Transcription mapping of human herpesvirus 8 genes encoding viral interferon regulatory factors

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The human herpesvirus 8 (HHV-8) genome contains four tandemly arranged genes encoding viral interferon regulatory factors (vIRF-1 to 4) located between genes 57 and 58. Transcript mapping techniques were employed to determine the sizes, ends and splicing patterns of mRNAs specified by these genes in HHV-8-infected cell lines untreated or chemically induced into the lytic growth cycle. Depending on the cell line used, vIRF-3 transcription was minimally or not induced (i.e. expressed with latent kinetics), whereas the other vIRFs were inducible (i.e. expressed with lytic kinetics). Each gene possessed its own promoter (or promoters) and polyadenylation sites, and all but vIRF-1 were spliced from two exons. vIRF-1 was transcribed in uninduced and induced cells from a single initiation site preceded by a TATA box, with the possible use of an additional TATA box and initiation site in uninduced cells. In induced cells, vIRF-2 was transcribed from a single major initiation site preceded by a TATA box, and vIRF-4 was expressed from two sites each preceded by a TATA box. Transcripts for these genes were insufficiently abundant in uninduced cells to map the 5’-ends. vIRF-3 lacks an obvious TATA box and exhibited heterogeneous 5’-ends in uninduced and induced cells. These data clarify and extend our understanding of the structure and transcription of the HHV-8 vIRF genes.

INTRODUCTION

Genome sequences have been published for two strains of human herpesvirus 8 [HHV-8; also known as Kaposi’s sarcoma-associated herpesvirus (KSHV)] by Russo et al. (1996) (accession no. U75698) and Neipel et al. (1997) (U93872). In addition to genes that are common to the members of the genus Rhadinovirus, several genes are unique to HHV-8 or found only in closely related species (for reviews, see Schulz, 1998; Moore & Chang, 2001). A cluster of such genes is located in a 10 kbp region between open reading frames (ORFs) 57 and 58. Russo et al. (1996) described this region as containing an ORF (K9) whose encoded protein is related to cellular interferon regulatory factors (IRFs), plus two other protein-coding regions (K10 and K11), as illustrated in the upper part of Fig. 1. Other regions bearing amino acid similarity to IRFs were also noted, two upstream from K10 and one upstream from K11. Neipel et al. (1997) identified another ORF between K10 and K11, termed K10.1 (or K10.5), which corresponds closely to one of the IRF-like regions identified by Russo et al. (1996). Data from several groups subsequently indicated that this region of the genome contains four vIRF genes, one unspliced (vIRF-1 from K9) and three spliced (vIRF-2 from K11, vIRF-3 from K10.5 and vIRF4 from K10), as illustrated in the lower part of Fig. 1. The vIRF genes presumably arose by capture of cellular sequences followed by gene duplication events. The only other herpesvirus known to possess vIRFs is rhesus rhadinovirus (RRV), a close relative of HHV-8 (Searles et al., 1999; Alexander et al., 2000). RRV possesses eight tandem vIRFs, none of which appears to be spliced.

Expression of HHV-8 genes is usually studied in cell lines derived from primary effusion lymphomas (PELs) that contain the genome of HHV-8 and often in addition that of Epstein–Barr virus (EBV; human herpesvirus 4). The great majority of PEL cells are latently infected with HHV-8, but may be induced into the lytic cycle by treatment with compounds such as sodium butyrate or phorbol 12-myristate 13-acetate (PMA) (Renne et al., 1996). Broadly, genes that are constitutively expressed are considered to be in the latent class, and inducible genes in the lytic class. vIRF-1 has been shown to be inducible in PEL cell lines by Northern blotting (Moore et al., 1996; Sarid et al., 1998) and microarray techniques (Paulose-Murphy et al., 2001; Jenner et al., 2001), and the transcript was detected in KS tissue by RT-PCR (Yen-Moore et al., 2000) but not by Northern blotting (Gao et al., 1997). The gene is assigned to the early kinetic class since its expression is resistant to inhibition of DNA replication (Wang et al., 2001). The mRNA is 1.5 kb in size (Moore et al., 1996; Sarid et al., 1998) and appears to be unspliced. The 5’-end of the predominant transcript has been mapped by RACE and primer extension, and the
core promoter and regulatory regions have been dissected functionally (Inagi et al., 1999; Chen et al., 2000; Wang et al., 2001, 2002; Ueda et al., 2002). The 50 kDa vIRF-1 protein is predominantly nuclear (Inagi et al., 1999). The gene is not required for the generation of stable HHV-8-infected cell lines by transfection of a bacterial artificial chromosome containing the HHV-8 genome (Zhou et al., 2002). No mutagenesis data are available for the other vIRFs.

Cellular IRFs operate in interferon signal transduction, acting as transcriptional activators or repressors that are regulated by class I interferon receptor signalling. They consist of a conserved N-terminal DNA-binding domain and a C-terminal regulatory domain. The DNA-binding domain is also partially conserved in vIRF-1, although the protein appears to inhibit interferon-induced transcription in reporter transfection studies by mechanisms other than binding directly to DNA (Gao et al., 1997; Li et al., 1998; Zimring et al., 1998; Flowers et al., 1998; Burysek et al., 1999a). These mechanisms involve binding to some of the cellular IRFs and to coadaptors that link transcription factors to the RNA polymerase holoenzyme (Jayachandra et al., 1999; Burysek et al., 1999a; Seo et al., 2000; Li et al., 2000; Lin et al., 2001). The multifunctional properties of vIRF-1 are illustrated further by the observation that, although the protein is a transcription inhibitor, in some settings it can act as an activator (Roan et al., 1999). In addition, vIRF-1 is able to transform cells in culture which are then able to form tumours in nude mice (Gao et al., 1997). It exerts anti-apoptotic properties through transcriptional repression (Kirchhoff et al., 2002) and by binding to the p53 tumour suppressor (Nakamura et al., 2001; Seo et al., 2001) and a cell death regulator, GRIM19 (Seo et al., 2002).

Burysek et al. (1999b) carried out initial studies on an ORF of 163 codons that was subsequently identified as the first exon of vIRF-2. In order to distinguish this ORF from the spliced vIRF-2 gene, we term it vIRF-2x1. The vIRF-2x1 protein is related to the DNA-binding domain of cellular IRFs. Burysek et al. (1999b) showed from Northern blotting that even the smallest transcript detected (2-2 kb) is considerably larger than the vIRF-2x1 ORF, and from RT-PCR that transcription was only marginally inducible. A recombinant version of the vIRF-2x1 protein could form homodimers, and bound the consensus NF-kB-binding site. In reporter transfection assays, the vIRF-2x1 protein bound to several cellular IRFs and a transcriptional coadaptor, and inhibited interferon-induced transcription. Burysek & Pitha (2001) concluded that vIRF-2x1 is transcribed constitutively and characterized the protein as a 20 kDa species that is localized to the nucleus. The vIRF-2x1 protein was shown to inhibit the antiviral effect of interferon by binding to and inhibiting double-stranded RNA-activated protein kinase. Kirchhoff et al. (2002) showed that vIRF-2x1 is able to inhibit apoptosis by specific transcriptional repression.

Despite these studies, the expression pattern of vIRF-2 has remained controversial. Jenner et al. (2001) detected the spliced transcript by RT-PCR, whereas Burysek & Pitha (2001) excluded splicing by similar experiments. Moreover, in contrast to Burysek et al. (1999b) and Burysek & Pitha (2001), Jenner et al. (2001) and Fakhari & Dittmer (2002) catalogued vIRF-2 as an inducible gene from microarray and quantitative PCR studies, respectively. In another microarray study, Paulose-Murphy et al. (2001) noted that K11, which forms the second exon of vIRF-2, is inducible. Similarly, Sarid et al. (1998) listed K11 as inducible from Northern blot experiments. In addition to these conflicting conclusions regarding splicing and inducibility of vIRF-2, the locations of the mRNA ends were not determined.

vIRF-3 specifies a spliced mRNA of 2.2 or 1.8 kb (Lubyova & Pitha, 2000; Rivas et al., 2001; Jenner et al., 2001). Lubyova & Pitha (2000) and Jenner et al. (2001) characterized vIRF-3 as inducible. In contrast, Rivas et al. (2001) and Fakhari & Dittmer (2002) showed that the gene is expressed constitutively. Lubyova & Pitha (2000) noted a potential TATA box and polyadenylation signal in the DNA sequence, but the 5'-end of a single cDNA clone analysed by Rivas et al. (2001) does not correspond with use of this TATA box. The vIRF-3 protein (LANA-2) is expressed in the nuclei of PEL cell lines but not in KS tissue, and appears to be involved in inhibiting p53-mediated apoptosis (Rivas et al., 2001).

The splicing pattern of vIRF-4 has been demonstrated (Jenner et al., 2001), and the transcript characterized as inducible (Sarid et al., 1998; Paulose-Murphy et al., 2001; Jenner et al., 2001; Fakhari & Dittmer, 2002). The size of the mRNA and the locations of its ends have not been determined.

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**Fig. 1.** Gene arrangement in the vIRF region. The region between genes 57 and 58 (83–95 kbp) is shown in inverse orientation with respect to the HHV-8 DNA sequence (Russo et al., 1996). The upper part shows the arrangement of ORFs (shaded arrows) and two of the IRF-like regions (shaded boxes) proposed by Russo et al. (1996) and Neipel et al. (1997). The third IRF-like region corresponds closely to K10.5 and is not shown. The lower portion shows the expression pattern deduced from subsequent publications (discussed in the text) and the present work, with protein coding exons shown as shaded rectangles and arrows, and RNAs and introns as thick black lines. Polyadenylation signals are shown as vertical lines near the 3'-ends of mRNAs. Transcription data for gene 57 are from Bello et al. (1999).
In summary, the deduced transcriptional patterns of the vIRFs are incompletely understood and in some respects contentious. In this paper, we have evaluated transcription of all four genes in PEL cell lines by Northern blot hybridization to determine the sizes and inducibility of mRNAs, RT-PCR to examine splicing patterns, and RACE to detect mRNA ends. We have also confirmed the splicing pattern of vIRF-2 in HHV-8-infected endothelial cells.

METHODS

Growth and chemical induction of cell lines. HHV-8-containing PEL cell lines included HBL-6 (Gaidano et al., 1996), JSC-1 (Cannon et al., 2000), BCP-1 (Gao et al., 1996) and BCBL-1 (Renne et al., 1996). HBL-6 and JSC-1 cells also contain EBV. The Raji lymphoblastoid cell line, which contains EBV but not HHV-8, was used as a control. The cell lines were cultured in RPMI 1640 growth medium (BioWhittaker) containing 10% (v/v) heat-inactivated (56˚C, 30 min) foetal bovine serum (20% for BCP-1), antibiotics (penicillin, 100 µg ml⁻¹; streptomycin, 100 µg ml⁻¹) and 2 mM glutamine. Cell viability was monitored routinely by trypan blue exclusion. BCP-1 and HBL-6 cells at 4 × 10⁵ viable cells ml⁻¹ were induced into lytic replication by treatment for 48 h with 20 ng PMA (Sigma) ml⁻¹ or 3 mM sodium butyrate (Sigma), respectively. JSC-1 cells at 4 × 10⁵ viable cells ml⁻¹ were induced with 1 mM sodium butyrate for 24 h.

Transformed primary human dermal microvascular endothelial cells (tDMVECs) were cultured as described by Moses et al. (1999) in EGM-2 MV medium (BioWhittaker) and infected with HHV-8 prepared from JSC-1 cells treated with 1 mM sodium butyrate, essentially as described by Cannon et al. (2000). Briefly, JSC-1 cells were diluted to 2 × 10⁵ viable cells ml⁻¹ and cultured for 3 days, resuspended at 8 × 10⁵ viable cells ml⁻¹ and treated with sodium butyrate for 18 h. The cells were then resuspended in the original volume of culture medium for 4 days in the absence of sodium butyrate. The medium was filtered (0-45 µm), and virus was harvested from the filtrate by centrifugation at 16,900 g for 2 h at 4 ºC and resuspended in EGM-2 MV medium. The virus suspension was used to infect tDMVECs that had been cultured for 48 h in the absence of heparin and G418 and pre-treated with 100 µg polybrene (hexadimethrine bromide; Sigma) ml⁻¹ for 1 h. The infected cells were subcultured after incubation for 48 h, and induced with 20 ng PMA ml⁻¹.

Purification of polyadenylated RNA. Total cellular RNA was extracted with Trizol (Invitrogen) at 10⁵ cells ml⁻¹ according to the manufacturer’s instructions. Except for experiments involving tDMVECs, polyadenylated [poly(A)] RNA was isolated by resuspending the total cellular RNA from approximately 1.5 × 10⁹ cells in 2.7 ml TE/5 (2 mM Tris/HCl pH 7.5, 0.2 mM EDTA), heated at 55 ºC for 10 min, mixed with 300 µl 5 M NaCl, 15 µl 2 M Tris/HCl pH 7.5 and 120 mg oligo(dT)–cellulose (Sigma) and incubated at room temperature for 30 min. Poly(A) RNA bound to oligo(dT)–cellulose was pelleted by centrifugation for 10 min at 2000 g and washed twice by resuspending in 2 ml binding buffer (10 mM Tris/ HCl pH 7.5, 0.5 M NaCl) and centrifuging. The pellet was resuspended in 2 ml binding buffer, distributed between six Spin-X tubes (Costar) and centrifuged for 2 min at 6000 g. The pellets were then washed twice by resuspending in 400 µl washing buffer (10 mM Tris/HCl pH 7.5, 0.25 M NaCl) and centrifuging. The Spin-X columns were transferred to fresh tubes and poly(A) RNA was eluted by resuspending twice in 200 µl elution buffer (10 mM Tris/HCl pH 7.5) and centrifuging. The two fractions from each column were combined, mixed with 2 µl Pellet Paint (Novagen), 40 µl 3 M sodium acetate pH 5.2 and 800 µl ethanol, incubated at room temperature for 5 min and centrifuged at 12000 g for 5 min. The RNA pellets were washed by resuspending and centrifuging in 200 µl 70% (v/v) ethanol and then in 100% ethanol. Each pellet was air-dried and resuspended in 10 µl TE/5. The aliquots were combined and the RNA concentration was estimated by spectrophotometry.

RT-PCR. Except for experiments involving RNA from tDMVECs, RT-PCR was carried out in 50 µl reaction volumes using the Titan kit (Boehringer) with poly(A) RNA and primers shown in Fig. 2. The conditions consisted of heating at 50 ºC for 30 min, ten cycles at 94 ºC for 30 s, 55 ºC for 3 s and 68 ºC for 60 s, 25 cycles under the same conditions except that the final step was prolonged by increments of 5 s at each cycle, and a final cycle with an extension step of 7 min. RT-PCR analyses of infected tDMVECs were performed using total cellular RNA essentially as described by Blackbourn et al. (1992). An aliquot (5 µl) of each reaction was subjected to agarose gel electrophoresis and ethidium bromide staining, and gels photographed under shortwave UV irradiation. Product sizes were estimated by comparison with marker DNA ladders (New England Biolabs). The remainder of each reaction was subjected to gel electrophoresis in low melting point agarose and ethidium bromide staining, and fragments were excised under longwave UV irradiation. DNA fragments were recovered by treatment with β-agarase (New England Biolabs) and cloned using a pGEM-T Vector System 1 kit (Promega). Plasmid DNA was prepared by standard protocols and the sizes of inserts were estimated by agarose gel electrophoresis of DNA digested with Sulf and Sphl (which each cut the vector once, on opposite sides of the insert). Sequences were obtained for several clones of each fragment with universal and custom primers, using an ABI PRISM 377 instrument.

RACE. RACE experiments employed three commercial kits: Marathon (Clontech), Smart RACE (Clontech) and GeneRacer (Invitrogen). Primers are shown in Fig. 2. The Marathon approach involved full-length reverse transcription of mRNA using an oligo(dT)-containing primer, ligation of a partially double-stranded adapter to the ends of the double-stranded cDNA, and PCR of 5’- or 3’-ends using adapter-specific and gene-specific primers. The Smart RACE approach involved reverse transcription of mRNA using an oligo(dT)-containing primer under conditions that allow the intrinsic terminal transferase activity of the reverse transcriptase to add a tract of C residues at the 3’-end, second cDNA strand synthesis using the Smart oligonucleotide which has a tract of G residues at the 3’-end, and PCR of 5’-ends using Smart-specific and gene-specific primers. The GeneRacer approach involved enzymatic removal of 5’-phosphate residues from uncapped RNA, enzymatic decapping of RNA to leave a 5’-phosphate residue at the ends of authentic mRNAs, ligation of the GeneRacer RNA oligonucleotide to the 5’-ends, reverse transcription using an oligo(dT)-containing primer, and PCR of 5’-ends using GeneRacer-specific and gene-specific primers. In each approach, there was the possibility of conducting nested PCR utilizing additional kit-specific and gene-specific primers.

The conditions for PCR and nested PCR consisted of five cycles at 94 ºC for 30 s and 72 ºC for 180 s, five cycles at 94 ºC for 30 s, 70 ºC for 30 s and 72 ºC for 180 s, and 20–27 cycles at 94 ºC for 30 s, 68 ºC for 30 s and 72 ºC for 180 s. RACE products were isolated by agarose gel electrophoresis, cloned into pGEM-T and characterized and sequenced as described above.

Northern blotting. Aliquots of 4 µg poly(A) RNA were electrophoresed in formaldehyde–agarose gels alongside synthetic poly(A) RNA markers (Life Technologies). The markers were visualized by photographing the ethidium bromide-stained gel under shortwave UV irradiation alongside a ruler. The RNA was transferred to Nytran Supercharge membranes (Schleicher & Schuell) by standard methods, and the positions of markers marked. A ³²P-labelled double-stranded DNA probe was made using a random DNA nonprimer.
kit (Appligene) from inserts purified from plasmids. A control probe for cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (approx. 1.4 kb) was also generated. Hybridization was carried out by standard procedures and the results were visualized using a phosphorimager (Bio-Rad Personal Molecular Biology Imager FX). Induction ratios for vIRF mRNAs, standardized to GAPDH mRNA levels established by reprobing the blot with the control probe, were obtained using the phosphorimager software.
HHV-8 vIRF mRNAs

500 bp section of VIRF-3 omitted

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1600 bp section of VIRF-4 omitted
RESULTS

Splicing and induction of vIRF genes assessed by RT-PCR

RT-PCR experiments were carried out to assess splicing of the vIRF genes. The primers used are shown in Fig. 2, and a compilation of results is shown in Fig. 3. Fragment sizes in the following description were estimated from agarose gel electrophoresis and supported by sequencing.

As expected, Raji cells generated no specific HHV-8 products. Amplification of the 5'-portions of vIRF-4, vIRF-3 and vIRF-2 generated products of 680, 673 and 713 bp, respectively (marked A in Fig. 3), corresponding to sequences spliced as shown in Fig. 2. With the caveat that RT-PCR is not reliable as a quantitative method, the results suggested that vIRF-4 is induced in both cell lines, vIRF-2 is induced to a lesser degree and more convincingly in HBL-6 cells (which are considered to exhibit tighter control of HHV-8 latency; Sarid et al., 1998), and vIRF-3 is induced marginally in HBL-6 but not BCP-1 cells. Products corresponding to the unspliced sequences were also generated (marked B in Fig. 3). Production of these fragments was resistant to treatment of the RNA preparations with RNase or omission of reverse transcriptase (data not shown), indicating that they were probably due to residual HHV-8 respectively.
DNA. A fragment migrating slightly more slowly than the unspliced sequence was also produced for each gene (marked C in Fig. 3). This probably corresponds to a partially single-stranded duplex comprising the spliced and unspliced products. Larger fragments produced using vIRF-4 and vIRF-2 primers were not investigated. RT-PCR of the entire protein-coding region of vIRF-1 from a different RNA preparation containing undetectable amounts of contaminating DNA yielded a 1353 bp unspliced product from induced BCP-1 and HBL-6 (H) cells. The central and flanking marker lanes contain 100 and 123 bp ladders, respectively, with sizes (bp) indicated. The labels A, B and C are explained in the text.

Fig. 3. Agarose gel showing evidence for splicing of vIRF genes. RT-PCR reactions were performed with primers K9/1 and K9/2 (vIRF-1), K10/1 and K10/2 (vIRF-4), K10.5/1 and K10.5/2 (vIRF-3), K11/1 and K11/2 (vIRF-2), using RNA from uninduced (U) or induced (I) Raji (R), BCP-1 (B) and HBL-6 (H) cells. The central and flanking marker lanes contain 100 and 123 bp ladders, respectively, with sizes (bp) indicated. The labels A, B and C are explained in the text.

An experiment was undertaken to determine whether vIRF-2 is spliced in other cell lines. Fig. 4 shows that HHV-8 DNA yielded an unspliced product of 402 bp (B) and that a major spliced fragment of 281 bp (A) was produced from the JSC-1 and BCBL-1 PEL cell lines and also from tDMVECs infected with HHV-8 from JSC-1 cells. Very small amounts of unspliced (B) and heteroduplex (C) products were detected. Marginally more spliced product was generated from induced JSC-1 cells and tDMVECs than from uninduced cells.

These results indicate that vIRF-1 is unspliced and that each of the other three vIRF genes contains a single intron in the protein-coding region. They suggest that vIRF-1 and vIRF-4 are induced strongly, vIRF-2 is induced moderately, and vIRF-3 is induced minimally or not at all. Splicing of vIRF-4 and vIRF-3 occurs in two PEL cell lines, and of vIRF-2 in four PEL cell lines and infected tDMVECs.

Sizes and inducibility of vIRF mRNAs assessed by Northern blot hybridization

Representative results of Northern blot hybridization of RNA from uninduced and induced BCP-1 and HBL-6 cells using probes encompassing the entire protein-coding regions of the vIRF genes are shown in Fig. 5. The sizes of the major RNAs were 2-4 kb for vIRF-2, 1-9 kb for vIRF-3, 2-9 kb for vIRF-4 and 1-5 kb for vIRF-1. This implies that each gene possesses its own promoter and polyadenylation site. Although quantitative comparisons were not carried out, levels of transcription of the spliced genes appeared
low even in induced cells. Several minor inducible transcripts were detected for the spliced genes that may represent read-through RNAs or spurious hybridization. The relative levels of induction given at the foot of Fig. 5 indicate that vIRF-2, vIRF-4 and vIRF-1 were induced in BCP-1 and HBL-6 cells. The accuracy of these values is particularly sensitive to the low levels of transcripts in uninduced cells. vIRF-3 was not induced in HBL-6 cells, and was induced weakly in BCP-1 cells.

Locations of the ends of vIRF mRNAs assessed by RACE

Three RACE kits were used to map the ends of the vIRF mRNAs. 5'- and 3'-RACE were carried out using appropriate combinations of adaptor-specific and gene-specific primers, and products were cloned and sequenced. In some experiments, nested PCR was performed. A summary of the results is shown in Table 1.

The 5'-end of induced vIRF-1 mRNA has been mapped by two groups, and provided an opportunity to assess the three kits used in our study. Inagi et al. (1999) employed a RACE kit (Invitrogen) involving priming of the cDNA by a genespecific primer, addition of a poly(dC) tract by terminal transferase, priming of the second cDNA strand by an oligonucleotide with a dG tract at the 3'-end, and PCR using adaptor-specific and gene-specific primers. Chen et al. (2000), from the same group, also used primer extension. Wang et al. (2001) utilized first-strand cDNA priming with a gene-specific primer, addition of a poly(dA) tract to the 3'-end and PCR using an oligo(dT) primer and a second gene-specific primer. Wang et al. (2001) mapped the major 5'-end to an A residue in the sequence TATCTGA and Chen et al. (2000) to two closely adjacent nucleotides (TATC5A). These 5'-ends are located 21–25 nucleotides downstream from a TATA box (TATATA). In the present study, the major 5'-end in induced mRNA was mapped using the GeneRacer kit to the same A residue as that reported by Wang et al. (2001). This end was also detected using the Smart RACE kit, but an alternative end at the downstream A residue (TATCTGA) was more abundant. 5'-Ends mapped using the Marathon kit did not correspond to any of these ends, and instead mapped downstream. These results indicate that the GeneRacer and Smart RACE

![Fig. 5. Phosphorimage of a Northern blot of RNA from uninduced (U) and induced (I) BCP-1 (B) and HBL-6 (H) cells probed with vIRF (upper part) and control GAPDH sequences (lower part). The vIRF probes were inserts purified from plasmids generated via RT-PCR of induced HBL-6 RNA using the following primers: vIRF-1, K9/1 and K9/2; spliced vIRF-4, K10/1 and K10/3; spliced vIRF-3, K10.5/1 and K10.5/3; and spliced vIRF-2, K11/1 and K11/3. The size (kb) of the major transcript is indicated to the right of each panel. The level of induction of the relevant vIRF mRNA is underlined at the foot of each pair of lanes, followed by levels measured in other experiments.](image-url)
Table 1. Locations of (A) 5' - and (B) 3' -ends of vIRF mRNAs determined by RACE

(A)

<table>
<thead>
<tr>
<th>RNA</th>
<th>Type*</th>
<th>Kit</th>
<th>Primer</th>
<th>Closest</th>
<th>Locations of 5' ends with respect to DNA sequence</th>
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<tr>
<td>vIRF-2</td>
<td></td>
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<td>vIRF-2, 94295–94125 in (A), 91784–91747 in (B); vIRF-3, 89078–88908 in (A), 86030–86003 in (B); vIRF-1, 85377–85207 in (A), 83808–83786 in (B).</td>
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(I, induced; U, uninduced. BCP-1 cells were induced with PMA, and HBL-6 cells with sodium butyrate with the exception of one experiment in which they were induced with PMA (P).)

†The RACE kits used were Marathon (MAR), Smart RACE (SMA) and GeneRacer (GEN).

‡Gene-specific primer used to amplify RACE products in conjunction with the appropriate primer supplied with the kit. Primers before and after arrows indicate primary and nested PCR primers, respectively. S and L indicate smaller and larger fragments, respectively, obtained by nested PCR.

§The number of plasmid clones included in the results is given in relation to the number analysed. Excluded plasmids represented artefacts (cellular RNAs, RNAs from elsewhere in the viral genome or, in the case of vIRF-4, cryptically spliced RNAs).

*Number of plasmid inserts indicating an mRNA end at the location indicated (14 and 23). In (A), TATA boxes are in bold and the initiation codon is underlined. In (B), polyadenylation signals are in bold and the region containing the templated 3'-nucleotide is underlined. Sequences are from Russo et al. (1996): vIRF-2, 94295–94125 in (A), 91784–91747 in (B); vIRF-3, 91562–91392 in (A), 89392–89371 in (B); vIRF-4, 89078–88908 in (A), 86030–86003 in (B); vIRF-1, 85377–85207 in (A), 83808–83786 in (B).
kits were capable of accurately locating the 5′-end of vIRF-1 mRNA. The GeneRacer kit appeared the more reliable, in that the major 5′-end corresponded precisely with that identified previously by Wang et al. (2001). In these experiments, use of the Marathon kit failed to map the 5′-end.

Chen et al. (2000) reported a minor vIRF-1 transcript in uninduced RNA commencing 84 nucleotides upstream from the major 5′-end (TACCCA near the 5′-end of the sequence in Table 1). In nested PCR experiments using the Smart RACE kit with uninduced RNA (data not shown), a minor RACE product (denoted L in Table 1) was generated that was 60–70 bp larger than the product corresponding to the major 5′-end (denoted S). As shown in Table 1, the corresponding 5′-ends were distributed over a region upstream from the major end, but none corresponded to the minor end of Chen et al. (2000). No distinct product indicating 5′-ends upstream of the major end were identified using the GeneRacer kit followed by nested PCR, and cloned products from the relevant region of the gel exhibited 5′-ends no different from those of the major product. However, 5′-ends close downstream from the major end were identified in the S product made using the GeneRacer kit. These are located 25–26 nucleotides downstream from a TATA box (TATATC). Corresponding 5′-ends were not detected using the Smart RACE kit. Therefore, the existence and relevance of minor vIRF-1 transcripts in uninduced cells should be considered inconclusive. The results for vIRF-1 indicated that the locations of 5′-ends derived using the GeneRacer kit are the most reliable, and can be supported by data obtained using the Smart RACE kit.

Table 1 shows that the 5′-end of vIRF-2 mRNA in induced cells is located 23 nucleotides downstream from a TATA box (TATATT). vIRF-4 mRNA has two inducible promoters, with the 5′-ends located 25 and 22–26 nucleotides downstream, respectively, from TATA boxes (TATAAA and TATATAAG). Neither vIRF-2 nor vIRF-4 was expressed at sufficient levels in uninduced cells to permit identification of 5′-ends. In contrast, the 5′-ends of vIRF-3 transcripts from uninduced and induced cells were spread over a region, and the major end in both is not located downstream from a TATA box.

As expected, in experiments where the gene-specific RACE primer was positioned downstream from the intron in the three spliced genes, the spliced transcript was detected. Alternative splicing was also evident in induced vIRF-4 mRNA, occurring between a donor site upstream and an acceptor site downstream of the initiation codon, as well as in the usual intron (Fig. 2). Jenner et al. (2001) reported this splicing pattern for uninduced vIRF-4 mRNA and noted that the translation product would lack the N-terminal portion of the vIRF-4 protein. In addition, we found that other sequences were also spliced to the vIRF-4 region via the alternative acceptor site, some from other regions of the HHV-8 genome and some from cellular sequences.

The Marathon kit was used to map the 3′-ends of vIRF genes (Table 1). The data are considered reliable as, in contrast to 5′-end mapping, the method does not depend on the generation of full-length cDNAs. Each 3′-end is located close downstream from a polyadenylation signal (AATAAA or ATTAAA). These data confirm that each vIRF gene has its own promoter (or promoters) and polyadenylation site. No evidence was found for splicing between different vIRF genes.

**DISCUSSION**

We have characterized and mapped the HHV-8 vIRF mRNAs by RT-PCR, Northern blotting and RACE. vIRF-3 transcription was not induced in BCP-1 cells (i.e. it was expressed with latent kinetics) and induced minimally in HBL-6 cells, whereas the other vIRFs were clearly inducible (i.e. they were expressed with lytic kinetics) in both cell lines. As shown in the lower part of Fig. 1, each gene possesses its own promoter (or promoters) and polyadenylation sites, and all but vIRF-1 are spliced from two protein-coding exons. vIRF-1 was transcribed in uninduced and induced cells from a single initiation site preceded by a TATA box, with the possible use of an additional promoter detected in uninduced cells. In induced cells, vIRF-2 was transcribed from a single initiation site preceded by a TATA box, and vIRF-4 was expressed from two sites about 100 bp apart, each preceded by a TATA box. vIRF-3 lacks an obvious TATA box and exhibited heterogeneous 5′-ends in uninduced and induced cells. The vIRF region is now among the best transcriptionally characterized parts of the HHV-8 genome, and the data presented here will aid studies on functional aspects of these genes.

Comparisons of three RACE kits for mapping the 5′-end of vIRF-1 revealed that the Smart RACE and GeneRacer kits were reliable, the latter probably performing with greater accuracy. The situation regarding the use of alternative promoters to generate minor vIRF-1 transcripts in uninduced cells remains unresolved. We were unable to confirm the minor end reported by Chen et al. (2000), and instead detected a different minor end, but with only one kit. The pattern of vIRF-1 expression in uninduced cells would thus bear greater scrutiny. Nonetheless, the success of the Smart RACE and GeneRacer kits with the major vIRF-1 mRNA was taken as strong support for the validity of the 5′-ends mapped for other vIRF genes.

There is disagreement in the literature regarding vIRF-2 structure and transcription. Our data confirm that the vIRF-2 mRNA is inducible and spliced. It is possible that the gene expresses two products, the full-length protein from spliced mRNA and a C-terminally truncated form (the vIRF-2x1 protein) from unspliced mRNA. However, Jenner et al. (2001) did not detect the unspliced RNA in induced cells, and the RT-PCR results in Fig. 4 show that little or no unspliced RNA is present in uninduced or induced cells. Taken together, these data indicate that the vIRF-2x1
protein is probably produced in very small amounts, if at all. Nonetheless, it must be registered that Burysek & Pitha (2001) detected by Western blotting a protein in several PEL cell lines that had a size consistent with it being the product of vIRF-2x1, and apparently did not detect the larger protein anticipated from the spliced mRNA.

Of the four genes investigated, 5'-ends were the most difficult to detect for vIRF-4. Transcription of vIRF-4 was also unusual in that alternatively spliced transcripts were detected in induced mRNA. It should be noted that these RNAs were detected as contaminants of 5'-RACE products originating from the conventionally spliced transcripts. They were expressed by splicing of a variety of exons to vIRF-4 sequences via a range of donor sites, and consequently possessed a variety of 5'-sequences. They were probably fragmented products of longer RNAs that are unlikely to specify vIRF-4-related proteins. In any case, these RNAs would at best specify the C-terminal portion of the vIRF-4 protein. Therefore, it is our opinion that these transcripts are likely to be biologically irrelevant, and were detected because of the relatively low level of vIRF-4 transcription even in induced cells and the fortuitous presence of an alternative acceptor site near the 5'-end of the gene.

The vIRF-3 (LANA-2) promoter is unusual in that it contains no obvious TATA box and directs transcriptional initiation over a relatively wide region, although a single major 5'-end was evident. The putative TATA box identified by Lubyova & Pitha (2000) is located 500 bp further upstream and appears not to direct initiation. The 5'-ends of another latent mRNA, encoding LANA-1, have been

**Fig. 6.** Amino acid sequence alignment of the conserved region in HHV-8 vIRFs, RRV vIRFs (R9-1 to R9-8) and human IRFs and IRF-related proteins. The number of the first residue in each sequence is indicated. Residues that are conserved in at least eight of the nine human sequences and corresponding residues conserved in the viral sequences, and residues that are conserved in all of the viral sequences, are shown in bold. Residues in IRF-1 that contact DNA are underlined (Escalante et al., 1998). Sequences were derived from accessions U75698 (HHV-8), AF210726 (RRV), P10914 (IRF-1), A53340 (IRF-2), NP_001562 (IRF-3), Q15306 (IRF-4), NP_002191 (IRF-5), O14896 (IRF-6), Q92985 (IRF-7), NP_002154 (ICSBP) and Q00978 (ISGF3).
mapped to a region that is an unusually large distance (34–55 nucleotides) from a proposed TATA box (Dittmer et al., 1998; Talbot et al., 1999; Sarid et al., 1999). Sarid et al. (1999) mapped another 5′-end about 50 bp upstream that lacks a TATA box. Various potential transcriptional regulatory sites have been identified in the LANA-1 promoter region, one of which is an Oct-1/TAATGARAT element (AAGGTAATGAAAT) identified by Talbot et al. (1999) about 250 bp upstream from the initiation site. A closely related sequence (AAGGTAATGAGGT) is located a similar distance upstream from the major vIRF-3 initiation site (Fig. 2). We note that the Oct-1/TAATGARAT element has been characterized through its involvement in expression of herpes simplex virus immediate early genes (O’Hare, 1993).

Fig. 6 shows an alignment of the conserved domain in HHV-8 and RRV vIRFs in relation to the corresponding DNA-binding region of human IRFs, updated and expanded from alignments presented by Moore et al. (1996) and Jenner et al. (2001). Some of the residues involved in interactions between IRF1 and its binding site (Escalante et al., 1998) are conserved in vIRFs, but much conservation concerns other residues. Although it is clear that the DNA-binding domain of a cellular IRF has been captured by an ancestor of HHV-8 and RRV, it remains to be determined whether the viral proteins have retained functions that are dependent on binding DNA.

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