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CELL-TYPE-SPECIFIC INDUCTION OF THE UL9 GENE OF HSV-1 BY CELL SIGNALING PATHWAY

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The origin-binding protein, encoded by the UL9 gene of herpes simplex virus-1 (HSV-1), has the properties of an initiator of DNA replication. In this communication, we report that the UL9 promoter contains a cAMP-response element (CRE). Transient expression analyses show that dibutyryl cyclic AMP, known to elevate intracellular cAMP level, can induce the UL9 promoter in a rat pheochromocytoma cell line (PC12) but not in a non-neuronal human cell line (HeLa). Interestingly, a transcription factor that increases expression of a neuropeptide gene by interacting with CRE can also activate the UL9 promoter independent of cell type. Thus, our data suggest that extracellular stimuli, capable of interacting with the signaling pathway in neuronal cells, can activate UL9 gene expression, and different proteins may regulate UL9 expression in different cell types.

Among seven viral genes required for origin-dependent replication of HSV-1, only the product of the UL9 gene specifically recognizes the viral origin of replication (1-3), and acts as the initiator. Since origin recognition is one of the earliest events in DNA replication, the initiation of HSV-1 replication and the onset of the viral lytic cycle, in turn, should depend on the UL9 gene expression. The UL9 ptomoter is activated by viral immediate-early gene products (4). Involvement of any other viral or host transcription factors in the expression of the UL9 gene in neuronal and non-neuronal cells has not been reported. Previously (4) we have reported that the upstream 395-bp of the UL9 gene contains all the start sites for its mRNAs, and perform as a promoter in transient assays (4)

Sequence analysis indicated a cAMP response element (CRE) 68-bp upstream of the UL9 translation initiation site. This sequence is identical to the CRE present in various eukaryotic promoters, e.g., the promoter of the human proenkephalin gene (5), a neuropeptide, substance P precursor gene (6), human c-*fos* gene (7) and the latency associated transcript (LAT) gene of HSV-1 (8). Extracellular stimuli, such as growth factors, generate intracellular

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signals that often activate specific protein kinases (9,10). Dibutyryl cyclic AMP (Bt_2cAMP) activates protein kinase A (PKA) by elevating the intracellular cAMP level directly. The protein kinases are known to regulate gene transcription by modulating the phosphorylation state of transcription factors. The transcription factors that are the final targets of the protein kinases regulate transcription by interacting with CRE or CRE-like sequences (11,12). Interestingly, HSV remains latent in neuronal cells, and the latent virus gets reactivated under various stimuli (13,14). The presence of a CRE within an upstream sequence of the UL9 gene thus led us to investigate the regulation of UL9 gene expression in cells of neuronal and non-neuronal origin and the role of cellular signal transduction pathway in the process.

In this communication, using the UL9 promoter-chloramphenicol acetyl transferase (CAT) constructs we demonstrate induction of the UL9 promoter in Bt_2cAMP-treated PC12 cells but not in treated HeLa cells. This observation suggests the involvement of a cell type-specific transcription factor, modulated by the signaling pathway, in the expression of the UL9 gene in neuronal cells. A ubiquitous CAAT/enhancer binding protein (C/EBP)-like factor has been shown to enhance transcription by interacting with a CRE in neuronal cells (6). This transcription factor also induces the UL9 promoter. These data suggest that multiple host transcription factors are involved in cell type-specific and cell type-independent induction of the UL9 gene. To our knowledge this is the first report showing modulation of UL9 gene expression by cell-type specific signal transduction pathways.

MATERIALS AND METHODS

Cell culture. Vero, HeLa and PC12 cells were obtained from ATCC and were maintained as prescribed by the company.

Plasmid construction. The plasmids pCAT.A9 and pCAT.A9-Pst have been described earlier (4). pCAT.A9-Sac and pCAT.Sac-Pst were cloned by deleting the SacI-EcoRV, and XbaI-SacI fragments respectively, from pCAT.A9. The plasmids pEK4 (15) and pIGA15 (16, 17) were gifts from Drs. David Knipe and Gary Hayward, respectively. The plasmids pEK4 and pIGA15 can express viral immediate-early proteins IE175 (ICP4) and IE110 (ICP0), respectively. The plasmid pSVT7CELF (6) was a gift from Dr. Ryoichiro Kageyama, and can express CELF.

Transfection and CAT assay. Transfection and CAT assay procedures, including the use of the pSV β-gal to normalize transfection efficiencies, were as described earlier (4). As needed, cells were treated with 1 mM dibutyryl cyclic AMP (Bt_2cAMP) in fresh media 15 hours after transfection. The CAT assays were quantitated by cutting the spots from thin layer chromatography paper and measuring the radioactivity by liquid scintillation counting. All transfection experiments were repeated several times. Qualitatively similar results were obtained in all cases. Representative examples are depicted in the figures.

RESULTS

Bt_2cAMP, a protein kinase A activator, can induce the UL9 promoter in PC12 but not in HeLa cells. To investigate whether the CRE on the UL9 promoter (4) responds to cAMP, a

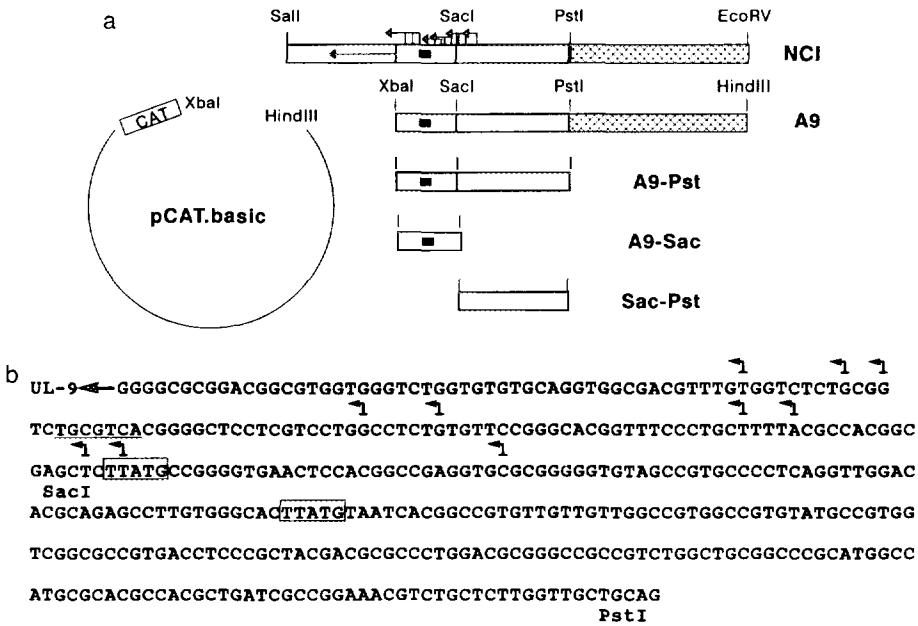

Figure 1. **(a)** Schematic representation of CAT plasmids used in this study. NCI is the HSV-1 sequence Sal I (22875) to EcoR V (24982) fragment from HSV-1. The rectangle with a long arrow is part of the UL9 reading frame. The arrow begins at the translation initiation site of the UL9 gene and shows the direction of the gene. This part of the UL9 coding region was deleted to isolate the 395-bp upstream sequence. The smaller arrows upstream of the UL9 coding region represent the transcription start sites. The black box represents the CRE. The restriction sites are shown. The figure is not drawn to scale. **(b)** The 395-bp UL9 upstream sequence. The arrows indicate the transcription start sites obtained from the experiment previously reported (4). The CRE is shown by underline. The 5'-CATAA-3' sequences are shown by boxes.

cAMP-responsive cell line (PC12) was transfected with pCAT.A9-Pst (Figure 1) in the presence and absence of the plasmids that express viral immediate-early proteins. PC12 cells are neural crest derived and show characteristics of sympathetic neurons (9). The transfected PC12 cells were then treated with 1 mM Bt_2cAMP that increases the intracellular cAMP level directly to activate cellular PKA (9). The results of CAT assays show that treatment with Bt_2cAMP increases CAT expression from pCAT.A9-Pst about 4-fold (Figure 2a). This suggests that UL9 gene expression may be controlled by a transcription factor modulated by a cAMP-dependent protein kinase of the cell signaling pathway (14,23). Interestingly, in PC12 cells the presence or absence of viral immediate-early proteins did not make a significant difference in the level of CAT expression. Our data suggests that either the immediate-early proteins are not expressed in sufficient amount to induce UL9 gene expression in PC12 cells, or that the function of the immediate-early proteins is inhibited in this cell line. These data show that Bt_2cAMP can induce CAT expression from pCAT.A9-Pst in the absence of viral immediate-early proteins.

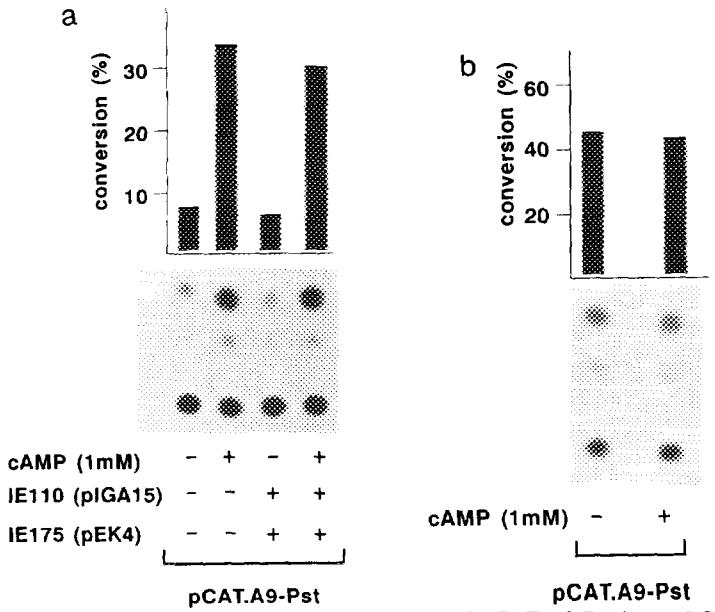

Figure 2. Transfection and transient expression analysis of pCAT.A9-Pst in (a) PC12 and (b) HeLa cells. Additions are shown at the bottom. Addition of Bt_2cAMP is shown as cAMP in the figure. The bar graph shows the percentage of input radioactive chloramphenicol acetylated in each case.

To determine if induction by Bt_2cAMP is found in a cAMP-responsive non-neuronal cell line, pCAT.A9-Pst was transfected into HeLa cells, and the cells were then treated with Bt_2cAMP. This treatment did not increase CAT expression from pCAT.A9-Pst (Figure 2b). Thus, Bt_2cAMP can induce CAT expression from pCAT.A9-Pst in PC12 cells but not in HeLa cells. These results suggest that the cAMP-mediated induction of pCAT.A9-Pst may be a cell type-specific phenomenon, not exhibited by cAMP-responsive HeLa cells. The results also show that basal CAT expression by pCAT.A9-Pst is higher in HeLa cells than in PC12 cells, suggesting a constitutive response of pCAT.A9-Pst to Bt_2cAMP in HeLa cells.

Bt_2cAMP activates reporter gene expression by the UL9 upstream sequences that harbor the CRE. To delimit sequences required for cAMP-mediated induction, the A9-Pst fragment was divided into two fragments at the Sac I restriction site (Figure 1) and cloned into pCAT.basic to produce pCAT.A9-Sac and pCAT.Sac-Pst (Figure 1). The A9-Sac segment has the CRE. The Sac-Pst fragment does not contain a similar sequence element (Figure 1). Since both the fragments (A9-Sac and Sac-Pst) have transcription start sites (Figure 1b) (4), both the fragments were analyzed for their ability to express the CAT reporter. To determine the effect of cAMP on CAT expression from A9-Sac and Sac-Pst, PC12 cells were transfected with pCAT.A9-Sac or pCAT.Sac-Pst, followed by treatment with 1 mM Bt_2cAMP. The results showed that the cells transfected with pCAT.A9-Sac have higher CAT activity than cells

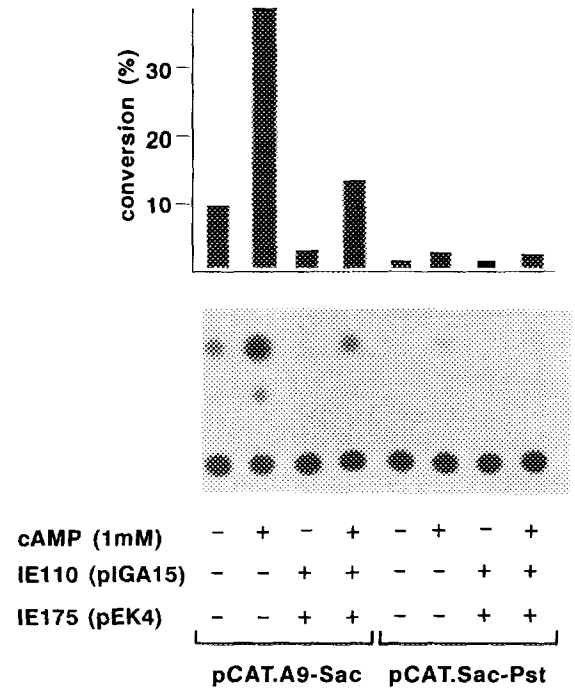

Figure 3. Transfection and transient expression analysis of pCAT.A9-Sac and pCAT.Sac-Pst in PC12 cells. Additions are shown at the bottom. The bar graphs show the percentage of radioactive chloramphenicol acetylated. The treatment with Bt_2cAMP is shown by cAMP.

transfected with pCAT.Sac-Pst. The presence of pEK4 and pIGA15 did not increase the CAT activity any further (Figure 3b). Cells transfected with pCAT.A9-Sac also showed about a 4-fold induction with cAMP in the absence of pEK4 or pIGA15 (Figure 3b). Cells transfected with pCAT.Sac-Pst did not show appreciable induction with Bt_2cAMP. Since Bt_2cAMP was able to induce pCAT.A9-Sac (Figure 3b) to the same level as pCAT.A9-Pst (Figure 2a), we conclude that sequences from Sac I to Pst I (HSV-1 sequences 23400 to 23655) are dispensable for cAMP-induction. The small increase in CAT expression of pCAT.Sac-Pst in the presence of Bt_2cAMP is probably due to a higher transcriptional activity as a result of expression of cellular immediate-early genes (14). The Bt_2cAMP-induced CAT expression from pCAT.A9-Sac, however is about 20-fold higher than that of pCAT.Sac-Pst (Figure 3b).

A C/EBP-like factor (CELF) activates the UL9 promoter in PC12 and HeLa cells. The CRE sequences present in various eukaryotic genes have been shown to interact with CCAAT/enhancer binding protein (C/EBP) or C/EBP-like factors to induce transcription (18). It has been reported that a C/EBP-like factor (CELF) interacts with a CRE on the promoter of substance P precursor gene to enhance transcription (6). Since CELF-mediated transcriptional control has been shown for a gene expressed in neuronal cells, and since the sequence element conferring this response is identical to the CRE present in the upstream region of the UL9 gene,

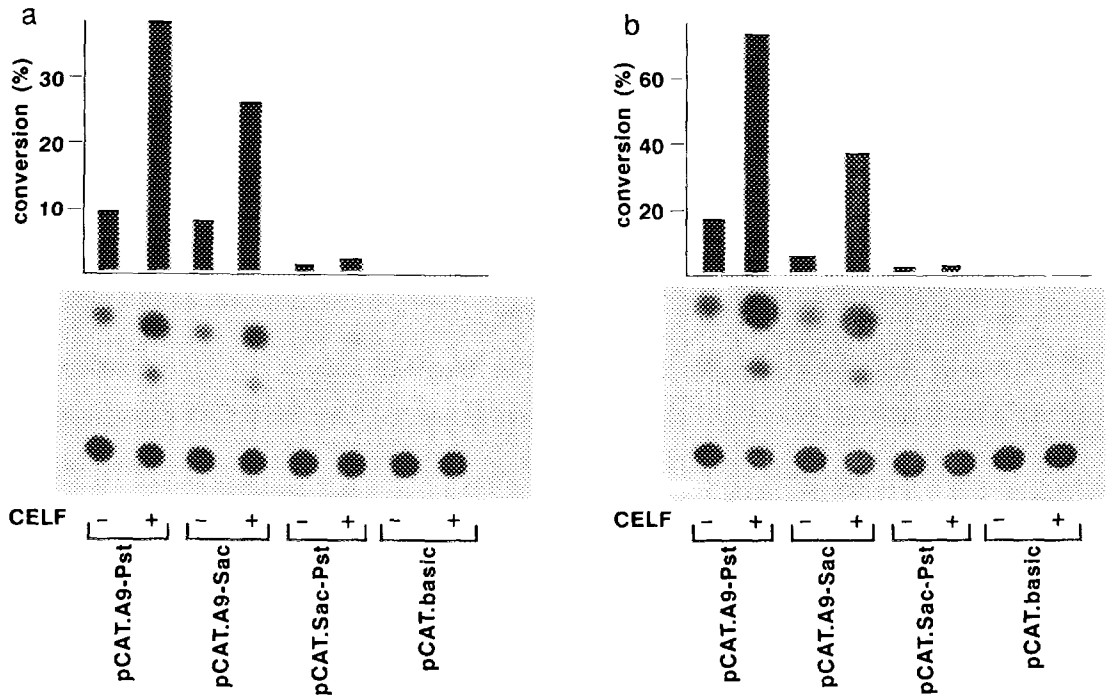

Figure 4. Effect of CELF on CAT expression by pCAT.A9-Pst, pCAT.A9-Sac, and pCAT.Sac-Pst in (a) PC12 and (b) HeLa cells. The addition of plasmid pSVT7CELF (plasmid that expresses CELF) is shown as CELF in these figures.

we tested possible effects of CELF on UL9 gene expression in PC12 cells. PC12 cells were cotransfected with pCAT.A9-Pst and a plasmid expressing CELF (pSVT7CELF). Cotransfection with pSVT7CELF enhanced CAT activity 4-to 5-fold (Figure 4a). Plasmid pCAT.A9-Sac, which contains the CRE, showed a similar induction of CAT activity. The plasmid pCAT.Sac-Pst, which lacks CRE, did not show any induction. This experimental result indicates that the transcription factor CELF induces the UL9 promoter in PC12 cells. As in the case of induction by Bt_2cAMP (Figure 3b), the sequences from Sac I to Pst I sites are dispensable for this effect (Figure 4a).

The constitutive response of pCAT.A9-Pst to Bt_2cAMP in HeLa cells (Figure 2b) led us to investigate whether the C/EBP-like factor, CELF, would generate a similar constitutive response in CAT expression by pCAT.A9-Pst in HeLa cells. To test this possibility, HeLa cells were cotransfected with pCAT.A9-Pst and pSVT7CELF. The results show that cotransfection with pSVT7CELF increased CAT expression from pCAT.A9-Pst by 4-to 5-fold. The plasmid pCAT.A9-Sac that contains the CRE also showed a similar increase in CAT activity in the presence of CELF. The plasmid pCAT.Sac-Pst that does not contain the CRE and the vector pCAT.basic do not show this effect (Figure 4b). Since exogenous CELF could still activate the UL9 promoter, these results suggest that the CELF-mediated increase in CAT expression by the

UL9 promoter is not constitutive in transformed HeLa cells. Since CELF has been shown to be present in both PC12 and HeLa cells (6), and since it increases CAT expression from pCAT.A9-Pst in both cell lines, CELF-mediated induction does not account for the Bt_2cAMP-mediated activation of the UL9 promoter observed only in PC12 cells. The results of these experiments therefore suggest the involvement of multiple host factors in UL9 gene expression in neuronal and non-neuronal cells.

DISCUSSION

Results shown in this report demonstrate that Bt_2cAMP induces the UL9 promoter (pCAT.A9-Pst) in PC12, but not in HeLa cells (Figures 2a and b). Since basal CAT expression from pCAT.A9-Pst is higher in HeLa cells than in PC12 cells, it is possible that the cAMP-induced pathway in PC12 cells is constitutive in HeLa cells. Induction of the *c-fos* promoter by cAMP occurs by a similar pathway (7). We also could not detect any significant induction of pCAT.A9-Pst by Bt_2cAMP in a monkey kidney cell line (Vero) (Swati Palit Deb, unpublished observation). Increase of UL9 promoter activity by cAMP in PC12 cells indicate that the 395-bp A9-Pst fragment is probably the target of a factor activated by the PKA pathway. These analyses thus suggest involvement of cell type-specific transcription factors, modulated by cellular signaling pathway, in UL9 gene expression in PC12 cells.

Cotransfection of pCAT.A9-Pst with viral immediate-early proteins in PC12 cells did not increase CAT expression. It is known that IE110 and IE175 can transactivate heterologous promoters (19). Cotransfection of PC12 cells with pCAT.A9-Pst and pSVβgal (the β-galactosidase gene driven by the SV40 early promoter) in the presence and absence of plasmids expressing the immediate-early proteins PIGA15 and PEK4 shows that IE110 and IE175 transactivate SV40 early promoter (Swati Palit deb, unpublished observation). This observation suggests that the promoter of the UL9 gene is relatively insensitive to the induction by viral immediate-early genes in PC12 cells, even when the genes are expressed to a level that produces detectable transactivation of heterologous promoters. Taken together, our data suggest that cAMP induces CAT expression from pCAT.A9-Pst, independent of viral immediate-early proteins.

The C/EBP-like factor, CELF, that increases transcription from the substance P precursor gene by interacting with the CRE also increased CAT expression from the UL9 promoter (Figure 4a). This induction is not specific for PC12 cells; HeLa cells also show this effect (Figure 4b). It has been reported that CELF is ubiquitous, and is not phosphorylated directly by protein kinase A. It can function independent of phosphorylation (6). Therefore, the CELF-mediated increase in UL9 gene expression seems to be distinct from modulation mediated by Bt_2cAMP in PC12 cells.

The analyses described above suggest that multiple host transcription factors interact with the upstream sequence of UL9, leading to the expression of the UL9 gene. Since many external stimuli may induce or downregulate PKA-mediated cell signaling pathway (20,21), it would be of interest to determine in the future whether the PKA-mediated induction of the UL9 gene in PC12 cells constitutes one of the earliest events during reactivation of the virus from latency.

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