A polypyrimidine tract facilitates the expression of Kaposi’s sarcoma-associated herpesvirus vFLIP through an internal ribosome entry site

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We have identified a novel internal ribosome entry site (IRES) within a latently expressed Kaposi’s sarcoma-associated herpesvirus (KSHV) gene (vCyclin) that controls the expression of a downstream open reading frame encoding an inhibitor of apoptosis (vFLIP). This IRES is the first such element to be identified in a DNA virus and may represent a novel mechanism through which this virus controls gene expression. We have used a dual luciferase reporter assay to identify important sequence elements essential for the activity of the IRES. A sequence of 32 nucleotides incorporating a polypyrimidine tract (PPT) was found to be required for the proper functioning of the IRES. We also show, using an electrophoretic mobility shift assay (EMSA), that proteins specific to a KSHV-infected cell line (BCP-1) but not a KSHV-negative cell line (HEK293) were able to form complexes with the IRES. By using an in vitro RNA binding assay, the cellular polypyrimidine tract binding protein (PTB, hnRNP-I) was found to bind to the IRES RNA. These results suggest that the interaction of PTB with the PPT may contribute to the correct functioning of the KSHV IRES in infected cells.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), is the most recently identified member of the herpesvirus family to infect humans (Chang et al., 1994). KSHV, a gamma-2-herpesvirus, has been proposed as the aetiological agent for Kaposi’s sarcoma as well as other malignancies such as primary effusion lymphoma (PEL) (Cesarman et al., 1995) and multicentric Castleman’s disease (MCD) (Soulier et al., 1995).

KSHV is closely related to three other herpesviruses with oncogenic potential: herpesvirus saimiri (HVS), murine gammaherpesvirus (MHV-68) and, more distantly, to Epstein–Barr virus (EBV). The complete nucleotide sequence of KSHV DNA has revealed several genes which have probably been captured from the host cell during virus evolution, and whose products could also play a role in cellular transformation and tumour induction (Neipel et al., 1997; Russo et al., 1996). The three genes encoded by open reading frames (ORFs) K13, 72 and 73 [vFLIP (Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein) vCyclin and LANA] are transcribed from a common transcription start site in cell lines latently infected with KSHV. The resulting transcript is spliced to yield a 5.32 kb message encoding LANA, vCyclin, vFLIP and a 1.7 kb bicistronic message encoding vCyclin and vFLIP (Dittmer et al., 1998; Talbot et al., 1999).

The observation that a bicistronic transcript (Talbot et al., 1999) encodes vCyclin and vFLIP led to the investigation of the mechanism of translation of the vFLIP ORF. We (Bieleski & Talbot, 2001) and others (Grundhoff & Ganem, 2001; Low et al., 2001) were able to identify a novel internal ribosome entry site (IRES) within the latently expressed vCyclin gene that controls the expression of the downstream vFLIP ORF. This IRES is the first such element to be identified in a DNA virus. Recently, an IRES element has been described in the untranslated region of the Epstein–Barr nuclear antigen-1 (EBNA1) gene, which may contribute to the regulation of latent gene expression (Isaksson et al., 2003).

IRES elements were first identified in the 5’ untranslated regions (UTR) of picornaviruses and are essential for the cap-independent translation of the viral polyprotein (Jang et al., 1988; Pelletier & Sonenberg, 1988). More recently IRES elements have been characterized in several cellular genes which encode growth factors (FGF-2, VEGF) (Stein et al., 1998; Vagner et al., 1995), proto-oncogenes (c-myc) (Naburu et al., 1997) and an inhibitor of apoptosis (XIAP) (Holcik & Korneluk, 2000). IRES-dependent translation of these mRNAs may be essential for the survival and proliferation of cells under stressful conditions (Holcik et al., 2000). IRES elements in two cellular mRNAs [encoding ornithine decarboxylase (Cornelis et al., 2000) and PITSLRE protein kinase (Pyronnet et al., 2000)] have been identified, and are regulated in a cell cycle-dependent manner. These
data reveal a novel role for IRES elements in the translational regulation of protein expression during cell cycle progression. The IRES element that we have identified potentially controls the expression of a virus-encoded anti-apoptotic protein, vFLIP (Thome et al., 1997), which is intimately linked to the expression of a cell growth promoting protein, vCyclin (Cesarman et al., 1996; Godden-Kent et al., 1997).

This paper investigates sequence elements within the KSHV IRES essential for efficient translation of the downstream ORF. In addition the cell type-specific activity of the IRES are investigated.

**METHODS**

**Cells.** The KSHV-positive primary effusion lymphoma (PEL) B-cell line, BCP-1 (Boshoff et al., 1998), was grown in RPMI (Invitrogen) supplemented with 20 % (v/v) fetal calf serum (FCS), 2 mM glutamine, 60 µg penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. HEK293 cells (Graham et al., 1977) were grown in DMEM (Invitrogen) supplemented with 10 % (v/v) FCS, 2 mM glutamine, 60 µg penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Cells were incubated at 37 °C under 4 % CO₂.

**Plasmids.** The plasmids pdLUC and pdLUC-SL were constructed as described previously (Bielecki & Talbot, 2001). The IRES sequence from encephalomyocarditis virus (EMCV) or fragments of KSHV vCyclin/vFLIP were cloned into the Smal–NcoI or XhoI–NcoI sites of pdLUC (Fig. 1a). The following primers were used to PCR amplify specific IRES sequences:

**Primers**

Primer 1: GCATCTCGAGAGGGTCTTCTTCTTG

Primer 2: GCATCTCGAGGTCTTCTTCTTG

**Transfection of cells.** BCP-1 cells (1 × 10⁵ cells per well), HEK293 (5 × 10⁴ cells per well) were seeded in 24-well trays and incubated overnight. The cells were infected with vTF7-3 (Fuerst et al., 1986), a recombinant vaccinia virus expressing T7 RNA polymerase, at 5 p.f.u. per cell in 200 µl serum free medium (OptiMEM; Gibco-BRL) for 60 min at 37 °C. The inoculum was removed and the cells washed once with OptiMEM. The cells were then transfected with 0.5 µg of linearized (AfII and NotI) plasmid DNA and 1.5 µl Transfast transfection reagent per well according to the manufacturer’s instructions (Promega). After incubation at 37 °C for 60 min, 1 ml of growth medium was added to the wells. The cells were assayed for luciferase activity 24 h later as described below.

**Dual luciferase assays.** Transfected cells were washed twice in PBS, and then lysed by addition of 200 µl of passive lysis buffer (PLB, Promega). After incubation for 15 min at room temperature the cell lysates were transferred to Eppendorf tubes and snap-frozen on dry ice. The lysates were then thawed, vortexed for 1 min and the cell debris removed by spinning at 10,000 r.p.m. for 1 min. The
activity of Renilla and firefly luciferase was assayed using the dual luciferase system as described by the manufacturer (Promega). Luciferase activities were measured using a Labsystems benchtop luminometer and the ratio of firefly luciferase to Renilla luciferase activity was calculated and used as a measure of IRES function.

**Electrophoretic mobility shift assays (EMSA)**. RNA was transcribed in vitro from 0.2 µg of linearized plasmid using T3 RNA polymerase and labelled internally with 32P-UTP according to manufacturer’s instructions (Life Science). Whole-cell lysate was prepared from BCP-1 or HEK293 cells by sonication for 15 min at 4 °C in binding buffer [20 mM HEPES/KOH pH 7.5, 50 mM KCl, 10 mM MgCl2, 0.01% (v/v) NP40, 5% (v/v) glycerol], and the cellular debris was removed by centrifugation at 10 000 g for 5 min. The binding reaction was carried out in binding buffer containing 25 000 c.p.m. of labelled RNA, 6 µg protein, 1 unit RNasin, and 0.05 µg poly(dI–dT), in a total volume of 20 µl in the presence or absence of 10-fold excess of unlabelled competitor transcripts. After 15 min incubation at 20 °C, the samples were loaded on a 5% (w/v) non-denaturing polyacrylamide gel containing 0.5× TBE. The gel was run at 0.5× TBE for 1 h at 30 mA before exposure to x-ray film (Hyperfilm) at −80 °C with an intensifying screen.

**Preparation of S10 cell extract**. Cells (1×106) were centrifuged and washed three times with is isotonic buffer (35 mM HEPES pH 7.4, 146 mM NaCl, 11 mM glucose), resuspended in 2 vols of hypotonic buffer (20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM magnesium acetate, 1 mM DTT) and incubated on ice for 10 min. The cells were disrupted with 25 strokes of a Dounce homogenizer (on ice), before addition of 0.1 vols of 10× buffer (0.2 M HEPES pH 7.4, 1:2 M potassium acetate, 40 mM magnesium acetate, 50 mM DTT). The nuclei were removed by centrifugation at 2000 r.p.m. for 10 min at 4 °C, followed by addition of CaCl2 (1 mM) and 75 units of S7 nuclease (Roche) per ml of supernatant. After incubation at 20 °C for 15 min the S7 nuclease was inactivated by adding EGTA to 2 mM. The S10 supernatant was centrifuged at 10 000 r.p.m. at 4 °C for 15 min before freezing in aliquots at −80 °C.

**RNA–protein pull-down assays**. Plasmids derived from the pSP64Poly(A) vector were linearized with EcoRI, and then used as templates for transcription reactions. RNA transcripts [containing a 3' poly(A) tail of 30 residues] were produced and purified according to the manufacturer’s instructions (Ambion; SP6 megascript). The KSHV vCyclin IRES RNA (sense or antisense) was captured onto oligo(dT) Dynabeads (Dynal, 0.5 ml) as described previously (Stassinopoulos & Belsham, 2001). The immobilized RNA transcripts were then incubated with BCP-1 cell S10 extract at 4 °C for 60 min on a rotating wheel. The magnetic beads were captured and the depleted S10 was removed. The beads–RNA–protein complex was washed twice in binding buffer, resuspended in SDS sample buffer, and incubated at 4 °C for 10 min. These samples were analysed by SDS-PAGE and Western blot analysis. The anti-PTB polyclonal antibody was a gift from R. J. Jackson (University of Cambridge, UK) (Hunt & Jackson, 1999; Mitchell et al., 2001).

**RESULTS**

**A polypurimidine tract is essential for the activity of the vCyclin IRES**

We have described previously a 233 nucleotide sequence within the vCyclin gene of KSHV that efficiently promotes the translation of the downstream vFLIP orf via internal ribosome entry (Bielecki & Talbot, 2001). We noted the presence of two sequence elements within the IRES that could potentially modulate its activity. The first was a polypurimidine-rich sequence and the second an 11 nucleotide sequence complementary to a sequence in 18s rRNA (Fig. 1b). We have used a combination of restriction enzyme-directed deletion and PCR to determine the minimal sequence necessary for the activity of the IRES. These sequences were cloned into the pdLUC plasmid as shown in Fig. 1(a). These plasmids were transfected into the BCP-1 cell line (latently infected with KSHV), which had been infected with vaccinia vTF7-3 (Fuerst et al., 1986) at an m.o.i. of 5. The Renilla luciferase and firefly luciferase activities were measured in cell lysates 24 h post-transfection. The ratio of firefly luciferase to Renilla luciferase activity was calculated and used as a measure of IRES function. As shown in Table 1 we were able to delete the 18s RNA sequence without significantly affecting IRES function, but deletion of the polypurimidine tract (PPT) resulted in loss of IRES function. Two of these constructs (Primer 1&4 and 1&3) revealed a higher IRES activity than the other constructs and equivalent to the activity of the EMCV IRES. This may be due to the removal of inhibitory sequences or the presentation of the KSHV IRES in a more favourable structural conformation. To confirm that the PPT was necessary and sufficient for IRES activity we cloned this sequence alone into the pdLUC plasmid using oligonucleotides encompassing the PPT (Fig. 1c). As seen in Table 1, the PPT alone was able to direct efficient expression of the downstream firefly luciferase. The cap-independent activity of this PPT sequence was confirmed using an equivalent reporter construct that contained an inverted repeat, with the potential to form a stable 28 bp stem–loop structure in the 5' UTR immediately upstream from the Renilla luciferase start codon. Translation of the first cistron was efficiently inhibited by the presence of the stable stem–loop structure, whereas translation of the second cistron via the PPT sequence

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Activity of IRES [FF:RL (%)]*</th>
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<tbody>
<tr>
<td>EMCV IRES</td>
<td>12.3 ± 1.1</td>
</tr>
<tr>
<td>SacII–Eco47III</td>
<td>7.0 ± 1.7</td>
</tr>
<tr>
<td>Faul–Faul</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>Smll–Eco47III</td>
<td>5.1 ± 1.3</td>
</tr>
<tr>
<td>Mrnl–Mrnl</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Aarfl–Eco47III</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Primers 1 and 4</td>
<td>14.2 ± 1.9</td>
</tr>
<tr>
<td>Primers 1 and 3</td>
<td>13.7 ± 1.4</td>
</tr>
<tr>
<td>Primers 2 and 4</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>Primers 2 and 3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>PPT†</td>
<td>6.9 ± 0.7</td>
</tr>
</tbody>
</table>

*The ratio (%) of the firefly (FF) and Renilla (RL) luciferase activities are shown from an experiment performed in triplicate.†PPT (polypurimidine tract); see Fig. 1(c).
within the vCyclin gene was unaffected by the presence of the stem–loop (data not shown).

**Cell-type specificity of KSHV IRES activity**

We noted previously that the IRES activity was high in the PEL cell line BCP-1 (latently infected with KSHV) but that there was little or no activity in the cell lines HEK293, HeLa or KSIMM (Bieleski & Talbot, 2001). This suggests that cell-specific and/or KSHV-specific factors play an important role in the modulation of IRES activity. EMSA is a technique used to study the interaction of proteins with specific nucleic acid targets (DNA or RNA). Radiolabelled RNA encompassing the IRES was produced by *in vitro* transcription using T7 RNA polymerase mixed with protein (whole-cell lysate) and then electrophoresed through a non-denaturing polyacrylamide gel. Any RNA–protein complexes that are formed run with a slower mobility through the gel in comparison with non-complexed RNA. We have used this system to investigate the possibility of proteins interacting with the KSHV IRES. As shown in Fig. 2, several specific RNA–protein complexes (indicated by *) are formed when *in vitro*-transcribed IRES RNA is mixed with crude cell lysate from BCP-1 cells but not with cell lysate from HEK293 cells. The specificity of these RNA–protein interactions was confirmed by the fact that excess unlabelled IRES RNA successfully competed out these complexes. These data confirm the presence of specific protein factors present in the KSHV-positive BCP-1 cell line that may be essential for the activity of the IRES.

**Specificity of proteins interacting with IRES**

We have defined a minimal fragment of vCyclin coding sequence that is able to function as an IRES in our dual luciferase assay. The most striking feature of this sequence is a PPT, 17 nucleotides in length. The presence of a PPT has been noted in several other IRES elements in viruses and cellular mRNAs (Pyronnet et al., 2000). Pyrimidine-rich sequences have been shown to interact with the PTB (hnRNP-I), and this protein is known to affect the function of certain IRES elements (Gosert et al., 2000; Hunt & Jackson, 1999; Mitchell et al., 2001). To test the possibility that PTB may bind to the KSHV IRES, we used an *in vitro* binding assay (Stassinopoulos & Belsham, 2001). The KSHV IRES (Fig. 1b; SacII and Eco47III) was cloned in both the sense and antisense direction into the plasmid pSP64poly(A) allowing the production of RNA tailed with a 30 nucleotide poly(A) sequence. This RNA was bound to oligo(dT) Dynabeads and the complex incubated with S10 protein extract from BCP-1 cells. Proteins bound to the RNA were analysed by SDS-PAGE and Western blot. As shown in Fig. 3, PTB was found to bind specifically to the KSHV IRES in the sense but not the antisense orientation.

**DISCUSSION**

We have previously reported the presence of an IRES element within the coding region of the vCyclin ORF encoded by KSHV that directs the expression of the vFLIP ORF on a bicistronic message (Bieleski & Talbot, 2001). We determined that the sequence requirements for the correct functioning of the IRES fell within a 233 nucleotide fragment. We noted that there were two potentially interesting sequence motifs within the IRES that might contribute to its efficient functioning. These were an 11 nucleotide sequence complementary to a sequence in 18S rRNA, and a 20 nucleotide PPT. Deletion of the 18S rRNA sequence had no effect on the functioning of the IRES, whereas deletion of the PPT abolished IRES activity in a dual-luciferase reporter assay. To confirm the essential role for the PPT, we tested this sequence (32 nucleotides) in isolation for IRES activity and found that it efficiently directed expression of a downstream ORF on a bicistronic message. The presence of pyrimidine-rich sequences has
been noted in several other IRES elements in viruses and cellular mRNAs (Pyronnet et al., 2000).

We have used an EMSA to define IRES–protein complexes within permissive (BCP-1) and non-permissive cells (HEK293). Four distinct IRES–protein complexes were observed with BCP-1 lysate, whereas no distinct species were identified in HEK293 cells. In order to identify potential interacting proteins we used an in vitro binding assay using poly(A)-tailed IRES RNA and oligo(dT) magnetic beads. Proteins interacting with the IRES RNA were enriched from BCP-1 S10 extract and analysed by SDS-PAGE and Western blot. Using this technique we were able to show that the cellular PTB (hnRNP-I) selectively bound to KSHV IRES RNA, but not to the antisense IRES RNA used as a control. Clearly, PTB is not the sole determinant of IRES activity since it is expressed in a wide variety of cell types including HEK293 cells in which the KSHV IRES is non-functional. The potential interaction of PTB with the PPT of the IRES may provide a framework for the binding of further cellular and/or viral factors. We tested whether the expression of PTB in an in vitro transcription/translation system (rabbit reticulo-cyte lysate) would enhance the activity of the IRES, but found that it had no effect (data not shown). This also supports the idea that multiple protein factors are required for efficient IRES activity.

Some viral IRESs, e.g. the EMCV IRES, do not appear to require proteins other than canonical translation initiation factors for function (Pestova et al., 1996), while others require an additional complex set of factors for activity. Such factors include PTB, which binds specifically to several viral IRESs, although the absolute requirement of viral IRESs for this factor differs. For example, PTB stimulates the initiation of translation by internal ribosome entry from hepatitis C and A virus RNA in vivo (Gosert et al., 2000) and from the human rhinovirus (HRV) and poliovirus IRESes in vitro (Hunt & Jackson, 1999) but is not necessary for the activity of wild-type EMCV (Kaminski & Jackson, 1998). PTB is a cellular protein known to be involved in splicing and branch point selection (Grossman et al., 1998). It has been shown that the IRES element controlling the expression of the cellular gene Apaf-1, involved in the apoptotic cascade, requires both PTB and, upstream of N-ras (unr), two cellular RNA-binding proteins previously identified to be required for rhinovirus IRES activity (Mitchell et al., 2001). This study showed that PTB binding to the Apaf-1 IRES occurred only if unr was present.

We have shown that a PPT is essential for the activity of the KSHV IRES and that PTB interacts with KSHV IRES RNA in vitro. Additional IRES RNA–protein complexes were observed using an EMSA that are yet to be identified. Further investigations will be required to determine other binding and regulatory components necessary for the correct functioning of the KSHV IRES.

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REFERENCES


