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Herpesvirus quiescence in neuronal cells. V: Forskolin-responsiveness of the herpes simplex virus type 1 α0 promoter and contribution of the putative cAMP response element

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The herpes simplex virus (HSV)-1 α0 promoter contains a putative cAMP response element (CRE) located at positions −68 to −60 with respect to the initiation of transcription. In this report, the authors examined the functionality of this element using (1) luciferase reporter gene assays in nerve growth factor–differentiated (ND)–PC12 cells and (2) virus-induced activation from quiescently infected (QIF)-PC12 cells. The putative α0 CRE was completely eliminated by digestion with the restriction enzyme Tsp45I followed by mung bean nuclease treatment. The mutated region was verified by DNA sequencing and was inserted into the α0-luciferase reporter plasmid (pRDα0-LUC) creating (pRDα0ΔCRE-LUC), and into the HSV-1 genome of strain 17$^+$(α0ΔCRE). Insertion into both copies of the α0 promoter was verified by Southern blot analysis. ND-PC12 cells transfected with pRDα0-LUC and pRDα0ΔCRE-LUC plasmids responded similarly to forskolin (50 μM), with approximately 250% increases in luciferase activity compared to mock-treated cultures as measured 3 days following treatment. When QIF-PC12 cultures established with HSV-1 strain 17$^+$ and α0ΔCRE were treated with forskolin (50 μM) 17 days post infection, virus was detected in 9/24 (37.5%) and 13/24 (54.2%) of induced cultures by day 8 post treatment, respectively. In contrast, virus was detected in 0/23 and 1/24 (4.2%) of mock-treated cultures by day 8 post treatment for wild-type and mutant viruses, respectively. These findings indicate that the α0 promoter is forskolin responsive, the purported CRE of the α0 promoter does not confer forskolin responsiveness in ND-PC12 cells, and this element is not required for reactivation of HSV-1 from QIF-PC12 cells. *Journal of NeuroVirology* (2003) **9,** 489–497.

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Introduction

Herpes simplex virus type 1 (HSV-1) is a large DNA virus that establishes latency in sensory neurons for the life of the host. Reactivation of virus from latency and the resulting recurrent disease are the defining hallmarks of this pathogen (Stevens, 1989). During the lytic infection, the regulated cascade of gene transcription from immediate-early (IE, α), early (E, β),

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and late (L, γ) have been well defined (Honess and Roizman, 1974, 1975). In contrast, transcription of the viral genome during the latent state appears to be extremely repressed. Only the latency-associated transcripts (LATs) are detected in abundance (Stevens *et al*, 1988), and the sequence of events that regulate latency and reactivation remain obscure.

The process of reactivation from latency requires several steps that include transcription of viral (α and β) genes and replication of viral DNA. Reports indicate that transcription of α0 facilitates reactivation and is an early event in the process (Zhu *et al*, 1990; Minagawa *et al*, 1994; Halford *et al*, 1996, 2001; Tal-Singer *et al*, 1997; Halford and Schaffer, 2001). ICP0, encoded by the α0 gene from within the HSV-1 DNA long repeats, is a promiscuous *trans*-activator protein (Gelman and Silverstein, 1985; Everett, 1989; Smith and Cheung, 1998). It can *trans*-activate all three classes of HSV-1 promoters (Mavromara-Nazos *et al*, 1986; Cai and Schaffer, 1992; Chen and Silverstein, 1992) and activate previously silent gene expression in heterologous systems (Mosca *et al*, 1987; Nabel *et al*, 1988; Samaniego *et al*, 1998; Everett *et al*, 1998). The sufficiency of ICP0 for reactivation of HSV-1 in both *in vitro* and *in vivo* models of latency suggest its importance in reactivation (Russell *et al*, 1987; Everett, 1989; Leib *et al*, 1989; Gordon *et al*, 1990; Halford *et al*, 2001).

Many studies examining the role of α0 in reactivation from latency have utilized null mutants. Such viruses, lacking functional ICP0, replicate less efficiently and have reduced ability to reactivate from latency than their wild-type counterparts (Clements and Stow, 1989; Leib *et al*, 1989; Cai *et al*, 1993; Halford and Schaffer, 2001). Growth impairment of these mutants, however, complicates the interpretation of ICP0's involvement in initiating reactivation. To date, relatively few studies have examined the role of the α0 promoter in reactivation and its response to stress in neurons (Wheatley *et al*, 1992; Davido and Leib, 1996; Ralph *et al*, 1994) This is true despite the importance of stress in reactivation (Fraser and Valyi-Nagy, 1993; Miller *et al*, 1998).

The α0 promoter contains TAATGARAT motifs and several potential transcription factor–binding domains, including, but not limited to, activating transcriptor protein (ATP)/cAMP response element binding protein (CREB), CAAT-enhancer binding protein (C/EBP), GA binding protein (GABP), nuclear factor (NF)-κB, Olf-1, Oct-1, and Sp1 (Kristie and Roizman, 1988; Devireddy and Jones, 2000; Tsavachidou *et al*, 2001). The α0 TAATGARAT motifs are not required for efficient ICP0 promoter activity in mouse neuroblastoma cells (Devireddy and Jones, 2000) and sequences from -420 to -70 relative to the start site of transcription are unneeded for reactivation in mice (Davido and Leib, 1996). Downstream of the -70 site lies the putative cAMP response element (CRE) binding domain. CRE may be important for reactivation because agents that activate the adenylate cyclase pathway (i.e., epinephrine, forskolin, and PACAP) induce HSV-1 from a quiescent state *in vivo* (Leib *et al*, 1991; Bloom *et al*, 1994) and *in vitro* (Smith *et al*, 1992; Danaher *et al*, 1999b, 2001). The CRE site has been purported to be located between two Sp1 sites at positions -60 to -68, with its orientation opposite that of the promoter. This putative sequence has five of eight base matches with the consensus binding site for CREB/ATF (GTGACGTA/CA/G) (Roesler *et al*, 1988; Lin and Green, 1988). Sequences of the α0 promoter encompassing the CRE have been shown to bind cell extracts from ND7 cells in a manner similar to the human alpha-chorionic gonadotrophin CRE and confer induction by elevated cAMP levels in a transient transfection assay (Wheatley *et al*, 1992). However, the response of the promoter to activation via the protein kinase A (PKA) pathway appears to be cell and/or inducer specific (Davido and Leib, 1998).

Because the α0 gene is a potential initiator of reactivation and its promoter contains a CRE-like sequence, the significance of this site in activation from quiescently infected, neurally differentiated PC12 cells was investigated. These cells support HSV-1 in a cryptic (quiescent) state that is reversible by several induction stimuli (Danaher *et al*, 1999a, 1999b, 2001). In this report, the significance of the putative CRE site was evaluated following forskolin induction in reporter gene assays and in quiescently infected (QIF)-PC12 cells. The former system allowed us to assess the viral gene response to adenylate cyclase activating stimuli (i.e., forskolin) under neuronal conditions without the influence of other viral gene products. The latter permitted evaluation of the putative CRE site in a functional assay of reactivation from quiescence.

Results

Forskolin-induced HSV-1 activation is dose dependent

We previously demonstrated that agents that induce the PKA pathway (i.e., forskolin and PACAP) activate HSV-1 from QIF-PC12 cultures and that the level of virus production is reduced in the presence of a PKA inhibitor (Danaher *et al*, 2001). To further examine the inductive mechanisms of forskolin, we first determined the optimal concentration for activation of HSV-1. QIF cultures were established in 12-well plates as previously described (Danaher *et al*, 1999b) and nonproductive cultures were treated with increasing concentrations of forskolin on day 17 post infection (pi). Forskolin treatment induced virus activation in a dose-dependent manner (Figure 1). Peak induction occurred at 50 μM, with the majority of cultures (62%) producing detectable levels of virus by day 5 following forskolin treatment. This compared with the approximate 40% efficiency that resulted from 2 and 10 μM treatment and 13% efficiency at

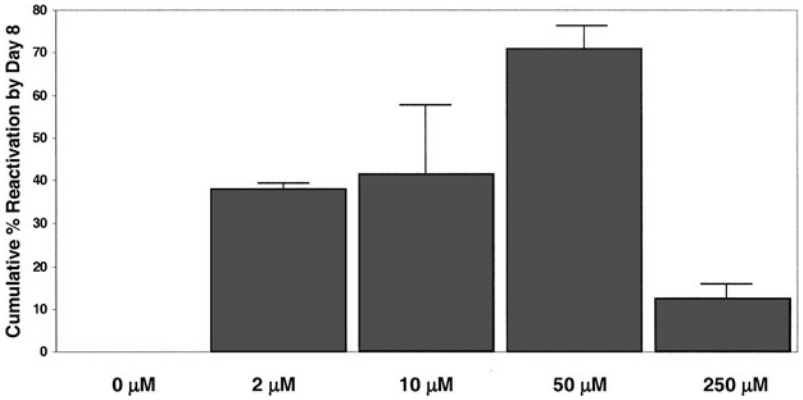

Figure 1 Effect of forskolin dose on HSV-1 activation from QIF. QIF-PC12 cells were established with strain 17^+ and treated with forskolin on day 17 pi. The cumulative proportion of quiescent cultures that reactivated virus during the 8 days following treatment as determined from culture supernatants in direct plaque assays are shown. Data represent the means of experiments performed in duplicate in 12-well plates per experimental group.

250 μM. The reduction at 250 μM appeared to be due to toxicity as culture viability deteriorated at this concentration.

The HSV-1 α0 promoter is forskolin responsive

Because expression of α0 facilitates reactivation from latency (Russell *et al*, 1987; Zhu *et al*, 1990; Cai *et al*, 1993; Leib *et al*, 1989; Halford and Schaffer, 2001) and the promoter has been shown to be responsive to PKA-dependent pathways (Wheatley *et al*, 1992), the response of this promoter to forskolin was assessed in neurally differentiated (ND)-PC12 cells. ND-PC12 cells support a cryptic HSV-1 genome (Danaher *et al*, 1999b), thus allowing for the assessment of α promoters under conditions that mimic aspects of latency. Preliminary studies in our lab have shown that α gene promoter activity is low and LAT promoter activity is high in ND-PC12 cells (data not shown). In this regard, this system more closely mimics the latent transcriptional state than other cell lines such as C1300 (Kemp *et al*, 1990; Mador *et al*, 1995; unpublished observation).

PC12 cells were seeded in T75 flasks in RPMI 1640 supplemented with nerve growth factor (NGF) and transfected with pRDα0-LUC (−800 *Sac*I to +154 *Nco*I region encompassing the α0 promoter; Figure 2) and the promoter-less plasmid pGL3-Basic as described in Materials and Methods. All transfections included cotransfection with the control renilla plasmid pRL-Null. Four days after transfection, cultures were induced with forskolin 50 μM. Firefly and renilla luciferase levels were readily detected in lysates from all cultures. The relative level of luciferase activity from pRDα0-LUC–transfected cell cultures increased threefold by day 3 following forskolin treatment, whereas pGL3-Basic–transfected cultures did not respond to forskolin treatment (data not shown).

The putative CRE sequence of the α0 promoter does not contribute to the response to forskolin

Our work and the work of others (Wheatley *et al*, 1992) demonstrate that the α0 gene promoter is activated in neuronal cells by agents that elevates levels of intracellular cAMP. A region within the α0 promoter encompassing a putative CRE site is capable of binding a transcription factor of the CREB/ATF family (Wheatley *et al*, 1992). However, the forskolin responsiveness of this site has not been shown. To examine the functionality of the purported CRE in the α0 promoter, the ND-PC12 transfection model was used. A reporter plasmid lacking the putative CRE (pRDα0ΔCRE-LUC) was generated by mung bean treatment of partially *Tsp*45I-digested pRDα0-LUC followed by ligation with T4 DNA ligase. The complete loss of the 5-base overhangs was verified by sequence analysis, indicating the entire loss of the CRE consensus nucleotides. PC12 cells were neurally differentiated and transfected with pRDα0-LUC, pRDα0ΔCRE-LUC, pGL3-Basic, and the renilla control plasmid pRL-Null. On day 3 post transfection, cultures were forskolin or sham treated for 3 days. Analysis of lysates by luminometry revealed that the deletion of the CRE site did not alter the basal level of the α0 promoter (data not shown). Activity of pRDα0ΔCRE-LUC was similar with that of pRDα0-LUC following forskolin treatment (Figure 3). In contrast, pGL3-Basic–transfected cultures did not respond to forskolin treatment. These results indicate that elimination of the putative CRE sequence did not alter the response of the promoter to treatment with forskolin, and that the forskolin response elements are located elsewhere in the promoter.

The purported α0 CRE sequence is not required for reactivation from QIF-PC12 cultures

To further examine the functionality of the putative α0 CRE sequence, the deletional mutant promoter

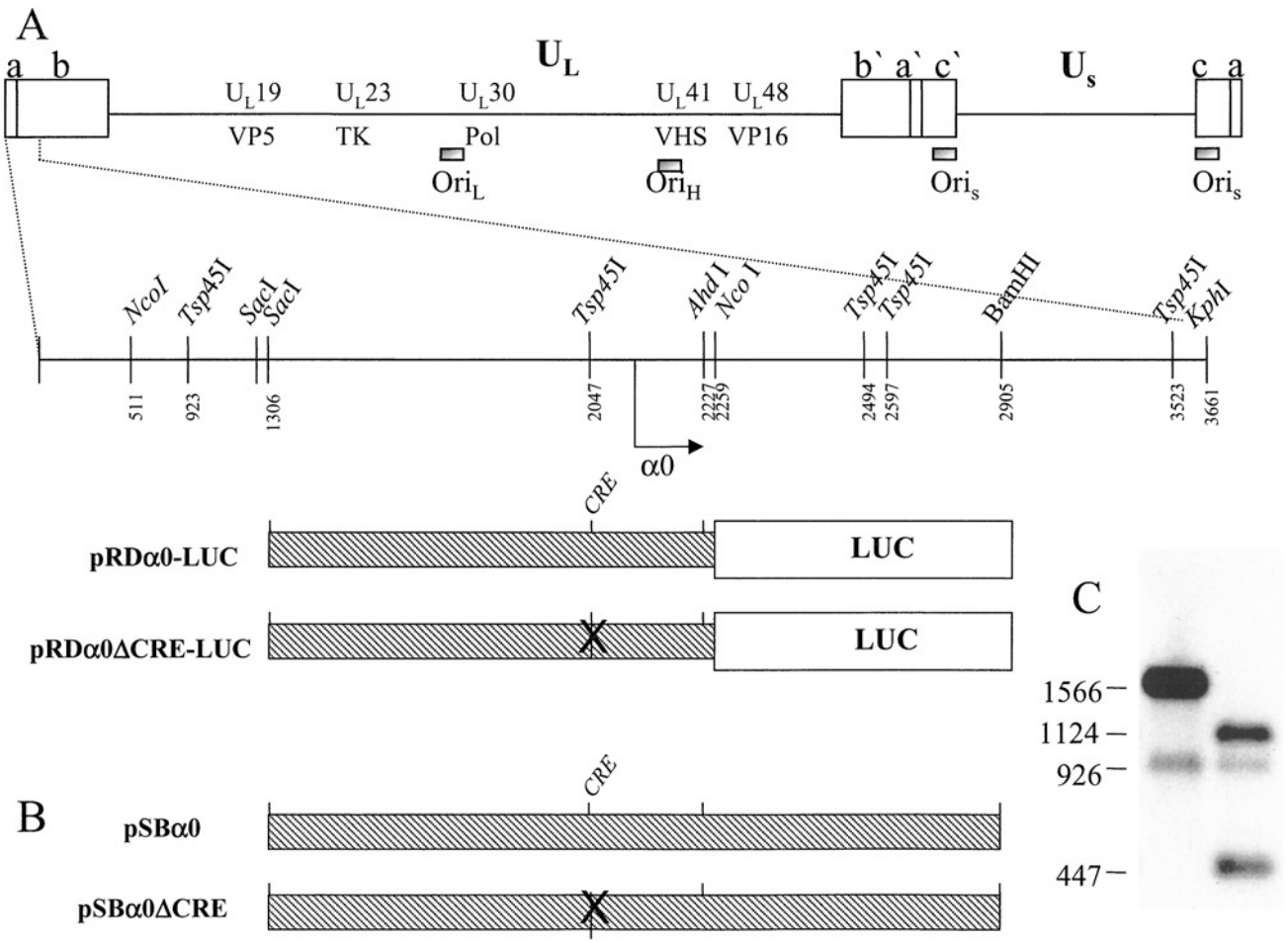

Figure 2 Schematic representation of HSV-1 and the construction of the expression plasmids and mutated region. (**A**) Map of the genome, enlargement of region containing the $\alpha 0$ promoter, and location of insert into luciferase plasmids. The numbers represent the base position from the left side of the genome. (**B**) Region used to generate the recombinant virus with deleted putative CRE. (**C**) Southern blot of the CRE deletion recombinant virus ($\alpha 0\Delta$CRE). Viral DNA isolated from Vero cells infected with $\alpha 0\Delta$CRE (*left lane*) and 17^+ (*right lane*) was digested with *Tsp*45I and electrophoresed on a 0.8% agarose gel. DNA was probed with a digoxigenin-labeled insert of pSB$\alpha 0$ as indicated in B.

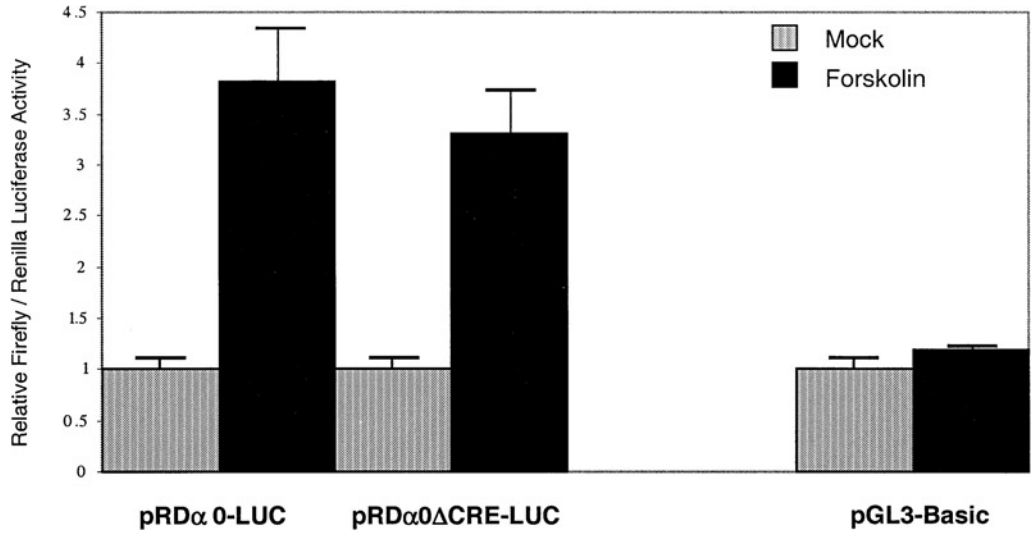

Figure 3 Analysis of forskolin responsiveness of the $\alpha 0$ promoter lacking the putative CRE binding site. The designated plasmids were transfected into ND-PC12 cells. Cells were cotransfected with pRL-Null, pooled, and replated into 35-mm wells. Cultures were treated on day 3 post replate. Three days after 50 μM forskolin induction, the ratio of the LUC to renilla activity was measured as described in the Material and Methods. Data shown represent the mean of triplicates from duplicate assays. Bars indicate the standard error of the means.

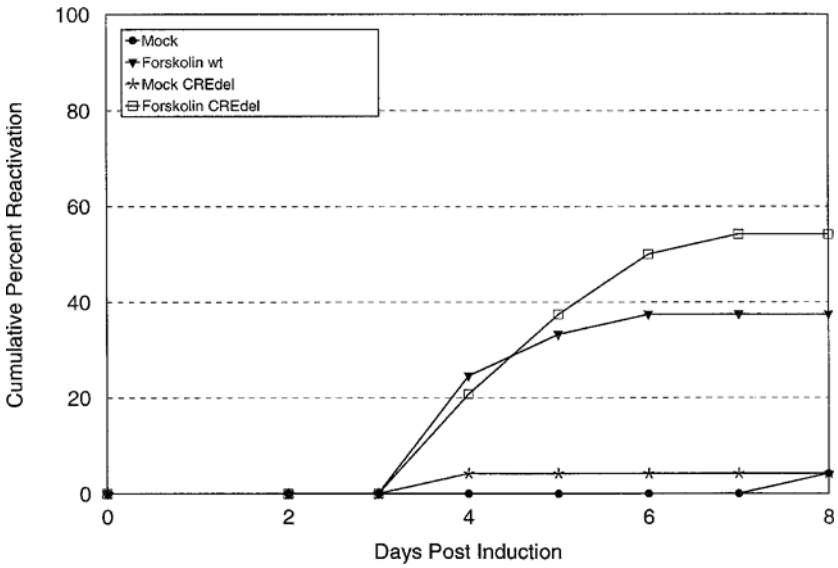

Figure 4 Reactivation of α0ΔCRE from QIF-PC12 cells in response to forskolin induction. QIF cultures were established in duplicate 12-well plates as described in the Material and Methods using the α0ΔCRE and wild-type virus. On day 17 pi, nonproductive cultures were induced with forskolin 50 μM. The cumulative proportion of quiescent cultures that reactivated virus during the days following treatment as determined from culture supernatants in direct plaque assays are shown.

was introduced into the viral genome as described in the Material and Methods and reactivation of the resulting recombinant (α0ΔCRE) from QIF-PC12 cultures was assessed. Southern blot analysis confirmed that the mutation was introduced into both copies of the viral genome (Figure 2). Analysis of the growth of α0ΔCRE in Vero cells revealed no difference from that of wild type (wt) virus (data not shown).

In as much as we have previously shown that reactivation efficiency correlates with multiplicity of infection (MOI) (Danaher *et al*, 1999a), we established QIF cultures with α0ΔCRE and wt virus at an MOI predicted to yield reactivation in 50% of cultures. On day 17 pi, nonproductive cultures were induced with forskolin 50 μM. Figure 4 shows that the α0ΔCRE reactivated from 54.2% (13/24) QIF cultures whereas wt viruses reactivated from (9/24) 37.5%, indicating that reactivation occurred with similar kinetics and efficiency as wt virus. We conclude that the putative α0 CRE is not needed in the forskolin response of the α0 promoter in transient transfection assays involving ND-PC12 cells and for efficient reactivation from QIF-PC12 cultures.

Discussion

This study examined the forskolin-responsiveness of the α0 promoter and the role of the promoter in HSV-1 activation from a quiescent state. The α0 gene encodes ICP0 a promiscuous *trans*-activator not essential for virus growth in tissue culture, but sufficient to induce HSV-1 reactivation from latently infected TG cell cultures (Russell *et al*, 1987; Halford *et al*, 2001). Defects in ICP0 have been shown to affect viral growth and reactivation (Cai *et al*, 1993; Clements and Stow, 1989; Leib *et al*, 1989; Halford and Schaffer, 2001). Few studies, however, have examined the role of the α0 promoter in response to stress and reactivation (Wheatley *et al*, 1992; Davido and Leib, 1996, 1998; Devireddy and Jones, 2000; Lu and Misra, 2000; Tsavachidou *et al*, 2001). In this study, the α0 promoter was shown to be forskolin responsive in neuronal cells. ND-PC12 cells support HSV-1 in a cryptic state and permit activation of virus following forskolin treatment. We deleted the putative CRE element of the α0 promoter and inserted the mutated regions into the α0-LUC fusion plasmid and the genome of strain 17^+. Loss of the putatitve CRE site had no demonstrable effect on forskolin induction of the α0 promoter as assessed by transient transfection experiments in ND-PC12 cells. The mutation, present in both copies of the α0 promoter, also did not alter the reactivation kinetics following forskolin-induced reactivation.

The mechanism by which cellular stress signals (e.g., forskolin) and the PKA pathway trigger HSV-1 reactivation are not yet understood. The proposed pathway involves adenylate cyclase activation, cAMP migration, PKA activation, CREB binding to CRE, and increased transcription. cAMP and the PKA pathway accelerate reactivation (Smith *et al*, 1992; Bloom *et al*, 1994; Leib *et al*, 1991; Davido and Leib, 1996; Danaher *et al*, 2001), and the CRE in the LAT promoter has been reported to be important in reactivation from latently infected rabbits and mice (Bloom *et al*, 1997; Marquart *et al*, 2001). Because (1) the α0 gene promoter has been reported to demonstrate increased activity following a rise in intracellular levels of cAMP (Wheatley *et al*, 1992), (2) the *cis*-elements

between −70 and the transcription start site may up-regulate ICP0 during reactivation (Davido and Leib, 1998), and (3) the region has been reported to contain a CRE (Wheatley *et al*, 1992; Lu and Misra, 2000), it was important to test the function of this element in reactivation.

The putative CRE is located between −60 and −68. The sequence in the putative α0 CRE (TGACGATT) has a 5 of 8 base match with the consensus CRE (TGACGTCA) (Roesler *et al*, 1988). The lack of sequence homology may explain its lack of activity in the assays performed. Alternatively, the cAMP response unit of the α0 promoter (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001) may involve sequences approximating the region, be cell type specific, or be facilitated by interaction with a accessory (viral or virally induced cellular) protein not activated during transient transfection assays in ND-PC12 cells (Lu and Misra, 2000).

Our findings, in part, support the finding of Wheatley *et al* (1992) in that the HSV-1 α0 promoter contains elements that confer forskolin responsiveness. However, the putative CRE did not confer the response. The findings that putative CRE sequence had no demonstrable role in forskolin-induced activation of the α0 promoter and did not affect forskolin-induced reactivation are consistent with the findings of Davido and Leib (1998) who reported that the promoter does not bind to purified CREB. However, it contrasts with their data showing the α0 promoter was unresponsive to a cAMP analog. The difference may be explained by the fact that in our study PC12 cells were neurally differentiated and the inducer was forskolin. We have found that induction of the α0 promoter is greater with forskolin treatment than cAMP analogs (e.g., CPT-cAMP and 8-bromo-cAMP) and when treatment is for 2 or 3 days versus 1 day (data not shown). Although the elements conferring the response are not yet identified, our findings support the hypothesis that HSV-1 reactivation may result from the up-regulation of ICP0 by the PKA pathway. Current studies in our laboratory are ongoing to identify the regions that confer the response and the mechanisms involved.

Material and methods

Virus and cells

Rat pheochromocytoma (PC12) and Vero cells were grown as previously described (Danaher *et al*, 1999a). HSV-1 strain 17^+ was a kind gift of N. Fraser (Wistar Institute, Philadelphia, PA). Viral stocks were prepared in Vero cells and maintained at −85°C. Virus production was determined using supernatants from infected cultures in a direct plaque assay (DPA) on monolayers of Vero cells as previously described (Miller and Smith, 1991).

Morphologic differentiation

Differentiated PC12 cells were maintained in RPMI 1640 supplemented with 0.1% bovine serum albumin (BSA), fraction V, and 50 ng/ml of 2.5S mouse NGF (Becton Dickinson, Franklin Lakes, NJ) (maintenance medium) beginning on the day of plating. Cells were plated, following two rinses with RPMI 1640 containing 0.1% BSA and dissociation by passage through a 22-guage needle, in 12-well tissue culture dishes (Becton Dickinson) coated with rat tail collagen type 1 (Becton Dickinson) at 2.2×10^5 cells/well in maintenance medium as previously described (Danaher *et al*, 1999b). Morphologic differentiation was confirmed by microscopic visualization of dendritic processes. Medium was changed every 3 days unless indicated.

Establishment of a quiescent infection (QIF-PC12)

Neurally differentiated PC12 cells (ND-PC12) were infected with virus at a MOI of 5 to 10 unless otherwise indicated, in a volume of 0.4 ml/well in 12-well plates, without agitation, overnight at 37°C. Acycloguanosine (ACV) 100 μM (Sigma; St. Louis, MO) was maintained in the medium from 1 day prior to infection to 10 days pi. After ACV withdrawal, a quiescent state (i.e., free of detectable infectious virus in culture supernatants) was maintained for 7 days prior to induction.

Activation stimuli

HSV-1 QIF-PC12 cells that were free of detectable infectious virus were subjected to maintenance medium supplemented with forskolin (Sigma) prepared in DMSO (Sigma) as recommended by Huang *et al* (1982).

Plasmids

The regions of the HSV genome and reporter plasmid relevant to this work are illustrated in Figure 2. Plasmid pRDα0-LUC was generated by replacing the *Sac*I-*Sma*I fragment of reporter plasmid pGL3-Basic with the −800 (*Sac*I) to +154 (*Nco*I) HSV region encompassing the α0 promoter. Note that the *Nco*I end was altered by mung bean nuclease treatment. Sequencing of the *Nco*I:*Sma*I junction revealed that only two of the four base overhangs of the *Nco*I end were eliminated and that the 5′ C residue of the *Sma*I end of the vector was lost, resulting in the junction sequence of ACGACCCCCAggctcgag, with HSV and vector sequence in upper and lower case, respectively. A reporter plasmid lacking the putative CRE, pRDα0ΔCRE-LUC, was generated by mung bean treatment of partially *Tsp*45I-digested pRDα0-LUC, followed by ligation with T4 DNA ligase. The complete loss of the five base overhangs was verified by sequence analysis, indicating the entire loss of the CRE consensus nucleotides. Plasmid pSBα0 contains the 1.6-kb *Sac*I *BamH*I fragment, encompassing

the $\alpha 0$ promoter in the *Sac*I and *BamH*I sites of the cloning vector pUC19. Plasmid pSBα0ΔCRE was generated by replacing the 925-bp *Sac*I-*Ahd*I fragment of pSBα0 with the mutated fragment of pRDα0ΔCRE-LUC.

Luciferase assay

Transfection of PC12 was performed with LipofectAmine (GIBCO) as recommended by the manufacturer, with slight modifications. Briefly, nearly confluent PC12 cells were rinsed twice with RPMI 1640 maintenance medium lacking NGF and collected into maintenance medium. Cells were passed through a 22-gauge needle 12 times and seeded into rat-tail collagen-coated T75 flasks at 8×10^6 cells per flask in RPMI 1640 medium supplemented with NGF only. The following day, cells were cotransfected with 3.9 μg of each of the indicated experimental reporter plasmids and pRL-Null control plasmid in the presence of 39 μl LipofectAmine. Transfections were performed in RPMI 1640 medium supplemented solely with NGF. Following incubation at 37°C for 5 h, an equal volume of RPMI 1640 medium supplemented with 5% fetal bovine serum, 10% heat-inactivated horse serum, 100 units/ml penicillin, and 100 μ/ml streptomycin (PS) and NGF was added and incubation continued overnight. The following day, cells were washed once with maintenance medium and collected by scraping in maintenance media and reseeded to three collagen-coated 6-well dishes per flask.

Cultures were treated as indicated 3 days following replate and harvested 3 days later in passive lysis buffer (Promega). Lysates were stored at −85°C until use. Measurements of firefly and renilla luciferase activity present in each lysate was determined with the Dual-Luciferase Reporter Assay System (Promega) using a Sirius Luminometer equipped with dual injectors. Data were displayed as ratios of relative light units (RLU) of firefly luciferase to RLU of renilla luciferase activities relative to mock-treated cultures. In all cases, the level of activity of both reporters was in the linear range of analysis.

Recombinant virus

A recombinant virus containing the deleted CRE sequence was generated and identified as follows. Plasmid pSBα0ΔCRE was linearized with *Pst*I and transfected with HSV-1 strain 17^+ purified DNA into Vero cells using LA2000 (Gibco). DNA was isolated from individual transfectants and analyzed for the mutant $\alpha 0$ promoter by polymerase chain reaction (PCR) with primers specific for the mutant sequence. The primer sequences were α0CRE-1: TGGGCAACCCCGGTATTC; α0CRE-2: CCAAAGGGGCGGCAGATTC, with the five nucleotides of the 3′ end of the α0CRE-2 primer being specific only for template containing the deleted CRE sequence. Introduction of the mutation into both copies of the genome was verified by Southern blot analysis. The CRE mutant recombinant virus (α0ΔCRE), was triple plaque purified and the reactivation phenotype was assessed in QIF-PC12 cultures.

References

Bloom DC, Devi-Rao GB, Hill JM, Stevens JG, Wagner EK (1994). Molecular analysis of herpes simplex virus type 1 during epinephrine-induced reactivation of latently infected rabbits in vivo. *J Virol* **68:** 1283–1292.

Bloom DC, Stevens JG, Hill JM, Tran RK (1997). Mutagenesis of a cAMP response element within the latency-associated transcript promoter of HSV-1 reduces adrenergic reactivation. *Virology* **236:** 202–207.

Cai W, Astor TL, Kiptak LM, Cho C, Coen DM, Schaffer PA (1993). The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. *J Virol* **67:** 7501–7512.

Cai W, Schaffer P (1992). Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. *J Virol* **66:** 2904–2915.

Chen J, Silverstein S (1992). Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. *J Virol* **66:** 2916–2927.

Clements GB, Stow ND (1989). A herpes simplex virus type 1 mutant containing a deletion with immediate early gene 1 is latency-competent in mice. *J Gen Virol* **70:** 2501–2506.

Danaher RJ, Jacob RJ, Chorak MD, Freeman CS, Miller CS (1999a). Heat stress induce reactivation of herpes simplex virus type 1 from quiescently infected neurally differentiated PC12 cells. *J NeuroVirol* **5:** 374–383.

Danaher RJ, Jacob RJ, Miller CS (1999b). Establishment of a quiescent herpes simplex virus type 1 infection in neurally differentiated PC12 cells. *J NeuroVirol* **5:** 258–267.

Danaher RJ, Savells-Arb A, Black SA Jr, Jacob RJ, Miller CS (2001). Herpesvirus quiescence in neuronal cells. IV: virus activation induced by pituitary adenylate cyclase-activating polypeptide (PACAP) involves the protein kinase A pathway. *J NeuroVirol* **7:** 163–168.

Davido DJ, Leib DA (1996). Role of cis-acting sequences of the ICP0 promoter of herpes simplex virus type 1 in viral pathogenesis, latency and reactivation. *J Gen Virol* **77:** 1853–1863.

Davido DJ, Leib DA (1998). Analysis of the basal and inducible activities of the ICPO promoter of herpes simplex virus type 1. *J Gen Virol* **79:** 2093–2098.

Devireddy LR, Jones CJ (2000). Olf-1, a neuron-specific transcription factor, can activate the herpes simplex virus type 1-infected cell protein 0 promoter. *J Biol Chem* **275:** 77–81.

Everett R (1989). Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J Gen Virol* **70:** 1185–1202.

Everett RD, Orr A, Preston CM (1998). A viral activator of gene expression functions via the ubiquitin-proteasome pathway. *EMBO J* **17:** 7161–7169.

Fraser NW, Valyi-Nagy T (1993). Viral, neuronal and immune factors which may influence herpes simiplex virus (HSV) latency and reactivation. *Microb Pathogen* **15:** 83–91.

Gelman IH, Silverstein S (1985). Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc Natl Acad Sci U S A* **82:** 5265–5269.

Gordon YJ, McKnight JL, Ostrove JM, Romanowski E, Araullo-Cruz T (1990). Host species and strain differences affect the ability of an HSV-1 ICP0 deletion mutant to establish latency and spontaneously reactivate in vivo. *Virology* **178:** 469–477.

Halford WP, Gebhardt BM, Carr DJ (1996). Mechanisms of herpes simplex virus type 1 reactivation. *J Virol* **70:** 5051–5056.

Halford WP, Kemp CD, Isler JA, Davido DJ, Schaffer PA (2001). ICP0, ICP4, or VP16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. *J Virol* **75:** 6143–6153.

Halford WP, Schaffer P (2001). ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. *J Virol* **75:** 3240–3249.

Honess RW, Roizman B (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14:** 8–19.

Honess RW, Roizman B (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc Natl Acad Sci U S A* **72:** 1276–1280.

Huang RD, Smith MF, Zahler WL (1982). Inhibition of forskolin-activated adenylate cyclase by ethanol and other solvents. *J Cyclic Nucleotide Res* **8:** 385–394.

Kemp LM, Gelman IH, Silverstein SJ, Latchman DS (1990). Regulation of herpes simplex virus immediate-early gene promoters in mouse neuroblastoma cells. *Neurosci Lett* **118:** 185–188.

Kristie T, Roizman B (1988). Differentiation and DNA contact points of host proteins binding at the cis site for virion-mediated induction of alpha genes of herpes simplex virus 1. *J Virol* **62:** 1145–1157.

Leib DA, Coen DM, Bogard CL, Hicks KA, Yager DR, Knipe DM, Tyler KL, Schaffer PA (1989). Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *J Virol* **63:** 759–768.

Leib DA, Nadeau KC, Rundle SA, Schaffer PA (1991). The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. *Proc Natl Acad Sci U S A* **88:** 48–52.

Lin Y-S, Green MR (1988). Interaction of a common cellular transcription factor with regulatory elements in both Ela and cyclic AMP inducible promoters. *Proc Natl Acad Sci U S A* **85:** 3396–3400.

Lu R, Misra V (2000). Potential role for luman, the cellular homologue of herpes simplex virus VP16 (A gene transinducing factor), in herpesvirus latency. *J Virol* **74:** 934–943.

Mador N, Panet A, Latchman D, Steiner I (1995). Expression and splicing of the latency-associated transcripts of herpes simplex virus type 1 in neuronal and non-neuronal cell lines. *J Biochem (Tokyo)* **117:** 1288–1297.

Marquart ME, Zheng X, Tran RK, Thompson HW, Bloom DC, Hill JM (2001). A cAMP response element within the latency-associated transcript promoter of HSV-1 facilitates induced ocular reactivation in a mouse hyperthermia model. *Virology* **284:** 62–69.

Mavromara-Nazos P, Silver S, Hubenthal-Voss J, McKnight JL, Roizman B (1986). Regulation of herpes simplex virus 1 genes: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late (gamma 2) promoters. *Virology* **149:** 152–164.

Mayr B, Montminy M (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* **2:** 599–609.

Miller CS, Danaher RJ, Jacob RJ (1998). Molecular aspects of herpes simplex virus I latency, reactivation and recurrence. *Crit Rev Oral Biol Med* **9:** 541–562.

Miller CS, Smith KO (1991). Enhanced replication of herpes simplex virus type 1 in human cells. *J Dent Res* **70:** 111–117.

Minagawa H, Tanaka S, Toh Y, Mori R (1994). Detection of herpes simplex virus type1-encoded RNA by polymerase chain reaction: different pattern of viral RNA detection in latently infected murine trigeminal ganglia following in vitro or in vivo reactivation. *J Gen Virol* **75:** 647–650.

Mosca JD, Bednarik DP, Raj NB, Rosen CA, Sodroski JG, Haseltine WA, Hayward GS, Pitha PM (1987). Activation of human immunodeficiency virus by herpesvirus infection: identification of a region within the long terminal repeat that responds to a trans-acting factor encoded by herpes simplex virus 1. *Proc Natl Acad Sci U S A* **84:** 7408–7412.

Nabel GJ, Rice SA, Knipe DM, Baltimore D (1988). Alternative mechanisms for activation of human immunodeficiency virus enhancer in T cells. *Science* **239:** 1299–1302.

Ralph WM Jr, Cabatingan MS, Schaffer PA (1994). Induction of herpes simplex virus type 1 immediate-early gene expression by a cellular activity expressed in Vero and NB41A3 cells after growth arrest-release. *J Virol* **68:** 6871–6882.

Roesler WJ, Vandenbark GR, Hanson RW (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J Biol Chem* **263:** 9063–9066.

Russell J, Stow ND, Stow EC, Preston CM (1987). Herpes simplex virus genes involved in latency in vitro. *J Gen Virol* **68:** 3009–3018.

Samaniego LA, Neiderhiser AL, DeLuca NA (1998). Persistence and expression of the herpes simplex virus genome in the absence of immediate early proteins. *J Virol* **72:** 3307–3320.

Shaywitz AJ, Greenberg ME (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* **68:** 821–861.

Smith RL, Pizer LI, Johnson EM Jr, Wilcox CL (1992). Activation of second-messenger pathways reactivates latent herpes simplex virus in neuronal cultures. *Virology* **188:** 311–318.

Smith TA, Cheung A (1998). Herpes simplex virus type 1 ICP-0 induces reactivation of pseudorabies virus from latently infected trigeminal ganglia explant cultures. *Arch Virol* **143:** 591–599.

Stevens J (1989). Human herpesviruses: a consideration of the latent state. *Microbiol Rev* **53:** 318–332.

Stevens JG, Haarr L, Porter DD, Cook ML, Wagner EK (1988). Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. *J Infect Dis* **158:** 117–123.

Tal-Singer R, Lasner TM, Podrzucki W, Skokotas A, Leary JJ, Berger SL, Fraser NW (1997). Gene expression during reactivation of herpes simplex virus type 1 from latency in the peripheral nervous system is different from that during lytic infection of tissue cultures. *J Virol* **71:** 5268–5276.

Tsavachidou D, Podrzucki W, Seykora J, Berger SL (2001). Gene array analysis reveals changes in peripheral nervous system gene expression following stimuli that result in reactivation of latent herpes simplex virus type 1: induction of transcription factor Bcl-3. *J Virol* **75:** 9909–9917.

Wheatley SC, Dent CL, Wood JN, Latchman DS (1992). Elevation of cyclic AMP levels in cell lines derived from latently infectable sensory neurons increases their permissivity for herpes virus infection by activating the viral immediate-early 1 gene promoter. *Mol Cell Brain Res* **12:** 149–154.

Zhu XX, Chen JX, Young CS, Silverstein S (1990). Reactivation of latent herpes simplex virus by adenovirus recombinants encoding mutant EI-0 gene products. *J Virol* **64:** 4489–4498.