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Transcriptome profile of murine gammaherpesvirus-68 lytic infection

Bahram Ebrahimi,[1]‡ Bernadette M. Dutia,[1] Kim L. Roberts,[1] Jose J. Garcia-Ramirez,[2]† Paul Dickinson,[2,3] James P. Stewart,[1]‡ Peter Ghazal,[2] Douglas J. Roy[2] and Anthony A. Nash[1]

Laboratory for Clinical and Molecular Virology[1], Scottish Centre for Genomic Technology and Informatics[2] and MRC Centre for Inflammation Research[3], The University of Edinburgh, Summerhall Square, Edinburgh EH9 1QH, UK

Correspondence
Bahram Ebrahimi
ebrahimi@staffmail.ed.ac.uk

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The murine gammaherpesvirus-68 genome encodes 73 protein-coding open reading frames with extensive similarities to human γ_2 herpesviruses, as well as unique genes and cellular homologues. We performed transcriptome analysis of stage-specific viral RNA during permissive infection using an oligonucleotide-based microarray. Using this approach, M4, K3, ORF38, ORF50, ORF57 and ORF73 were designated as immediate-early genes based on cycloheximide treatment. The microarray analysis also identified 10 transcripts with early expression kinetics, 32 transcripts with early-late expression kinetics and 29 transcripts with late expression kinetics. The latter group consisted mainly of structural proteins, and showed high expression levels relative to other viral transcripts. Moreover, we detected all eight tRNA-like transcripts in the presence of cycloheximide and phosphonoacetic acid. Lytic infection with MHV-68 also resulted in a significant reduction in the expression of cellular transcripts included in the DNA chip. This global approach to viral transcript analysis offers a powerful system for examining molecular transitions between lytic and latent virus infections associated with disease pathogenesis.

INTRODUCTION

Murine gammaherpesvirus-68 (MHV-68) is closely related to the human herpesviruses Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Efstathiou *et al.*, 1990a; Virgin *et al.*, 1997). MHV-68 is a natural pathogen of small murid rodents, and infection via the respiratory route results in an initial lytic infection of lung epithelial cells followed by virus dissemination and latent infection of B lymphocytes as well as macrophages. The peak of virus latency in the spleen coincides with splenomegaly followed by an infectious mononucleosis-like syndrome, and lymphoproliferative disease (reviewed by Nash *et al.*, 2001).

The MHV-68 genome contains 118 311 bases of unique sequence with variable numbers of internal repeats flanked by multiple copies of terminal repeats. The genome encodes eight tRNA-like sequences and 73 protein-coding open reading frames (ORF) and is broadly contiguous with other herpesviruses. Also present are genes unique to MHV-68 (M1–M4), as well as homologues of cellular genes such as complement regulator protein, Bcl-2, cyclin-D and G-protein-coupled receptor, believed to play a role in establishment of infection and evasion of host immune responses (Bowden *et al.*, 1997; Virgin *et al.*, 1997; Kapadia *et al.*, 1999; van Dyk *et al.*, 1999; Wang *et al.*, 1999; van Berkel *et al.*, 1999; Roy *et al.*, 2000; Parry *et al.*, 2000; Nash *et al.*, 2001).

Herpesvirus replication follows a regulated pattern of viral gene expression. Using either sensitivity to drug inhibitors and/or measurements of viral gene expression, it is feasible to classify these viral proteins into immediate-early (IE), early (E) and late (L). IE genes encode predominantly transcription factors, associated with transactivation, whereas E genes include enzymes associated with DNA replication. L genes are mainly associated with structural components of viral particles.

DNA microarrays offer a powerful platform for parallel analysis on a genome-wide scale. DNA microarrays for viral pathogens can facilitate identification of transcription patterns during replication, persistence and latency. The parallel and comprehensive transcriptome analysis by DNA microarrays will be useful in exploring transitions between different stages of virus infection and disease pathogenesis.

Analysis of MHV-68 gene expression has been limited to a small set of genes. In this paper we sought to: (a) design and validate a DNA microarray specific for MHV-68, (b)

†Present address: Departamento de Bioquimica y Biología Molecular, Facultad de Medicina, 02071 Albacete, Spain.

‡Present address: Department of Medical Microbiology, The University of Liverpool, Liverpool L69 3GA, UK.

develop new PCR assays for tRNA-like genes of MHV-68 and (c) provide an overview of the stage-specific kinetic class of all viral genes during permissive infection *in vitro*.

We show the simultaneous expression of MHV-68 genes by using classical drug inhibitors. Data obtained from the microarray platform and complemented with RT-PCR assays constitute a comprehensive transcriptome analysis of MHV-68.

METHODS

Cell lines and virus infection. Murine epithelial cells (C127, ATCC CRL 1616) were cultured in Dulbecco's Modified Eagle's Medium supplemented with foetal calf serum (10 %, v/v), glutamine (2 mmol l^{-1}), potassium benzylpenicillin (100 U ml^{-1}) and streptomycin sulphate (100 μg ml^{-1}). Drug treatments were performed as previously described (Stewart *et al.*, 1994). Briefly, cells were infected with MHV-68 (strain g2.4) at 5 p.f.u. per cell. To select for RNA enriched for viral IE transcripts, cells were pre-treated for 2 h with cycloheximide (CHX; 100 μg ml^{-1}), to act as an inhibitor of *de novo* protein synthesis. Infection was carried out in fresh medium containing CHX for up to 8 h. To select for viral E transcripts, cells were pre-treated for 2 h with phosphonoacetic acid (PAA; 100 μg ml^{-1}), an inhibitor of DNA polymerase. This was then followed by infection with MHV-68 in the presence of PAA for a further 8 h. Viral RNA was also obtained from infected cells in the absence of drug inhibitors. This approach differentiates between E and L transcripts. Mock-infected cultures included cells subjected to drug treatment regimens but without virus infection and cells without either drug inhibitors or virus infection.

Synthesis, isolation and labelling of nucleic acids. Cloned *Bacillus subtilis* genes obtained from the ATCC (pGIBS-TRP, accession #K01391; pGIBS-DAP, accession #L38424; pGIBS-PHE, accession #M24537; pGIBS-LYS, accession #X17013) were linearized and mRNA transcribed *in vitro* using the MEGAscript kit (Ambion) according to the manufacturer's protocol.

Viral DNA was extracted from purified MHV-68 virions as described previously (Efstathiou *et al.*, 1990b). The purity and integrity of viral DNA were checked by digestion with *Eco*RI, *Bam*HI and *Hin*dIII followed by gel electrophoresis (data not shown).

Purified viral DNA (1 μg) was digested with 5 mU DNase 1 (Life Technologies) at 15 °C for 15 min, and heat-inactivated at 65 °C for 20 min. To generate labelled DNA fragments, digested DNA was incubated with 10 U *E. coli* DNA polymerase 1 (Life Technologies), dATP, dGTP, dTTP (4 mM each) and 20 nM FluoroLink Cy3-dCTP (Amersham Pharmacia). Labelled DNA was mixed with 20 μg COT-1 human DNA (Life Technologies) and excess label was removed by YM-30 microfiltration (Amicon). This step was also used to reduce the final reaction volume of labelled DNA to 12 μl.

Total RNA was harvested from cells using RNeasy columns (Qiagen) according to the manufacturer's recommendations. Briefly, adherent cells were trypsinized and the cell pellet was resuspended in RLT lysis buffer (Qiagen). Contaminating DNA was digested with 10 U AmpGrade DNase 1 (Life Technologies) for 15 min at room temperature. RNA was recovered and purified using RNeasy columns and the quality of RNA was checked by spectrophotometry and gel electrophoresis. Purified RNA (25 μg in 11 μl) was mixed with 4 μl (2 μg) of poly$(dT)_{21}$, heated to 70 °C for 10 min and snap-cooled on ice for 5 min. Direct labelling was carried out in a 30 μl reaction volume containing 400 U Superscript II reverse transcriptase (Life Technologies), 6 μl of 5× first-strand buffer, 3 μl of DTT (10 mM), 0·6 μl of dNTP mix (dATP, dGTP and dTTP at 0·5 mM; dCTP at 0·3 mM) and 3 μl of Cy3-dCTP (0·1 mM) at 42 °C for 2 h. This was followed by addition of 15 μl 0·1 M NaOH at 70 °C for 10 min, and then neutralized by addition of 15 μl 0·1N HCl. From this stage onwards, labelled cDNA was treated in an identical manner to labelled viral genomic DNA.

Probe design and array fabrication. Probes were designed using the Oligo6 primer design software (Molecular Biology Insights: www.oligo.net/contact.htm). ORF- and strand-specific viral probe sequences were based on the g2.4 strain of MHV-68 (accession #AF105037) (Table 1). Design parameters for probe selection included: lack of homo-oligomers or sequence repeats, 40–60 % GC-rich and a melting temperature range of 85–95 °C. Control elements on the array included 54 murine cellular genes, five negative control genes (plant and bacterial), four *B. subtilis* genes for spike RNA and printing buffer without nucleic acids (Table 2). Probes were synthesized by MWG-biotech, resuspended at 0·4 μg ml^{-1} in printing buffer (3× SSC) and printed in triplicate using an Affymetrix 417 arrayer onto poly-L-lysine-coated glass slides (Eisen & Brown, 1999). Arrays were stored in desiccators at room temperature prior to use.

Array hybridization. Prior to hybridization, arrays were post-processed in succinic anhydride/*N*-methyl-2-pyrrolidinone solution (Eisen & Brown, 1999), and then incubated in pre-hybridization solution (1 %, w/v, BSA/5× SSC/0·1 % SDS) at 42 °C for 45 min, rinsed in water and dehydrated in isopropanol.

Labelled target DNA was resuspended in 15 μl of hybridization solution (3·5× SSC/0·3 % SDS), and incubated on the array at 65 °C overnight. Following this, arrays were rinsed in 1× SSC/0·2 % SDS, followed by 0·1× SSC/0·2 % SDS, and 0·1× SSC. All stringency washes were done at room temperature.

Primer design and RT-PCR for tRNA-like sequences. MHV-68 encodes eight tRNA-like sequences which lack poly(A) tracts and are therefore unsuitable for oligo(dT)-directed reverse transcription. We therefore designed PCR primers for tRNA1–tRNA8 to complement the array data (Table 3). The design parameters included: 40–60 % GC content; 3′-end stability; and a melting temperature of 84–90 °C. RNA was isolated and treated with DNase 1 as for microarray experiments and was used in RT-PCR assays. PCR included 1 U per reaction of *Taq* DNA polymerase (Life Technologies), 100 nM dNTP and 100 pmol of each primer. Cycling parameters were: hot-start at 95 °C for 5 min followed by addition of *Taq* DNA polymerase; 94 °C for 60 s; appropriate annealing temperature for 60 s; 72 °C for 60 s; followed by 35 cycles with a final extension at 72 °C for 5 min. PCR products were resolved by agarose gel electrophoresis and DNA visualized by UV transillumination.

Data analysis. All probes on the array were printed in triplicate. QUANTARRAY (Packard Biosciences) image analysis software was used for initial data capture and filtration. This algorithm defines a background correction value and a signal intensity value for each spot. Therefore, the background was subtracted from the corresponding spot intensity and the median value for each probe was then calculated ($n=3$).

To define the linear dynamic range of the fluorescence dye and to control for scanning parameters (laser settings and gain), arrays were scanned at five combinations of increased laser/gain settings; therefore generating five datasets. These datasets were then analysed against each other by scatter plots. Scan settings that gave the optimum scatter without signal saturation (assessed by least-squares fit analysis) were chosen for subsequent analysis. Normalization (scaling) between arrays was based on four *B. subtilis* genes transcribed *in vitro*. The *in vitro*-transcribed spike mRNA was added to the test RNA prior to reverse transcription. This approach normalizes for intensity differences

Table 1. MHV-68 microarray probes

75-mer oligonucleotide probes were designed based on the g2.4 strain (accession #AF105037).

Gene	Sequence	Genome coordinates
M1	CAGGCCCCACCGCTACAATACTGCGTGGTCTTTATACATAGAGGAAATTATGGCTTCTTTAGAACCCGACAACGG	3001–3075
M2	CTCGACTGACAGTCCAGAAAATCTAGGCCCACCTACAAGACCTTTGCCTAAACTCCCGAACCAACACCCCATGAA	4175–4101
M3	TGTGCTCTACAGATGACAAGTCATGATGGGGTGTGGACTTCAACTTCCAGTGAGCAGTGTCCCATTAGACTGGTT	6255–6181
M4	TGGCCATGTTATACCAACTACAGAAGTTCGCCAGTACCAGAGATTGTCATTCATGAAAATATTCTCCTAGAGGGA	9472–9546
ORF4	ACCACCACCTGGAGCACCCGAAAGTTAACCAGTAACACTCCACCCGACCCTGAAGTGCAAACCCTGGGTAGCTAT	10762–10836
ORF6	ATTCTATCTGGGAGAGTTTTCATTTATGGTGAATGATGTCATGGAGGTTCTCGAAACCTTGAAGGCTTCGGGGTG	14272–14346
ORF7	CAGGCTCCCAGCCCAGCCAAAAACTCACTCAAGAAAATGCCCCCCAACAGAAAAACCCTGAAAAGGGCTTTGCCT	15623–15697
ORF8	CTCCTAATTATTGGTGGAATAATCTTCTTGGTGGTAGTCCTAAATAGAAGAAACTCACAGTTTCACGATGCACCC	18717–18791
ORF9	CATGACAGAATACCTTATGTATTCATAGACGGTAGTGAAAAGGGACTAAAGTCTGACTTGGCAGAGCACCCCGCC	22002–22076
ORF10	GGAGTGTCTTATGAGACTCTCCAATGTGAGATATTATTAGAACTGGTTCAGGGCCCCACAAATTTCATATTTCCA	22960–23034
ORF11	TTTTATGGGATTTGTCAACAGTGTGAATATAACTGGAACTATCCTCAGGGTGTACTATTCTGGTGTCAGTGAAGC	24118–24192
ORF12 (k3)	TGAACTCCATTGATGAGGCGATCGCGACCCAGCAGCCCACAAAGACTGACCTTGGATTAGCCCGTGAGACCCTCT	24876–24802
M5	AAAATATGGGGCAGTAGGGAGGGGCACACCATATTGGATATACTATAGGAAGACCCTCTACCTGGAAATCCCCTT	26430–26504
ORF17	CACCAGAAACAGTTCCAACGGTCCCTGTAGCCCAAAGTGTGGAGCGGGAACCAGATGCTCTGGTGACTACCAAAA	28493–28419
ORF18	CTACGTGGGAAAGTTCTTCCTCTTGTACTTGATTCTGGCTGGTTATCTTCTCCCCAGGAAAGAGTGTGTGGAATT	30502–30576
ORF19	CATGGCACAAGATGTTTATGATTATGTTTATTTTTGTGTGTTGGGGGCACTTCCTGTTACAGTGGCCGTGGTGTA	30799–30725
ORF20	CAGCACAGTACCACCAGAGAAGCACAAAAGGTAGAGGGTAGCAACCAGCTGAGGGACTCTGCCAAGGCGCTAGCA	32581–32507
ORF21	TGAAGTTATTGTGGATGCTATGCTTTCCCAGACTTCAGTTAAGACTGTGTTTCAGAAGGCTGGCTTTGTTGCACC	34448–34522
ORF22	TATCAGTGATAGCAGATGTCAATTTTGTGGTTCGATCATCATGAGTTATGATGAATCACAGGGATTTCTGGCAGC	36714–36788
ORF23	TTATGAACCATGCCCACAACGGACTAGACTTCTTTCCCAGCGATTCTGACATTAATGACATGCAGCAGATGTTTT	37528–37454
ORF24	CGAAACCGCTTTCAGATTCAAACCTTGCACAAAAGATTCCTAGAGTGCTTGTTTGAGTCATGTGTGGCTAGCAAA	38469–38395
ORF25	TCATGGCAGAGTATGAAGAGTGGCACAAGTCTCCCATGTCAAGTATGTGAGAGATTGTGATGTGCAACCTCAGG	43205–43279
ORF26	AAATCCTCATGTACAATTTGTATTCTAACCTTTTGGCAGCGGAGCGCAACCATGACAATGTTAATGCACTAGAAC	44875–44949
ORF27	TTGTGTGTCCATTTGAAATAAAACATTTGTATGGTACTTGGCAGAGTGGAGACTCTATATTGGCTGGAGAAGTGG	45508–45582
ORF29b	GGATTGCTCTGTGCCAGCCAAACCGTGGTATCTAACACTGTCCTGCTGTCTAGTGACCCAATCTCCTATCTGATT	46629–46555
ORF30	TTTAACAGACCATTACATGCTCTCATTGCTGAGGTGTCAAAAACCACTTCTGATTTGGAACTTGTCAGGTCAACC	47586–47660
ORF31	GTACCTTTCCAATGTGCGCCAACATCATTAATTGTAATAGCCAAGGCTATTCCATACTTTGTAGCATGTTTGTCC	48114–48188
ORF32	GGATACTGACTTCCTCCACGCTGTTCAAAATAGATTCATCGACTCAATTGATCTCCCTGGTGTCAAAGATGGTGG	49095–49169
ORF33	GTTGAGTACAGAACATCAGATGATGTGTCCGTCCCTACCAGTGTTTCCCTCCCTGTCTAAGATCCTCAATATTAT	50095–50169
ORF29a	TGTCCTTGGCAGTTTTCCTGGAAATCTCAAACATTCTGCTGGCCTGGTTCCCAAGAAAGAATATTGACATTAAAA	50716–50642
ORF34	GGGGACACTTCAGTGAATTCTTTTGCCCATCACCTGTGTCAGTATGTGAAACATTTGTATGCTGATTATGAACCA	52051–52125
ORF35	AAGAGAGTTGAGTGATTGCGTAAGTGAGCTGACTCATCTTAAAGAGATATGCCAAAACTTTTCAGTGGAAGATGC	52630–52704
ORF36	CCAATGTACTGGAGGACACCACAGGAGGGACTAAGATTGGACTTGTGCTGTCTTTATTATTCCTTTCAGGACTGT	53703–53777
ORF37	CACATGGAAAACTTCCCTCAGAAAGTGACTATCTAATCACCCACTCCATGAACTGGAAAACCAGCAACAAGAGAA	55001–55075
ORF38	GGGACAACTCATTGACCTGACAAAGGACTTTGAACTAATTTCTGAAAACACTGGACTTTTGGTTGAAGACCCCTC	55601–55675
ORF39	TGCAGTGTTTGGTGCTGTCAACCTATTCCTCATCATATCTCTGGTGTGGCATATCGGGGCAGAAGTGTTCCTGGC	56202–56128
ORF40	CATGGTGGGACCCGACCTGGTGAATGATACACAGTTCCTGGATGAAATATTCCACAGACTCTCATCAGTTTATAA	58315–58389
ORF42	CAGAATGGACAATAACATAGATGATTTTCAAAACCCAGCGGGGACATTCACACTTCTGTGCTTGCCCACCATTTT	59036–58962
ORF43	CCCAAGGAAAGATGATATGTGTTAGGTACACCCCAGACACTGTTTCACTTTTACTTGGACCATTCACTTATGTAA	59885–59811
ORF44	TGCTGGGCTGCACAGGAGATTATGGAATAAGTTCAAAATTGGCTATGACGATTGTCAAGGCTCAGGGCATGTCTC	63357–63431
ORF45	TATGTGAGCAGTGAAGACTCTGATGATGAAGCACAAGAACATGCGGATAACCCGGGACACTAATGACCAGGTCAGC	64075–64001
ORF46	AAGGGACGGCCTGGTTCTCACCACAAACTTGGATGGGGATGGTTCACAGACTATGTCATTTCTACAATCTCTGAA	64575–64501
ORF47	CACTTAGAATGTGCAAATGGTTTTGGACTCATGGGCTTTCTTGAAGCCGTGGTCAGCAGGTTTAGAGACCCCACC	65210–65136
ORF48	GGACTGCATGTCTCAGCCAACAGCTCTGCTTCGGGATTGTATTCAGAAGGGTGCAAAGAATAATGGACTCCAGCT	65897–65823
ORF49	AAGCGTCTTTAAACTGTTCAGTGTTTGTGTCCAAAGTCAGTTTCTGCTGGACAGCCTTGTGGCATTTAATGCCCT	67075–67001
ORF50	CAGAAGAAATCATTAACCTGGACCCTCTACACAATACCACTGAACCCTGTTTGTATCAAATGTTTTCAGAGGCGG	68506–68580
Gp150	CCTACAGCAGAACCTCCCACCTCCAATGCAGATGTTTCTACTGAACACGTTGATGAGACAGAACCCGAGTCACCA	69926–70000
ORF52	GTTAAGTCTTCAGGCGCTGTCTCATCAGATGATAGCATCTTGACAGCTGCCAAGAGAGAAAGTATCATTGTCTCT	71275–71201
ORF53	CATACGTCCCTCAGTGGGGGAGTGTCTCTAGTGTGTTGGCAATAATTAATGGGTCTATAGTCACAGTGTCTAGTG	71575–71501
ORF54	ATTGCTGAAGAAAAGAATAGGCGATACAGCAAACAGAGGCCAGGAAAGCCTTCTAGGGAGGGGCGGGGGTTTGGG	72614–72688
ORF55	CTGGCCGTGCAGGCAAATAATTTAACAGAGCACATCAAGAGATTTACCTTGGGTGAGATACCAGAAGAATGTAAA	73134–73060
ORF56	AGGGTCGCTGCATTCGCCTGCCACACACCTACAAATTTACATCTACAGGATTGCCTGAGAGACTGCTAAAATTGT	75172–75246
M8	CTGCTTAGAGATACAACAAAGTATGAGCCTGCACCCAAAAGAAGCTATTCTTACCACCCCAGACGCAGTCCACAG	76201–76275
ORF57	CAGTGTAACAAAGACTGTGATAAATGTAAATATATTTTGGATCCCCTGTTGGGACAAACACACCGCACCAAGGGG	77068–77142
ORF58	GCTGGCTGCTTTCTGGAATGTATGCGTGTCAAGTTAAGGAGAGCCATAAATGGTGTCTATTTTGTTTATCTAGTA	77340–77266
ORF59	AGATCATCCCAAAGAATCCCTCACTAGCGACTCTGATTCCTCAGTTCCTGGGCCACGAAGTACTGACCTTTCCTC	78475–78401
ORF60	CCAATGATTACATTTCTAAAGATGAACTGCTACACACGCGAGCCGCTGCTGTTCTGTACAATAGTCTAATACCGG	79933–79859
ORF61	GAACCCAGCCTGTATGTACGCAGAGCTATGCTTAGGGCACCATTCATTGATCAGAGCCAGTCTATGACCTTGTTT	80865–80791
ORF62	AGAAGATTCATTGCTGACCAGAACCAGAGTTTCCATCAAGGGTGGTGGGTTGTCTTTAATTGAAATTCCTGACTT	82977–82903
ORF63	TACAGCACGGTGCTCTCACTTCTTCAAGACCTAGAGGCATATAAACAGCATGAGTTGGGGATAGAATTTGACACC	86002–86076
ORF64	CATGGTAGATGCGGCCCTGGCAAAGAAAACTCAGTCAACCACAGAATATAACACCCATCTACTGAAGATTGTCAA	90284–90358
ORF65 (M9)	CTATGTCTGACGGGAACAGCTTTAGGGTTATTCCAGACCCCTCATCTCTAACAGACAAGGATGACCAGAACTTGG	94078–94004
ORF66	AATCTAGGTCTTGCAGTATTAAGCTGAGGGCGACATTTAGGGAGCTACTTTATTTGACAGCGTCTGTTGATAATT	94625–94551
ORF67	TCCTGAGGTTCAAGAGCTACCATGTGTTTCCCAAGGACGTACCAATCTATAACTCTATGGTGGTATGTAGAACCT	96025–95951
ORF68	CCGCCTGAGTTTGTTATCTAATACCATCTGTAATGTCCAGTCTGCTGCTCACATACAAGGTATCTGGTCCAGCTC	97201–97275
ORF69	CGATCTGCTTCACTTGCACGAGTCCTACCTTCGTCTTCTCATCGATAATCTGGGCAATTATGACATTTCAGTGGA	98691–98765
*M10/1	CTCGTCTTTCTCAGCAATAAATTTTTTTCTTTCTAACTACCTGCTAATAGTG	98921–98972
M10/2	ACAGCAGAGGTCCGTCCAGTAGCGAGGCGCGGCGCAGAATGAGCCCCCACAGAAATCAGGGTCTATATATAACCT	101287–101361
M10/3	TTTAAACTACCAACAGGTGATTCATAAAAACCGCAAAAGGTATCAACAGGTAAGGAGGTCAGGGGTTGACATACC	101393–101467
Cyclin–D (ORF72)	TATGCGATGGCAGGTCAGCCATGAAACGCCCAGTGCTAATCACACTGGCCTGTATGCACTTAACTATGAACCAAA	102738–102664
Bcl–2 (M11)	CCCTGCCCTCAAGAACTGGAAAAGAGACCTGTCCAAACTTTTCACCTCGTTGTTTGTAGATGTCATCAACAGTGG	103701–103775
ORF73	CACCAGTTAGCTGTTTACCCTTGGTACCAGGCACAACACAACAGTGTGTAACATACTATTTACTTTCATTTGTTG	104380–104306
GPCR (ORF74)	ACATGGTGCGTTTATATGTGCTATGGAGCTAGGAGGGACCACTAGGGTAAGTGTGAGATTAGCCCAACAATTTCT	105733–105807
ORF75c	ATGGAAGGGCTACTGCTATGCTAATTGATCCCTCCCAGAGCTTCTTCCCTTGGCAGTGGCAATATGATGCAGAAA	106313–106239
ORF75b	GGGCTTCTCTAAATCTGAGACAGGTGTGGTCTGACATCATTACTCATCCAGCGGTTGAAAGCAAGGAATACATCC	112327–112253
ORF75a	TATTCTAGGATTTGGAGAGTTTGCCACCCAAGTACTCCTTGCTCTTAACTTAACAGAATGGAGGTGTGATCGCCC	114679–114605
M12	AAATATGCGTGATACCAAACCTGGCCCCGAGGAGTGCTGCGACGCGGAGACATACCTAGTCGGCGCTACTCACGA	118101–118175

*51 bases due to sequence overlap between M10a–c.

between arrays because of scan settings and hybridization efficiencies and is also independent of potential changes in cellular transcripts because of virus infection. Signal intensities of viral and cellular genes above the arithmetic mean plus 2 SD of signal intensities for negative control genes were used for further analyses. Each data point was obtained from three experiments ($n=3$).

Table 2. Microarray probes for murine housekeeping genes, spike RNA and negative controls

(A) Forty-eight 75-mer oligonucleotide probes were designed for cellular transcripts. The array also included probes for (B) four *B. subtilis* genes for spike RNA and (C) negative controls.

	Gene	Sequence
A	AB009392	ACTGGGAGTGAAGCGGCCAACCTCTGTCAAAGTATTTTCAGGCAAAAGCGAGCGGAGCTCCTCTGGGCTGCTGGA
	AB015613	TGGGTAACAACATTTGTCAACCATCCACAAGTGTCTGCACTGCTTGGGGAGGAGGACGAGGAGGCTCTGCATTAT
	AB027565	GGTTTCCCACCATACACGGCCTGGCCCTGCAGCTTCTTTTCTTGCCCTCCCCTTTGCCTGTCCCCACCTGCAGTA
	AB039919	TGCTGCAGAGCTGCGGACACTGGGGATATATTCCACCTGAGGAGCAAACTGCCCCTGATCCTTGAGATAAAACTA
	AF004927	GCCCTTCCCTTCATCCCTTTCCTCACCATATGCCTTATCTCCATTCCAATCCTTTCCTATCCATATGCCCTTATC
	AF007267	TCCTGACGAGACGCATACCTGCTCTCTACCCTGATGGTCCTGGCCTCTGATGCTGCTGGTTTTGGGGACAGAACA
	AF019661	GCAAGTCATGGAGGAGAAGCTGAATGCAACCAACATCGAGCTAGCCACGGTGCAGCCTGGTCAGAATTTCCACAT
	AF021031	GTGTGGGGGCTACTGTCCAGCTGCCAACAGGTCTGATCTCTCTGAGGGGCTGATTAGATCCTGGCTATGCCTGCT
	AJ000059	GGAGACCTGCCAATACCTCAAGAATTACCTAACTCAGCTGCTGGTTCCCTACATAGTCAACGTGTCCTGGGCCAC
	D00472	ACCGACACCCCTACTCCGTATCCCTCCCCATCCCATGCTGCCAACTTCTAACCACAATAGTGACTCTGTGCTTGT
	D38117	GATCACTCTGACCAACTGAGCCAGGTGTGCCGTCTGCAAGAAGATCGTGATAAACCCCAGGGACTGTGCCTACAG
	KO2060	TGCCTGATGAACTTCTTGGTCCTCTTTTTGACGATCTTGGGCTTCACCAGAGGCCGGAGGGCAGCCATG
	L16846	GCTAGGGAGTGAAGTCGAAGGGTGGTTTCAGTTTCTCCCAGACCTTATACCCAATTTTGCACAGCAGTCCCTTTA
	M20480	AAGGAGAAAGAGGAGGGAGAGGAAGAGGAAGCGGCTGAGGAGGAAGAAGCTGCCAAGGATGAGTCTGAAGACACA
	M21495	CCCTGTGCATATCTTGATTTAGTCCTTAGTTCATGTGGCTCGGTCACTTGGGGGCTGGGGAGAGCACGCTGTAGA
	M22432	AAACGACCCACCAATGGAAGCAGCTGGCTTCACTGCTCAGGTGATTATCCTGAACCATCCAGGCCAAATCAGTGC
	M24509	CACGGTGATGAGAGCTAAGCTGACGTCCCCAAGGCCATGTGACTTTACTGGTCAACTGAGGCAGTGCTGCATGTC
	M28727	AAAGCCTACCATGAGCAGCTTTCTGTAGCAGAGATCACCAATGCCTGCTTTGAGCCAGCCAACCAGATGGTGAAA
	M32599	GTCGTGGATCTGACGTGCCGCCTGGAGAAACCTGCCAAGTATGATGACATCAAGAAGGTGGTGAAGCAGGCATCT
	M60419	AATTACCGACGCAGACGCCCAGAGAACCCTAAACCACAAGATGGCAAAGAGACAAAAGCAGCCGATCCACCAGCT
	M99167	AGGTGGCTATGGCGGTTCCAGCAGCAGCAGTAGCTATGGCAGTGGCAGGAGGTTCTAATTACATACAGCCAGGAA
	NM_008084	GCCCTATCCCAACTCGGCCCCCAACACTGAGCATCTCCCTCACAATTTCCATCCCAGACCCCCATAATAACAGCA
	NM_008363	GCGTCCTTCAGAGAGGCTAGCTGTACCCAGGGAGGCACTACCAGAGAATCAAGTGTGAGGAGTAGCCCAGGCTTC
	NM_008618	CAGAGGGAGAGTTCGGTGCGATGGGTGTTATCTCTGATGGCAACTCCTATGGTGTCCCTGATGACCTGCTCTACT
	NM_009159	CTTTTTCTGTGGGGGTGGGATTTGGAGGGGGTTGGGCTGGATTTCTTTGTAAGATGTGGACCACCAAGGGGTTGT
	NM_010310	CACTACTGCTACCCTCACTTTACCTGCGCCGTGGACACTGAGAACATCCGCCGTGTCTTCAACGACTGCCGTGAC
	NM_010699	GCAGTTCTAAAGTCTTCCCCGTGTCCTAGCACTTCACTGTCCAGGCTGCAGCAGGGCTTCTAGGCAGACCACACC
	NM_010817	AAAGGACGGATCTCAGCCCTACGATACAGCTTCAGTTGCATGAGTTGGGGGTGTGGTGGCTCAGGTGTCCGACTT
	NM_011653	CCGTGAGGACATGGCTGCCCTAGAGAAGGATTATGAGGAGGTTGGTGTGGATTCTGTGGAAGGCGAGGGGGAGGA
	NM_011694	CCTGCGTGAAAGGGATGTTTTTAGACTGATCCTCACACCCTGTTCCCATCGTGCCCTGTTCCCATCCTAGCCCAT
	NM_011701	TTCTCTGCCTCTGCCAACCTTTTCTTCCCTGAACCTGAGAGAAACTAACCTGGAGTCACTTCCTCTGGTTGACAC
	NM_018858	CTCTGAAAAAGGAAGCACTCCGCCAGGATCAGGTCTAGAGGAGAGCTGGAGCTGGTGGTGTCTGTGAATGGTTGA
	NM_019827	CTAATGCTGGAGACCGTGGACAGACCAATAACGCCGCTTCTGCATCAGCTTCCAACTCCACCTGAACAGCCCCCA
	NM_019912	CCAGAAACATTCATGTGAACTAGGCTAGTTACCTCCCCTCGTCCCCTTTCCTAACCTAAATGTAAGCCAGGCCAG
	NM_021278	TCCCCTGCCTCTCCCACCCATCTGATGGTCTGGCTAGCAGAGAGGGAAAAGAACTTGCATGTTGGTGAAGGAAAA
	U13393	GGTCCTACATCGCCAATCGAGTGACTGACAAGCTGACCCCTATCCACGTACACATCTTCTGCTGCCGCTCAGGCT
	U22107	GCACCTTCCCCTTCAACTCGTTCCTCTCTGGCTTCATCTCTTGTGTGGGCAGCTTCATCCTAGCGGTTTGCCTGA
	U29402	TCATCCTGCACGACGACGAGGTGACGGTCACGGAGGATAAGATCAATGCCCTCATTAAAGCAGCTGGTGTCAGCG
	U53456	AGGAGTGACCTGCCTGCCGCCAGCCCTGCCCGACTATCATGAAGCACACTCCTTCCCACTGCACATTTAATAATG
	U68526	ATGAGCCCTGCGTGGGTGTTGGTATGGTCACACAAGCCCTCCTGTACGTCATTCTGTGCCCTGTGGCGTCCTACT
	X03672	TCCCAAGTATCCATGAAATAAGTGGTTACAGGAAGTCCCTCACCCTCCCAAAAGCCACCCCCACTCCTAAGAGGA
	X13661	GCCCATGTGTGTTGAGAGCTTCTCTGACTACCCTCCACTTGGTCGCTTTGCTGTTCGTGACATGAGGCAGACAGT
	X15848	CCCTGGAGATGGATGACACCGAGACTGGGCTACTTAGTGCTATCTGCCTCATCTGTGGAGACCGAATGGACCTGG
	X16646	AAGCCTCCCAAGAATGAATCCTTGGAGACTTACCCACTGATGATGAAGTATAATCCAAATGTCCTGCCTGTTCAG
	X52803	AGCTCTGAGCACTGGAGAGAAAGGATTTGGCTATAAGGGTTCCTCCTTTCACAGAATTATTCCAGGATTCATGTG
	X55315	CCAGTTAATGCAGATGGCACAATTCTCCAGCAAGTTACAGTCCCTGTTTCAGGCATGATCACCATCCCAGCAGCC
	X60671	TCACTGCAGGCAGGTGTCACACTTGGCTCTTTAGTTCTCTTAAGTTTAGACACCCCCTTGCTGTGTTCCAGTCCC
	X67677	GGCTCTACCCTAATTTCAGTGGCCCAGCTTTTATACAGAACCTCTTAGAACACCAAGTTTCAAATCCCTGTTGGA
	X70067	AAGGGTTCTGGCTTGTAGAGCTACTGTTGGTTCACGGCTGCTCCCATAGAGGTGCCCTGTAGTTTTCTGGCTAGA
	X72230	TTTTTCTCTCCCTTCCTTCCGTATTCCCTCCCTTCCTTGCTCCTTCGTTGCTTTCTTCCTTCCTATCGCTGTTCT
	X73331	GTGCCTCCCTCCGGAAAATGGTGAAGAAAATTGAAATCAGCCAGCACGCCAAGTACACTTGCTCCTTCTGTGGCA
	X98207	GGGTCAAAGAAGAGGTCAAGTTGGCCTGAGCAAGGTCTGCAGCCCCATCTTCTCCCCAAGTTCCTCACTCTCCCT
	Z71189	ATGGCCAGTCTGCAGTCCAGCCCTCAGCATCAGGAGCTCTTCCGGAACTTCAGAAGCATCTCCAAGGCCATGGTG
	Z85979	CCGCAAGCTCCCCTTTCAGCGTCTGGTGCGAGAAATTGCTCAGGACTTCAAAACAGATCTGCGCTTCCAGAGTGC
B	X17013	CGATCTTCATAACGGACAAACTGAACGGGCCATTGAACAAGTATTACAATCGGAACACATTCAGCTGCTGGGTGT
	K01391	AGCTACCGAAAAGTATCTCCGCAAGATGTGAAAAAGTGGCTGAGTCAAGTTTGTGTCGAAAAACAGGTTGCAGGG
	M24537	GGTCCAGCAGCTACATTTACACATCTAGCAGTCAGTTCTTGTTTTCAAAACGGCGCCGAACATGTTGCTTACGCG
	L38424	GGGGAGAAGAGCCGAATGTATAATGCTGACGTTCTTGCTTTTGGCGCCCACAGTGATGATGTCGAGATCGGAATG
C	NM_117849	AAAGATGTGTTGAGAAATTAACGGAAGAGAATCGGCGGCTCGAGAAAGAGGCAGCAGCGGAACTAAGAGCATTAA
	NM_117780	GCTGAAGCAAACGGAGGTAGACTGCGAGTTCTTACGGAGATGCTGCGAGAATCTAACGGAAGAGAACCGTCGGCT
	AF320624	CTTGCTCCTCCTCCTCCTCCTCCTTCAGCATCGCAAGAATCCGTGGAAAATGGCATTAAGATCGGGTCTTGAAAA
	BF069666	GACATAATCAAGCCAAGCTGTGTCAACACTGACCCCATCTTTCCCAACAACAAGCTCACCGGAACCGATGTGGGT
	X51944	CGAGAAGTACGGCACTACTCTTTACTGCGGTGAATACGGTGTGATCGATGTCGTGTCCGCCGAGGATTCGCTGAA

RESULTS

Design and validation of MHV-68-specific DNA microarray

In order to obtain the transcription profile of the MHV-68 genome, we designed and validated an oligonucleotide-based DNA microarray specific for this murine gammaherpesvirus. We first tested the specificity of viral probes by hybridization with MHV-68 genomic DNA. All viral probes tested positive with purified viral genomic DNA, indicating specificity of selected probes. We then tested the likelihood of false-positive signals because of cellular sequences by hybridizing the array with RNA isolated from mock-infected cells. Only probes for the M6, M13 and M14 genes reacted positively with labelled cDNA from mock-infected samples. These probes were eliminated from the probe set. M13 and M14 sequences reside within the terminal repeats and are highly G/C-rich. A number of PCR primer sets for M13 and M14 genes gave false-positive signals when tested on RNA isolated from mock-infected samples (data not shown). These ORFs were not analysed further.

Table 3. PCR primer sets for MHV-68 tRNA and M6 genes

Gene	Forward primer	Reverse primer	T_a (°C)*	[Mg^{2+}] (mM)†	Size (bp)
tRNA-1	TAGAGCAACAGGTCACCGATC	TGGACCCACTTCCTCGACCAG	56	1·5	70
tRNA-2	GGTAGAGCAGCGGTTCCT	ACTCCCCCTCTCAACCA	56	1·5	69
tRNA-3	TGGCAGGCCAACATA	CGTGCTCCTCGATGGTCA	55	1·5	63
tRNA-4	GCGGCAGGCTCATC	ATCTCAACTCTGCGTCGG	56	1·5	62
tRNA-5	CTCCACCTTTAACCAG	TAGAGCATCAGGCTAGTA	51	1·5	63
tRNA-6	GCGTAGCTCAATTGT	GGCCACTCAACAGAC	53	2·5	92
tRNA-7	GAGCGGCAGACACCA	TAGCTGGCCAGGACT	58	1·5	68
tRNA-8	CCCATCCTGTTGGTT	CGCGGGTAGCTAGTC	51	1·5	54
M6	GGACCCTCCATTCTATAAAC	GACCCAGACAAGCTCGTGAA	56	1·5	166
GAPDH‡	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	55	1·5	195

*Optimum annealing temperature.
†$MgCl_2$.
‡Glyceraldehyde-3-phosphate dehydrogenase (accession #M32599).

Immediate-early transcripts of MHV-68

Herpesviruses encode a number of IE (or α) genes transcribed immediately after infection and independently of *de novo* protein synthesis. In this study, viral transcripts whose median fluorescence signals were increased at least twofold in the presence of inhibitor (CHX) when compared with CHX-treated but uninfected cultures were grouped as IE transcripts. No viral transcripts were detected in the presence of CHX at 2 and 4 h post-infection. In contrast, six transcripts were detected 8 h post-infection in the presence of CHX. These IE transcripts were M4, ORF12 (K3), ORF38, ORF50, ORF57 and ORF73 (Fig. 1).

ORF12 (K3) shows sequence homology to KSHV and bovine herpesvirus-4 IE genes. This gene contains an Rta-responsive element, and the K3 protein interferes with MHC class I presentation of peptide antigens (Stevenson *et al.*, 2000). ORF50 encodes the main transactivator with homologies to KSHV ORF50 and EBV Rta (Liu *et al.*, 2000). ORF73 is a homologue of KSHV LANA (Virgin *et al.*, 1997). LANA, a latency-associated gene product, has also been described

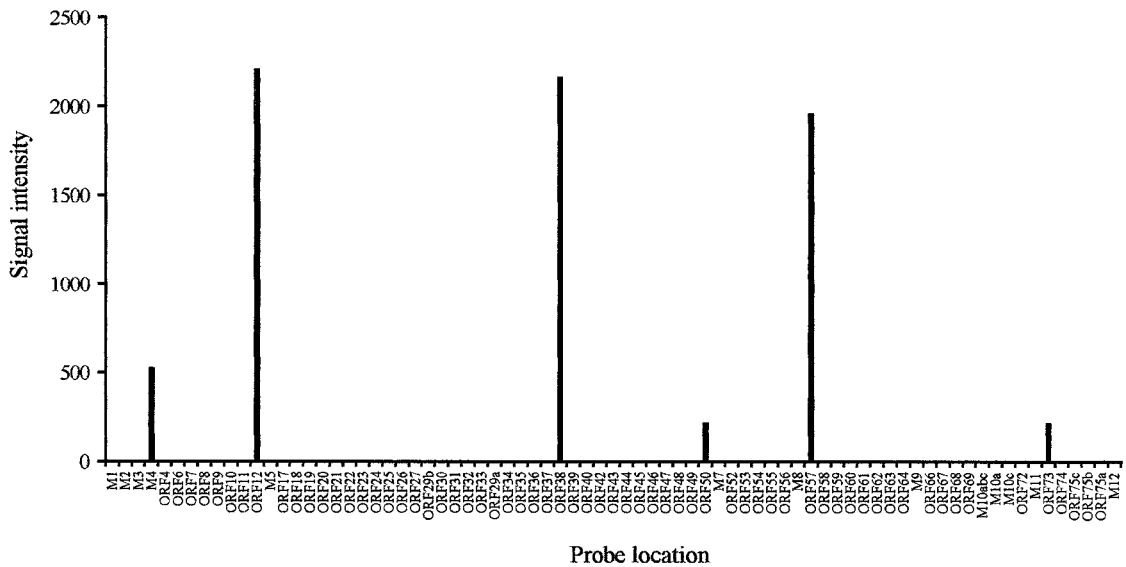

Fig. 1. MHV-68 transcripts in the presence of cycloheximide. C127 cells were pre-treated with inhibitor CHX followed by infection with MHV-68. Total RNA (25 μg) was isolated at 8 h post-infection and gene expression analysed by DNA microarrays. Note that only a small number of viral transcripts were detected in the presence of CHX. The histogram shows median signal intensity above background (mean + 2 SD of five negative control genes). Data were normalized against spiked RNA. Each data point is median signal intensity ($n = 3$).

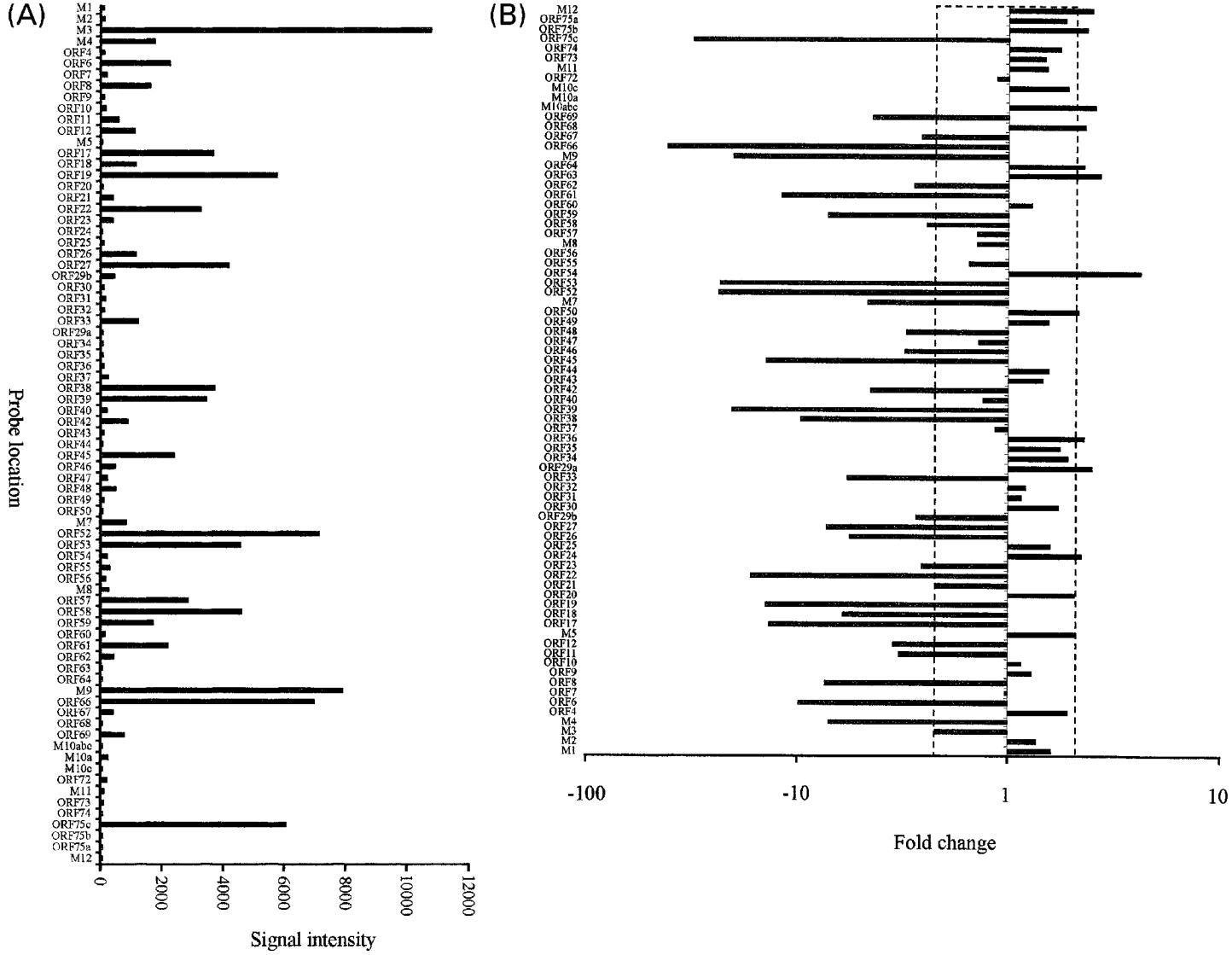

Fig. 2. Early and late transcripts of MHV-68. (A) After overnight infection with MHV-68 (5 p.f.u. per cell), total RNA was isolated and analysed by DNA microarrays. To identify E transcripts, the expression profile of the MHV-68 genome in the absence of PAA was compared with that of PAA-treated cells. PAA-pre-treated cells were infected with MHV-68 and total RNA was isolated at 8 h post-infection. Total RNA was then spiked with control RNA and analysed by DNA microarrays. Each data point is the median signal intensity ($n=3$). (B) The histogram shows -fold changes in the expression of viral transcripts between MHV-68-infected cultures without PAA and with PAA. The rectangle delineates the twofold change in gene expression because of PAA treatment.

as an IE gene detected in a KSHV-infected BC-1 line (Sun *et al.*, 1999). ORF57 shows sequence homology to the KSHV ORF57 IE gene and to EBV Mta. In KSHV-infected BC-3 cells, treatment with chemical inducers caused rapid expression of ORF57 transcript, indicative of early expression kinetics (Jenner *et al.*, 2001). The M4 ORF is one of the unique genes of MHV-68. This transcript was also detected in the presence of CHX by Northern analysis (data not shown).

Early, early-late and late transcripts of MHV-68

We used PAA to generate stage-specific RNA to identify the kinetic class of E and L genes. Most E gene products are involved in viral nucleic acid metabolism. Classically, E genes show increased expression in the presence of PAA (compared to virus-infected cells without PAA) and are sensitive to CHX treatment. These E transcripts included ORF24, ORF29a (packaging), ORF36 (kinase), ORF54

Table 4. MHV-68 transcription kinetics determined by DNA microarray and comparison with other methods

Gene	Microarray	Others†	Gene	Microarray	Others†
tRNA1–8	IE*		ORF43 (capsid)	E-L	
M1	E-L		ORF44 (helicase)	E-L	
M2	E-L	L(3)	ORF45	L	
M3	E-L	E-L (3)	ORF46 (DNA Gly)	L	L (1)
M4	IE		ORF47 (gL)	E-L	
ORF4	E-L	L (5)	ORF48	L	
ORF6	L	E (1); EL (5)	ORF49	E-L	
ORF7	E-L		ORF50 (Rta)	IE	IE (2, 3)
ORF8 (gB)	L	L (1, 3, 4)	Gp150 (M7)	L	L (9)
ORF9 (DNA Pol)	E-L	E (3)	ORF52	L	
ORF10	E-L		ORF53	L	
ORF11	L		ORF54 (dUTPase)	E	
ORF12 (K3)	IE	IE (3)	ORF55	E-L	
M5	E-L		ORF56 (DNA repair)	E-L	
M6	IE*		M8	E-L	IE (3)
ORF17 (capsid)	L		ORF57	IE	
ORF18	L		ORF58	L	
ORF19 (tegument)	L		ORF59 (DNA repair)	L	
ORF20	E-L		ORF60 (ribonucleotide reductase)	E-L	
ORF21 (TK)	E-L		ORF61 (ribonucleotide reductase)	L	
ORF22 (gH)	L		ORF62 (Assembly/maturation)	L	
ORF23	L		ORF63 (tegument)	E	
ORF24	E		ORF64 (tegument)	E	
ORF25 (major capsid)	E-L		ORF65 (M9)	L	L (3)
ORF26 (capsid)	L		ORF66 (capsid)	L	
ORF27	L		ORF67 (tegument)	L	
ORF29b (packaging)	L		ORF68	E	
ORF30	E-L		ORF69	L	
ORF31	E-L		M10abc	E	
ORF32 (tegument)	E-L		M10a	E-L	
ORF33	L		M10c	E-L	
ORF29a (packaging)	E		ORF72 (Cyclin-D)	E-L	E-L (6, 8)
ORF34	E-L		M11 (bcl-2)	E-L	E-L (3, 8)
ORF35 (tegument)	E-L		ORF73	IE	IE (3, 8)
ORF36 (kinase)	E		ORF74 (GPCR)	E-L	E-L (3, 7)
ORF37 (alkaline exonuclease)	E-L		ORF75c	L	
ORF38 (tegument)	IE		ORF75b	E	
ORF39 (gM)	L		ORF75a	E-L	
ORF40 (helicase/primase)	E-L		M12	E	
ORF42	L				

*By RT-PCR analysis.

†(1) Mackett *et al.* (1997); (2) Liu *et al.* (2000); (3) Rochford *et al.* (2001); (4) Stewart *et al.* (1994); (5) Kapadia *et al.* (1999); (6) van Dyk *et al.* (1999); (7) Wakeling *et al.* (2001); (8) Roy *et al.* (2000); (9) Stewart *et al.* (1996).

(dUTPase), ORF63 (tegument), ORF64 (tegument), ORF68, ORF75b and M12 (Fig. 2).

The median expression of a number of viral genes remained within a twofold range after PAA treatment when compared to MHV-68-infected cells without PAA (Fig. 2). These genes show E-L expression kinetics (also referred to as delayed early, βγ or leaky γ). The MHV-68 genes showing early-late (E-L) kinetics included the abundantly expressed M3 transcripts that encode the chemokine-binding protein and M9. The MHV-68 genome encodes a number of cellular homologues. These include ORF72 (vCyclin-D), M11 (vBcl-2) and ORF74 (vGPCR), clustered within the virus genome. This locus has been associated with viral latency and cellular transformation (Husain *et al.*, 1999; Virgin *et al.*, 1999; Rochford *et al.*, 2001). M11 and ORF74 showed similar E-L expression kinetics. This is in accord with the low expression levels of these genes as determined by Northern analyses (Roy *et al.*, 2000; Virgin *et al.*, 1999). The M1 (ORF1) gene of MHV-68 shows limited sequence homology to poxvirus serpins (data summarized in Table 4).

Broadly, L genes are expressed after the onset of viral DNA replication. L genes encode mainly, but not exclusively, structural proteins. The genes with L transcription kinetics included ORF8 (gB), ORF17 (capsid), ORF19 (tegument), ORF22 (gH), ORF26 (capsid), ORF29b (packaging), ORF39 (gM), ORF46 (DNA Gly) and gp150 (M7). Moreover, most L genes showed high levels of expression and included the most highly expressed genes: ORF19, ORF27, ORF52, ORF53, ORF58, ORF65 (M9), ORF66 and ORF75c (Fig. 2).

Based on sequence comparisons with other herpesviruses, ORF75a, ORF75b and ORF75c may encode tegument proteins (Virgin *et al.*, 1997). Interestingly, the array analysis shows a hierarchy of expression for these genes, with abundant expression of ORF75c transcript compared to ORF75a and ORF75b (Fig. 2).

Development of PCR primers and kinetics of viral tRNA genes

The MHV-68 genome encodes eight tRNA-like sequences which lack poly(A) tracts and are therefore unsuitable for analysis by our microarray approach. Therefore, we designed and validated primers specific for these viral sequences and identified the expression kinetics of these unique MHV-68 genes in C127 cells in the presence and absence of inhibitors. All eight tRNA-like transcripts were detected in RNA representing the three kinetic stages of MHV-68 transcription (Fig. 3).

Expression of cellular genes during MHV-68 infection

We included 48 probes for cellular transcripts in our microarray to analyse the impact of MHV-68 lytic infection on cellular RNA expression. It is known that infection with herpesviruses, for example herpes simplex virus (HSV-1),

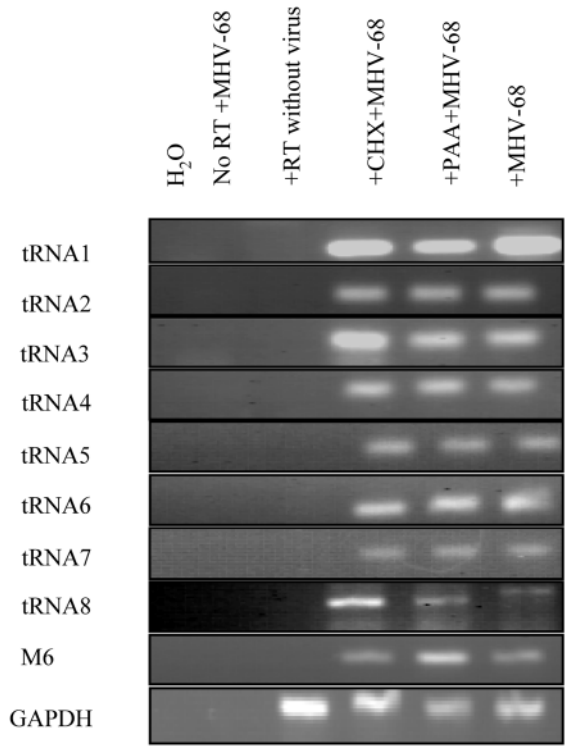

Fig. 3. RT-PCR analysis of viral tRNA-like genes and M6. Total RNA isolated from infected cultures with drug treatment (CHX and PAA) or without drug treatment was reverse-transcribed into cDNA for PCR analysis. Negative controls were: water; RNA from infected cells without reverse transcriptase; and RNA from mock-infected cells. After amplification (35 cycles), 10 % (v/v) aliquots from PCR reactions were resolved by gel electrophoresis. Note that all tRNA-like transcripts and the transcript for M6 could be detected in the presence of drug inhibitors.

results in accelerated degradation of cellular transcripts (Stingley *et al.*, 2000). Similarly, MHV-68 lytic infection significantly reduced the expression of cellular transcripts (Fig. 4). This observation suggests that interference with cellular transcription machinery may be a feature during the lytic phase of herpesvirus infections.

DISCUSSION

We have designed and validated a microarray for MHV-68 using ORF- and strand-specific 75-base-long oligonucleotides. This is the first oligonucleotide-based microarray for this gammaherpesvirus. The use of longer probes presents useful advantages including ease of design, consistency of probe synthesis and polarity. The latter is of particular importance because bi-directional transcription is a feature of herpesvirus replication. By combining the DNA microarray with RT-PCR assays, we have mapped the transcription profile of the MHV-68 genome in C127 cells infected at a high m.o.i. and in the presence of inhibitors of protein synthesis and DNA polymerase (Table 4).

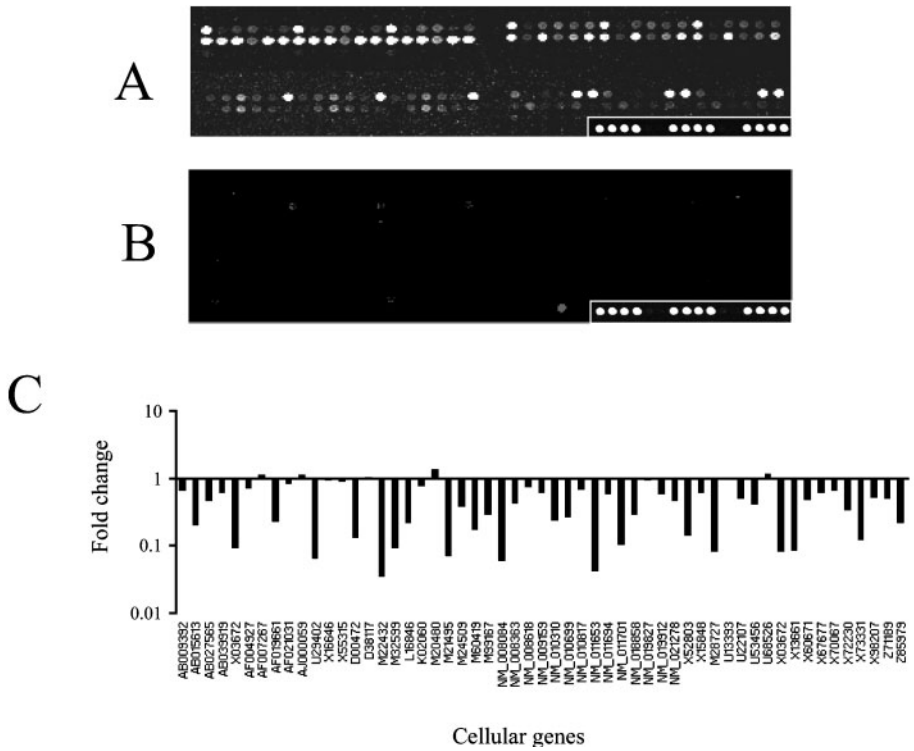

Fig. 4. Effect of MHV-68 lytic infection on cellular transcripts. MHV-68 infection reduced overall levels of cellular transcripts. The scanned array images show the expression of cellular transcripts in (A) mock-infected and (B) MHV-68 infected cells. The insets show relative stability of spiked RNA in both control and infected cultures. (C) This panel shows -fold changes in cellular mRNA levels between uninfected and MHV-68-infected cultures.

Drug inhibitors were used to map the expression kinetics of MHV-68 genes during the lytic cycle of infection *in vitro*. Viral transcripts were not detected at 2 and 4 h post-infection with MHV-68 in the presence of CHX by DNA microarrays. However, six transcripts were detected in the presence of CHX at 8 h post-infection. These included previously identified IE genes K3, ORF50 and ORF73 (Liu *et al.*, 2000; Rochford *et al.*, 2001; Virgin *et al.*, 1999), as well as M4, ORF38 and ORF 57. ORF50 is homologous to Rta genes of other gammaherpesviruses and encodes the major transactivator of MHV-68 lytic cycle antigens as well as playing a role in reactivation from latency (Wu *et al.*, 2000, 2001). The M4 ORF of MHV-68 contains a heparan sulphate-binding domain, which may have a role in initial virion interaction with the cell membrane via proteoglycan heparan sulphate. In BC-3 cells, the KSHV K8.1A gene, which also contains a heparan sulphate-binding domain, showed an early expression profile after induction (Jenner *et al.*, 2001). However, expression of this gene in the presence of CHX may indicate other roles for this protein during the infection cycle.

A subset of MHV-68 genes showed L expression kinetics and includes previously identified L genes ORF8 (gB), gp150, ORF65 (M9) and ORF46 (DNA Gly). In total, 28 transcripts showed L kinetics. These data are summarized in Table 4.

A recent sequence re-evaluation of the MHV-68 genome suggested a number of viral genes unlikely to encode proteins (Nash *et al.*, 2001). These include M5, M6, M8, M10a–c, M12, M13 and M14. We were able to detect transcripts for M5, M6, M8, M10a–c and M12 by a combination of microarray and PCR assays. However, it is not clear whether these transcripts encode proteins.

Our analysis by an oligonucleotide-based microarray compares favourably with a recently published PCR-based microarray (Ahn *et al.*, 2002). For example, 22 transcripts which showed L expression kinetics were also grouped as γ transcripts by the PCR microarray. Similarly, 15 transcripts with β expression kinetics as determined by the PCR microarray also showed E or E-L expression kinetics by the oligonucleotide microarray. Moreover, the simultaneous analysis of the MHV-68 transcriptome by both microarray studies supports previous observations of highly expressed genes such as M3 and M9 and genes expressed at a low level, for example M11 and ORF74 (Virgin *et al.*, 1999; Parry *et al.*, 2000; Rochford *et al.*, 2001; Bridgeman *et al.*, 2001; Roy *et al.*, 2000; Ahn *et al.*, 2002).

Overall, the array data parallel previously published data obtained by other methodologies in terms of specificity and sensitivity of the assay (Table 4). There are, however, some discrepancies. These are very likely a consequence of

different experimental parameters – for example, use of double-stranded PCR probes, as well as the cell types and the criteria used in the classification of virus transcripts, i.e. whether based on temporal expression rather than sensitivity to drug inhibitors only or a combination of both.

MHV-68 encodes eight tRNA-like sequences for which we developed gene-specific PCR primers. Using these assays, we were able to detect all eight tRNA-like transcripts during the three stages (IE, E, L) of MHV-68 infection in C127 cells.

Small RNA sequences that have been identified in HSV-1, EBV and adenoviruses may have a role in virus evasion of host responses and/or pathogenesis (Albrecht & Fleckenstein, 1992; Howe & Shu, 1989; Mathews, 1995). In EBV, two Epstein–Barr early RNAs (EBERs), which reside adjacent to the *OriP* origin of replication, are expressed during the latent but not lytic phase of infection (Albrecht & Fleckenstein, 1992). This expression pattern is in contrast to tRNA1–4 transcripts, detected during lytic as well as latent phases of MHV-68 infection (Simas *et al.*, 1998).

Alphaherpesviruses encode homologues of host shut-off proteins associated with degradation of cellular RNA. In HSV-1, U_L41, which encodes the host shut-off protein, was implicated in the decline of cellular transcripts (Schek & Bachenheimer, 1985). More recently, DNA microarray analysis showed a global reduction in cellular transcripts following HSV-1 infection (Stingley *et al.*, 2000). U_L41 is an endonuclease and interacts with translation initiation factor eIF4H. No homologue of U_L41 exists in the MHV-68 genome. This would suggest that either MHV-68 encodes a cryptic host shut-off gene product or that the loss of cellular transcripts is independent of viral gene products.

The present study is a global analysis of the MHV-68 genome using DNA microarrays and adds to the growing kinetic analysis of herpesvirus transcriptomes (Chambers *et al.*, 1999; Stingley *et al.*, 2000; Jenner *et al.*, 2001; Paulose-Murphy *et al.*, 2001, Sarid *et al.*, 1998; Ahn *et al.*, 2002). Although not a substitute for other established methodologies, DNA microarrays provide a useful first-step platform for simultaneous and parallel analysis of large numbers of genes. This approach will help identify global transcription patterns (viral and cellular) associated with virus latency, reactivation and disease pathogenesis.

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