Olf-1, a Neuron-specific Transcription Factor, Can Activate the Herpes Simplex Virus Type 1-Infected Cell Protein 0 Promoter*

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Herpes simplex virus type 1 (HSV-1) is a large DNA virus that establishes latency in sensory neurons. Periodically, latent virus reactivates resulting in recurrent disease and transmission to uninfected individuals (reviewed in Refs. 1 and 2). During productive infection, viral gene expression is sequentially activated as follows: immediate early (IE), early, and late. IE gene expression is activated by VP16, a trans-activation domain, which forms a complex with the ubiquitous cellular transcription factor Oct-1 and host cell factor (3–5). This complex binds to the DNA sequence TAATGARAT, which is present in all IE promoters. DNase I footprinting assays indicated that three distinct motifs in the ICP0 promoter are bound by nuclear factors. One of these motifs contains a binding site for a novel helix-loop-helix olfactory neuron-specific transcription factor (Olf-1). Gel shift assays and supershift assays using an Olf-1-specific antibody demonstrated that mouse neuroblastoma cells express Olf-1, which is bound to the Olf-1-like site in the ICP0 promoter. Deletion of the putative Olf-1 motif reduced ICP0 promoter activity more than 5-fold in mouse neuroblastoma cells and prevented trans-activation by an Olf-1 expression vector. We hypothesize that the Olf-1-binding site activates ICP0 promoter activity in neurons during reactivation from latency.

Herpes simplex virus type 1 (HSV-1)$^1$ is a large DNA virus that establishes latency in sensory neurons. Periodically, latent virus reactivates resulting in recurrent disease and transmission to uninfected individuals (reviewed in Refs. 1 and 2). During productive infection, viral gene expression is sequentially activated as follows: immediate early (IE), early, and late. IE gene expression is activated by VP16, a trans-activation domain, which forms a complex with the ubiquitous cellular transcription factor Oct-1 and host cell factor (3–5). This complex binds to the DNA sequence TAATGARAT, which is present in all IE promoters. Abundant IE gene expression does not occur during latent infection, and mutants that do not express individual IE genes establish latent infections at reduced efficiency (reviewed in Refs. 1 and 2).

ICP0 RNA is expressed under IE conditions, and the transcript encodes a protein that activates expression of all classes of viral genes (6, 7). One domain in the ICP0 protein activates IE gene expression and a second activates early and late gene expression (8). Viral mutants that contain deletions in the ICP0 gene exhibit substantial impairment in infectivity (6). ICP0 also facilitates reactivation of HSV-1 from a latent infection in animal models (9, 10) and an in vitro tissue culture latency model (11). Several distinct cis-acting elements in the ICP0 promoter have been identified (12, 13). Sequences between −70 and −420 of the ICP0 promoter are important for expression during productive infection of Vero cells and virulence in mice but not for explant-induced reactivation.

The central and peripheral nervous system consists of approximately 10^12 cells, which are distinct in biochemical and functional properties. During development and following stress, gene expression undergoes many changes in the nervous system (reviewed in Ref. 14). The peripheral olfactory system has the unique ability to generate new neurons from precursor cells, thus offering a system to study neuron-specific gene expression (15, 16). Olfactory marker protein expression is involved with smell, and its expression is tightly controlled, in part because of the olfactory neuron-specific transcription factor (Olf-1) (15–18). Olf-1 is a helix-loop-helix (HLH) protein, which is implicated in olfactory gene regulation and B-cell development (19, 20). Olf-1 specifically binds to a consensus DNA sequence, TCCCCAT/NGGAG (15, 18). The Olf-1 transcript is alternatively spliced and thus may encode protein isoforms with novel biological properties (21).

We have identified a trans-acting factor that regulates ICP0 promoter activity in mouse neuroblastoma (neuro-2A) cells. TAATGARAT motifs are dispensable for efficient ICP0 promoter activity in neuro-2A cells. High constitutive promoter activity is dependent on a cis-acting element that closely matches the Olf-1-binding site. Cellular factors that bind to the Olf-1 site are different in neuro-2A cells compared with non-neuronal cells suggesting tissue-specific factors can regulate ICP0 promoter activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**A plasmid that contains part of the HSV-1 ICP0 promoter (−100 to +150) is designated pAB5 (4). Plasmid pAB5 contains only one TAATGARAT motif. Plasmid pAB2 contains the region of the ICP0 promoter spanning −130 to +150 but lacks the TAATGARAT motif in pAB5. The VP16 expression plasmid contains the HSV-1 VP16 coding sequences linked to the Moloney murine leukemia virus-long terminal repeat (22). Plasmid pSV2 CAT (SV40 enhancer-promoter linked to the CAT gene) was provided by Bruce Howard, National Institutes of Health. pcDNA3.1 CAT (CMV/CAT), pSV2-β-galactosidase, and pUC19 were purchased from Invitrogen, CLONTECH, and New England Biolabs, respectively. Randall Reed, The Johns Hopkins University, provided pCMV/Olf-1 cDNA. The ICP0 promoter was also cloned into pUC19 using standard molecular biology techniques.

The Olf-1-like site in pAB5 was deleted using a polymerase chain
reaction that employed a primer spanning ICP0 promoter sequences downstream of this site (primer 1) and a downstream primer (primer 2). Primer 1 has the same sense as the coding strand, is located between −77 to −58, and has a HindIII site at its 5’ end. Primer 2 is complementary to +145 to +121 and has a terminal XhoI site. These primers were used to amplify the ICP0 promoter using pAB5 as a template in a Hybaid thermal cycler under the following conditions: 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and 72 °C for 7 min to allow for final extension. The resulting polymerase chain reaction product was purified as described previously (23), digested with HindIII and XhoI, and subsequently cloned into pBlCAT 6 vector (provided by Gunther Schütz, German Cancer Institute, Heidelberg, Germany).

Cells and Transfection Procedures—Mouse neuroblastoma (neuro-2A), COS-7, CV-1, and NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained in Earle’s minimum essential medium supplemented with 10% fetal bovine serum. Transfections were performed in 100-mm plates with the indicated amounts of DNA by calcium phosphate precipitation as described previously (24).

Chloramphenicol Acetyltransferase (CAT) Assay—Cells were cotransfected with the pSV2-β-galactosidase expression plasmid and the designated CAT reporter plasmid. The amount of DNA transfected was kept constant in all experiments with pUC19. Cells were harvested 48 h after transfection and CAT enzymatic levels were measured as described previously (24). The amount of extract used for measuring CAT was adjusted based on β-galactosidase activity as described previously (25). To measure β-galactosidase activity, a portion of the cell extract (10 μl) was incubated for 4 min with 40 μl of 4 mg/ml ortho-nitrophenyl β-d-galactoparanoside (Sigma) in 200 μl of buffer (60 mM Na2PO4, 40 mM NaH2PO4, 10 mM KC1, 1 mM MgSO4, 50 mM β-mercaptoethanol). To stop the reaction, 100 μl of 1 M Na2CO3 was added and the absorbance of the supernatant measured (420 nm).

Preparation of Nuclear Extract—Nuclear extract (NE) was prepared as described previously (26). Protein concentration in nuclear extract was determined using a kit from Bio-Rad according to the manufacturer’s instructions.

DNase I Protection Assays—DNase I protection assays were performed using the Sure track footprinting system (catalogue number 27-9101-01; Amersham Pharmacia Biotech) following the manufacturer’s instructions. Radiolabeled fragments for footprinting the ICP0 promoter were generated from plasmid pUC19-ICP0 containing nucleotides −165 to +150 of the ICP0 promoter cloned into BanHI and XhoI sites. The ICP0 promoter was liberated by cutting with PstI and EcoRI (to detect binding on the top strand) or KpnI and SulI (to detect binding on the bottom strand). Probes for DNase I footprinting were labeled with Super-Script reverse transcriptase (Life Technologies, Inc.) in the presence of [α-32P]dATP (Amersham Pharmacia Biotech). Labeled inserts were purified by polyacrylamide gel electrophoresis. Footprinting was performed in a volume of 100 μl by incubating 15–30 μg of nuclear extract and 100 pg of the probe in a binding buffer, supplied by the manufacturer. After 20 min incubation, the designated concentration of DNase I was added and the incubation continued for 1 min at room temperature. Reactions were terminated by addition of an equal volume of stop buffer supplied by the manufacturer. The mixture was then phenol-extracted and ethanol-precipitated. Products were denatured at 95 °C, resolved on a 6% urea-polyacrylamide gel, and subjected to autoradiography.

Electrophoretic Mobility Shift Assays (EMSA)—Oligonucleotides were purchased from Integrated DNA Technology (Corvallis, IA). The sequences of these oligonucleotides are as follows.

| 5’ | 3’ |
|---|---|
| 5’AGCTTGGCTCCCTCGTGAGGCGCTC 3’ (+) | 3’ AACCAGGCGGCACCTGCCGACATC 5’ (−) |
| OLIGONUCLEOTIDE WT ICP0 OLF-1 |
| 5’ | 3’ |
| 5’AGCTTGGCTCCCTCGTGAGGCGCTC 3’ (+) | 3’ AACCAGGCGGCACCTGCCGACATC 5’ (−) |
| OLIGONUCLEOTIDE MUTANT 1 |
| 5’ | 3’ |
| 5’AGCTTGGCTCAGCTTGGCGCTC 3’ (+) | 3’ AACCAGGCGGCACCTGCCGACATC 5’ (−) |
| OLIGONUCLEOTIDE MUTANT 2 |

The + and − oligonucleotides have terminal HindIII and XhoI sites, respectively, and these sites are designated by italics. The restriction sites were introduced to facilitate end labeling using reverse transcriptase. The underlined nucleotides are the HSV-1 sequences from −97 to −79 in the ICP0 promoter. The bold nucleotide is the only base that differs from the Olf-1 consensus site. Double underlined nucleotides are those bases that differ from the WT sequence and eliminate Olf-1 binding (15).

EMSA were performed as described in Ref. 27 with the following modifications. The probe was prepared by end labeling with Super-Script reverse transcriptase (Life Technologies, Inc.) in the presence of [α-32P]dATP (Amersham Pharmacia Biotech). NE was treated with Nonidet P-40 (Sigma) to a final concentration of 1% on ice (28). Binding reactions contained 10 mM HEPES (pH 7.9), 10 mM MgCl2, 10% glycerol, 50 mM NaCl, 0.5 mM dithiothreitol, 2 μg of poly(dI-dC), 5–15 μg of protein, and 200 pg of 32P-labeled probe. After 10 min incubation on ice, reactions were electrophoresed on a 4% non-denaturing polyacrylamide gel in 0.25 X TBE and subjected to autoradiography.

For supershift assays, binding reactions were incubated with 1 μl of Olf-1 antibody (provided by Randall Reed, The Johns Hopkins University), normal rabbit serum, or Bcl-2 antibody (catalogue number sc-783; Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 10 min before loading onto the gel.

RESULTS

Analysis of ICP0 Promoter Activity in Neuro-2A Cells—IE gene expression is activated by VP16 during productive infection (reviewed in Ref. 5). Since the ICP0 gene is important for reactivation from latency, cis-acting sequences in the ICP0 promoter may be regulated by neuronal factors in the absence of VP16. To test this hypothesis, a chimeric CAT gene containing the ICP0 promoter spanning −165 to +150 (pAB5; Ref. 4) was transfected into neuro-2A cells, and its activity compared with the SV40 (pSV2-CAT) and CMV (CMV/CAT) promoters.

Neuro-2A cells are murine neuroblastoma cells that have certain neuronal characteristics and can be readily transfected. At least 10-fold higher levels of CAT activity were obtained with pAB5 in neuro-2A cells compared with COS-7, CV-1, or NIH 3T3 cells (Fig. 1). In contrast, pSV2 CAT and pcDNA3.1 CAT contained similar levels of CAT activity in the respective cell lines (Fig. 1). In neuro-2A cells, pAB5 promoter activity was slightly higher than the SV40 promoter but lower than the CMV IE promoter.

Interaction of Nuclear Factors with the ICP0 Promoter—The results in Fig. 1 suggested that novel cellular transcription factors in neuro-2A cells interacted with and subsequently activated the ICP0 promoter. To begin to test this possibility, DNase I footprinting assays were performed using NE prepared from neuro-2A cells and the ICP0 promoter. DNA sequences containing an Olf-1 like binding site (15, 18) were protected from DNase I digestion (Fig. 2A and summarized in Fig. 2B). The other prominent DNase I-protected sites were similar to a GC box (29) and initiator-like sequences (reviewed in Ref. 30). Although the DNase I footprinting assay suggested...
that binding occurred with the Olf-1-like binding site in neuro-2A cells, there was no obvious cell type differences that correlated with enhanced promoter activity in neuro-2A cells (data not shown). Furthermore, the ICP0 Olf-1-like binding site has a C in the last base and not a G as described for the consensus (TCCC(A/T)NGGAG; Refs. 15 and 18).

EMSA were subsequently performed with NE prepared from neuro-2A, COS-7, CV-1, or NIH 3T3 cells using an oligonucleotide containing the ICP0 Olf-1-binding site. A diffuse complex was observed with NE from neuro-2A cells (Fig. 3A). However, NE from COS-7 or CV-1 cells yielded a faster migrating complex, and the shifted complex was nearly undetectable in NIH 3T3 cells. Two mutant Olf-1 oligonucleotides were synthesized to examine the specificity of binding. A 100-fold excess of WT Olf-1 oligonucleotide reduced the shifted complex in neuro-2A cells (Fig. 3B). However, Mutant 1 and 2 oligonucleotides (Fig. 3B, M1 and M2, respectively) had little effect on the shifted complex. WT, M1, or M2 oligonucleotides reduced the intensity of the faster migrating shifted band in CV-1 NE (Fig. 3C). The intensity of the slower migrating shifted band was nearly eliminated by the WT Olf-1 oligonucleotide but to a lesser degree by the mutant oligonucleotides suggesting that binding to the
slower migrating shifted band was specific. In summary, EMSA indicated that novel nuclear proteins in neuro-2A cells specifically bind to the ICP0 Olf-1-like binding site.

To test whether the Olf-1 protein was expressed in neuro-2A cells and was bound to the Olf-1 oligonucleotide, supershift assays were performed using an Olf-1-specific antibody. The Olf-1 antibody specifically binds to a 60–70-kDa protein in neuronal cell types (Ref. 18 and data not shown). Incubation with the Olf-1 antibody retarded mobility of the shifted complex in neuro-2A cells (Fig. 4A, Olf-1 lane). In contrast, incubation of NE from CV-1 cells with the Olf-1 antibody did not alter the mobility of the shifted band (Fig. 4B). Normal rabbit serum (Fig. 4A, NRS) and a Bcl-2 antibody had no effect on complex formation. Thus, in neuro-2A cells Olf-1 was expressed and bound to the ICP0 Olf-1 site.

Olf-1 trans-Activates the ICP0 Promoter—The ability of Olf-1 to trans-activate the ICP0 promoter in COS-7, CV-1, or NIH 3T3 cells was subsequently tested. Co-transfection of the Olf-1 cDNA expression plasmid with pAB5 resulted in 5-fold trans-activation in COS-7 cells and 3-fold trans-activation in NIH 3T3 cells (Fig. 5A). In contrast, the Olf-1 cDNA did not activate the ICP0 promoter in CV-1 cells. To evaluate the role of the TAATGARAT motif in Olf-1 trans-activation, the ICP0 promoter (pAB2) lacking this motif was co-transfected with Olf-1 cDNA. The Olf-1 cDNA trans-activated pAB2 in COS-7 and NIH 3T3 cells but not in CV-1 cells (Fig. 5B).

To test whether the Olf-1-binding site in the ICP0 promoter was required for Olf-1 trans-activation, the Olf-1 site was deleted (Fig. 6A, ΔOlf-1). ΔOlf-1 was not responsive to Olf-1-
mediated trans-activation in COS-7 cells (Fig. 6B). Furthermore, Olf-1 promoter activity was nearly 7-fold less in neuro-2A cells relative to pAB2 (Fig. 6C). In summary, these studies demonstrated that the Olf-1 site, but not the TAATGAGAT site, was necessary for Olf-1-mediated trans-activation of the ICP0 promoter.

**DISCUSSION**

This study demonstrated that sequences closely resembling a consensus Olf-1-binding site in the ICP0 promoter were necessary for constitutive activity in neuro-2A cells. DNase I footprinting assays protected a 19-base pair motif located from position −97 to −79 that was nearly identical to the consensus Olf-1-binding site. The Olf-1 antibody supershifted the ICP0 Olf-1 DNA protein complex in neuro-2A cells, and an Olf-1 cDNA expression construct activated ICP0 promoter activity. Taken together, these results demonstrated that Olf-1 activated ICP0 promoter activity in transiently transfected cells.

Olf-1 expression occurs during mouse embryogenesis in dorsal root ganglia, trigeminal ganglia, cranial, and glossopharyngeal nerve ganglia (21, 31). We hypothesize that neuronal specific activation of ICP0 expression during reactivation from latency may be regulated by Olf-1. Except for the ICP0 promoter, no additional Olf-1-binding sites were detected in the HSV-1 genome suggesting that ICP0 is the only viral gene that is directly trans-activated by Olf-1. It will be of interest to determine if site-directed mutagenesis of the Olf-1-binding site has any role in HSV-1 pathogenesis or latency. It will also be of interest to determine if stimuli that induce reactivation from latency induce expression of Olf-1 in the peripheral nervous system. Studies aimed at addressing these issues are in progress.

Olf-1 is a novel member of the HLH family of transcription factors that binds to the promoter region of genes involved in odorant transduction pathway (15, 18, 20, 21). Olf-1 was also identified independently and cloned as early B-cell factor that regulates the mb-1 gene (19). Transcriptional activation and DNA binding by Olf-1 is facilitated by homodimerization of Olf-1 (21). Since Olf-1 is an HLH protein, dimerization of Olf-1 with other proteins may also regulate its activity. A novel Olf-1 associated zinc finger protein (Roaz) has been identified that is expressed in precursor cells of olfactory neurons. The Roaz/Olf-1 heterodimer inhibits promoters containing the Olf-1-binding site (32). We hypothesize that Roaz and/or similar factors exist, and these factors prevented activation of the ICP0 promoter by Olf-1 in CV-1 cells.

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