuman primates and human herpesvirus 8 [HHV-8] associated with Kaposi's sarcoma).

Over the past decade chemokines have been shown to play an important role in inflammation, hematopoiesis, and angiogenesis, as well as in atherosclerosis, tumor growth, and several other pathological conditions (27, 34). Chemokines represent a family of structurally related molecules whose conserved cysteine residues define the two major subgroups of CXC and CC chemokines (7). Such molecules interact with seven transmembrane-spanning receptors and signal through the activation of heterotrimeric G proteins, thus modulating several cellular functions (58).

Several herpesviruses have been found to encode chemokines and/or chemokine receptor genes that may affect host immune responses or virus tissue tropism and/or dissemination, thus contributing to viral pathogenesis. A number of herpesvirus-encoded chemokine receptors have been reported to be functional and able to bind known chemokines (29, 38). Thus, HVS open reading frame (ORF) 74, also known as ECRF3, is homologous to the CXCR2 receptor and binds and signals in response to Groα, NAP-2, and interleukin-8 (IL-8) (3). Similarly, the cytomegalovirus (CMV) ORF US28, homologous to CCR1, binds MIP-1α, MIP-1β, MCP-1, and RANTES and signals in response to these ligands (20, 41). HHV-8 ORF 74 has been extensively studied and shown to be a CXC chemokine receptor homolog which, interestingly, while it exhibits constitutive signalling, may be further activated by Groα and IL-8 and inhibited by SDF-1, IP-10, and Mig. This receptor is expressed in Kaposi's sarcoma lesions and appears to act as a viral oncogene, inducing cell proliferation, transformation, and tumor angiogenesis (6, 8, 21).

Equine herpesvirus 2 (EHV-2) is a lymphotropic gammaherpesvirus with a high prevalence rate in horse populations (1). The complete genome of EHV-2 (strain 86/67) has been determined, demonstrating that it is more similar to γ2 herpesviruses (e.g., HVS) than γ1 herpesviruses (e.g., EBV) (52). While its role as a pathogen is still unclear, EHV-2 infection has been implicated in immunosuppression in foals, in conjunctivitis, and in respiratory inflammatory processes and poor racing performance (14, 30, 39, 47). EHV-2 has been isolated from peripheral blood mononuclear cells of foals (39), from the respiratory tracts of animals with clinical signs of disease, and from draining lymph nodes, potentially representing the main viral reservoirs. Moreover, the virus has been detected, at lower frequency, in both the peripheral and central nervous systems, mostly in the trigeminal ganglion, a putative site for EHV-2 latency (47). In addition, EHV-2 has been proposed to act as a *trans*-activating factor, which may either trigger or upregulate EHV-1 and EHV-4 expression from a latent state (44, 59). EHV-2 contains 79 ORFs that encode 77 distinct molecules, several of which show striking homology to cellular genes. These include an IL-10 homolog (E7); two proteins which interact with apoptosis-signalling pathways, v-FLIP (E8) (55) and v-CARMEN (E10) (54); and three putative G protein-coupled receptors (GPCRs). EHV-2 ORF 74 is colinear and conserved (ca. 20% homology) with the corresponding ORF of HVS (53). This ORF is a characteristic feature of the γ2 genus, being conserved in the majority characterized to date (namely, HVS, EHV-2, HHV-8, and murine gammaherpesvirus 68 (MHV-68) (57), with the exception of alcephaline herpesvirus type 1 (18). ORF E6 is predicted to encode a protein with seven transmembrane domains and other features characteristic of GPCRs (53) and is homologous to the BILF1 ORF of EBV (16). The third GPCR homolog, ORF E1, is the

* Corresponding author. Mailing address: Laboratorio di Patologia Vascolare, Istituto Dermopatico dell'Immacolata-IRCCS, Via Monti di Creta 104, 00167 Rome, Italy. Phone: 39-06-66462431. Fax: 39-06-66462430. E-mail: m.napolitano@idi.it.

only identified diploid ORF within the EHV-2 genome, being encoded within the terminal direct repeat elements.

The E1 ORF shows the highest degree of homology with cellular chemokine receptors compared with other viral GPCRs and, among viral products, is conserved most closely with the human CMV ORF US28 (53). Its structural relatedness to this class of molecules suggests that it may also share biological responses of classical chemokine receptors. We therefore investigated whether E1 may be functionally active in response to chemokines when expressed in eucaryotic cell lines and found that this receptor responds to eotaxin in both calcium mobilization and chemotaxis assays, suggesting a role of chemokines during EHV-2 infection.

MATERIALS AND METHODS

Recombinant proteins. Human I-309 was purchased from R&D Systems (Minneapolis, Minn.) and human eotaxin, RANTES, MIP-1α, MIP-1β, MCP-3, BCA-1, IP-10, and Mig were from PeproTech (London, United Kingdom).

Molecular cloning of the E1 ORF into expression vectors. Both nucleotide and protein sequences corresponding to known chemokine receptors were used for comparative analysis of GenBank and other databases to search for homologous receptors. Our bioinformatic screening of nonredundant databases identified the EHV-2 E1 ORF (accession number U20824) (53) as a putative chemokine receptor.

The entire E1 ORF was amplified from EHV-2 (strain 86/67) genomic DNA (the kind gift of A. J. Davison, University of Glasgow) by PCR with primers containing *Eco*RI restriction sites at their 5′ ends. The primer sequences were 5′-CAG AAT TCA TGG CAA CCA CTT CAG C-3′ (forward primer) and 5′-CAG AAT TCC ATG CTG GTG GTC CAT C-3′ (backward primer). After an initial denaturation step (5 min at 94°C), PCR was performed with AmpliTaq DNA polymerase (Perkin-Elmer/Roche, Branchburg, N.J.) for a total of 35 cycles (45 s at 94°C, 1 min at 60°C, and 1 min at 72°C), with a final extension at 72°C for 10 min. PCR products were digested with *Eco*RI, gel purified, and subcloned in the pcDNA3 expression vector (Invitrogen, Groningen, The Netherlands) by standard procedures.

Creation of stably transfected cell lines. 293 human embryonic kidney (HEK) cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 2 mM glutamine; 300-19 murine pre-B cells were grown in complete medium (RPMI, 10% FBS, glutamine, and 50 μM β-mercaptoethanol). Plasmids were transfected into 293 cells by the calcium phosphate method as previously described (11) and into 300-19 cells by electroporation. Briefly, 3×10^6 cells/400 μl were mixed with 10 μg of plasmid DNA, incubated on ice for 10 min, transferred to a 0.4-cm electroporation cuvette, and subjected to a single pulse at 280 V and 960 μF (Gene Pulser II Apparatus; Bio-Rad Laboratories, Hercules, Calif.). The electroporated cells were left on ice for 15 min and resuspended in complete medium. At 48 h posttransfection, with both methods, cells were placed under selection in medium containing 1 mg of G418 (Life Technologies, Inc., Grand Island, N.Y.) per ml for several weeks to generate stable transfectants.

RNA extraction and RT-PCR analysis. Total RNA was extracted from transfected cells by using the TRIzol Reagent (Life Technologies) according to the manufacturer's instructions, digested with RNase-free DNase (Promega, Madison, Wis.) for 1 h at 37°C, and purified. First-strand cDNA was obtained by using Superscript II reverse transcriptase (RT; Life Technologies) according to the manufacturer's instruction. Briefly, 5 μg of total RNA was reverse transcribed in a 20-μl reaction mixture. Then, 1 μl of the cDNA template was PCR amplified by using primer pairs and the cycling conditions described above. The same amount of template was subjected to 25 cycles of PCR amplification by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers as a control for normalization. The samples were then run on a 1.2% agarose gel in the presence of ethidium bromide and detected by using a GelDoc 1000 apparatus (Bio-Rad Laboratories).

Intracellular $[Ca^{2+}]$ measurement. 300-19 cells were loaded with Fluo4-AM (Molecular Probes, Inc., Eugene, Oreg.) according to the manufacturer's instructions, with minor modifications. Briefly, cells (5×10^6/ml) were incubated in Hanks buffered saline solution without Ca^{2+} and Mg^{2+}, supplemented with 10 mM HEPES (pH 7.4), and containing 2.5 μM Fluo4-AM for 40 min at 37°C. Cells were subsequently washed with Hanks solution and incubated at 37°C for 30 min in Hanks solution containing 10 mM HEPES, 5% FBS, and 2 mM $CaCl_2$ (HHF) to allow for complete fluorochrome de-esterification. Cells where then washed twice in HHF and resuspended in HHF at 2×10^6 cells/ml. Intracellular $[Ca^{2+}]$ changes were evaluated by using a FACScan cytofluorimeter (Becton-Dickinson, Mountain View, Calif.) equipped with an argon laser (emission at 488 nm). After basal levels of fluorescence were attained, cells were stimulated with chemokines (at 100 ng/ml), and the fluorescence increase in the emission spectrum of Fluo4 (516 nm) was recorded every 10 s for 3 min. Intracellular $[Ca^{2+}]$ levels were expressed as the fluorescence fold increase, calculated by dividing the mean fluorescence intensities at each time point of stimulation by the mean fluorescence intensity recorded at time zero.

Chemotaxis assay. The migration of 293 cells expressing the E1 receptor was assessed as previously described (10, 46). Briefly, cells were trypsinized, incubated in DMEM–10% FBS for 1 h at room temperature, washed in RPMI supplemented with 1% bovine serum albumin–25 mM HEPES (migration medium [MM]), and placed in the upper wells of a 48-well chemotaxis chamber (Neuro Probe, Inc., Cabin John, Md.) at 0.5×10^6 cells/ml in triplicates in a final volume of 50 μl. Chemokines were placed in the lower wells in a 27-μl volume of MM. Polyvinylpyrrolidone-free polycarbonate membranes (12-μm pores) (Costar, Corning Inc., Corning, N.J.) were coated with 20 μg of mouse collagen type IV (Collaborative Biomedical Product; Becton-Dickinson Labware, Bedford, Mass.) per ml for 2 h at 37°C. The chemotaxis assay was performed for 6 h at 37°C; the filter was fixed and stained with a Diff-Quik Kit (DADE, Dudingen, Switzerland), and the cells were counted at ×400 magnification in four randomly selected fields. Migration indices were calculated by dividing the average number of cells migrated in the presence of chemokines by the number of cells migrated in migration medium alone.

RT-PCR analysis of E1 expression in EHV-2-infected cells. Equine embryonic kidney cell monolayers (1.5×10^6 cells) were infected with approximately 0.01 PFU of EHV2 isolate 33839 (provided by the Animal Health Trust Diagnostic Services laboratory) per cell and, after infection, maintained in minimal essential medium containing 5% FBS, glutamine, penicillin, and streptomycin. Cells were harvested 5 days postinfection, and total RNA was extracted by using the guanidine thiocyanate-based DNA/RNA Isolation Kit (USB, Cleveland, Ohio) according to the manufacturer's instructions. Purification of poly(A)$^+$ RNA from 50 μl of total RNA (≤250 μg of total RNA) was performed by using Oligotex (Qiagen, GmbH) according to the manufacturer's instructions. RT-PCR analysis of EHV-2 infected-cell poly(A)$^+$ RNA was performed as follows. First, 5 μl of poly(A)$^+$ RNA was mixed with 1 μl (40 U) of RNasin (Promega) and 2 μl (200 ng) of Oligo$(dT)_{15}$ Primer (Promega), heated at 65°C for 5 min, and then quenched on ice for 2 min. Then, reverse transcription was performed by using M-MLV Reverse Transcriptase (Promega), and the samples were incubated at 37°C for 60 min, 42°C for 60 min, and 95°C for 5 min. After reverse transcription, the E1 ORF was amplified by PCR by using the forward primer E1Ef (5′-TT CGA ATT CAC AGT AAA ATG GCA ACC AC-3′) and the reverse primer E1Er (5′-T TCG AAT TCA AAT GCG GGT GGG CCC CT-3′) as follows. A 5-μl portion of the above RT reaction mixture was subjected to PCR amplification by using AmpliTaq DNA Polymerase (Perkin-Elmer). After an initial denaturation step (94°C for 4 min), PCR was performed for a total of 33 cycles (94°C for 30 s, 58°C for 1 min, and 72°C for 2 min), with a final extension at 72°C for 10 min. Amplified products were run on a 1.5% agarose in 1× TBE gel, and products were visualized by using ethidium bromide.

RESULTS

Cloning of EHV-2 E1 ORF and generation of cellular transfectants. In an effort to search for new members of the chemokine receptor superfamily, we used selected amino acid sequences corresponding either to complete ORFs or to conserved regions of these receptors, in order to screen nucleotide and protein databases of both expressed sequence tags (36) and nonredundant sequences. This method allowed us to identify a number of molecules whose predicted sequence matched GPCRs possibly belonging to the chemokine receptor family. Among those we chose to characterize, the EHV-2 E1 ORF was predicted to encode a 383-amino-acid (aa) seven-transmembrane protein with significant homology with human CC-chemokine receptors, specifically, CCR3 (47%), CCR1 (44%), CCR5 (40%), and CCR8 (35%) (Table 1). In Fig. 1 the amino acid alignment of E1 and human CCR3 by a CLUSTALW analysis is shown. Like most GPCRs, the E1 sequence shows an N-terminal extracellular domain, seven transmembrane regions, three extracellular and intracellular loops, and a C-terminal cytoplasmic tail. As for most chemokine receptors, the N-terminal region is poorly conserved and is, in addition, considerably longer compared to members of the chemokine receptor family. E1 shows three potential N-linked glycosylation sites in its N-terminal region (aa 11 to 13, 22 to 24, and 42 to 44) and a protein kinase C (PKC) phosphorylation site in its third intracellular loop (aa 270 to 272) (Fig. 1).

To characterize the role of E1 as a chemokine receptor, we cloned the entire E1 ORF, whose DNA was PCR amplified by

TABLE 1. Amino acid homology between EHV-2 E1 ORF and human chemokine receptors

Receptor	Identity (%)	Positivity[a] (%)	No. of residues
CCR3	47	63	355
CCR1	44	60	355
CCR2	41	56	374
CCR5	40	56	352
CCR8	35	57	355
CCR4	33	51	360
CCR6	33	47	374
CCR7	31	49	378
CXCR2	29	53	360
CXCR1	29	51	350
CXCR4	27	48	352

[a] Positivity indicates the amino acid similarity.

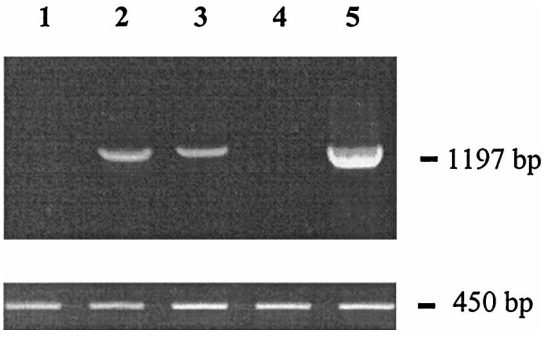

FIG. 2. RT-PCR analysis of E1 mRNA expression in E1-transfected cells. (Upper panel) A single E1 mRNA transcript (1,197 bp) was detected in two 300-19/E1 bulk transfectants (lane 2 and 3) and 293/E1 (lane 5) cells but not in 300-19/mock (lane 1) and 293/mock (lane 4) cells. (Lower panel) GAPDH amplification (450 bp) was used for sample normalization.

using oligonucleotides based on the viral DNA sequence, into the pcDNA3 eucaryotic expression vector and, after cell transfection, generated cell lines stably expressing the E1 protein. Both a lymphoid (300-19) and an epithelial (293/HEK) cell line were utilized. After G418 selection, total RNA was extracted from stably transfected cells and subjected to RT-PCR amplification to verify E1 mRNA expression. As depicted in Fig. 2 (upper panel), 300-19- and 293-transfected cells showed the expression of a single band of 1,197 bp (lanes 2, 3, and 5) corresponding to an amplified message containing the entire E1 ORF and flanking regions in comparison to the control cells (lanes 1 and 4). PCR amplification of GAPDH, showed in the lower panel of Fig. 2, was used as a control for normalization.

Functional characterization of transfected cells. In order to demonstrate that E1 may behave as a functional receptor and to identify its potential agonists, we measured both calcium mobilization and chemotaxis of E1- and mock-transfected cells in response to a panel of CC and CXC chemokines. Both assays are widely utilized for the characterization of chemokine receptors, since their ligands typically induce such functional responses in target cells.

We first analyzed 300-19-transfected cells for calcium mobilization in response to chemokines. As shown in Fig. 3, 300-19 E1 cells, loaded with Fluo4, functionally responded to eotaxin at a concentration of 100 ng/ml. Eotaxin induced an increase in the intracellular calcium concentration, as measured by Fluo4 fluorescence intensity, after 30 s of chemokine addition, which reached a twofold increase over background levels and then declined progressively. By contrast, mock-transfected cells did not show any change in Fluo4 fluorescence upon eotaxin addition (Fig. 3), thus identifying eotaxin as a functional ligand for EHV-2 E1. Conversely, the CC chemokines RANTES,

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                    ***                 ***                 ***
EHV2E1 MATTSATSTVNTSSLATTMTTNFTSLLTSVVTTIASLVPSTNSSEDYYDDLDDVDYEESA
       :          :.:: :. : . ::                    ::::.  .
hCCR3  M----------TTSLDTVETFGTTS--------------------YYDDVGLL------

                    TM 1                                    
EHV2E1 PCYKSDTTRLAAQVVPALYLLVFLFGLLGNILVVIIVIRYMKIKNLTNMLLLNLAISDLL
       : :.::  : :: :: :: :::  :::::.:::.::.:: ... .::. ::::::::::
hCCR3  -CEKADTRALMAQFVPPLYSLVFTVGLLGNVVVVMILIKYRRLRIMTNIYLLNLAISDLL

       TM 2                TM 3
EHV2E1 FLLTLPFWMHYIGMYHDWTFGISLCKLLRGVCYMSLYSQVFCIILLTVDRYLAVVYAVTA
       ::.::::::.::.   :.:.:: ..:::: :  . .::::.: ::::.:::::.:.:: :
hCCR3  FLVTLPFWIHYV-RGHNWVFGHGMCKLLSGFYHTGLYSEIFFIILLTIDRYLAIVHAVFA

                TM 4                                  
EHV2E1 LRFRTVTCGIVTCVCTWFLAGLLSLPEFFFHGHQDDNGRVQCDPYYPEMSTNVWRRAHVA
       :: :::: :.:: . :: :: : .::::.:.  ..  .. :.  ::: ..  :: :.
hCCR3  LRARTVTFGVITSIVTWGLAVLAALPEFIFYETEELFEETLCSALYPEDTVYSWRHFHTL

       TM 5                ooo         TM 6
EHV2E1 KVIMLSLILPLLIMAVCYYVIIRRLLRRPSKKKYKAIRLIFVIMVAYFVFWTPYNIVLLL
       .. .. :.:::::::::::  ::. ::: :::::::::::::::::::::::::::::::
hCCR3  RMTIFCLVLPLLVMAICYTGIIKTLLRCPSKKKYKAIRLIPVIMAVFFIFWTPYNVAILL

       TM 7
EHV2E1 STFHATLLNLQCALSSNLDMALLITKTVAYTHCCINPVIYAFVGEKFRRHLYHFFHTYVA
       :.... :.. .:  :.:::::.:.:::.:.:::.:::::::::::::.:::: :::: ..
hCCR3  SSYQSILFGNDCERSKHLDLVMLVTEVIAYSHCCMNPVIYAFVGERFRKYLRHFFHRHLL

EHV2E1 IYLCKYIPFLSGDGEGKEG---PTR--------I
       ..: .:::::: ..   . .   :.         .
hCCR3  MHLGRYIPFLPSEKLERTSSVSPSTAEPELSIVF
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FIG. 1. Amino acid homology between EHV-2 E1 and human CCR3. Alignment was done by using the CLUSTALW program. Two dots denote identities, whereas single dots indicate conservative substitutions. N-linked glycosylation (***) and PKC phosphorylation (ooo) sites are marked. Gaps (dashes) were inserted from the program to obtain maximum alignment.

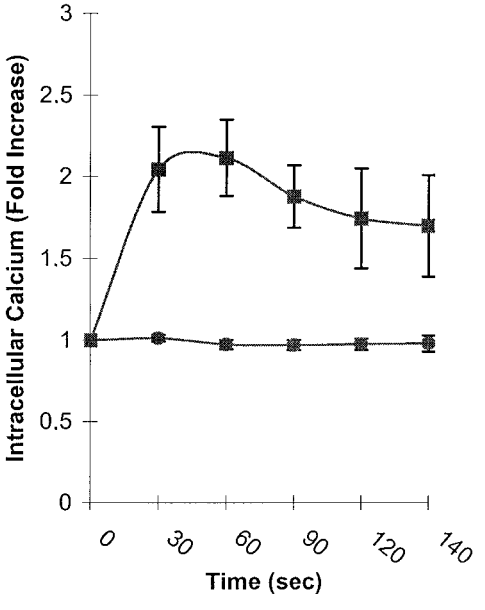

FIG. 3. Intracellular calcium mobilization in E1-transfected cells. Fluo4-loaded mock (closed circles)- and E1 (closed squares)-transfected cells were analyzed by flow cytometry for calcium mobilization after the addition of eotaxin (100 ng/ml). Results are expressed as the fold increase of Fluo4 mean fluorescence intensities compared to the emission at time zero. The data represent the average (± the standard error of the mean [SEM]) of four independent experiments.

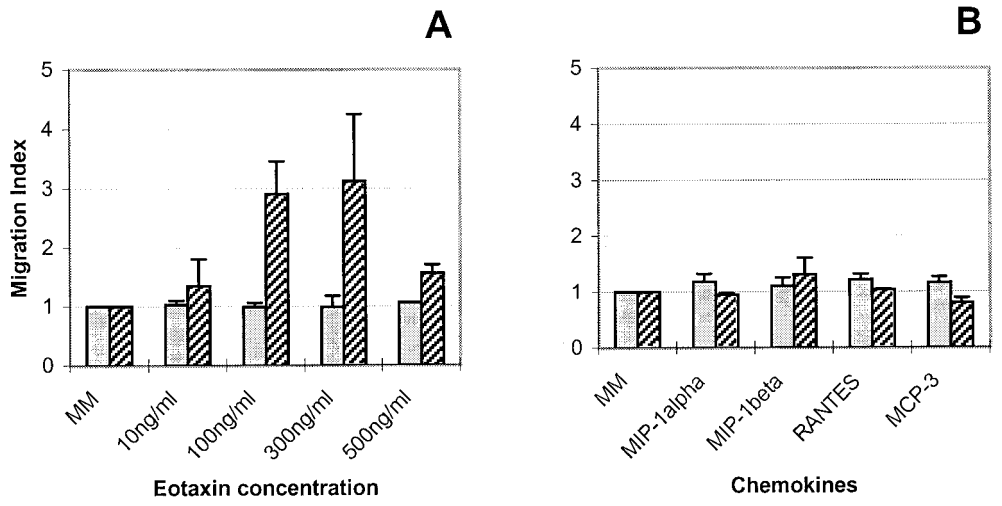

FIG. 4. Chemotaxis of E1-transfected cells. (A) A range of concentrations of eotaxin (from 10 to 500 ng/ml) was used in a 48-well microchamber migration assay on mock (shaded columns)- and E1 (dashed columns)-transfected cells. Migration indexes (± the SEM) are shown, and the data represent the average of three to five independent experiments. (B) Chemotaxis of mock (shaded columns)- and E1 (dashed columns)-transfected cells in response to 100 ng of several CC chemokines per ml. Migration indices (± the SEM) are shown for three to five independent experiments.

MIP-1α, MCP-3, and I-309 and the CXC chemokines IP-10, Mig, and BCA-1 induced a barely detectable increase of mean fluorescence intensities in both mock- and E1-transfected cells (data not shown).

We then measured the chemotactic responses of 293 cells to a panel of chemokines in a 48-well microchamber assay. Such transfectants were chosen for their high level of E1 expression, as shown in Fig. 2.

Eotaxin was tested in a wide range of concentrations on both mock- and E1-transfected cells to confirm its activity as a chemoattractant. As shown in Fig. 4A, eotaxin induced a potent chemotactic response (migration index, 2 to 4) in 293/E1-transfected cells, with a typical bell-shaped dose-response curve and maximal activity of agonist at a concentration of 100 to 300 ng/ml. These concentrations did not elicit a chemotactic response in mock-transfected cells (Fig. 4A). Further, the CC chemokines RANTES, MIP-1α, MIP-1β, and MCP-3 did not induce a significant chemotactic response in either mock- or E1-transfected 293 cells (Fig. 4B).

In conclusion, while eotaxin induced both calcium mobilization and chemotaxis in E1 transfectants, RANTES, MIP-1α, MIP-1β, MCP-3, I-309, BCA-1, IP-10, and Mig did not elicit functional activation of E1 transfectants in either calcium or chemotaxis assays.

RT-PCR analysis of E1 expression in EHV-2-infected cells. In order to confirm that the E1 ORF is transcribed in EHV-2-infected cells, we determined whether E1 mRNA is expressed by RT-PCR analysis. Primary equine embryonic kidney cells were infected with a field isolate of EHV-2 and harvested 5 days postinfection (after the development of significant cytopathic effect). Cell lysates were processed for the preparation of poly(A)$^+$ RNA and analyzed by RT-PCR by using oligo(dT) to prime cDNA synthesis, followed by PCR with E1 specific primers. As shown in Fig. 5, E1-specific mRNA was detected in EHV-2-infected (lane 4) but not in mock-infected (lane 5) cells. Thus, mRNA of the correct orientation for expression of E1 is transcribed in EHV-2-infected equine cells.

DISCUSSION

EHV-2 has been found to encode ORFs with significant homology to cellular immunomodulatory proteins, similarly to other gammaherpesviruses. These include an IL-10 homolog (E7) that may be involved in the suppression of antiviral immune functions and three GPCR homologs (ORFs E1, E6, and 74) that potentially interact with cellular chemokines (53). To date, however, there has been no direct demonstration of activity for the proteins encoded by these ORFs or of their gene expression during infection.

E1 has been identified as a putative CC chemokine receptor that is highly related to CCR3 (47% identities) and other chemokine receptors. The high degree of homology shown by E1 with cellular receptors belonging to this family is striking compared with other viral GPCRs, which are usually more distantly related to eucaryotic counterparts, and suggests that this ORF has been acquired relatively recently. The location of

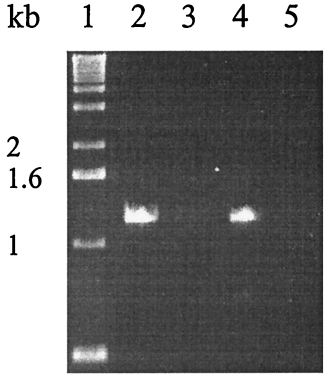

FIG. 5. RT-PCR detection of E1 mRNA in EHV-2-infected cells. Virus-infected cells were probed for the expression of mRNA encoding E1 by RT-PCR with oligo(dT)-primed cDNA synthesis, followed by PCR amplification with E1-specific primers. Lanes: 1, kilobase ladder; 2, PCR of EHV-2 DNA template; 3, RT-PCR of poly(A)$^+$ RNA from EHV-2-infected cells in the absence of RT treatment; 4, RT-PCR of poly(A)$^+$ RNA from EHV-2-infected cells in the presence of RT treatment; 5, RT-PCR of total RNA from uninfected cells.

E1 in the viral genome, namely, within the terminal repeat elements, distal to the "core" blocks of genes conserved with other gammaherpesviruses, further supports this hypothesis.

Our studies show that the chemokine receptor-like E1 ORF encodes a functional receptor, since we identified the CC chemokine eotaxin as a ligand able to induce both calcium mobilization and chemotaxis in cellular transfectants overexpressing the E1 protein, whereas a number of other chemokines, either CC or CXC, were ineffective. Furthermore, we have demonstrated that mRNA encoding E1 is expressed upon EHV-2 infection of equine cells in tissue culture.

A certain degree of functional redundancy may be hypothesized for chemokine receptors (7), since they often share common ligands and biological functions (i.e., chemotaxis) and are distributed on similar leukocyte subpopulations. However, there are several examples of discrete roles attributed either to selected ligands (15, 23) or to chemokine receptors (13, 25, 51, 60) by the use of genetically modified mice, as well as the demonstration of discrete ligand and receptor expression on leukocyte populations in pathophysiological states (34). Eotaxin represents a typical example of a "specialized" function, playing an important role during allergic reactions and parasitic infections; this chemokine is produced locally by inflamed tissues and is a potent activator of eosinophils and basophils (26, 31, 43). Moreover, eotaxin-deficient mice show an impaired allergen-induced eosinophil infiltration in the lungs (48), and eotaxin has a role in the growth of granulocytic progenitors and in the differentiation of embryonic mast cell progenitors (45).

Eotaxin is a selective ligand for CCR3, while its other agonists, RANTES, eotaxin-2, and MCP-2, -3, and -4 (19, 42, 56), are also able to bind other chemokine receptors. CCR3 is mostly expressed by eosinophils, basophils (42, 56), and Th2 lymphocytes (50) that are recruited at sites of allergic inflammation; therefore, this ligand-receptor pair is crucially involved in the generation of allergic reactions, antihelminth responses and, potentially, the modulation of responses after other infections.

Examples of virus-host interactions have been described for several classes of viruses and, in particular, several viral ORFs encode for chemokines and chemokine receptors. This suggests strongly that these molecules may possess important regulatory functions for viral escape from, or interaction with, immune responses (2, 16, 22).

Human CMV (37) encodes four potential chemokine receptors: UL33, UL78, US27, and US28. US28 is a functionally active molecule (20, 41), and it has been recently shown that its expression may alter chemokine levels in the supernatant of infected cells, possibly by a sequestration mechanism (12), potentially affecting immune responses, cellular proliferation and, ultimately, the course of viral infection. UL33, conserved in murine and rat CMV (M33 and R33, respectively) encodes a chemokine receptor-like molecule that, when mutated, does not affect viral replication in tissue culture but is important for murine and rat CMV replication in salivary glands, suggesting a potential role of this receptor in viral tropism (9, 17, 35).

Poxviruses provide additional evidence for the importance of chemokines in controlling viral spread through the identification of distinct viral mechanisms for interfering with chemokine function. Examples are represented by a chemokine homolog encoded by molluscum contagiosum virus (MC148R), functional as an antagonist for several chemokines (32), and myxoma virus MT-7, a low-affinity chemokine-binding protein that interacts with the heparin-binding domain of chemokines, thereby potentially disrupting their normal association with the extracellular matrix (33). Similarly, the vCKBP protein of the T1/35K family, expressed by myxoma, vaccinia, cowpox and camelpox viruses, binds to CXC, C, and CC chemokines, thereby blocking their interaction with chemokine receptors (5, 24). Functions of vCKBP have been demonstrated, namely, the inhibition of eotaxin-induced eosinophil infiltration in an in vivo model of allergic inflammation and the inhibition of leukocyte recruitment to foci of rabbitpox virus infection (24).

Our demonstration of a functional activity of the EHV-2 E1 ORF indicates that this virally encoded chemokine receptor may play a role in the subversion of immune functions during EHV-2 infection. Possible functions include the sequestration of cellular chemokines at sites of infection, the modulation of virus replication in the presence of secreted chemokines, or the altered trafficking of virus-infected cells in response to chemokine gradients. Elucidation of the biological significance of E1 during virus infection may be achieved through characterization of mutant EHV-2 viruses with the E1 ORF deleted, thereby determining the influence of E1 upon virus replication and tissue tropism in vitro and in vivo.

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REFERENCES

1. **Agius, C. T., and M. J. Studdert.** 1994. Equine herpesviruses 2 and 5: comparisons with other members of the subfamily gammaherpesvirinae. Adv. Virus Res. **44:**357–379.
2. **Ahuja, S. K., J. L. Gao, and P. M. Murphy.** 1994. Chemokine receptors and molecular mimicry. Immunol. Today **15:**281–287.
3. **Ahuja, S. K., and P. M. Murphy.** 1993. Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri. J. Biol. Chem. **268:** 20691–20694.
4. **Albrecht, J. C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wittmann, M. A. Craxton, H. Coleman, B. Fleckenstein, and K. W. Honess.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66:**5047–5058.
5. **Alcamì, A., J. A. Symons, P. D. Collins, T. J. Williams, and G. L. Smith.** 1998. Blockade of chemokine activity by a soluble chemokine binding protein from vaccinia virus. J. Immunol. **160:**624–633.
6. **Arvanitakis, L., E. Geras-Raaka, A. Varma, M. C. Gershengorn, and E. Cesarman.** 1997. Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. Nature **385:**347–350.
7. **Baggiolini, M., B. Dewald, and B. Moser.** 1997. Human chemokines: an update. Annu. Rev. Immunol. **15:**675–705.
8. **Bais, C., B. Santomasso, O. Coso, L. Arvanitakis, E. Geras-Raaka, J. S. Gutkind, A. S. Asch, E. Cesarman, M. C. Gershengorn, and E. A. Mesri.** 1998. G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature **391:**86–89.
9. **Beisser, P. S., C. Vink, J. G. Van Dam, G. Grauls, S. J. Vanherle, and C. A. Bruggeman.** 1998. The R33 G protein-coupled receptor gene of rat cytomegalovirus plays an essential role in the pathogenesis of viral infection. J. Virol. **72:**2352–2363.
10. **Ben-Baruch, A., L. Xu, P. R. Young, K. Bengali, J. J. Oppenheim, and J. M. Wang.** 1995. Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors. C-C CKR1, a receptor for macrophage inflammatory protein-1 α/Rantes, is also a functional receptor for MCP3. J. Biol. Chem. **270:**22123–22128.
11. **Bernardini, G., J. Hedrick, S. Sozzani, W. Luini, G. Spinetti, M. Weiss, S. Menon, A. Zlotnik, A. Mantovani, A. Santoni, and M. Napolitano.** 1998. Identification of the CC chemokines TARC and macrophage inflammatory protein-1β as novel functional ligands for the CCR8 receptor. Eur. J. Immunol. **28:**582–588.
12. **Bodaghi, B., T. R. Jones, D. Zipeto, C. Vita, L. Sun, L. Laurent, F. Arenzana-Seisdedos, J. L. Virelizier, and S. Michelson.** 1998. Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. J. Exp. Med. **188:**855–866.
13. **Boring, L., J. Gosling, M. Cleary, and I. F. Charo.** 1998. Decreased lesion formation in $CCR2^{-/-}$ mice reveals a role for chemokines in the initiation of atherosclerosis. Nature **394:**894–897.
14. **Collinson, P. N., J. L. O'Rielly, N. Ficorilli, and M. J. Studdert.** 1994.

Isolation of equine herpesvirus type 2 (equine gammaherpesvirus 2) from foals with keratoconjunctivitis. J. Am. Vet. Med. Assoc. **205:**329–331.
15. **Cook, D. N., M. A. Beck, T. M. Coffman, S. L. Kirby, J. F. Sheridan, I. B. Pragnell, and O. Smithies.** 1995. Requirement of MIP-1α for inflammatory response to viral infection. Science **269:**1583–1585.
16. **Davis-Poynter, N. J., and H. E. Farrell.** 1996. Masters of deception: a review of herpesvirus immune evasion strategies. Immunol. Cell. Biol. **74:**513–522.
17. **Davis-Poynter, N. J., D. M. Lynch, H. Vally, G. R. Shellam, W. D. Rawlinson, B. G. Barrell, and H. E. Farrell.** 1997. Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus. J. Virol. **71:**1521–1529.
18. **Ensser, A., R. Pflanz, and B. Fleckenstein.** 1997. Primary structure of the alcelaphine herpesvirus 1 genome. J. Virol. **71:**6517–6525.
19. **Forssmann, U., M. Uguccioni, P. Loetscher, C. A. Dahinden, H. Langen, M. Thelen, and M. Baggiolini.** 1997. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. J. Exp. Med. **185:**2171–2176.
20. **Gao, J. L., and P. M. Murphy.** 1994. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. J. Biol. Chem. **269:**28539–28542.
21. **Geras-Raaka, E., L. Arvanitakis, C. Bais, E. Cesarman, E. A. Mesri, and M. C. Gershengorn.** 1998. Inhibition of constitutive signaling of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor by protein kinases in mammalian cells in culture. J. Exp. Med. **187:**801–806.
22. **Gooding, L. R.** 1992. Virus proteins that counteract host immune defenses. Cell **71:**5–7.
23. **Graham, G. J., E. G. Wright, R. Hewick, S. D. Wolpe, N. M. Wilkie, D. Donaldson, S. Lorimore, and I. B. Pragnell.** 1990. Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. Nature **344:**442–444.
24. **Graham, K. A., A. S. Lalani, J. L. Macen, T. L. Ness, M. Barry, L. Y. Liu, A. Lucas, I. Clark-Lewis, R. W. Moyer, and G. McFadden.** 1997. The T1/35kDa family of poxvirus-secreted proteins bind chemokines and modulate leukocyte influx into virus-infected tissues. Virology **229:**12–24.
25. **Gu, L., Y. Okada, S. K. Clinton, C. Gerard, G. K. Sukhova, P. Libby, and B. J. Rollins.** 1998. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol. Cell **2:**275–281.
26. **Heath, H., S. Qin, P. Rao, L. Wu, G. LaRosa, N. Kassam, P. D. Ponath, and C. R. Mackay.** 1997. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J. Clin. Investig. **99:**178–184.
27. **Howard, O. M., A. Ben-Baruch, and J. J. Oppenheim.** 1996. Chemokines: progress toward identifying molecular targets for therapeutic agents. Trends Biotechnol. **14:**46–51.
28. **Hsu, D. H., R. de Waal Malefyt, D. F. Fiorentino, M. N. Dang, P. Vieira, J. de Vries, H. Spits, T. R. Mosmann, and K. W. Moore.** 1990. Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. Science **250:**830–832.
29. **Isegawa, Y., Z. Ping, K. Nakano, N. Sugimoto, and K. Yamanishi.** 1998. Human herpesvirus 6 open reading frame U12 encodes a functional β-chemokine receptor. J. Virol. **72:**6104–6112.
30. **Jensen-Waern, M., S. G. Persson, A. Nordengrahn, M. Merz, and C. Fossum.** 1998. Temporary suppression of cell-mediated immunity in standardbred horses with decreased athletic capacity. Acta Vet. Scand. **39:**25–33.
31. **Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams.** 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. J. Exp. Med. **179:**881–887.
32. **Krathwohl, M. D., R. Hromas, D. R. Brown, H. E. Broxmeyer, and K. H. Fife.** 1997. Functional characterization of the C-C chemokine-like molecules encoded by molluscum contagiosum virus types 1 and 2. Proc. Natl. Acad. Sci. USA **94:**9875–9880.
33. **Lalani, A. S., K. Graham, K. Mossman, K. Rajarathnam, I. Clark-Lewis, D. Kelvin, and G. McFadden.** 1997. The purified myxoma virus gamma interferon receptor homolog M-T7 interacts with the heparin-binding domains of chemokines. J. Virol. **71:**4356–4363.
34. **Luster, A. D.** 1998. Chemokines—chemotactic cytokines that mediate inflammation. N. Engl. J. Med. **338:**436–445.
35. **Margulies, B. J., H. Browne, and W. Gibson.** 1996. Identification of the human cytomegalovirus G protein-coupled receptor homologue encoded by UL33 in infected cells and enveloped virus particles. Virology **225:**111–125.
36. **Marra, M. A., L. Hillier, and R. H. Waterston.** 1998. Expressed sequence tags—ESTablishing bridges between genomes. Trends Genet. **14:**4–7.
37. **Mocarski, E. S.** 1996. Cytomegaloviruses and their replication, p. 2447–2487. *In* B. N. Fields et al. (ed.), Fields virology, 3rd ed. Lippincott-Raven, Philadelphia, Pa.
38. **Murphy, P. M.** 1997. Pirated genes in Kaposi's sarcoma. Nature **385:**296–299.
39. **Murray, M. J., E. S. Eichorn, E. J. Dubovi, W. B. Ley, and D. M. Cavey.** 1996. Equine herpesvirus type 2: prevalence and seroepidemiology in foals. Equine Vet. J. **28:**432–436.
40. **Nava, V. E., E. H. Cheng, M. Veliuona, S. Zou, R. J. Clem, M. L. Mayer, and J. M. Hardwick.** 1997. Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. J. Virol. **71:**4118–4122.
41. **Neote, K., D. DiGregorio, J. Y. Mak, R. Horuk, and T. J. Schall.** 1993. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. Cell **72:**415–425.
42. **Ponath, P. D., S. Qin, T. W. Post, J. Wang, L. Wu, N. P. Gerard, W. Newman, C. Gerard, and C. R. Mackay.** 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. J. Exp. Med. **183:**2437–2448.
43. **Ponath, P. D., S. Qin, D. J. Ringler, I. Clark-Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J. A. Gonzalo, W. Newman, J. C. Gutierrez-Ramos, and C. R. Mackay.** 1996. Cloning of the human eosinophil chemoattractant, eotaxin: expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. J. Clin. Investig. **97:**604–612.
44. **Purewal, A. S., A. V. Smallwood, A. Kaushal, D. Adegboye, and N. Edington.** 1992. Identification and control of the *cis*-acting elements of the immediate early gene of equid herpesvirus type 1. J. Gen. Virol. **73:**513–519.
45. **Quackenbush, E. J., B. K. Wershil, V. Aguirre, and J. C. Gutierrez-Ramos.** 1998. Eotaxin modulates myelopoiesis and mast cell development from embryonic hematopoietic progenitors. Blood **92:**1887–1897.
46. **Richmond, A., S. Mueller, J. R. White, and W. Schraw.** 1997. C-X-C chemokine receptor desensitization mediated through ligand-enhanced receptor phosphorylation on serine residues. Methods Enzymol. **288:**3–15.
47. **Rizvi, S. M., J. D. Slater, U. Wolfinger, K. Borchers, H. J. Field, and A. J. Slade.** 1997. Detection and distribution of equine herpesvirus 2 DNA in the central and peripheral nervous systems of ponies. J. Gen. Virol. **78:**1115–1118.
48. **Rothenberg, M. E., J. A. MacLean, E. Pearlman, A. D. Luster, and P. Leder.** 1997. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. J. Exp. Med. **185:**785–790.
49. **Russo, J. J., R. A. Bohenzky, M. C. Chien, J. Chen, M. Yan, D. Maddalena, J. P. Parry, D. Peruzzi, I. S. Edelman, Y. Chang, and P. S. Moore.** 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc. Natl. Acad. Sci. USA **93:**14862–14867.
50. **Sallusto, F., C. R. Mackay, and A. Lanzavecchia.** 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science **277:**2005–2007.
51. **Tachibana, K., S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, T. Kishimoto, and T. Nagasawa.** 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature **393:**591–594.
52. **Telford, E. A. R., M. J. Studdert, C. T. Agius, M. S. Watson, H. C. Aird, and A. J. Davison.** 1993. Equine herpesviruses 2 and 5 are γ-herpesviruses. Virology **195:**492–499.
53. **Telford, E. A. R., M. S. Watson, H. C. Aird, J. Perry, and A. J. Davison.** 1995. The DNA sequence of equine herpesvirus 2. J. Mol. Biol. **249:**520–528.
54. **Thome, M., F. Martinon, K. Hofmann, V. Rubio, V. Steiner, P. Schneider, C. Mattmann, and J. Tschopp.** 1999. Equine herpesvirus-2 E10 gene product, but not its cellular homologue, activates NF-κB transcription factor and c-Jun N-terminal kinase. J. Biol. Chem. **274:**9962–9968.
55. **Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meinl, F. Neipel, C. Mattmann, K. Burns, J. L. Bodmer, M. Schroter, C. Scaffidi, P. H. Krammer, M. E. Peter, and J. Tschopp.** 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature **386:**517–521.
56. **Uguccioni, M., C. R. Mackay, B. Ochensberger, P. Loetscher, S. Rhis, G. J. LaRosa, P. Rao, P. D. Ponath, M. Baggiolini, and C. A. Dahinden.** 1997. High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. J. Clin. Investig. **100:**1137–1143.
57. **Virgin, H. W., IV, P. Latreille, P. Wamsley, K. Hallsworth, K. E. Weck, A. J. Dal Canto, and S. H. Speck.** 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. **71:**5894–5904.
58. **Ward, S. G., K. Bacon, and J. Westwick.** 1998. Chemokines and T lymphocytes: more than an attraction. Immunity **9:**1–11.
59. **Welch, H. M., C. G. Bridges, A. M. Lyon, L. Griffiths, and N. Edington.** 1992. Latent equid herpesviruses 1 and 4: detection and distinction using the polymerase chain reaction and co-cultivation from lymphoid tissues. J. Gen. Virol. **73:**261–268.
60. **Zou, Y. R., A. H. Kottmann, M. Kuroda, I. Taniuchi, and D. R. Littman.** 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature **393:**595–599.