Role of the DR2 repeat array in the regulation of the ICP34.5 gene promoter of herpes simplex virus type 1 during productive infection

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Previous analyses using transient transfection assays indicated that the promoter for the gene encoding the herpes simplex virus type 1 (HSV-1) neurovirulence protein ICP34.5 can be divided into an essential core region of approximately 80 bp and two potent upstream silencer domains corresponding to the DR2 and DR6 repeat arrays. In order to examine the potential role of transcriptional silencing during productive HSV-1 infection, recombinant viruses were generated in which wild-type or mutant ICP34.5 promoters controlling the expression of a chloramphenicol acetyltransferase reporter gene were inserted into the thymidine kinase gene of the viral genome. The intact promoter in the virus HSV-Δ1CAT exhibited delayed-early kinetics of expression that were comparable to those of the ICP34.5 gene promoter at its native site in the genome. Deletion of the core promoter domain eliminated promoter activity in the virus HSV-Δ5CAT, indicating that this region was required for expression not only in transient transfections assays but also in the context of the viral genome. However, deletion of the DR2 repeat array from the ICP34.5 promoter in the virus HSV-Δ7CAT was found to increase promoter activity only minimally at late times, and even to reduce activity at early times. Thus, in marked contrast to its behaviour in transient expression assays, the DR2 repeat array does not appear to act as a transcriptional silencer in the context of the HSV-1 genome during productive infection.

Introduction

The gene encoding infected cell polypeptide 34.5 (ICP34.5) is one of several diploid genes in the herpes simplex virus type 1 (HSV-1) genome, as it maps within the inverted repeat sequences of the viral DNA (Fig. 1). It encodes a 44 kDa polypeptide (Ackermann et al., 1986) that appears to represent a critical neurovirulence factor for HSV-1 infections in vivo, as it facilitates replication of the virus in the central nervous system of mice (Chou et al., 1990). However, the exact mechanism by which the ICP34.5 protein functions is unknown. Studies on mutant derivatives of ICP34.5 have shown that a carboxy-terminal domain which possesses homology to the mammalian growth arrest and differentiation proteins MyD116 and GADD34 is critical for function (Chou & Roizman, 1994), and that the homologous sequences in the MyD116 protein can functionally substitute for the carboxy-terminal domain of ICP34.5 (He et al., 1996). The ICP34.5 protein may act to prevent the premature shut-off of host cell protein synthesis during infection through interaction with protein phosphatase α in a pathway which involves dephosphorylation of the α subunit of translation initiation factor eIF-2 (Chou et al., 1995; He et al., 1997).

The promoter for the ICP34.5 gene lies within the α sequence (Chou & Roizman, 1986) (Fig. 1), which also contains the signals required for cleavage and encapsidation of newly replicated viral DNA (Deiss et al., 1986; Varmuza & Smiley, 1985). Two transcriptional start sites have been identified for the ICP34.5 gene which map adjacent to the terminal DR1 repeats of the α sequence (Chou & Roizman, 1986). The ICP34.5 promoter is remarkable in that it lacks nearly all of the features common to most HSV-1 promoters, such as consensus TATA and initiator elements and upstream binding sites for cellular transcription factors (Cohen et al., 1986; Everett, 1984; Goodart et al., 1992; Homa et al., 1988; Steffy & Weir, 1991). However, a recent mutational analysis of the ICP34.5 promoter in transient transfection assays did reveal several interesting features, including a core element contained within the 80 bp region immediately upstream of the transcriptional start sites.
and two upstream transcriptional silencer domains (Sarisky & Weber, 1994). The latter coincided with the DR2 and DR6 repeat arrays of the a and c sequences, respectively, and mediated transcriptional repression only if they were allowed to form an unusual unwound conformation termed anisomorphic DNA. Moreover, results from in vivo titration experiments suggested the existence of a cellular factor that could mediate transcriptional repression of the ICP34.5 promoter in a DNA conformation-dependent manner (Sarisky & Weber, 1994). Taken together, these studies indicated that the DR2 and DR6 tandem repeat arrays with their unusual secondary structure may play an important role in HSV-1 infections by mediating the downregulation of ICP34.5 gene transcription.

However, the evidence that the DR2 and DR6 repeats act as transcriptional silencers was generated entirely in plasmid transfection experiments (Sarisky & Weber, 1994), and it was thus necessary to characterize these elements in the more relevant context of the viral genome before any final conclusions could be reached concerning their contribution to the regulation of the ICP34.5 gene. Recombinant HSV-1 were therefore generated in which wild-type and mutant ICP34.5

**Fig. 1.** Structures of recombinant HSV-1 genomes containing ICP34.5 promoter–CAT fusions. (A) BamHI restriction maps of recombinant virus genomes. The structure of the HSV-1 genome is shown at the top, including the locations of the L and S components, the inverted repeat sequences a, b, and c, and the two copies of the ICP34.5 gene. The structure of the promoter/regulatory region of the ICP34.5 gene within the a sequence is detailed above the genome and includes the DR1 terminal repeats and the DR2 and DR4 repeat arrays. The positions of the two transcriptional start sites of the ICP34.5 gene (arrows), the core promoter and the DR2 repeat array silencer are also indicated. BamHI restriction maps are illustrated below the genome for the TK locus of wild-type HSV-1, HSV-Δ1CAT, HSV-Δ7CAT and HSV-Δ5CAT. The sizes of the BamHI fragments of each virus are shown below the maps. HSV-Δ1CAT and HSV-Δ7CAT contain additional BamHI fragments representing L and S genomic termini that result from cleavage and packaging at their a sequences. (B) Autoradiogram of BamHI-digested recombinant virus DNA hybridized with radiolabelled TK probe DNA. Sizes (in kb) of fragments are indicated at the right, along with the identification of 5′ TK, 3′ TK, or S-terminal fragments. The source of each fragment is discussed in the text.
promoters were inserted into the thymidine kinase (TK) gene of the viral genome. Each of these promoters directed the expression of a chloramphenicol acetyltransferase (CAT) reporter gene, so that they could be readily characterized during infection by simply analysing the kinetics of CAT production. These studies demonstrate that the DR2 repeat array of the ICP34.5 promoter does not appear to act as a transcriptional silencer during productive HSV-1 infection, in sharp contrast to its behaviour during transient expression assays.

Methods

- **Cell lines.** Vero (African green monkey kidney) cells and SH-SYSY neuroblastoma cells were maintained in Dulbecco's modified essential medium (Gibco-BRL). Primary human foreskin fibroblast cells were maintained in Fibroblast Basal Medium (Clonetics). All media were supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 292 µg/ml glutamine.

- **Plasmid constructions.** Three ICP34.5 promoter–CAT constructs created in a previous study (Sarisky & Weber, 1994) were selected for incorporation into the viral genome (Fig. 1). The construct p34.5∆CAT contained a wild-type ICP34.5 promoter with both the DR2 repeat array marker transfer transfections were purified first on CsCl gradients. Five promoters were inserted into the thymidine kinase (TK) gene of the viral genome. Each of these promoters directed the expression of a chloramphenicol acetyltransferase (CAT) reporter gene, so that they could be readily characterized during infection by simply analysing the kinetics of CAT production. These studies demonstrate that the DR2 repeat array of the ICP34.5 promoter does not appear to act as a transcriptional silencer during productive HSV-1 infection, in sharp contrast to its behaviour during transient expression assays.

Results and Discussion

**Construction of recombinant HSV-1 carrying ICP34.5 promoter–CAT fusions**

In order to study the behaviour of the DR2 silencer in the ICP34.5 promoter during HSV-1 infection, recombinant viruses were generated in which wild-type and mutant promoters were inserted into the TK gene of the viral genome. Each of these promoters directed the expression of a CAT reporter gene, so that their activity in the HSV-1 genome could be readily ascertained by quantification of CAT enzyme levels in infected cells. These promoter–CAT fusion constructs were originally created and characterized in transient transfection assays in a previous work (Sarisky & Weber, 1994), and included p34.5∆1CAT, p34.5∆5CAT and p34.5∆7CAT (Fig. 1). p34.5∆1CAT represented the wild-type ICP34.5 promoter and contained both the core promoter and DR2 silencer elements. p34.5∆7CAT was identical to p34.5∆1CAT, but lacked the DR2 repeats. p34.5∆5CAT served as a promoterless control construct and contained neither the core element nor the DR2 silencer elements.

The constructs p34.5∆1CAT, p34.5∆5CAT and p34.5∆7CAT were successfully incorporated into the TK gene of HSV-1 to generate the recombinant viruses HSV-∆1CAT, HSV-∆5CAT and HSV-∆7CAT, respectively. The structures of these recombinant genomes were confirmed by Southern blot analysis using a number of restriction enzymes; the results obtained for one of these enzymes, BamHI, are shown in Fig. 1. The ICP34.5 promoter–CAT fusions were inserted into the TK gene-containing 3-6 kb BamHI fragment of wild-type HSV-1 virus, which resulted in the creation of several novel BamHI fragments (Fig. 1 A). Major species that hybridized to a radiolabelled 3-6 kb BamHI fragment probe included the 4-4,
4.2 and 3.9 kb fragments of HSV-Δ1CAT, HSV-Δ7CAT and HSV-Δ5CAT, respectively; these contained the 3′ sequences of the TK gene, the ICP34.5 promoter and part of the CAT gene. Additionally, a 1.4 kb fragment was present in all three recombinant viral DNAs; this contained the 5′ sequences of the TK gene and the remainder of the CAT gene (Fig. 1B).

Several additional minor fragments were also detected in BamHI-digested HSV-Δ1CAT and HSV-Δ7CAT DNA (Fig. 1B). The 4.8 and 4.5 kb fragments represented derivatives of the 4.4 and 4.2 kb fragments of HSV-Δ1CAT and HSV-Δ7CAT, respectively, which had undergone amplification of their a sequences. Such amplification has been shown to be carried out by the same viral machinery that promotes normal cleavage and packaging at a sequences (Deiss et al., 1986; Varmuza & Smiley, 1985). In this regard, it is important to note that the molecular mass increases of the two bands resulting from amplification were different, and corresponded to the different sizes of the mutant a sequences present in HSV-Δ1CAT and HSV-Δ7CAT, and not to that of the wild-type a sequence. Thus recombination with native a sequences at the L–S junctions which could have restored the mutant ICP34.5 gene promoters was not evident in these viruses.

Finally, two smaller minor bands of 2.8 and 2.6 kb were seen in HSV-Δ1CAT and HSV-Δ7CAT DNA, respectively (Fig. 1B). These bands correspond to the fragments that resulted when the a sequences within the TK locus were cleaved to yield genomic termini. Since the ICP34.5 promoters of both HSV-Δ1CAT and HSV-Δ7CAT contained intact pac1 and pac2 elements, the viral cleavage and packaging machinery was able to introduce a double-strand break at these sites and create novel genomic termini (Deiss et al., 1986; Varmuza & Smiley, 1985). Although both L and S termini are generated in this reaction (Fig. 1A), only the S-terminal fragments were detected in this analysis since the BamHI site in the CAT gene separated the L-terminal fragments from sequences that would have hybridized to the TK probe employed. Unlike HSV-Δ1CAT and HSV-Δ7CAT, no amplification or cleavage of the corresponding 3.9 kb band of HSV-Δ5CAT was observed (Fig. 1B) because the necessary pac elements were not present in this construct (Fig. 1A).

Taken together, these results confirm that the three desired recombinant viruses had been successfully constructed, so that each of the ICP34.5 promoter–CAT fusions that had been originally characterized in plasmid transfection assays (Sarisky & Weber, 1994) were now present in the TK gene of the HSV-1 genome. While this site of incorporation differs from the native locus of the ICP34.5 promoter at the L–S junction, it would be difficult if not impossible to conduct studies at the latter site, since any mutations introduced at this position would also probably affect the sequence elements essential for cleavage and packaging of viral DNA which overlap the promoter (Deiss et al., 1986; Varmuza & Smiley, 1985). By examining mutant ICP34.5 promoters at the neutral locus of the TK gene, this potential for disruption of normal viral DNA propagation has been avoided. Furthermore, it should also be noted that a small but detectable fraction of the ICP34.5 promoters present in the HSV-Δ1CAT and HSV-Δ7CAT genomes do contain duplicated or cleaved a sequences, and are therefore technically not the same as those that were originally characterized in plasmid transfection assays. While this situation is unavoidable due to the propensity of the a sequence for undergoing such genetic alterations, it is clear from the analyses of these genomes shown that duplicated or cleaved promoters represent only a small subpopulation of the total number of ICP34.5 promoter templates (Fig. 1).

Analysis of promoter activity in recombinant HSV-1 carrying ICP34.5 promoter–CAT fusions

In order to determine if the ICP34.5 promoter–CAT fusions that were placed at the TK locus in the viral genome were active, extracts from Vero cells infected with each of the recombinant viruses at an m.o.i. of 1.0 were examined for the presence of the CAT enzyme. Cells were harvested at 3 and 16 h post-infection (p.i.) and the levels of CAT activity in the resulting extracts were determined (Fig. 2A). At 3 h p.i. only
HSV-1 ICP34.5 promoter regulation

Fig. 3. Kinetics of activation of the CAT gene in the recombinant viruses HSV-Δ1CAT and HSV-Δ7CAT. Lysates of infected Vero cells were prepared at the times indicated and processed to determine their levels of CAT activity (histogram) and glycoproteins C and D (Western blots). Each bar of the histogram represents the mean and standard deviation of quadruplicate experiments.

Fig. 4. Effect of the DNA replication inhibitor PAA on activation of the CAT gene in the recombinant viruses HSV-Δ1CAT and HSV-Δ7CAT. Lysates of Vero cells infected in the presence or absence of 400 µg/ml PAA were prepared at 15 h p.i. and processed to determine their levels of CAT activity (histogram) and glycoproteins C and D (Western blots). Each bar of the histogram represents the mean and standard deviation of duplicate experiments.

The results of these preliminary experiments indicated that there was a small but statistically significant difference (P < 0.03 for the Vero cell experiment using an unpaired t-test) in the activities of the ICP34.5 promoters in HSV-Δ1CAT and HSV-Δ7CAT, which differ only in the presence or absence of the DR2 repeat array. Therefore, the kinetics of activation of the two promoters were examined further to ascertain whether the DR2 repeats contributed to the temporal regulation of ICP34.5 gene expression during HSV-1 infection. The two recombinant viruses were used to infect Vero cells, and lysates were prepared at various times p.i. and assayed for CAT activity. The low level expression of CAT enzyme at early times followed by high level expression at late times after the onset of DNA replication (Fig. 3) indicated that the wild-type promoter in HSV-Δ1CAT displayed classic delayed-early kinetics (Honess & Roizman, 1974), which was consistent with the results of an earlier study of the ICP34.5 gene promoter (Chou & Roizman, 1986). This interpretation was supported by Western blot analyses which revealed that the kinetics of expression of a prototype delayed-early gene (glycoprotein D) but not a true late gene (glycoprotein C) closely paralleled the appearance of CAT activity in HSV-Δ1CAT-infected cells: the former was detectable at 6 h p.i. and accumulated to maximum levels between 12 and 14 h p.i., while the latter was not detected until 10 h p.i. (Fig. 3).

The kinetics of expression of the ICP34.5 promoter were examined further by testing the effect of the viral DNA replication inhibitor phosphonacetic acid (PAA) on promoter activity. Since the ICP34.5 promoter exhibited delayed-early kinetics, where activity was detectable at low levels before the onset of DNA replication and steadily increased after replication was initiated, the action of a DNA replication inhibitor such as PAA should have a significant effect on promoter function. Vero cells were infected in the presence or absence of 400 µg/ml PAA, harvested at 15 h p.i., and processed to determine CAT enzyme levels. The results of this experiment (Fig. 4) demonstrate that PAA decreased the activity of both the HSV-Δ1CAT and HSV-Δ7CAT promoters by 60%,
indicating that the CAT expression driven by these constructs was enhanced by, but not strictly dependent upon, DNA replication. Expression of the delayed-early gene encoding glycoprotein D was similarly partially inhibited by PAA, whereas expression of the true late gene encoding glycoprotein C was completely abolished in its presence (Fig. 4). Thus, the partial sensitivity to inhibition of DNA replication by PAA was another indication that the ICP34.5 promoter inserted at the TK locus in HSV-Δ1CAT behaved with the same delayed-early kinetics as the ICP34.5 promoter at its native site in wild-type HSV-1 (Chou & Roizman, 1986).

Several differences were observed in the kinetics of expression of the wild-type ICP34.5 promoter in HSV-Δ1CAT when compared to the mutant promoter lacking the DR2 repeats in HSV-Δ7CAT (Fig. 3). CAT activity was not detectable in either virus infection until 8 h p.i., whereupon HSV-Δ1CAT exhibited almost a threefold greater level of expression than that of HSV-Δ7CAT. This difference between the two viruses was significantly reduced by 10 h p.i., and by 12 h p.i. HSV-Δ7CAT expressed 20% more CAT activity than HSV-Δ1CAT. This trend increased at 14 h p.i., where HSV-Δ7CAT expressed 25% more CAT activity than HSV-Δ1CAT. These differences in CAT activity between the two viruses were found to be statistically significant at each time point (P < 0.05–0.001 using an unpaired t-test) and suggested that the DR2 repeat array was bifunctional, in that it activated gene expression at earlier times of infection and repressed gene expression at later times of infection. However, at no time during infection did the loss of this putative silencer result in the dramatic increase in expression that had been observed for this same ICP34.5 promoter–CAT construct in transfection assays (Sarisky & Weber, 1994). Conversely, the wild-type promoter in HSV-Δ1CAT produced significant levels of CAT enzyme during infection, despite the fact that it possessed a full DR2 repeat array silencer (Fig. 3). Thus, the behaviour of the DR2 repeat array in the viral genome contrasted sharply with the silencing effect that had previously been observed in transient expression assays (Sarisky & Weber, 1994).

In order to rule out the possibility that any differences in CAT activity induced in infected cells might be due to differences in the relative growth rates of the individual recombinant viruses, rather than differences in the structures of their ICP34.5 gene promoters, the replicative abilities of each recombinant virus were examined. Dishes (60 mm) containing 1 × 10^6 Vero cells were infected in duplicate with each virus at an m.o.i. of 0·1 and harvested by freezing and thawing at 24 h p.i. The titres of the resulting virus stocks were 7·9 × 10^5 (±0·5 × 10^5), 8·2 × 10^5 (±0·5 × 10^5) and 7·6 × 10^5 (±0·1 × 10^5) for HSV-Δ1CAT, HSV-Δ5CAT and HSV-Δ7CAT, respectively. Thus all three of the recombinant viruses replicated with comparable efficiency, so that the differences in the levels of CAT activity that were observed during infection directly reflected differences in the ability of the respective ICP34.5 promoters to drive expression of this enzyme.

**Implications for the regulation of the ICP34.5 promoter**

The equivocal behaviour of the DR2 repeats documented in this work clearly underscores the importance of extending transfection studies of putative negative regulatory elements to the viral genome before reaching any conclusions about their role in promoter function. This is a matter of increasing concern, as plasmid transfection assays have identified additional silencer-like sequences not only in HSV-1 but also in other herpesviruses (Frazier et al., 1996; Huang & Stinski, 1995; Jones et al., 1993; Schwarzmann et al., 1994; Thrower et al., 1996), and like the DR2 repeat array (Sarisky & Weber, 1994), host cell factors have been implicated in mediating the silencing effects associated with many of these sequences. The results of this study clearly demonstrate that although the DR2 element may act as a powerful silencer in plasmid transfection assays (Sarisky & Weber, 1994) it appears to possess only modest regulatory properties in the more relevant context of the HSV-1 genome (Fig. 3). The reason for this alteration in the behaviour of the DR2 repeat array is unclear, but may involve differences in the conformations of the templates in the two experimental systems. In transfection assays, the promoter is in the context of a supercoiled circular plasmid template, while at its native site in the viral genome, the promoter is likely to exist in a more relaxed linear molecule. However, the conformational state of the viral genome may be continuously altered by extensive transcription and replication during infection. For example, HSV-1 DNA has been shown to be associated with host topoisomerase II (Ebert et al., 1994), suggesting that supercoiling in the template may occur at later times in infection. This may be responsible for the nominal downregulation of the ICP34.5 promoter that was observed in HSV-Δ1CAT at 12–14 h p.i. (Fig. 3), through a mechanism analogous to that observed in transient expression assays. Moreover, a similar process may act to repress ICP34.5 promoter expression very early in infection, when the genome is still a circular unreplicated molecule that may become supercoiled as a result of movement of immediate-early transcription complexes.

Interestingly, the DR2 repeat elements have recently been shown to bind the cellular transcription factor Sp1 in vitro (Chung et al., 1995). Sp1 binding to the DR2 repeat array in vivo may have been responsible for the increase in CAT activity seen at early times in HSV-Δ1CAT, but would be inconsistent with the decrease in CAT activity seen in this virus at later times in infection (Fig. 3). Moreover, the DR2 repeat array had previously been shown to silence rather than activate transcription in two different promoters under conditions where Sp1-mediated activation was functional (Sarisky & Weber, 1994). Thus the potential contribution of Sp1 to the regulation of the ICP34.5 promoter during HSV-1 infection is unclear at present. However, additional studies of recombinant HSV-1 containing promoter–CAT fusions should prove useful...
in identifying those transcription factors which play a role in regulating this unique viral promoter.

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