Identification of a cis-acting element within the herpesvirus saimiri ORF 6 promoter that is responsive to the HVS.R transactivator

Adrian Whitehouse, Alex J. Stevenson, Matthew Cooper and David M. Meredith

Molecular Medicine Unit, University of Leeds, St James’s University Hospital, Leeds LS9 7TF, UK

We have previously demonstrated that two distinct transcripts are produced from ORF 50, the major transcriptional activating gene of herpesvirus saimiri. The products of these transcripts transactivate the delayed-early ORF 6 promoter, though to different degrees. Deletion analysis demonstrated that the ORF 50 responsive elements are contained in a 132 bp fragment situated 127–259 bp from the transcription initiation site within the ORF 6 promoter. This fragment conferred ORF 50-responsiveness on an enhancerless simian virus 40. Gel retardation analysis further mapped the responsive elements to a 38 bp fragment.

Herpesvirus saimiri (HVS) is a lymphotropic rhadinovirus (v2 herpesvirus) of squirrel monkeys (Saimiri sciureus) which persistently infects its natural host without causing any apparent disease. HVS infection of other species of New World primates causes fatal T-cell lymphomas and lymphoproliferative diseases (Fleckenstein & Desrosiers, 1982). The genome of HVS (strain A11) has been sequenced and analysis indicates that HVS is closely related to the gammaherpesviruses Epstein–Barr virus (EBV) (Albrecht & Fleckenstein, 1990; Albrecht et al., 1992; Albrecht et al., 1994; Rooney et al., 1994; Rooney et al., 1997). Another IE protein, the BRLF1 gene product (termed R) is a sequence specific transactivator (Gruffat et al., 1990). Analysis of the EBV.R transactivator has shown that it binds to specific DNA sequences which have the consensus sequence GNNCGGGNG (Gruffat et al., 1990, 1992; Gruffat & Sergeant, 1994; Kenney et al., 1989). The EcoRI-D or HVS.R protein transactivator encoded by ORF 50 shares limited homology with the EBV.R gene product (Albrecht et al., 1992; Nicholas et al., 1991). The HVS gene produces two transcripts, termed ORF 50a and ORF 50b; the first is spliced containing a single intron and is detected at early times in replication, whereas the second is expressed later and is produced from a promoter within the second exon. The product of the spliced transcript is 5-fold more potent in activating the delayed-early (DE) ORF 6 promoter, a component of the major DNA binding protein. The function of the non-spliced transcript is unclear (Whitehouse et al., 1997). HVS has no gene homologous to the Z protein gene (Albrecht et al., 1992), which suggests early gene expression is either activated by the ORF 50 gene products alone, or in conjunction with another, as yet unidentified protein.

To analyse the sequences within the ORF 6 promoter which are required for transactivation by the ORF 50 gene products, a 5′ deletion series of the ORF 6 promoter region was produced (Fig. 1). The complete ORF 6 gene is contained in the Kpn-I fragment of HVS-L DNA (Knust et al., 1983). A deletion series of the ORF 6 promoter was constructed by digesting pKpmI-F with the restriction enzymes shown in Fig. 1(a); blunt-ending was achieved with T4 polymerase or Klenow fragment (Life Technologies). These fragments were ligated with pCATBasic (Promega), previously digested with PstI, to derive pAWA1–6, respectively. Owl Monkey kidney (OMK) cell monolayers were transfected with 2 µg of each deletion plasmid, using DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer’s instructions. Cells were harvested after 48 h and assayed for CAT activity by standard methods (Gorman et al., 1982). No CAT expression was observed when either the full-length promoter or any of the deletion plasmids were used in the assay (data not shown). pCAT-Control, which has the CAT coding region under the control of the simian virus 40 (SV40)
Fig. 1. (a) Diagrammatic representation of the deletion series of the ORF 6 promoter. A series of 5’ mutants was constructed by digesting pKpnI-F with the restriction sites shown, blunt-ending with T4 polymerase or Klenow enzyme and ligated with pCATBasic. (b) The 132 bp RsaI–PleI fragment containing the ORF 50 responsive elements was inserted into pCAT (which contains CAT under the control of an enhancerless SV40 promoter), in either orientation.

promoter, served as a positive control to assess transfection efficiency.

Previous transfection experiments have demonstrated that ORF 50 transactivates the ORF 6 gene promoter. To determine which region of this promoter is responsive to either ORF 50 gene product, OMK cells were transfected with each deletion plasmid with either pORF50a (which encodes the ORF 50a gene product) or pAWHincII (which encodes the ORF 50b gene product) as previously described (Whitehouse et al., 1997). Cells were harvested after 48 h and assayed for CAT activity (Fig. 2a, b). Both ORF 50 products specifically require the ORF 6 region contained within a 132 bp RsaI–PleI fragment, situated 127–259 bp from the transcription initiation site (Nicholas et al., 1991). This fragment appears to be essential for transactivation of ORF 6 since deletions not containing this region were unresponsive to either ORF 50 gene product.

Primer extension analysis was performed to ascertain whether the increased level of CAT activity produced by ORF 50a and ORF 50b transactivation of the ORF 6 promoter was due to an increase in the levels of CAT mRNA, and whether the mRNA initiation site remained unchanged. OMK cells were either transfected with pAWA1 or co-transfected with either pAWA1 and pORF50a or pAWA1 and pAWHincII. Cells were harvested 24 h post-infection, hybridized to a 32P-
Fig. 3. Gel retardation analysis. (a) In order to locate the ORF50 response region three sets of oligonucleotides were produced which spanned the 132 bp fragment. (b) Gel retardation assays were performed on each set of oligonucleotides: (i) Set 1 (lane 1, untransfected cells; lane 2, pORF50a transfected cells; lane 3, pAWHincII transfected cells); (ii) Set 2 (lane 1, untransfected cells; lane 2, pORF50a transfected cells; lane 3, pAWHincII transfected cells); (iii) Set 3 (lane 1, untransfected cells; lane 2, pORF50a transfected cells; lane 3, pAWHincII transfected cells). The retarded complexes were separated on a 5% polyacrylamide gel, run in 1% TBE buffer and detected by autoradiography.

labelled primer homologous to the CAT coding region and total RNA was isolated. The expected primer extension product was 210 bp, if the correct transcription start site was used. A product of the correct size was observed suggesting that transactivation does not alter the mRNA initiation site (data not shown). Product levels indicated that more RNA was produced upon transactivation with ORF 50a than ORF 50b, confirming that ORF 50a is primarily responsible for transactivation of the ORF 6 promoter.

To assess the effect of the 132 bp RsaI–Plei fragment on a heterologous promoter, the fragment was ligated in both orientations with pCAT-Promoter, previously digested with BglII and blunt-ended, to derive pCAT-E1 and pCAT-E2, respectively (Fig. 1b). To assess the effect of this sequence upon the SV40 promoter, each construct was transfected into OMK cells, harvested after 48 h and assayed for CAT activity; CAT activity was compared to the levels of CAT produced from the reporter construct pCAT-Promoter (Fig. 2c). Results indicated that the 132 bp fragment had no effect on the SV40 promoter. However, when pCAT-E1 and pCAT-E2 were co-transfected with pORF50a, an increase in CAT activity was recorded, but this was not the case with pCAT-Promoter. Therefore, the sequence contained in the 132 bp RsaI–Plei fragment increases the responsiveness of the enhancerless SV40 promoter, in either orientation, to the ORF 50 gene products, suggesting that this sequence functions as an enhancer element.

To determine which sequences within the 132 bp fragment were necessary for ORF 50 responsiveness, gel retardation experiments were performed using a set of oligonucleotides which spanned the 132 bp fragment (Fig. 3a). Sets of oligonucleotides were annealed and labelled using T4 polynucleotide kinase (Life Technologies) in the presence of $[^{32}P]d$ATP, incubated with nuclear extracts of untransfected cells or cells transfected with pAWHincII or pORF50a [prepared by the method of Andrews & Faller (1991)], and the products separated on a polyacrylamide gel (Fig. 3b). Results from experiments using oligonucleotides Set 2 and extracts of cells transfected with pORF50a and pAWHincII show the formation of a retarded complex. No other complex was identified with other oligonucleotides with untransfected or transfected cells, indicating that the specific sequences for ORF 50 responsiveness are contained within the 38 bp of oligonucleotides Set 2, which map to 12386–12424 bp of the published sequence. Unlabelled oligonucleotide was shown to compete with this reaction (data not shown), further indicating that this 38 bp sequence contained the ORF 50 response elements.

We have previously shown that ORF 50 encodes two transcripts produced from two separate promoters which are active at different stages of the virus replication cycle (Whitehouse et al., 1997). In this study, we have identified response elements within the DE ORF 6 promoter which are required for transactivation by both ORF 50 transcripts. The
response elements identified show homology to the EBV.R response element consensus sequence, GNCCN\(_2\)GGNG. It has been shown by guanine methylation studies that the CCN\(_2\)GG motif is essential for EBV.R binding, suggesting that the R binds to adjacent major grooves of the DNA (Gruffat & Sergeant, 1994). The ORF 50 response elements contain a CCN\(_2\)GG motif; however, the flanking sequences are significantly different to the EBV.R response elements, suggesting the ORF 50 gene products have different sequences required for recognition and fixation of the proteins to its target. At present, we are unable to determine, using gel retardation analysis, if the ORF 50 gene products bind directly to the response elements, or whether the retarded complex identified is due to the recruitment of host cell proteins by ORF 50, required for the translation of the ORF 50 promoter present in the cellular extract. It is likely that ORF 50 gene products bind to the response sequences because of their homology with EBV.R protein, which has been purified and shown to specifically bind to its response elements (Gruffat & Sergeant, 1994). Production of purified ORF 50 gene products and examination of the consensus sequence by mutagenesis are required for further analysis of the transactivation process.

At present, the major DNA binding protein gene is the only gene known to be transactivated by ORF 50. The EBV.R protein has been shown to transactivate three promoters and we predict that the ORF 50 gene products transactivate multiple promoters. We have searched the HVS genome for additional ORF 50 response elements using the motif CCN\(_2\)GG and have identified 69 homologous sequences. However, only 10 of these reside in promoter regions. As both ORF 50 gene products recognize the same 38 bp sequence, we believe that both these gene products also use the same response elements. It will therefore be of interest to examine these genes for possible transactivation by both ORF 50 gene products and determine whether late genes are specifically transactivated by the later ORF 50b transcript. Alternatively, the ORF 50b gene product (which has been shown to transactivate ORF 6 to a lesser extent) may compete with ORF 50a for binding to the response elements, thus acting as a negative regulator of transactivation.

This work was supported in part from grants from the Medical Research Council, Yorkshire Cancer Research Campaign and the Wellcome Trust.

References


Received 17 December 1996; Accepted 14 February 1997