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Murine gammaherpesvirus 68 encodes tRNA-like sequences which are expressed during latency

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Murine gammaherpesvirus 68 (MHV-68) is a virus of wild rodents and is a convenient small animal model for studies of gammaherpesvirus pathogenesis. We have sequenced 6162 bp at the left end of the MHV-68 genome and identified two unique open reading frames (ORFs) (ORF2 and ORF3) and an ORF (ORF1) which displays similarity to poxvirus members of the serpin family. Interspersed with the ORFs is a family of eight novel tRNA-like sequences sharing tRNA-like predicted secondary structures and RNA polymerase III promoter elements. These sequences are expressed to high levels during lytic infection and are processed into mature tRNAs with post-transcriptionally added 3′ CCA termini, indicating their recognition as tRNAs by cellular machinery. Acidic Northern analysis of four tRNAs tested has demonstrated that they are not aminoacylated by aminoacyl-tRNA synthetases present in the infected cell. Thus, it is currently unclear what biological function these uncharged viral tRNA-like sequences may fulfil. *In situ* hybridization analysis has shown that in addition to being expressed within productively infected tissues during acute stages of infection, the tRNA-like sequences are abundantly expressed within splenic germinal centres of latently infected mice. Therefore, the MHV-68 viral tRNAs represent a marker for latent infection and constitute the first report of tRNA-like sequences encoded by a virus of eukaryotes.

Introduction

Murine gammaherpesvirus (MHV-68) is a natural pathogen of wild murid rodents (Blaskovic *et al.*, 1980) and is a convenient model with which to study gammaherpesvirus pathogenesis (Sunil-Chandra *et al.*, 1992*a*, *b*). In young laboratory mice intranasal inoculation of MHV-68 results primarily in an acute productive infection of the lungs and associated splenic lymphoproliferation (Sunil-Chandra *et al.*, 1992*a*; Usherwood *et al.*, 1996*a*). Splenic B cells are the major site of virus latency (Sunil-Chandra *et al.*, 1992*a*, *b*; Usherwood *et al.*, 1996*b*; Weck *et al.*, 1996), though virus has also been reported to persist in an as yet unidentified cell type of the lungs of latently infected animals (Rajcani *et al.*, 1985; Sunil-Chandra *et al.*, 1992*a*; Usherwood *et al.*, 1996*b*).

On the basis of both its genomic structure and limited sequence analyses, MHV-68 is a gammaherpesvirus more closely related to members of the γ_2 herpesviruses, typified by herpesvirus saimiri (HVS) and human herpesvirus 8 (HHV-8), than to Epstein–Barr virus (EBV), the γ_1 prototype (Efstathiou *et al.*, 1990*a*, *b*; McGeoch *et al.*, 1995; Pepper *et al.*, 1996; Mackett *et al.*, 1997). Thus, on the basis of its genetic relatedness to primate herpesviruses and characteristic biological properties, MHV-68 is a useful model with which to investigate the molecular basis of gammaherpesvirus pathogenesis.

In sequenced gammaherpesviruses the genes employed in lytic replication are collinearly arranged in five conserved blocks, allowing the alignment and comparison of different virus genomes (Bublot *et al.*, 1992). In general, the major differences observed between viruses are the presence, in closely related genomes, of unique genes encoding special functions, captured from host DNA relatively recently in evolutionary time. Examples with recognized cellular homologues are the various viral immunomodulatory proteins which are the result of separate gene captures in different gammaherpesvirus lineages (Albrecht *et al.*, 1992; Russo *et al.*, 1996).

Partial sequences have been derived for the rightmost and

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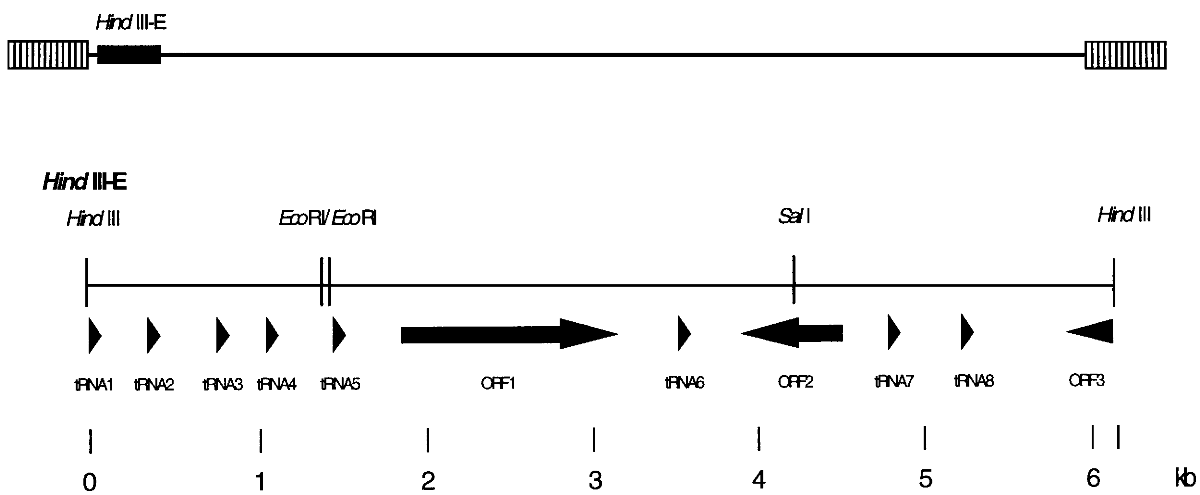
The EMBL accession number of the sequence reported in this paper is Y11705.

(*a*) **MHV68**



(*b*)

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               1          Box A              30                                            50     Box B          70
                        T G     A   GG                                                             G TC A    C
tRNA1   GCCAGAGTAG CTCAATTGGT AGAGCAACAG GTCACCG..........ATC CTGGTGGTTC TCGGTTCAAG TCCGAGCTCT GGTC   74
tRNA2   GCCAGGGTAG CTCAATCGGT AGAGCAGCGG TTCCTGG..........AGT CCGCTGGTTC TCGGTTCAAG CCCGAGCCCT GGTT   74
tRNA3   GTCGGGGTAG CTCAAATGGT AGAGTGGCAG GCCAACAT..........AG CCAGCAGATC TCGGTTCAAA CCCGAGCCCT GACC   74
tRNA4   GTCGGGGTAG CTCAATTGGT AGAGCGGCAG GCTCATC...........CC CTGCAGGTTC TCGGTTCAAT CCCGGGTCCC GACG   73
tRNA5   GCCAGGGTAG CTCAATTGGT AGAGCATCAG GCTAGTA...........TC CTGTCGGTTC .CGGTTCAAG TCCGGGCCCT GGTT   72
tRNA6   GCCAGCGTAG CTCAATTGTT AGAGCAGCGG CCACCAA...........GC CTGCAGGTTC TCGGTTCAAG TCCGGGCGCT GGCA   73
tRNA7   GCCAGGGTAG CTCAATTGGT AGAGCGGCAG ACACCACCTACGTGGTCTAG TCTGTGGATC TCGGTTCAAG TCCGAGTCCT GGCC   84
tRNA8   ACCAGAGTGG CTCACCTGGT AGAGCACCAG GCTGCCC..........ATC CTGTTGGTTC TCGGTTCAAA TCCGAGCTCT GGTG   74
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Fig. 1. For legend see facing page.

leftmost genes and other landmarks of the conserved set in MHV-68 in order to define the extent of the shared gene blocks and thus the likely positions of unique genes, an approach which was applied to the sequencing of the unconserved regions of the bovine herpesvirus 4 (BHV-4) genome (Bublot *et al.*, 1992; Lomonte *et al.*, 1996). Near the right end, a sequence from *Eco*RI F is homologous to the HVS membrane antigen orf75 (Efstathiou *et al.*, 1990*a*), which has homologues in all gammaherpesviruses thus far characterized (Efstathiou *et al.*, 1990*a*; Albrecht *et al.*, 1992; Bublot *et al.*, 1992). Approximately 11 kb from the left end, the MHV-68 *Hin*dIII G fragment contains sequences homologous to the major DNA-binding protein (MDBP), the leftmost gene common to all gammaherpesviruses (Efstathiou *et al.*, 1990*a*). Clustered at either end of other gammaherpesvirus genomes are sequences which are unique to particular viruses. For example, the left end of HVS contains the oncogene *stp* and sequences encoding the herpesvirus saimiri U-RNAs (HSURs) (Albrecht *et al.*, 1992), while the equivalent segment of EBV encodes the transforming protein LMP1 and part of the LMP2 gene (Fennewald *et al.*, 1984; Laux *et al.*, 1988). The other sequenced gammaherpesviruses alcelaphine herpesvirus 1 (AHV-1), equine herpesvirus 2 (EHV-2), BHV-4 and HHV-8 have distinct complements of left-terminal genes (Ensser & Fleckenstein, 1995; Telford *et al.*, 1995; Lomonte *et al.*, 1996; Russo *et al.*, 1996). Noting the relatively large space distal to the last universal landmark, MDBP, it seemed likely that MHV-68 might possess in an analogous position its own unique genes involved in functions other than replication.

In this communication we describe a sequence at the left end of the MHV-68 genome and report the identification and characterization of eight novel small RNAs with characteristics of tRNAs, two unique open reading frames (ORFs) and an ORF with significant homology to members of the poxvirus serpin family. Of significance is our observation that the tRNA-like sequences are abundantly expressed within B cell areas of the spleens of latently infected animals and therefore act as markers of latently infected cells.

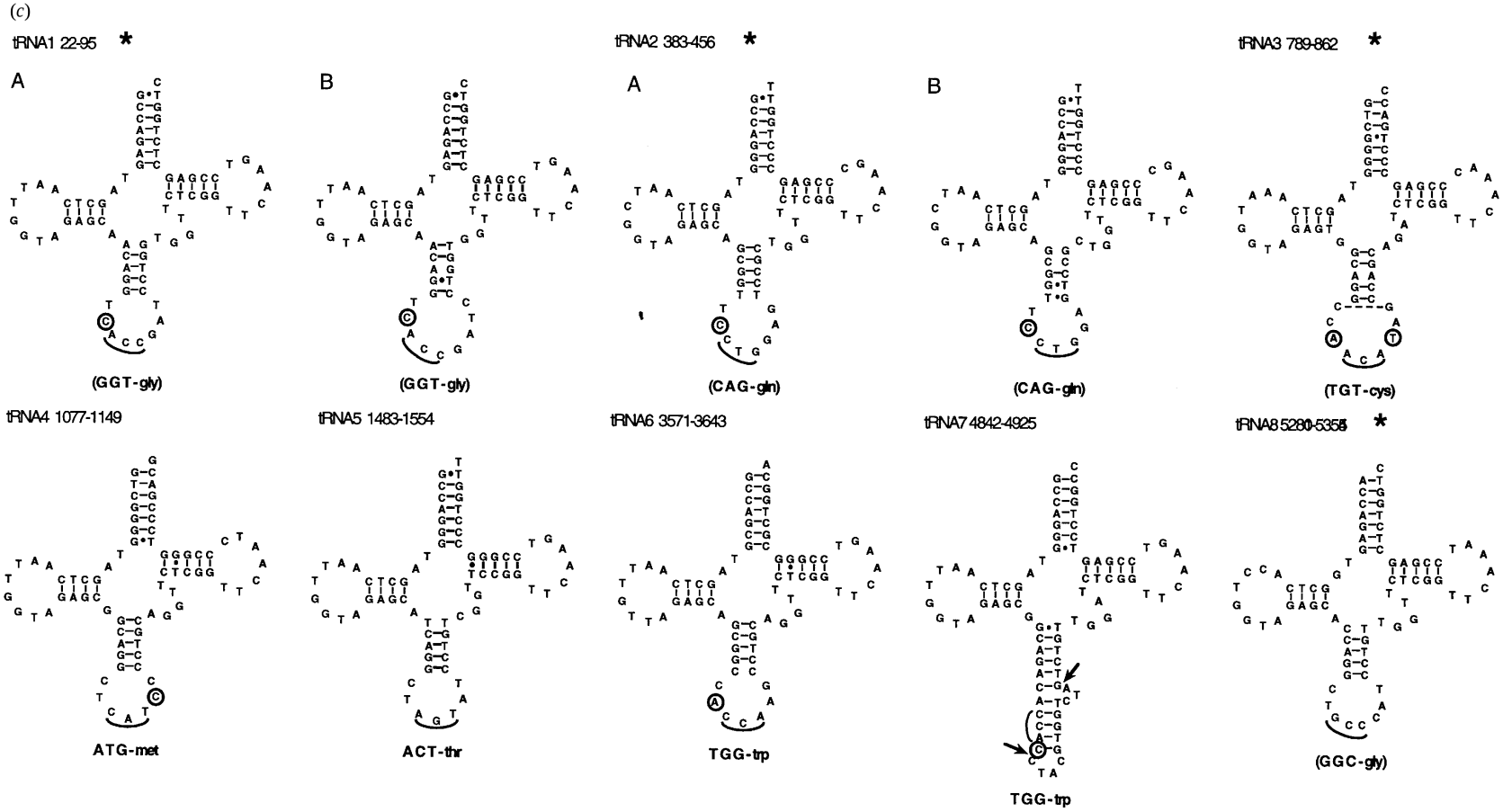


Fig. 1. *Hind*III E sequence analysis. (*a*) *Hind*III E (6162 bp), situated at the left end of the MHV-68 unique DNA, was sequenced as described in Methods. Analysis revealed eight tRNA-like sequences, two ORFs (420 aa and 199 aa) and a partial ORF (69 aa) overlapping the right end. (*b*) Alignment of MHV-68 tRNA-like sequences showing the polIII promoter elements common to tRNAs. The invariant bases of box A and box B are shown above the sequence and are conserved in all eight tRNAs. The positions of the putative anticodons are indicated. (*c*) Predicted cloverleaf structures of the eight *Hind*III E tRNA-like sequences. Putative anticodons and aminoacyl specificities are indicated, and non-conserved invariant or semi-invariant bases are circled. Asterisks indicate structures with atypical anticodon arm structure. For tRNAs 1 and 2, two possible structures (both unconventional) are shown. tRNA5 conserved all the functionally important bases of tRNAs as well as a canonical cloverleaf structure. tRNA7 was predicted to form a secondary structure similar to an unspliced intron-containing pre-tRNA, except that the position corresponding to the invariant purine 37 was filled by a cytosine, suggesting an inability to splice. Arrows indicate the putative splice sites.

Methods

■ **Cell lines, virus stocks and virus infections.** BHK-21 (baby hamster kidney) cells were grown in Glasgow's modified Eagle's medium with 10% newborn calf serum supplemented with tryptose phosphate broth. Virus stocks were prepared by low multiplicity infection of BHK cell monolayers. MHV-68 virions were purified on Ficoll 400 (Sigma) gradients. Stocks were assayed by plaquing on BHK cells. Three- to four-week-old female BALB/c mice (Harlan) under light metophane anaesthesia were inoculated intranasally with 4×10^5 p.f.u. of MHV-68 in a volume of 20 µl PBS. At various times after infection, mice were sacrificed by intraperitoneal injection of sodium pentobarbitone, and lungs and spleens were dissected, fixed in 10% buffered formalin solution and paraffin-embedded.

■ **RNA extraction.** Cytoplasmic RNA was isolated essentially as described by Ausubel *et al*. (1997). BHK cells were infected at 2 p.f.u./cell and harvested after 24 h. Infected or uninfected BHK cells were resuspended by scraping, collected by centrifugation at 4 °C in pre-chilled tubes and washed three times with cold PBS. Cytoplasmic membranes were lysed using NTE + NP40 (0·1 M NaCl, 0·01 M Tris pH 7·5, 1 mM EDTA pH 8·0 and 0·5% NP40) for 1 min on ice, and intact nuclei were removed by centrifugation. SDS was added to 1% and RNA was purified by two phenol and two chloroform extractions, concentrated by ethanol precipitation, resuspended in water and quantified spectrophotometrically. Cytoplasmic RNA was extracted for acidic Northern analysis using the same method but with the following modifications to maintain acidic conditions: cells were washed in 20 mM MES pH 6·0, 0·9% NaCl instead of PBS and lysed with NTE + NP40 in which the Tris had been substituted with 10 mM MES pH 6·0. Tris-buffered phenol with an aqueous phase pH of ~6·0 was used and after ethanol precipitation the RNA was resuspended in 0·01 M Na acetate pH 4·5 for storage at −70 °C.

■ **Plasmids, DNA sequencing and sequence analysis.** Three subclones of *Hin*dIII E (Efstathiou *et al*., 1990*b*) were constructed containing either the 1·4 kbp *Eco*RI–*Hin*dIII (pEH1.4), 2·8 kbp *Eco*RI–*Sal*I (pES2.8) or 2·0 kbp *Sal*I–*Hin*dIII (pSH2.0) fragment in pBS (Stratagene). The 1·2 kbp *Pst*I MHV-68 terminal repeat (Efstathiou *et al*., 1990*b*) was subcloned in pGEM-3Z (Promega). Plasmids were purified by the alkaline lysis technique followed by caesium chloride gradient centrifugation or Qiagen column purification (Diagen).

The three subclones of *Hin*dIII E were sequenced separately by dideoxy sequencing of shotgun cloned restriction fragments or nested deletion clones, with extra primers designed to resolve areas of ambiguity and to join the subclones. Sequencing chemistries used were ABI Prism cycle sequencing and manual sequencing using *Taq* polymerase cycle sequencing (Promega) and Sequenase T7 kits (USB). The sequence was assembled using the Staden package and was fully double-stranded with an average sixfold redundancy.

ORF and tRNA searches used the Staden package (Staden, 1980). Sequence similarity searches were done using DNAsis (Hitachi), GCG FastA (Pearson & Lipman, 1988) and on-line BLAST searching at the National Center for Biotechnology Information (Altschul *et al*., 1990).

Plasmids containing MHV-68 tRNAs 3, 4 and 6 (ptRNAs 3, 4, 6) were constructed by PCR with engineered sites for cloning into *Bam*HI-*Kpn*I-digested pUC119. *Eco*RI–*Hin*dIII inserts of ~133 bp from the appropriate ptRNA construct, containing the tRNA sequence and some flanking vector sequence, were used in random primed probes for acidic Northern analysis. tRNA5-specific probes were made from a 133 bp *Eco*RI–*Eco*RV fragment corresponding to nt 1466–1593 of *Hin*dIII E. The plasmid ptRNAlys5, containing a mammalian $tRNA^{lys}$ sequence under the control of a T7 promoter and terminated by a *Bst*NI site, was a gift from Ian Brierley (Division of Virology, University of Cambridge, UK). *Bgl*II-*Pvu*II digestion released a fragment of 130 bp, which was used to synthesize probes.

■ **Northern analysis.** Northern blot analysis was performed according to Ausubel *et al*. (1997). Hybridizations were at 42 °C (6 × SSC, 50% formamide) and washes were at 60 °C (2 × SSC, 0·1% SDS then 0·1 × SSC, 0·1% SDS).

Acidic Northern analysis using the method of Varshney *et al*. (1991) was as follows: before electrophoresis, duplicate RNAs were deacylated by incubation in 0·1 M Tris pH 9·0 for 45 min at 37 °C (deacylated fractions) or left on ice (acylated fractions). RNA (approx. 2 µg/lane) in 0·06 M Na acetate pH 5·0, 5 M urea with traces of bromophenol blue and xylene cyanol was fractionated on a 0·4 mm thick by 420 mm long 6·5% polyacrylamide gel with 8 M urea in 0·1 M Na acetate pH 5·0, run at 500 V at 4 °C until the bromophenol blue dye reached the bottom. The part of the gel between the dye fronts was transferred to a positively charged nylon membrane (Boehringer Mannheim) using a Bio-Rad semi-dry transfer cell in 40 mM Tris-acetate 2 mM EDTA pH 8·1 (10 V, 20 min). The membrane was rinsed with 2 × SSC and UV crosslinked. Membranes were hybridized with ^{32}P-labelled probes made by random priming of cloned tRNA sequences. Hybridizations were at 42 °C (6 × SSC, 50% formamide) and washes were at 60 °C (2 × SSC, 0·1% SDS then 0·1 × SSC, 0·1% SDS).

■ **S1 analysis.** Probes for S1 analysis were produced by arithmetic PCR with [α^{32}P]dATP or [α^{32}P]dCTP as label. The 5′ ends of probes were defined using specific primers within or downstream of the predicted transcription units, and the 3′ ends were defined by pre-digestion of the template DNA using restriction enzymes (see Fig. 4*a*). CCA^+ probes were synthesized using the M13 universal sequencing primer (USP) on *Hin*dIII-digested ptRNA4 or ptRNA6. Hybridization and S1 digestion were done according to Ausubel *et al*. (1997) using 5 µg lots of cytoplasmic RNA hybridized with 5×10^4 Cerenkov counts of probe. S1 digests were ethanol-precipitated, resuspended in TE (10 mM Tris, 0·1 mM EDTA, pH 8) and electrophoresed on 0·4 mm 6% polyacrylamide, 8 M urea sequencing gels alongside full-length probe controls. Dideoxy sequencing ladders generated from pES2.8 with primer 21 (see Fig. 4) or pUC119 with USP (see Fig. 5) were ethanol-precipitated and resuspended in TE before use as size markers.

■ ***In situ* hybridization.** Digoxigenin (DIG, Boehringer Mannheim)-labelled riboprobes were generated by T7 transcription of pEH1.4 or the 1·2 kbp MHV-68 terminal repeat. *In situ* hybridization was performed essentially as described by Arthur *et al*. (1993). Briefly, 5 µm sections were de-waxed in xylene, rehydrated through graded ethanol solutions, treated with 100 µg/ml proteinase K for 10 min at 37 °C and acetylated with 0·25% v/v acetic anhydride–0·1 M triethanolamine. For detection of viral DNA, sections were denatured in 50% v/v formamide in 0·1 × SSC at 80 °C for 15 min and quenched in 2 × SSC at 4 °C. Sections were hybridized with DIG-labelled riboprobes in 50% formamide, 1 × SSC overnight at 55 °C. The stringent wash (0·1 × SSC, 30% formamide, 10 mM Tris pH 7·5) was carried out at 58 °C. Hybridized probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments (Boehringer Mannheim) according to the manufacturer's instructions.

Results

Sequence analysis of MHV-68 *Hin*dIII E

The sequence of the *Hin*dIII E restriction fragment (6162 bp) was determined on both strands (Fig. 1*a*). Homology searches of this nucleotide sequence against a structural RNA

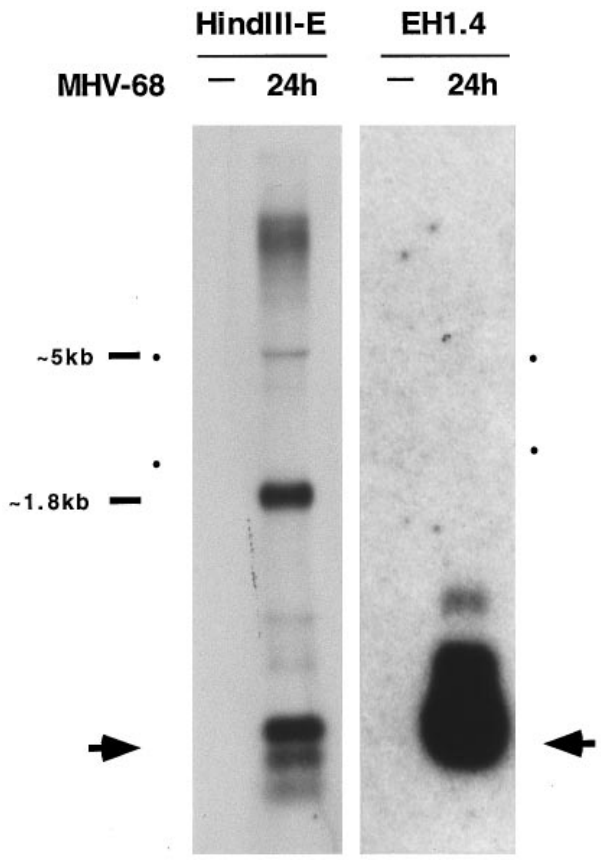


Fig. 2. MHV-68 transcription in cultured cells. BHK cells were infected with MHV-68 at 2 p.f.u./cell. Cytoplasmic RNA was extracted after 24 h, fractionated on formaldehyde agarose gels, blotted and hybridized with ^{32}P-labelled viral DNA. (*a*) A *Hin*dIII E probe hybridized to a range of RNAs from infected cells including highly abundant small RNAs (arrowed) and transcripts of ~ 5 kb and ~ 1·8 kb. (*b*) An EH1.4 probe containing tRNAs 1–4 detected only small RNAs. Dots indicate the positions of 28S (5 kb) and 18S (2 kb) ribosomal RNA.

database (DNASIS, Hitachi) revealed a family of eight novel sequences with a high level of identity ($\leqslant$ 75%) to some published tRNAs (Fig. 1*a*, *b*). Their arrangement was one of dispersed direct imperfect (75–80% identical) repeats, similar to mammalian tRNA gene clusters but distinguishable by the insertion of protein-coding sequences between the genes. In order to determine whether these sequences were transcribed during lytic infection, Northern blot analysis was performed (Fig. 2). Both *Hin*dIII E and EH1.4, which contained four of the novel sequences but no ORFs, detected small, highly abundant transcripts in MHV-68-infected BHK cell RNA. In addition, *Hin*dIII E detected transcripts of ~ 5 kb and ~ 1·8 kb. Interspersed between the tRNA-like sequences, *Hin*dIII E contains two ORFs (420 aa and 199 aa) and a partial ORF (69 aa), which we have designated ORFs 1, 2 and 3 respectively (Fig. 1*a*). Consistent with their position outside the gammaherpesvirus conserved gene blocks, none of the ORFs has identifiable homologues amongst known gammaherpesvirus genes. Further, ORF2 and ORF3 displayed no significant similarity to any available database sequences. BLAST searching revealed significant sequence homology between four segments of ORF1 and collinear segments of a number of poxvirus serpins (Fig. 3).

Expression and processing of MHV-68-encoded tRNAs

Analysis of *Hin*dIII E using the Staden tRNA search program (Staden, 1980) confirmed the identities of the eight tRNA-like sequences and demonstrated that they were capable of forming cloverleaf-like secondary structures, in which the large majority of invariant and semi-invariant bases typical of tRNAs were conserved (reviewed in Dirheimer *et al.*, 1995) (Fig. 1*c*), suggesting a tRNA-like function in the expressed sequences. Amongst the conserved bases were box A and box B motifs comprising the internal RNA polymerase III (polIII) promoters of cellular tRNAs (Sprague, 1995), implying, in the absence of upstream TATA boxes and given their interspersed arrangement in a protein-coding region, that the viral elements would be transcribed monocistronically by RNA polIII.

Assignment of the likely amino acid specificities of the tRNAs was hampered because several of the structures had atypical anticodon arms, diverging in size or sequence from recorded tRNAs. We predicted that the tRNA7 transcript, which appeared to contain a short intron, would fold into a pre-splicing intermediate whose processing would then be blocked by the absence of the biochemically essential purine residue (R37) 1 nt 3′ of the predicted anticodon (Lee & Knapp, 1985). Therefore, if the tRNAs in general act in translation, tRNA7 is likely to be an exception. We have not attempted to assay the processing or function of tRNA7. Comparison of respective determinant bases (nt 73) with those of published tRNAs sharing the same anticodon showed little conservation of this feature, known to be important in cognate recognition by many aminoacyl-tRNA synthetases (reviewed in Pallanck *et al.*, 1995). Likewise, each MHV-68 tRNA was no more similar overall to those known tRNAs sharing its anticodon than to other tRNAs (data not shown).

The transcripts produced from MHV-68 tRNA sequences 5 and 6 were mapped by S1 hybridization analysis using probes derived from cloned virus sequences (Fig. 4*a*). Probes spanning entire tRNA-like sequences were protected as 72–74 nt species (Fig. 4*b*), demonstrating the expression of viral products corresponding in size and sequence to tRNAs. There was also evidence for longer 3′-extended precursors, indicating inefficient processing or possible contamination with nuclear RNA. The *Hin*dIII E sequence revealed putative polIII termination signals (TTTT) 120–140 nt downstream of each tRNA sequence except tRNA3, which has the sequence TGTTT at a similar position. For neither tRNA5 nor tRNA6 was there evidence for extensions significantly past the 5′ terminus, consistent either with accurate promoter activity at the first base of the mature tRNA or cotranscriptional cleavage of 5′ flanking sequences.

Viral tRNAs 4 and 6 were S1-mapped using probes corresponding to their predicted mature tRNA products,

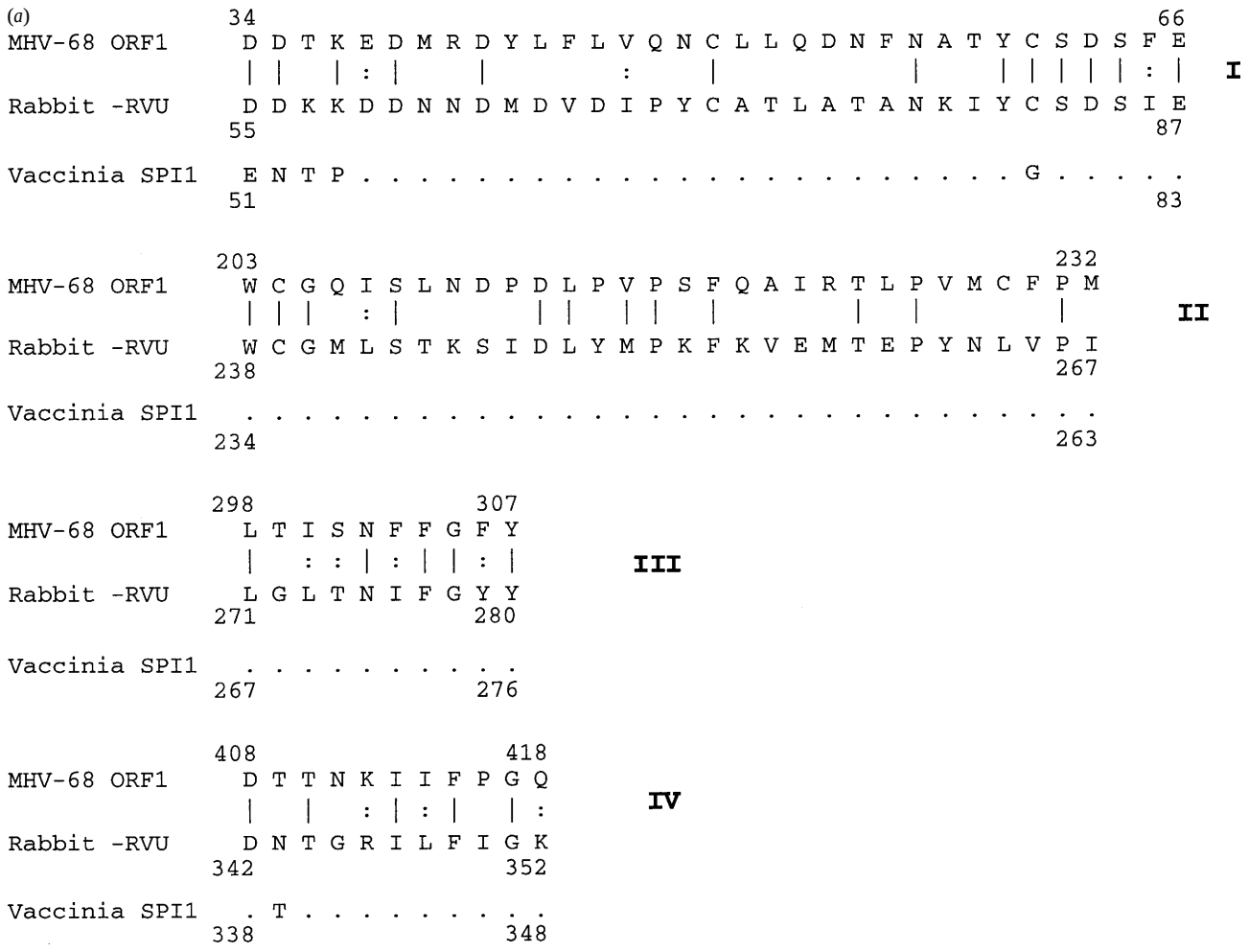


Fig. 3. ORF1–poxvirus serpin homology. BLAST searches revealed four collinear segments of similarity between ORF1 and some poxvirus serpin sequences. (*a*) Alignment of the regions of homology (I–IV) between ORF1 and rabbitpox virus serpin-1. Identical residues are indicated with vertical lines, double dots indicate similar amino acids. The equivalent segments of the vaccinia serpin-1 sequence are shown below, with only those residues which differ from the rabbitpox sequence shown. The amino acid positions within each sequence are indicated. (*b*) The four segments of homology identified by BLAST do not include the active site region of poxvirus serpins.

including the 3′ CCA sequence which is added post-transcriptionally to functional eukaryotic tRNAs (Fig. 5). Probes of this type protected correspondingly longer products than probes of virus origin, indicating that the cell is able to recognize and process at least two of the viral tRNAs into a mature form. Attempts to map tRNA3 in the same way yielded only weak hybridization signals, probably indicating low-level expression of this sequence.

The demonstration that the viral tRNAs are matured in the same way as cellular tRNAs and their higher level of sequence similarity to cellular tRNAs distinguish them from short interspersed repetitive elements (SINEs), ~ 300 bp retro-elements most examples of which comprise tRNA-derived sequences containing a polIII promoter with non-tRNA-derived downstream sequences (Daniels & Deininger, 1985). SINEs, though transcribed, are not known to be processed into tRNA-like molecules, and the *Hin*dIII E sequence does not contain the downstream vestigial polyA or A/T-rich region nor the short target site duplications characteristically flanking SINEs (reviewed by Okada, 1991).

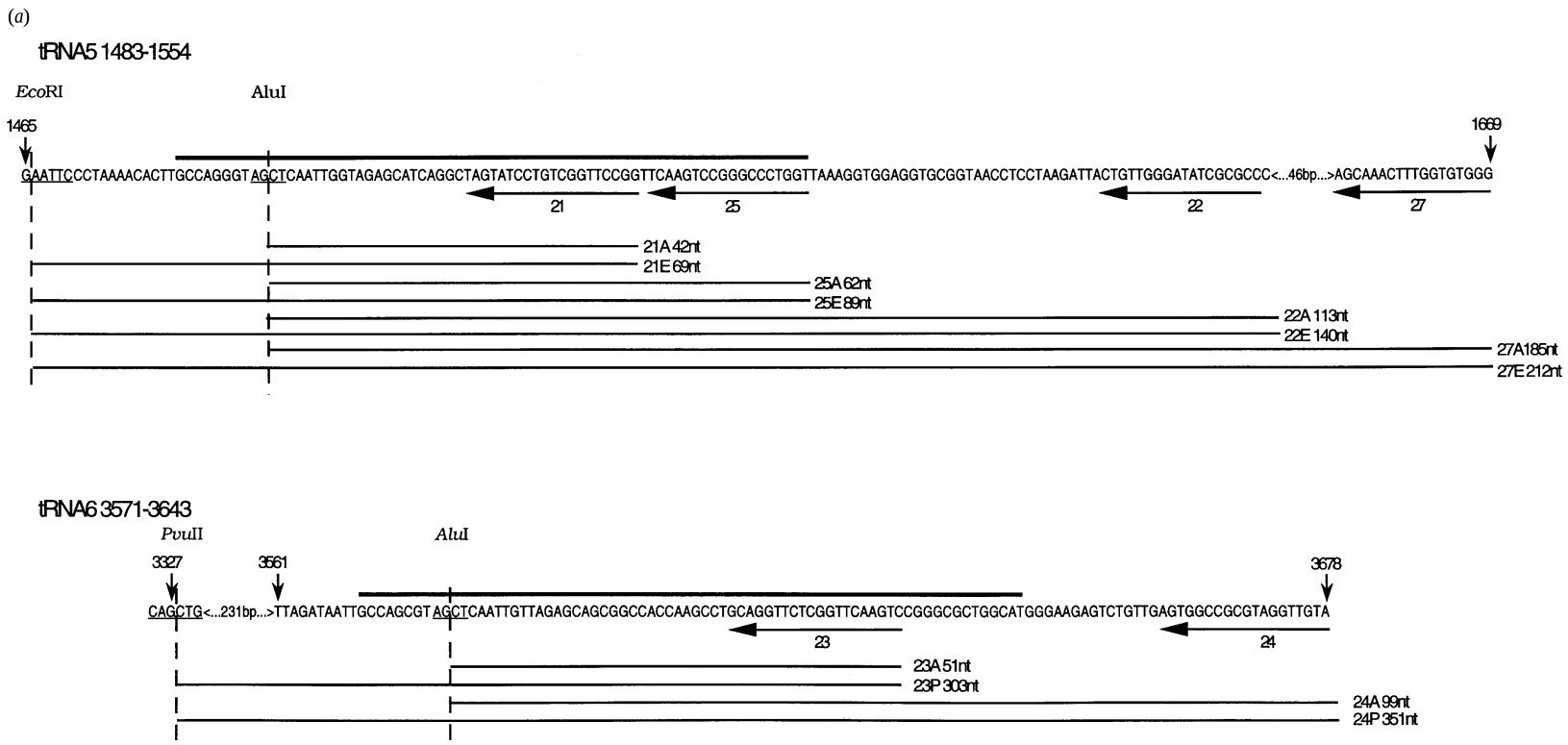


Fig. 4. S1 nuclease analysis of tRNAs 5 and 6. (*a*) Probes directed at tRNA5 or tRNA6. Predicted tRNAs are indicated by bold lines above the sequence, oligonucleotides used to prime A-PCR are indicated by arrows, and full-length probes and their lengths, by lines below the sequence. Relevant restriction sites and positions within the *Hin*dIII E sequence are shown. (*b*) Cytoplasmic RNA was hybridized with internally ^{32}P-labelled probes synthesized by arithmetic PCR. After S1 digestion the protected fragments were accurately sized using dideoxy sequencing ladders derived from sequencing the *Eco*RI–*Sal*I subfragment of *Hin*dIII E using primer 21. For each probe: P, full-length probe; U, uninfected BHK cells; M, MHV-68-infected BHK cells. Fine arrowheads indicate positions of full-length probes [sizes as in (*a*) except 27E, whose major fragment was sized at ~ 193 nt], wide arrows indicate major products with their sizes in nucleotides. Sizes of minor products are indicated by numbers alone.

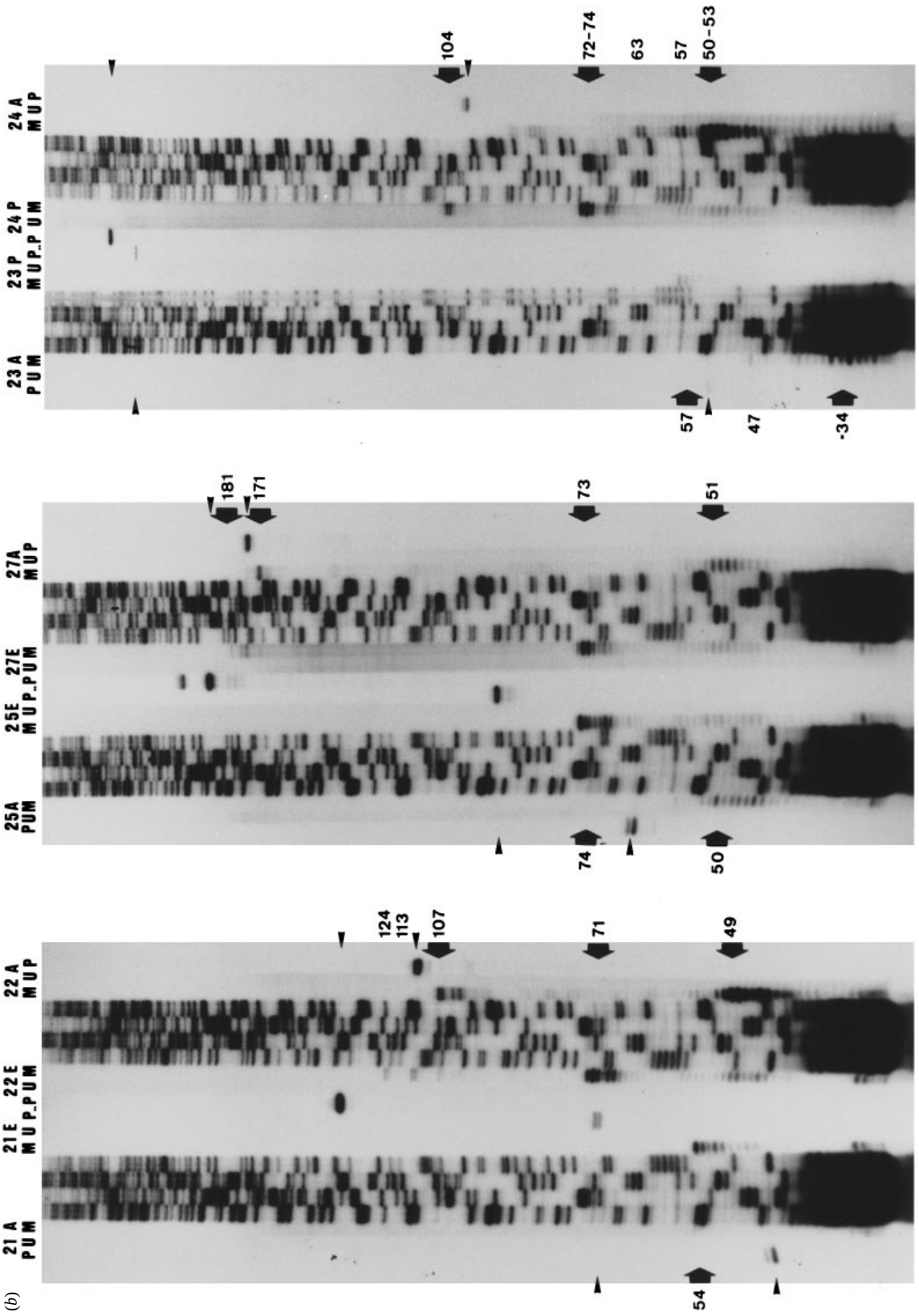
tRNA5 (nt 1483–1554) was mapped using eight probes. 21A gave only very weak bands, possibly due to its small size. 21E, designed to map the 5′ terminus, was protected as a species of ~ 54 nt, placing the 5′ end of tRNA5 at nt 1481, 2 nt upstream from that predicted by sequence analysis. 25E (74 nt product) confirmed this result.

Probes 22E and 27E were designed to span the full tRNA5 sequence. Both 22E (71–72 nt) and 27E (72–73 nt) gave products corresponding closely to the tRNA-like sequence (72 nt). In conjunction with results from 21E and 25E, which confirm the approximate predicted location of the 5′ end, they can be used to infer that the 3′ terminus lies within 2 nt of its predicted position. 22E (full-length probe 140 nt) was also protected as a fragment of ~ 124 nt, corresponding to the distance from the predicted 5′ end of the transcript to the 5′ end of the probe (122 nt), indicating that a 3′-extended form of tRNA5 exists. This result was confirmed by other probes overlapping the 3′ end of tRNA5.

Probes 22A and 27A were designed to map the 3′ end of tRNA5. Both gave fragments of ~ 49–51 nt, results which suggested that intra-molecular basepairing of the aminoacyl stem nucleotides of tRNA5 might have shortened the sequence available for hybridization with the probe.

tRNA6 (nt 3571–3643) was mapped using a similar strategy to tRNA5. Probe 24P was protected as 72–74 nt fragments, corresponding to a mature tRNA without a 3′ CCA. 23P gave 54–58 nt protected products, placing the 5′ terminus at nt 3574, three bases downstream of the predicted end. 24A gave multiple bands, suggesting heterogeneity about the 3′ terminus, including possible RNAs smaller and larger than, as well as those corresponding to, the predicted size, though 24P bands definitively suggest a 3′ terminus at nt 3643–3645, close to the predicted position. Minor bands in 24P indicated an extra species extending at least as far 3′ as primer 24 (nt 3678).

[Fig. 4. *Continued overleaf*]


24 A MUP
23 P MUP.PUM
23 A PUM
104
72-74
63
57
50-53
57
47
-34
27A MUP
25E 27E MUP.PUM
25A PUM
181
171
73
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124
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(b)

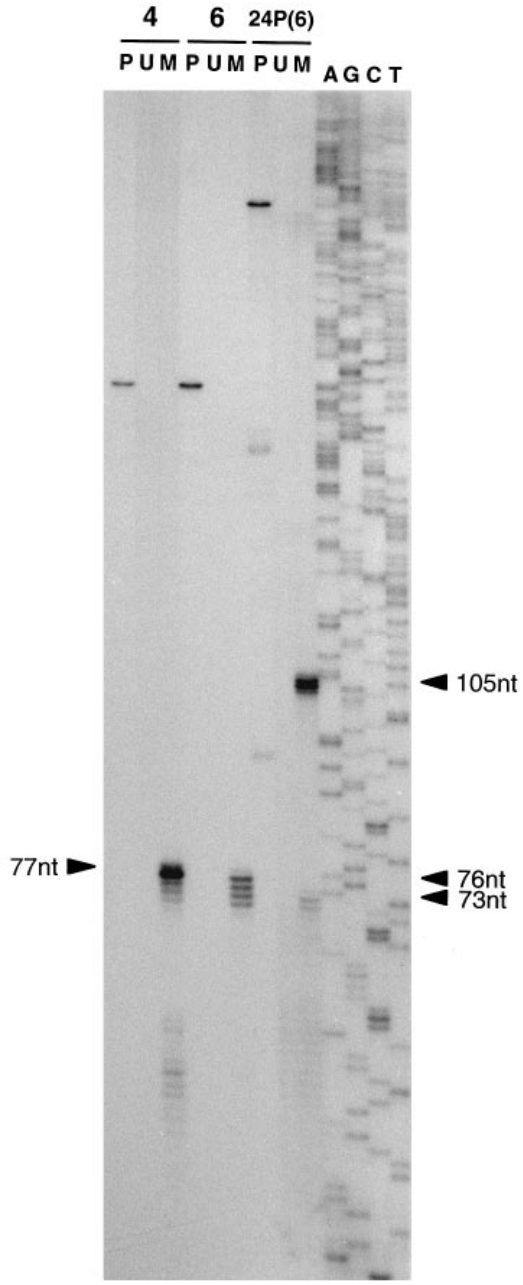

Fig. 5. S1 analysis using probes directed at mature tRNA-like sequences. Probes synthesized by arithmetic PCR from clones of tRNA4 and tRNA6 engineered to include the 3′ CCA characteristic of mature tRNAs were used in S1 hybridization analysis to determine whether viral tRNAs are post-transcriptionally CCA-modified. For each probe: P, full-length probe; U, uninfected BHK cells; M, MHV-68-infected BHK cells. 24P (see legend to Fig. 4) is a control probe directed at tRNA6 without the added CCA sequence and gives rise to a ~ 73 nt product. In contrast, tRNA6 protects a correspondingly longer species of ~ 76 nt with the CCA^+ probe. tRNA4 protects a 77 nt fragment of its CCA^+ probe, consistent with CCA modification (predicted CCA^- product, 73 nt). Neither CCA^+ probe detects a longer precursor-specific product similar to that in 24P.

Direct assay for tRNA aminoacylation

In order to function conventionally in translation, the MHV-68 tRNAs would need to be recognized by cognate aminoacyl-tRNA synthetases and charged with the appropriate amino acid. As mentioned previously, assignment of aminoacyl specificities to the viral tRNAs was difficult because of their atypical anticodon arm structures and lack of particular similarity to mammalian tRNAs sharing the same anticodon. Therefore, in order to determine whether the MHV-68 tRNAs could be charged, we attempted to assay the levels of aminoacylation of specific tRNAs during virus infection. Cytoplasmic RNA was purified from infected and uninfected cells using a set of reagents modified to maintain acidic conditions and thus prevent deacylation of the extracted tRNA. Samples of the RNAs were deacylated artificially by treatment with Tris pH 9·0, and native and deacylated fractions of RNAs from infected and uninfected cells were subjected to Northern analysis according to the method of Varshney *et al.* (1991) using denaturing acidic PAGE. Replicate filters were hybridized with probes derived from cloned tRNA sequences corresponding to MHV-68 tRNAs 3, 4, 5 and 6 and cellular $tRNA^{lys}$. Fig. 6 demonstrates that while cellular $tRNA^{lys}$ was almost completely aminoacylated in both infected and uninfected cells, and was deacylated by the pH 9 incubation, deacylation had no effect on the mobility of the viral tRNAs tested. Thus, no significant charging of the viral tRNA-like sequences could be detected using this assay. Remarkably, tRNA5 conserves all the nucleotides thought to be responsible for tRNA function in a structure highly similar to functional tRNAs (Fig. 1*c*) and yet in this definitive assay cannot be shown to be charged with any amino acid, indicating that it does not compete effectively with cellular tRNAs in the aminoacylation pathway. By Northern hybridization, as in S1 analysis, tRNA3 was only weakly detectable, confirming that it is expressed at lower levels than the other sequences examined.

Although determination of functionality would necessitate further experimentation to see whether the MHV-68 tRNAs interacted with some part of the translation machinery, it was noted that ORF1 appears to have a relative under-representation of the $tRNA5^{thr}_{ACT}$ codon and excess of the thr-ACC triplet compared with published lytic genes and with ORF2 and ORF3 (data not shown).

Expression of MHV-68 tRNAs during *in vivo* infection

In order to investigate whether MHV-68-encoded tRNA-like species were transcribed during infection *in vivo*, *in situ* hybridization experiments were performed using an antisense riboprobe derived from the 1·4 kbp *Hin*dIII–*Eco*RI subfragment of *Hin*dIII E-encoding tRNAs 1–4. In parallel, a riboprobe corresponding to the 1·2 kbp terminal repeat fragment of MHV-68, present at > 20 copies per genome (Efstathiou *et al.*, 1990*b*) was used to detect viral DNA. BALB/c mice, 3–4 weeks old, were infected intranasally with 4×10^5 p.f.u. of MHV-68. Mice were killed 5 and 21 days post-infection (p.i.) and lungs and spleens were processed for *in situ* analyses. At 5 days p.i. widespread virus genome-positive cells were detected

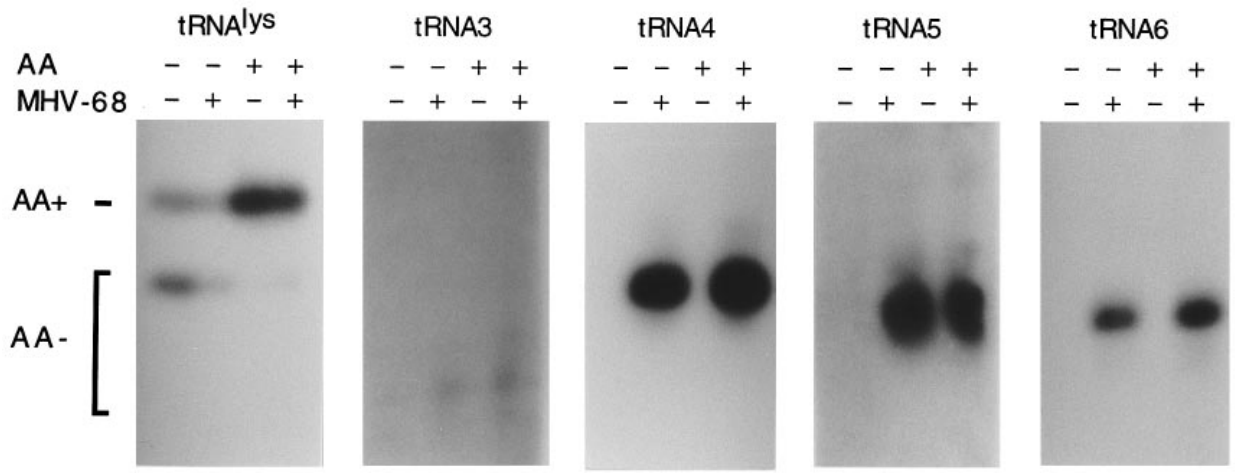

Fig. 6. Acidic Northern analysis of MHV-68 tRNAs. Cytoplasmic RNA extracted in acidic conditions from infected or uninfected BHK cells and duplicate RNAs which were isolated then deacylated were fractionated on an acidic denaturing polyacrylamide gel and electro-transferred to charged nylon membrane. Five replicate filters were hybridized with probes specific for cellular $tRNA^{lys}$ or MHV-68 tRNAs 3, 4, 5 or 6. Above each panel: AA+, acidic extraction; AA−, acidic extraction then deacylation; MHV-68−, uninfected; MHV-68+; infected. To the left of each panel: AA+, position of aminoacylated tRNAlys; AA− range of mobilities of uncharged RNAs. Judging from the control $tRNA^{lys}$, deacylation was not complete in this experiment. Each viral tRNA was detected only in infected cells as a single species whose mobility was not affected by pH 9·0 treatment.

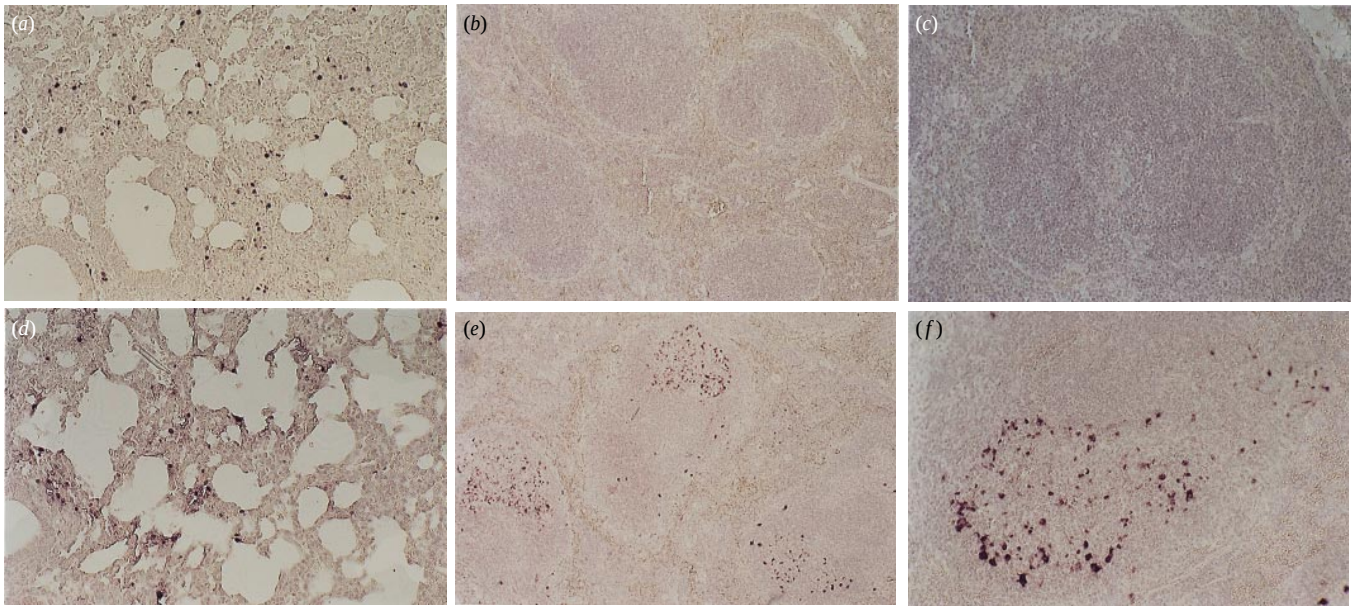

Fig. 7. Expression of MHV-68 tRNAs *in vivo*. *In situ* hybridization to detect MHV-68 DNA (*a*–*c*) and MHV-68 tRNAs 1–4 (*d*–*f*) in lung tissue (*a*, *d*) and spleen tissue (*b*, *c*, *e*, *f*) from BALB/c mice following intranasal infection. Tissues were lightly stained with haematoxylin. Widespread virus genome-positive cells (*a*) and cells expressing viral tRNAs (*d*) were readily detected in lung tissue 5 days p.i. In contrast, only viral tRNAs were detected in spleen tissues from day 21 (*e*, *f*). *In vivo* tRNA expression was widespread in splenic germinal centres.

in lung tissue, and hybridization to the MHV-68 tRNA probe was similarly widespread (Fig. 7). At 21 days p.i. hybridization to the genomic terminal repeat probe was not detected in any of the lung or spleen tissues analysed. Similarly, no MHV-68 tRNA expression could be detected in any lungs analysed after 5 days p.i. In contrast, abundant MHV-68 tRNA expression was detected in germinal centres of all spleens analysed at 21 days p.i. (six of six spleens tested) (Fig. 7). *In situ* hybridization was performed with either probe alternately on 10 serial sections from each of two spleens sampled at 21 days p.i. Whereas MHV-68 tRNAs were abundantly expressed in all the sections examined, no hybridization-positive cells were

detected in any section with the terminal repeat probe for viral DNA, confirming the expression of tRNAs 1–4 in spleens during latent infection in the absence of detectable viral DNA (data not shown).

While we have not addressed this issue experimentally, the high level of similarity to functional tRNAs and specifically the conservation of tRNA-like internal promoter elements suggests that the MHV-68 tRNAs are most likely transcribed by RNA polIII. Their expression during lytic and latent infection is consistent with constitutive transcription, a likely property of a tRNA gene in the absence of other tissue-specific *cis*-acting sequences, but contrasting with the polIII-transcribed Epstein–Barr virus-encoded RNAs (EBERs) of EBV which are not expressed during productive infection (Barletta *et al.*, 1993).

Discussion

In this manuscript we report the sequence characteristics of the 6162 bp *Hin*dIII E restriction fragment located at the left end of the MHV-68 genome. This sequenced region contains two ORFs (ORF2 and partial ORF3) which are unique to this gammaherpesvirus, and ORF1, which has amino acid similarity to members of the poxvirus serpins. The regions of ORF1 with greatest similarity to serpins correspond to four domains, designated I to IV, which are highly conserved between the serpin 1 proteins of rabbitpox, vaccinia, cowpox and variola viruses (Boursnell *et al.*, 1988; Smith *et al.*, 1989; Ali *et al.*, 1994). Thus, ORF1 constitutes the first reported serpin homologue encoded by a herpesvirus.

Interspersed with the ORFs is a family of eight sequences with characteristics of tRNAs. They contain polIII promoter elements and are abundantly expressed as small transcripts predicted to form tRNA-like cloverleaf structures. For those sequences tested, there is evidence that the infected cell machinery is able to recognize these tRNA-like structures within longer precursors, processing them into mature tRNA species, including the addition of the 3′ CCA sequence. These characteristics are all typical of the transcription and processing of functional cellular tRNAs.

On the basis of their flanking sequences, the MHV-68 tRNAs may be distinguished from SINEs. This distinction, the diversity of the sequences including the presence of a cryptic intervening sequence in tRNA7 and the similarity of their arrangement to cellular tRNA gene clusters implies that their origin may have been via capture of cellular DNA rather than a retroposition event involving an RNA intermediate.

We have shown definitively by acidic Northern analysis that at least the four tRNAs tested are not significantly aminoacylated by the aminoacyl-tRNA synthetases present in the infected cell. It is unclear what function these uncharged viral tRNAs may fulfil. Possible roles for the MHV-68 tRNAs based on previous reports of related molecules can be divided into two categories: those functions involving interactions with other cellular or viral components 'in *trans*' and those 'in *cis*' interactions related to the DNA from which they are transcribed.

Addressing firstly the interactions with other cellular components, we note that this is the first report of tRNA genes in a virus of eukaryotes, though functional tRNA genes have previously been described in bacteriophages T4 and T5 (Desai *et al.*, 1986; Calendar, 1988). T4 tRNAs are thought to act by skewing the translational capability of the infected cell towards the particular codon usage of the phage mRNAs, while T5 possesses a full range of tRNAs. While the MHV-68 tRNAs are not aminoacylated, it remains undetermined whether they might control the expression of viral or cellular genes by influencing the efficiency of decoding of particular codons, for example by interaction with the ribosome. Therefore, at this stage we cannot exclude the possibility that MHV-68 has developed a set of genes whose translation is modulated by the presence of particular atypical tRNAs.

There are other precedents for non-tRNAs maintaining a tRNA-like structure; plant mosaic viruses have pseudoknotted 3′ structures which are aminoacylated in order to associate with protein factors involved in translation and possibly transcription (Dreher *et al.*, 1996), and group I introns have recently been reported to have tRNA-like three-dimensional structures which are recognized by the tyrosyl-tRNA synthetase involved in their splicing (Caprara *et al.*, 1996). These examples are included to demonstrate that other RNAs have tRNA-like structures which allow them to fulfil their well-defined functions. A similar example is the SINE retroposons, most of which are tRNA-derived selfish DNA sequences which maintain tRNA promoters as a means of transcription essential to their propagation through the host genome (reviewed by Okada, 1991).

Secondly, to consider possible 'in *cis*' interactions, we turn to an example from the yeast genome where a number of mechanisms of interaction between neighbouring genetic elements have been described which might have relevance in large DNA viruses. It has been shown that yeast tRNA gene expression can exert an effect on the transcription of nearby polII genes (Hull *et al.*, 1994), a mode of gene control which would be plausible especially during the latent cycle in a virus which, like yeast, has constraints on intergenic distance and overall genome size. The *Hin*dIII E fragment contains three ORFs which are candidates for this type of control.

The small RNAs of gammaherpesviruses are structurally diverse and include the HSURs expressed by HVS in transformed T cells, the EBERs characteristic of EBV latency and now eight MHV-68 tRNA-like sequences. HVS encodes seven nuclear HSURs of 76–140 nt which like cellular U-RNAs are transcribed from specialized polII promoter sequences and associate with Sm snRNPs (Lee *et al.*, 1988; Wassarman *et al.*, 1989). HSURs are latency-specific and at least some may be deleted without affecting the ability of the virus to transform T cells (Murthy *et al.*, 1989). The EBERs are two $\sim$ 170 nt nuclear-localized EBV polIII transcripts which are dispensable

both for lytic replication and B cell immortalization (Swaminathan *et al.*, 1991) and share some structural similarity with adenoviral VAI RNAs, whose function in the evasion of the α/β-interferon-mediated cellular antiviral response they can partially complement (Mathews & Shenk, 1991), though EBER-deleted EBV recombinants did not differ from wild-type in their sensitivity to interferons (Swaminathan *et al.*, 1992). The EBV and HSV small RNA loci are both in close proximity to their respective origins of plasmid replication (Baer *et al.*, 1984; Kung & Medveczky, 1996). With reference to the conserved gene blocks of the gammaherpesviruses, the MHV-68 tRNA genes are positionally analogous to the HVS HSURs, and are in a similar location to the EBV EBERs, which are situated at the opposite genomic terminus.

Based on its positional and structural similarities with the small RNA loci in EBV and HVS, we believe that *Hin*dIII E may harbour an MHV-68 plasmid origin. The sequence about nt 5910 of *Hin*dIII E contains an imperfect dyad repeat analogous to other gammaherpesvirus sequences with known origin function, and our own unpublished data show that the adjacent fragment *Hin*dIII J is unstable in *E. coli*, implying the presence of nearby repetitive sequences, another hallmark of herpesvirus origins of replication. Returning to possible functional analogies in yeast, it has been proposed that polIII transcription units can modulate the movement of replication forks and hence control DNA replication (Deshpande & Newlon, 1996) and that polII transcription termination signals may coincide with origins of replication (Chen *et al.*, 1996), so the proximity of latently active small RNA genes to the plasmid origins of EBV and HVS and possibly a positionally analogous origin in MHV-68 suggests the possibility of similar interactions. In any case it is tempting to speculate that the activity of a gammaherpesvirus origin might be affected by the chromatin changes associated with constitutive transcription nearby, for example via hypomethylation, which has been reported for EBER genes but whose significance to the adjacent oriP is unknown. Experiments are underway to address the question of autonomous replication of MHV-68 plasmid clones in infected B cells.

The expression of the MHV-68 tRNA-like sequences within splenic germinal centres of latently infected animals identifies these RNAs as readily identifiable markers of latently infected cells in a manner reminiscent of the small RNAs encoded by HVS and EBV. Viral tRNA detection in a significant proportion of cells also demonstrates that *in situ* hybridization is likely to prove a more sensitive method for the enumeration of latent virus load within the spleens of latently infected animals than conventional explant co-cultivation methods.

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