Herpes simplex virus type 1 immediate early gene expression is stimulated by inhibition of protein synthesis

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Herpes simplex virus type 1 (HSV-1) transcription can be arrested at the immediate early (IE) stage by continuous treatment of cells with inhibitors of protein synthesis, usually cycloheximide, from the time of infection. We have analysed the effect of cycloheximide on IE gene expression with HSV-1 mutants deficient in the production of functional levels of the three major transactivators, the virion protein (VP16) and two IE proteins (ICP0 and ICP4). Expression from the HSV-1 IE promoters that control synthesis of ICP0 and ICP27 was, unexpectedly, stimulated by inhibition of protein synthesis. The effect was observed for the ICP0 promoter in its normal genome location and also when cloned upstream of the Escherichia coli lacZ coding sequences and inserted into the viral thymidine kinase locus. Expression from the human cytomegalovirus major IE promoter, when cloned into the genome of HSV-1 mutants, was also increased by inhibition of protein synthesis. Cycloheximide did not affect the intracellular stability of lacZ-specific RNA, suggesting that the response represented an increase in mRNA production. Activation of the ICP0 promoter was observed when protein synthesis was blocked by alternative agents. Since inhibitors of protein synthesis are known to activate cellular signal transduction pathways, our findings demonstrate new mechanisms for the regulation of HSV-1 IE gene expression which may be important during latency and reactivation. The results also highlight previously unrecognized difficulties in analysing the intrinsic activities of promoters when cloned into the HSV-1 genome.

Introduction

Herpes simplex virus type 1 (HSV-1) gene expression is regulated in a co-ordinated manner, with transcription of the immediate early (IE) genes preceding the expression of early and late genes (Honess & Roizman, 1974). The IE proteins ICP4 (Vmw175), ICP0 (Vmw110) and ICP27 (Vmw63), individually and in concert, strongly activate the expression of early and late genes at transcriptional and post-transcriptional levels (Preston, 1979; Everett, 1984; O’Hare & Hayward, 1985; Sandri-Goldin & Mendoza, 1992). In addition, ICP4 autoregulates its own synthesis (Preston, 1979; Watson & Clements, 1980). Proteins ICP4 and ICP27 are essential for virus replication, whereas ICP0 is not essential but is important for the efficient entry into the lytic phase of infection (Stow & Stow, 1986; Sacks & Schaffer, 1987; Everett, 1989). Expression of the IE genes themselves is strongly stimulated by the action of VP16 (Vmw65), a virion protein which operates through the IE-specific sequence TAATGARAT (R is a purine nucleotide) (Post et al., 1981; Campbell et al., 1984). VP16, together with the cellular proteins Oct-1 and HCF, forms a complex on TAATGARAT elements and as a consequence the C-terminal activating domain of VP16 is brought into proximity with the transcription pre-initiation complex, thereby stimulating IE gene expression (reviewed by O’Hare, 1993).

Dissection of the regulatory events in HSV-1 gene expression has relied heavily on the use of inhibitors of protein synthesis to block virus replication at the IE stage (Honess & Roizman, 1974; Preston, 1979; Watson & Clements, 1980; Harris-Hamilton & Bachenheimer, 1985; Elshiekh et al., 1991). Although IE genes are the first to be transcribed, this temporal criterion alone is not adequate to define a gene as IE because the synthesis of early gene products follows shortly after the production of IE proteins, and thenceforth all virus proteins are synthesized concomitantly, albeit at different relative rates, during the replication cycle. Infection in the presence of a protein synthesis inhibitor, usually cycloheximide, prevents de novo production of IE proteins and thus firmly blocks the infection cycle at the IE stage. Transcription during infection in
ICP0 production was controlled either by the Moloney LTR promoter or the endogenous ICP0 promoter.

Evaluation of the importance of activation of IE transcription by VP16, in the context of virus infection, has been provided by studies with the HSV-1 derivative in1814 (Ace et al., 1989). This mutant contains a 12 bp insertion in the coding sequences of VP16 in a domain which is crucial for the formation of the multiprotein complex with Oct-1 and HCF (Hayes & O'Hare, 1993; Lai & Herr, 1997). As a consequence, in1814 cannot activate IE transcription through the VP16-mediated pathway and the mutant initiates productive replication inefficiently when infection is carried out at low m.o.i. We have modified the in1814 genome to reduce further the expression of ICP0 and ICP4, two IE proteins which activate gene expression strongly during infection with HSV-1. The Moloney murine leukaemia virus (Molulv) LTR promoter was substituted for the ICP0 promoter of in1814 to yield in1820, a mutant which behaved as if devoid of ICP0 in all cell types tested except BHK, and a temperature-sensitive mutation in ICP4 coding sequences derived from tk.

The experiments described here demonstrate that HSV-1 and human cytomegalovirus (HCMV) IE promoters, when cloned into the HSV-1 genome, are activated by inhibition of

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>ICP0 coding sequences</th>
<th>ICP0 promoter†</th>
<th>lacZ insert</th>
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<tr>
<td>in1820K</td>
<td>Intact</td>
<td>Momulv</td>
<td>None</td>
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<tr>
<td>in1331</td>
<td>Intact</td>
<td>Momulv</td>
<td>ICP0-lacZ in TK</td>
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<td>None</td>
</tr>
<tr>
<td>in1383</td>
<td>RING deleted</td>
<td>ICP0</td>
<td>ICP0-lacZ in TK</td>
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* All mutants contained the insertion in VP16 coding sequences derived from in1814 and the temperature-sensitive mutation in ICP4 coding sequences derived from tk.
† ICP0 production was controlled either by the Moloney LTR promoter or the endogenous ICP0 promoter.
protein synthesis in a manner similar to that of their cellular namesakes.

**Methods**

- **Cells.** BHK21 C13 cells were used for propagation of viruses and for most plaque isolations. For propagation of the HSV-1 mutants described here, infected cells were grown at 31 °C in the presence of 3 mM HMBA for the first 48 h after infection. Gene expression was analysed in monolayers of human foetal lung (HFL) cells (Flow 2002, Flow Laboratories) or HeLa cells at 38.5 °C. Cycloheximide, anisomycin, puromycin and emetine were used at final concentrations of 50, 10, 25 and 25 µg/ml, respectively. At these levels, cellular protein synthesis was inhibited by 99% (results not shown). Actinomycin D was used at a final concentration of 1 µg/ml.

- **Plasmids.** Plasmid pJR3 contains the ICP0 coding sequences cloned into pUC9 (Everett, 1984). The extent of HSV-1 sequences upstream of ICP0 was increased by cleaving pJR3 with EcoRI, end-filling with Klenow enzyme, cleaving with BamHI and ligating in a HindIII–BamHI fragment of the cloned joint-spanning fragment BamHI k. This procedure yielded pCP2461, in which the upstream boundary extended to nucleotide 128041 (McGeoch et al., 1988) in ICP0. Plasmid pCP2461 was cleaved with XhoI and SstI, and a XhoI fragment from a derivative of pMJ101 (Jameison et al., 1995) in which the upstream HindIII site of the HCMV IE promoter was converted to an XhoI site was cloned in, to yield pMJ84. Plasmid pMJ84 thus contains an insert of lacZ controlled by the HCMV IE promoter, replacing most of the ICP0 coding sequences. In addition, pCP2461 was cleaved with XhoI and Asp718, end-filled with Klenow enzyme and religated to yield pCP47614, in which the RING finger domain of ICP0 was completely deleted and the reading frame of the remainder of the protein was altered.

- **Viruses.** Mutants in1331 and in1332 contain lacZ controlled by the HSV-1 ICP0 promoter and the HCMV IE promoter, respectively, inserted into the thymidine kinase (TK) coding sequences of in1820K (Preston et al., 1997). Plasmid pMJ84 was linearized by cleavage with Scal and co-transfected with in1820K DNA. Plaques were initially tested on HFL cells, in which a deletion of ICP0 coding sequences should not affect the efficiency of plaque formation, and β-galactosidase positive plaques were selected. Small-scale DNA preparations were analysed by Southern hybridization and one isolate was obtained which was homozygous for the deletion in both copies of the long repeat region, with no parental sequences detectable on long autoradiographic exposures. This virus, in1311, was thus deleted for ICP0 coding sequences. The Momulv LTR was not replaced by the normal ICP0 promoter, even though this was a possible outcome of the experimental strategy. Plasmid pCP47614 was cleaved with Scal and co-transfected with in1311 DNA, and single plaque isolates screened for the reacquisition of ICP0 coding sequences. After three rounds of plaque purification and analysis by Southern hybridization and one isolate was obtained which was homozygous for the deletion in both copies of the long repeat region, with no parental sequences detectable on long autoradiographic exposures. This virus, in1332, thus contained the remainder of the ICP0 sequences. The recombination events resulted in replacement of the Momulv LTR with the ICP0 promoter sequences. Mutant in1332 was further modified by insertion of lacZ, controlled by the ICP0 promoter, into the TK locus. This was achieved by co-transfection of in1312 DNA with Scal-cleaved pMJ102, selection for TK-negative mutants which expressed β-galactosidase, plaque purification and analysis by Southern hybridization. The resultant virus was named in1333. For experiments, cells were infected with in1311 or in1332 at an m.o.i. of 5 p.f.u. per cell, based on the titre on BHK cells at 31 °C in the presence of 3 mM HMBA. For in1311 and in1332, which are deficient in ICP0 function, virus stocks had titres approximately 100-fold lower than their in1820K-based counterparts. Mutants were therefore titrated on BHK cells and co-infected with 5 p.f.u. of wild-type HSV-1 per cell. After incubation at 37 °C for 6 h, monolayers were fixed with 1% glutaraldehyde and stained for the presence of β-galactosidase, as described previously (Jameison et al., 1995). Positive cells were counted to yield a ‘titre’ in terms of the number of functional lacZ genes applied to cells. Values for ICP0-deficient mutants were compared in parallel with in1820K-based viruses and amounts equivalent to 5 p.f.u. of an in1820K-based mutant per cell were used for infection of monolayers.

- **RNA analysis.** Polyadenylated RNA was prepared, electrophoresed, blotted and analysed by hybridization of radiolabelled gene-specific probes, as described previously (Ace et al., 1989; Nicholl & Preston, 1996). The probe for ICP0 was a 1056 bp MluI–Asp718 fragment, derived from pJR3, which did not overlap any of the deleted region of lacZ.

**Results**

**Inhibition of protein synthesis activates IE gene expression**

In initial experiments, HFL cells were infected with in1814, which specifies mutant VP16, in the presence of cycloheximide and at 6 h post-infection monolayers were thoroughly washed. It was found that treatment with cycloheximide resulted in an increase (10–20 fold) in the titre of in1814 at 37 °C (results not shown). The most obvious explanation of this result, in light of existing knowledge, was that autoregulation by ICP4 did not occur during the 6 h period without protein synthesis and, therefore, that increased amounts of this protein were produced after removal of the inhibitor. An alternative mechanism became apparent when the accumulation of IE mRNAs was investigated using mutants more impaired for IE gene expression than in1814. The mutants were based on in1820K, in which VP16 and ICP4 were inactivated by mutations in the coding sequences for the gene products and the ICP0 promoter was replaced by the Momulv LTR promoter. Experiments were also carried out with in1311, which was derived from in1820K by replacement of the ICP0 coding sequences with a cassette consisting of the HCMV major IE promoter containing lacZ, and in1332, which is in1820K with ICP0 inactivated by deletion of the RING finger domain and frameshift of the remaining coding sequences, together with an insertion of lacZ controlled by the ICP0 promoter at the TK locus. During infection with in1311 or in1332, therefore, no functional ICP0 was synthesized, whereas with in1820K it remained possible that small amounts of the protein were produced, even though we have previously shown that such amounts were not sufficient to be functionally important for virus replication (Preston et al., 1997).

Monolayers of HFL or HeLa cells were infected with in1332, a derivative of in1820K containing HCMV-lacZ inserted at the TK locus, or in1311, and maintained at 38.5 °C either with no additions or in the continuous presence of cycloheximide. At 6 h post-infection, polyadenylated RNA
was prepared and analysed by hybridization of gene-specific probes to Northern blots (Fig. 1).

It was found that treatment with cycloheximide resulted in greater accumulation of IE mRNA encoding ICP27. The levels of lacZ-specific RNA were also increased after infection with in1332 or in1311, demonstrating that the activation was not through the TAATGARAT element, which is absent from the HCMV IE promoter (Nicholl & Preston, 1996). The results were essentially identical in both cell types tested, irrespective of whether in1332 or in1311 was used, demonstrating that ICP0 was not involved in the observed effects. To investigate whether the ICP0 promoter was also activated by treatment of cells with cycloheximide, mutant in1331 was tested. Mutant in1331 was derived from in1820K by insertion of lacZ, controlled by the ICP0 promoter, into the TK locus. It was found that synthesis of lacZ-specific mRNA was elevated by cycloheximide treatment of HFL cell monolayers (Fig. 2A). To confirm that the ICP0 promoter was activated when in its normal genomic location, HFL cells were infected with in1383 with and without cycloheximide and the synthesis of ICP0 and lacZ-specific RNA was determined (Fig. 2B). Again, activation was observed when the inhibitor was added to monolayers. The results from the experiments shown in Figs 1 and 2 therefore demonstrate that IE promoters regulating ICP0 and ICP27 are activated by inhibition of protein synthesis during infection of cells under conditions in which the three major transactivators, VP16, ICP4 and ICP0, are not functional.

One of the ways in which cycloheximide treatment increases the levels of cellular IE products is stabilization of mRNAs; the possibility that this mechanism was operative in the experiments described above was therefore investigated. The only virus-specified mRNA consistently detectable by Northern blots in the absence of cycloheximide was lacZ when controlled by the HCMV IE promoter. Thus, cells were
infected with \textit{in1332} and at 2 h post-infection treated with cycloheximide, actinomycin D, both compounds together or no inhibitors. RNA was extracted after a further 2 and 4 h and the amounts of \textit{lacZ}-specific RNA, relative to actin mRNA, measured (Fig. 3). Without inhibitors, the relative level of \textit{lacZ} RNA reached a maximum at 2 h post-infection and did not increase during the subsequent 4 h. In the presence of cycloheximide alone, continuous accumulation was observed.

When new RNA synthesis was prevented by addition of actinomycin D at 4 h post-infection, relative \textit{lacZ}-specific RNA levels remained constant regardless of the presence of cycloheximide. Therefore, within the time-frame of this experiment, cycloheximide treatment did not increase the stability of \textit{lacZ} mRNA. It should be noted that the data shown graphically represent levels of \textit{lacZ} mRNA relative to those of actin mRNA; the absolute amounts of both mRNAs dropped.
by approximately 30% during the 4 h chase, possibly due to degradation by the virion host shut-off protein.

To determine whether the effects on IE gene expression were due to the use of cycloheximide or to a general response to inhibition of protein synthesis, different compounds were tested. Anisomycin is an inducer of SAPK activity independently of its effects on translation, and is thus the most potent activator of cellular IE genes. Anisomycin and cycloheximide are targeted to the 60S ribosomal subunit, as opposed to emetine, which acts on the 40S subunit. Puromycin causes premature termination of polypeptide chain elongation. The latter pair give weaker activation of cellular IE genes, due presumably to the reduced activation of SAPK. HFL cell monolayers were infected with in1331 and maintained in the presence of inhibitors until 4 h post-infection, at which time RNA was extracted and analysed (Fig. 4). When probed for lacZ-specific RNA, it was found that treatment with anisomycin or cycloheximide resulted in greater stimulation than puromycin or emetine, although all four agents gave a detectable effect.

Discussion

We describe here an unexpected response of HSV-1 IE genes to inhibition of cellular protein synthesis, namely a stimulation which is similar to that exhibited by cellular IE genes. The effect was only revealed through the availability of HSV-1 mutants so impaired that VP16, ICP4 and ICP0 did not influence the activities of the IE promoters; if any of these transactivators had been present the IE promoters would have been turned on, thereby obscuring the response to cycloheximide. In fact, mRNAs expressed under the control of the ICP0 and ICP27 promoters were produced in relatively small amounts that could barely be detected on Northern blots in the absence of cycloheximide, emphasizing that the apparent potency of IE promoters observed in previous experiments in which cycloheximide was used to arrest infection at the IE stage may have been due, in part, to activation of cellular signal transduction pathways by the inhibitor. This was particularly critical in the initial analysis of in1814, where IE mRNAs surprisingly accumulated to high levels even though VP16 was not active (Ace et al., 1989). As pointed out by Poon & Roizman (1995), the reductions in IE mRNA synthesis by in1814, compared with wild-type HSV-1, were probably not sufficient to account for the impairment of replication exhibited by the mutant. The results presented here suggest that the RNA levels measured previously were exaggerated by the unrecognized effect of cycloheximide.

The finding that HSV-1 IE promoters respond to inhibition of protein synthesis gives problems in determining their intrinsic activities in the context of the viral genome; the use of cycloheximide artificially raises IE RNA levels, but without the inhibitor the IE proteins ICP4 and ICP0 are rapidly produced and then act (positively, negatively or both) on IE promoters. To address this issue, the only possible approaches currently available are transfection of plasmid DNA, which may not accurately reproduce the gross structure of the viral genome at early stages of infection, or the use of viruses very impaired for activation of gene expression. The multiple mutants described here are the most defective in this respect so far reported, and their use gives a unique opportunity to investigate, in future studies, other cellular pathways which may influence IE gene expression. As a more esoteric point, it may be that responsiveness to inhibition of protein synthesis is the best criterion for defining a promoter as IE in the context of the HSV-1 genome since the more obvious indicator, activation by VP16, does not apply to the HCMV IE promoter which nonetheless exhibits IE regulation, i.e. it is expressed in the absence of de novo protein synthesis.

Whatever the precise mode of action of cycloheximide and other inhibitors, it is clear that our findings have revealed new aspects of the regulation of IE gene expression. The results eliminate mRNA stabilization as the mechanism of action of cycloheximide on lacZ-specific RNA production in cells infected with in1332 and the same conclusion is assumed to apply to lacZ mRNA in in1331-infected cells, although we cannot rule out the unlikely possibility that the additional 36 bases at the 5′ terminus of lacZ mRNA (derived from the 5′ terminus of ICP0 mRNA) alter the stability of the in1331-specific transcript. By analogy, the effects on ICP0- and ICP27-specific RNAs are also unlikely to be at the level of RNA stability. It is probable, therefore, that the activities of the ICP0 and ICP27 promoters are increased by inhibition of cellular protein synthesis, and in considering possible mechanisms for the effect it is reasonable to extrapolate from our knowledge on the activation of cellular IE genes. Activation of cellular SAPK may contribute to the effect, since anisomycin and cycloheximide, which are known to be strong inducers, had greater effects than the poor inducers of SAPK puromycin and emetine. Nonetheless, the latter two compounds activated the ICP0 promoter, suggesting that the inhibition of protein synthesis per se contributes to the response. This may be a direct consequence of the block to translation, the failure to produce a repressor which shuts down transcription of viral IE genes, or a combination of both.

At present, the precise DNA sequence elements that mediate the responses of cellular IE genes to inhibition of protein synthesis are largely unknown. The HCMV IE promoter, when integrated into the cellular chromosome, is induced by cycloheximide (Boom et al., 1986). Since the compound is also known to activate the transcription factor NF-κB (Sen & Baltimore, 1986), which binds to the 18 bp repeats of the HCMV IE promoter (Sambucetti et al., 1989), it is likely that NF-κB is at least partly responsible for the effects on lacZ-specific RNA in cells infected with in1332. Nonetheless, we are not aware of direct evidence that the 18 bp element mediates the response of the HCMV IE promoter to cycloheximide and it is noteworthy that the ATF class of
factor, which binds to the 19 bp repeats of the HCMV IE promoter (Stamminger et al., 1990), is activated upon phosphorylation by SAPK (Livingstone et al., 1995), thus defining another pathway through which the effect may be mediated. The identities of the responsive elements in the ICP0 and ICP27 promoters are unclear, although both NF-κB-like and ATF-like binding sites have been recognized in the ICP0 promoter (Rong et al., 1992; Wheatley et al., 1992). It is also possible that cycloheximide activates HSV-1 IE gene expression in a manner that is not sequence specific but rather affects the transcription of the entire genome. The system that we have developed, using multiply defective HSV-1 mutants, should enable any responsive DNA sequences to be delineated by introducing components of, for example, the ICP0 promoter into the in1820K or in1312 genome and subsequently analysing the effects of cycloheximide on gene expression. Experiments of this nature may provide a powerful new approach for the identification of cellular factors which transmit the signal transduction pathways activated by inhibition of protein synthesis to specific promoters.

The significance of the effects that we describe here may be minimal during the early stages of productive infection, since VP16 powerfully activates IE transcription and would be expected to override any contribution from cellular signals. During the establishment of latency, however, VP16 may not be active; thus cellular factors may have greater influence on IE gene expression and hence the outcome of infection (Steiner et al., 1990; Sears et al., 1991; Hagmann et al., 1995). Reactivation from latency almost certainly occurs in the absence of VP16 and therefore cellular controls are thought to be crucial in this context (O’Rourke & O’Hare, 1993; Ralph et al., 1994). It has been shown that HSV-1 is reactivated from latency in cultured foetal rat neurons by transient treatment with cycloheximide (Wilcox et al., 1990) and the results presented here suggest that this effect is a consequence of activating HSV-1 IE promoters. In addition, a recent study speculates that stimulation of cellular IE gene expression may be an important prerequisite for reactivation of HSV-1 after explant of latently infected neurons (Tal-Singer et al., 1997). The signals that initiate reactivation are generally described as causing ‘stress’ to cells, so it is intriguing that IE promoters are positively regulated by inhibition of protein synthesis, a process which activates cellular responses mediated by SAPK.

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References


