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Role of Nuclear Factor Y in Stress-Induced Activation of the Herpes Simplex Virus Type 1 ICP0 Promoter▽†

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Herpesviruses are characterized by the ability to establish lifelong latent infections and to reactivate periodically, leading to recurrent disease. The herpes simplex virus type 1 (HSV-1) genome is maintained in a quiescent state in sensory neurons during latency, which is characterized by the absence of detectable viral protein synthesis. Cellular factors induced by stress may act directly on promoters within the latent viral genome to induce the transcription of viral genes and trigger reactivation. In order to identify which viral promoters are induced by stress and elucidate the cellular mechanism responsible for the induction, we generated a panel of HSV-1 promoter-luciferase constructs and measured their response to heat shock. Of the promoters tested, those of ICP0 and ICP22 were the most strongly upregulated after heat shock. Microarray analysis of lytically infected cells supported the upregulation of ICP0 and ICP22 promoters by heat shock. Mutagenic analysis of the ICP0 promoter identified two regions necessary for efficient heat-induced promoter activity, both containing predicted nuclear factor Y (NF-Y) sites, at bases −708 and −75 upstream of the transcriptional start site. While gel shift analysis confirmed NF-Y binding to both sites, only the site at −708 was important for efficient heat-induced activity. Reverse transcription-PCR analysis of selected viral transcripts in the presence of dominant-negative NF-Y confirmed the requirement for NF-Y in the induction of the ICP0 but not the ICP22 promoter by heat shock in lytically infected cells. These findings suggest that the immediate-early ICP0 gene may be among the first genes to be induced during the early events in HSV-1 reactivation, that NF-Y is important for this induction, and that other factors induce the ICP22 promoter.

Herpes simplex virus type 1 (HSV-1) latency is characterized by the absence of viral replication, the absence of detectable viral proteins, and the presence of abundant nuclear latency-associated transcripts (LATs) and limited amounts of ICP4 and thymidine kinase (TK) transcripts (14, 25). Periodically, the virus is induced to reenter the lytic replication cycle, or reactivate, by a poorly characterized reactivation-inducing stimulus, such as physical or emotional stress, fever, or UV irradiation, among other stresses (47).

The molecular mechanism of HSV-1 reactivation from latency remains one of the most clinically relevant, yet least well-characterized aspects of HSV-1 infection. We hypothesize that in the absence of detectable viral proteins in latently infected neurons, the reactivation-inducing stimulus likely acts on viral promoters to increase viral gene expression and, subsequently, viral replication. There is some evidence to suggest that the well-characterized temporal cascade of viral gene expression during lytic infection of immediate-early, early, delayed-early, and late (IE, E, DE, and L, respectively) may be altered during the initial events of reactivation in neurons (44). The results of reverse transcription-PCR (RT-PCR) of RNA from latently infected mouse TG induced to reactivate by explant demonstrated that E genes are transcribed before IE genes (44). This suggests that the gene expression cascade may be altered in reactivating TG compared to lytic replication in cell culture.

ICP0 has been shown to play an important role in reactivation from latency. Studies have demonstrated that infection of quiescently infected cultures with adenoviruses expressing ICP0, but not ICP0, with a mutation in its RING finger domain led to the reactivation of both HSV-1 and HSV-2, suggesting that ICP0 activity is important for inducing reactivation (16, 18). In addition, ICP0-null viruses were unable to reactivate in vivo after heat shock (46) and reactivated with reduced efficiency compared to wild-type virus from latently infected cultures in vitro (5, 17), indicating that ICP0 is a key player in reactivation. Taken together, these studies present considerable evidence of an important role for ICP0 in reactivation.

A number of models have been developed in order to study the HSV-1 and cellular events that occur during reactivation. Heat shock has been used by our lab and others to induce reactivation both in vitro and in vivo. Reactivation can be induced in vivo by hyperthermic shock of latently infected animals (43°C for 10 min) and UV irradiation of the cornea (38, 41) or in vitro by explant or heat shock of ganglia (43°C for 3 h) (15–17).

The cellular response to heat shock is split into two interacting branches. One is characterized by the function of heat shock proteins (HSPs), whereas the other depends on the activation of pro- and antiapoptotic signaling intermediates in the mitogen-activated protein kinase family. The two pathways interact and modulate each other to determine whether a cell will survive or die by apoptosis (13), yielding a complicated interplay of the cellular factors activated by heat shock.

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In order to test which viral promoter or class of promoters is induced by cellular stress, we generated a panel of viral promoters representing genes from each kinetic gene class fused to the luciferase gene. The response of the viral promoters to heat shock in transfected cells was then quantitated by luciferase assays. Among the promoters tested, we found the IE ICP0 and ICP22 promoters to be the most responsive to heat shock. Microarray analysis of HSV-1 transcription supported the findings of the luciferase assay in that the ICP0 and ICP22 promoters were induced after heat shock of lytically infected cells. Deletional analysis of the ICP0 promoter led to the identification of two regions important for heat shock-induced activity. Interestingly, both regions contain predicted nuclear factor Y (NF-Y) binding sites.

An NF-Y site 75 bp upstream of the transcriptional start site (TSS) of the ICP0 promoter has been previously shown to be important for constitutive ICP0 promoter activity (33). NF-Y is a ubiquitous, heterotrimeric CCAAT-box binding transcription factor necessary for the basal and induced activity of multiple stress response genes, including endoplasmic reticulum stress response genes (12) and, importantly, HSP70 (19, 27, 39, 42). The mechanism of NF-Y function within the *Xenopus* hsp70 promoter has been partially defined. NF-Y is constitutively bound to CCAAT sites on the hsp70 promoter by maintaining the chromatin in a transcriptionally permissive state by preventing nucleosome assembly and by the recruitment of histone acetyltransferases (HATs) (19, 26, 27).

Mutation of the distal NF-Y binding site led to a decrease in heat shock-induced ICP0 promoter activity. Moreover, inhibition of NF-Y activity through the use of adenovirus-delivered dominant-negative NF-Y constructs led to a decrease in ICP0 promoter activity in response to heat shock in the context of a lytic infection, demonstrating a role for NF-Y in heat shock induction of the ICP0 promoter both in the presence (microarray, RT-PCR) or absence (luciferase assay) of other viral factors. These results suggest that ICP0 may be upregulated early in response to a stressful stimulus early in reactivation and that NF-Y plays an important role in its upregulation.

MATERIALS AND METHODS

Cells and viruses. (i) Cells. Vero cells (ATCC CCL-81) and HEK293 cells (ATCC CRL-1573) were obtained from the American Type Culture Collection, Rockville, MD. All cells were maintained at 37°C in 5% CO_2. Cells were propagated in Dulbecco modified Eagle medium (DMEM) supplemented with 5 or 10% fetal bovine serum (Vero and HEK293 cells, respectively). Experiments in Vero and HEK293 cells were performed using DMEM supplemented with 10% fetal bovine serum. DMEM was also supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine.

(ii) Viruses. The wild-type virus used in these studies was HSV-1 strain KOS at passage 13 and was propagated as described previously (37). Adenoviral vectors expressing green fluorescent protein (Ad.GFP), and wild-type (Ad.YA) or dominant-negative (Ad.YAm) forms of NF-YA were obtained from R. Mantovani (University of Milan, Milan, Italy) (20, 31), propagated in HEK293 cells, and titrated by using an Adeno-X rapid titer kit (Clontech, Mountain View, CA).

Reporter plasmid construction. Unless otherwise indicated, HSV-1 promoter fragments were generated by PCR from infectious HSV-1 KOS DNA using primers that added restriction enzyme sites for ease of subsequent cloning. Construction of the reporter plasmids and the primers used are described in the supplemental material. Luciferase constructs containing the ICP0 promoter, p36 (the minimal, 36-bp basal prolactin gene promoter), and selected ICP0 promoter mutations have been described previously (8, 40).

ICP0 point mutations. Point mutations were introduced into the wild-type ICP0 CCAAT box sequence by using the pAlter-1 mutagenesis system. To clone the fragment containing the ICP0 promoter into the pAlter-1 vector (Promega), the ICP0 promoter was excised from the pGL3 vector with SacI and NcoI, blunt ended, and ligated into the SmaI site in pAlter-1, and the insert was screened for orientation, yielding the wild-type template for subsequent mutagenesis, ICP0pAlter. Point mutations introduced using pAlter-1 mutagenesis were previously shown to abrogate NF-Y binding (22, 28). The mutagenic primers were as follows, with the mutations indicated in boldface and the CCAAT box underlined: −75NFYmut1, 5′-CCC CTG GGA CGC GCG GCC **ACT CG**G GGA ATC GTC ACT GCC GC-3′; −75NFYmut2, 5′-CCC CTG GGA CGC GCG GCC **GCG TT**G GGA ATC GTC ACT GCC GC-3′; −708NFYmut1, 5′-CCT CGG GGG CGG GAC TGG **CGA GT**C GGC GGC CGC CAG CGC GGC-3′; and −708NFYmut2. ′-CCT CGG GGG CGG GAC TGG **AAC GC**C GGC GGC CGC CAG CGC GGC-3′. The presence of each point mutation was confirmed by sequencing. Constructs with confirmed mutations were excised from pAlter-1 with KpnI and HindIII and ligated into the corresponding sites in pGL3-Basic.

NF-Y double mutant. An ICP0 promoter mutated at both nucleotide (nt) −708 and nt −75 NF-Y sites was generated by subcloning. A unique StuI site between two NF-Y sites was used to ligate each single mutation into one double mutant. Double mut1 contains −708mut1 and −75mut1; double mut2 contains −708mut2 and −75mut2.

Luciferase assays. Vero cells were seeded at a density of 1.6×10^6 cells per six-well plate and transfected 1 day later with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol with a total of 2 μg of DNA (250 ng of each luciferase reporter plasmid and 1.75 μg of salmon sperm DNA) in duplicate. At 24 h after transfection, transfected cells were either heat shocked for 3 h at 43°C in 5% CO_2 or maintained at 37°C. Cells were then allowed to recover at 37°C for 4 h. Cells were washed twice with phosphate-buffered saline (PBS), lysed by rocking in 500 μl of passive lysis buffer (Promega, Madison, WI) per well for 15 min and assayed by using a luciferase assay system (Promega) with a Lumat LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN). Statistical analysis was performed by using GraphPad Prism (San Diego, CA) software with one-way analysis of variance (ANOVA) to determine the statistical significance of series, and Bonferroni's multiple comparison post-test to determine statistical significance of differences between values within the series.

Microarray analysis. DNA oligonucleotide arrays stamped with 60mers containing sequences from every HSV-1 open reading frame were custom designed in collaboration with SABiosciences (Frederick, MD). Vero cells were seeded at 3×10^6 cells per 10-cm plate and incubated overnight at 37°C. Cells were then infected with an HSV-1 KOS multiplicity of infection (MOI) of 10 PFU/cell for 30 min and washed twice with warm Hanks balanced salt solution (Invitrogen). The medium was replaced, and the cells were heat shocked or mock heat shocked for 1 h at 43°C and allowed to recover for 1 h at 37°C, at which time total RNA was isolated using an RNAqueous-4 PCR kit (Ambion, Inc., Austin, TX). The mRNA was reverse transcribed, biotin-labeled using the TrueLabeling-AMP 2.0 kit (SABiosciences), hybridized to the microarrays, and washed according to the manufacturer's instructions (SABiosciences). The arrays were visualized by using a chemiluminescent detection kit (SABiosciences) and images were captured on a Kodak Image Station IS4000R. The data were analyzed with GEArray Expression Analysis Suite 2.0. Arrays were standardized by using the interquartile method, and the data are presented as the fold change in expression of heat-shocked versus mock heat-shocked control. The data represent the averages of three independent experiments.

Preparation of nuclear extracts. Nuclear extracts were prepared as published previously (2) from 10^7 Vero cells. Briefly, cells were scraped into ice-cold PBS, washed once with ice-cold PBS, and resuspended in 400 μl of buffer A (10 mM HEPES-KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl) supplemented with 1 mM dithiothreitol (Sigma, St. Louis, MO) and protease inhibitors. The cells were incubated on ice for 10 min, and 20 μl of 10% NP-40 were then added. The nuclei were pelleted by centrifugation at 4°C (10 min at 3,000 × *g*), gently resuspended in 100 μl of buffer C (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, and protease inhibitors), and incubated on ice for a further 20 min with occasional gentle rocking. Debris was cleared by centrifugation (16,000 × *g*, 10 min at 4°C), and nuclear extracts were divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80°C. The protein concentration of the nuclear extracts was determined by the Bradford assay (Bio-Rad, Hercules, CA).

Gel shift analysis. Gel shifts were performed with 5 μg of nuclear extract per binding reaction as described previously (23), using 1.7 μg of poly(dA-dT) per reaction. For reactions, including antibody supershifts, the antibody (anti-NF-YA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the binding reaction for 1 h and incubated on ice. The annealed and polyacrylamide gel electrophoresis-purified radiolabeled probe was then added to the reaction. The binding reaction was allowed to proceed a further 30 min on ice, at which time

A.

Kinetic class	Promoter	Protein function
Positive control	HSE	Consensus HSE upstream of the SV40 promoter driving luciferase
Negative control	p36	Prolactin promoter TATA box
IE	ICP0	Major viral transactivator
	ICP4	Transcriptional regulation protein
	ICP22/47	Regulatory protein; Blocks presentation of viral antigens
	U_L54	Regulatory protein (ICP27)
E	U_L9	Origin binding protein (OBP)
	U_L23	Thymidine kinase (TK)
	U_L29	ssDNA binding (ICP8)
	U_L39	Large subunit of ribonucleotide reductase (ICP6)
DE	U_L48	Tegument protein (VP16)
	U_L19	Major capsid protein (VP5)
	LAT	Latency associated transcripts
L	U_L44	Glycoprotein C (gC)

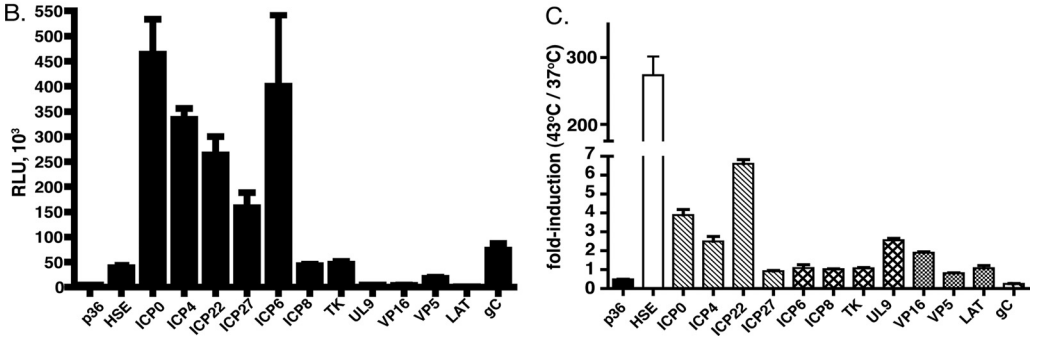

FIG. 1. Response of selected HSV-1 promoters to heat shock. (A) Viral promoter- and control-luciferase constructs used in these studies. Vero cells were transfected with the indicated HSV-1 promoter-firefly luciferase constructs in duplicate. At 24 h posttransfection, cells were heat shocked for 3 h at 43°C or maintained at 37°C (mock heat shocked). After a 4-h recovery period at 37°C, cells were harvested and subjected to the luciferase reporter assay (Promega). (B) Basal promoter activity (mock heat shocked) presented as relative light units (RLU). (C) Fold induction following heat shock. The data are presented as the fold change versus non-heat-shocked control (43°C/37°C) ± the standard error of the mean (SEM). IE genes are represented by hatched bars, E genes are represented by cross-hatched bars, DE genes are represented by small cross-hatched bars, and L genes are represented by a striped bar ($n \geq 3$). The luciferase activity of all samples was at least threefold higher than the background level (mock-transfected cells).

the protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gel electrophoresis gels in 0.5× Tris-borate-EDTA (TBE) at 4°C. The gels were dried and exposed on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) overnight.

The following probes were used in the binding reactions (the NF-Y binding site is underlined in each probe and mutations are indicated in boldface): −75WT ICP0, GAC GCG CGG CCA TTG GGG GAA TCG TCA; −75ICP0mut1, GAC GCG CGG CCA **CTC G**GG GAA TCG TCA; −75ICP0mut2, GAC GCG CGG CC**G CGT T**GG GAA TCG TCA; −708WTICP0, CGG GAC TGG CCA ATC GGC GGC CGC; −708ICP0mut1, CGG GAC TGG **CGA GT**C GGC GGC CGC; −708ICP0mut2 CGG GAC TGG **AAC GC**C GGC GGC CGC; and nonspecific probe, CCT GCC TCA GCT GTT CTG CGC GGC CAT. The consensus NF-Y probe was purchased from Santa Cruz Biotechnology (CBF consensus oligonucleotide [sc-2591]).

Real-time RT-PCR. Vero cells were seeded with 2.7×10^5 cells per well of a six-well plate and incubated overnight. Two wells each were infected with Ad.GFP, Ad.YA, or Ad.YAm-expressing adenoviruses at an MOI of 25 PFU/cell for 24 h. The cells were superinfected with HSV-1 KOS at an MOI of 1 PFU/cell for 30 min in order to maximize the number of cells infected and yet minimize the level of viral transcription, so as to better mimic latency. The cells were then washed twice with warm PBS, overlaid with warm DMEM, heat shocked (or mock heat shocked) for 1 h at 43°C, and allowed to recover for 1 h at 37°C. Total RNA was harvested by using an RNAqueous-4 PCR kit, and half was reverse transcribed with (RT^+) and without (RT^-) the addition of reverse transcriptase using a high-capacity cDNA RT kit (Applied Biosystems, Warrington, United Kingdom). RT^+ and RT^- samples were diluted twice with water and used directly in RT-PCRs. Reactions were set up in triplicate with 1.5 μl of template in a total volume of 15 μl, using SYBR green PCR master mix (Applied Biosystems). The reactions were run for 40 cycles (15 s at 95°C, 15 s at 55°C, and then 30 s at 68°C) on an Eppendorf Mastercycler ep Realplex machine. The resulting values were standardized to an actin control.

RT-PCR primers. ICP0 primers that span the ICP0 splice joint have been published elsewhere (6). The remaining primers were as follows: ICP22 forward, 5′-TGT GCA AGC TTC CTT GTT TG-3′; ICP22 reverse, 5′-GGC ATC GGA GAT TTC ATC AT-3′; Vero β-actin forward, 5′-AAG AGA GGC ATC CTC ACC CT-3′; and Vero β-actin reverse, 5′-TCT CCA TGT CGT CCC AGT TG-3′.

RESULTS

Among the promoters tested, the ICP0 and ICP22 promoters show the most potent induction by heat shock. To determine whether HSV-1 promoters can be induced by heat shock, a panel of viral promoter-luciferase constructs containing representative promoters from all four HSV-1 kinetic gene classes (Fig. 1A) was generated and tested for heat shock-inducible luciferase activity. A construct containing three heat shock elements (HSE) upstream of the simian virus 40 promoter was included in every assay as a positive control for the induction

of the cellular heat shock response. On average, the HSE control was induced 276-fold. The magnitude of the HSE response relative to response of the promoters made it impractical to include it in histograms of the results. This value is therefore included beneath each histogram. p36 contains a prolactin promoter TATA box and was used as a negative control for heat shock induction in these studies.

Vero cells were chosen for this initial study because they are easy to transfect, are responsive to heat shock, and are a common cell line used to monitor HSV-1 replication. Consequently, Vero cells were transfected with the indicated promoter-luciferase constructs, mock heat shocked or heat shocked for 43°C for 3 h and allowed to recover at 37°C for 4 h. The recovery period was necessary for the accumulation of luciferase and accurate determination of the final extent of viral promoter induction. The data are presented as the fold induction of activity relative to mock heat-shocked samples (43°C and 37°C).

In the course of these studies, we discovered that both *Renilla* and firefly luciferase and to a lesser degree, β-galactosidase, are highly heat labile, with *Renilla* luciferase more prone to denaturation and loss of activity than firefly luciferase, as noted previously (36). For this reason, we did not include an internal transfection control in the luciferase assays, relying instead on repetition of the assay to minimize the effects of experimental error. Each assay was therefore repeated at least three times in duplicate.

Among the HSV-1 promoters tested, IE gene promoters had the highest basal activity of the four kinetic classes tested (with the exception of the E ICP6 promoter, which exhibited the second highest level of basal activity, Fig. 1B) and were the most heat shock inducible, with the ICP0 and ICP22/47 (referred to subsequently as the ICP22 promoter) promoters demonstrating the highest upregulation in activity (Fig. 1C; approximately 3.9- and 6.6-fold, respectively). It is interesting that the ICP4 promoter did not respond to heat shock to the same extent as the ICP22 promoter (ICP4, ~2.5-fold). Since the ICP4 and ICP22 promoters differ only in their orientation and not their sequence, this finding suggests that heat shock induction is specific to the ICP22 promoter. Of the other gene classes, the E UL9 and DE VP16 promoters were modestly upregulated after heat shock (Fig. 1C). Collectively, these findings demonstrate that promoters of the IE gene class are the most responsive to heat shock in the absence of other viral factors and suggest that they may be among the first to be transactivated during the early stages of reactivation.

Microarray analysis of HSV-1 transcription in response to heat shock. To extend the luciferase assay findings, we utilized custom-designed oligonucleotide-based microarrays (SABiosciences) specific for all HSV-1 open reading frame sequences to assay changes in viral gene expression after heat shock during viral infection. This approach allowed an unbiased and global analysis of viral promoter activity after heat shock during lytic infection.

We found that heat shock protein 90 (HSP90) was upregulated with heat shock (3 h at 43°C), suggesting that our treatment was sufficient to induce the cellular heat shock response, as was noted in the luciferase studies (Fig. 2). Consistent with the results of the luciferase assay, the ICP0 and ICP22 promoters were upregulated by heat shock (ICP0, 9.4-fold upregulation [$P = 0.01$]; ICP22, 9.3-fold upregulation [$P = 0.007$]). The behavior of other viral genes in the microarray assay, however, differed from that seen with luciferase. ICP27 and ICP6 were upregulated after heat shock according to the microarray data but remained unchanged in luciferase assays. Potential reasons for this discrepancy are presented in the Discussion. These results demonstrate that the IE ICP0 and ICP22 promoters were upregulated by heat shock both in the absence (transfection, followed by luciferase assay) and presence (infection, followed by microarray assay) of other viral factors.

In order to characterize the regions of the ICP0 and ICP22 promoters necessary for promoter upregulation after heat shock, truncation mutants of each promoter were generated and assayed for the response to heat shock. Deletional analysis of the ICP22 promoter failed to yield defined regions necessary for induction of activity (data not shown). Therefore, the remainder of the present study is focused on characterizing the ICP0 promoter elements important for heat shock induction.

Regions of the ICP0 promoter necessary for heat shock-induced activity. (i) Broad 5′ truncations of the ICP0 promoter. To identify the regions of the ICP0 promoter that are necessary for heat shock-induced activity, sequential 5′ deletions spanning the length of the promoter were obtained (8) or generated by using PCR (Fig. 3). The truncation mutants were named for the location of the truncation relative to the TSS and tested for basal and heat shock-induced activity in the luciferase assay system as described above.

The basal activity of the ICP0 promoter truncations decreased stepwise relative to the full-length construct (Fig. 4A), in agreement with previously published data (8). Two regions of the ICP0 promoter were found to be important for efficient heat shock-induction. Deletion of sequences upstream of −669 TSS and between −231 TSS and −95 TSS lead to a two fold or greater decrease in heat shock-induced activity compared to the full-length construct (Fig. 4B, one-way ANOVA $P < 0.0001$, and $P < 0.01$, respectively), suggesting that these regions are important for efficient heat shock-induced activity.

(ii) Fine mapping of the ICP0 promoter. Having identified broad regions of the ICP0 promoter important for efficient heat shock-induced activity, we sought to identify the elements within those regions involved in the upregulation. This analysis could lead to the identification of cellular factors that interact with the ICP0 promoter and contribute to heat shock-induced activity. To this end, we engineered fine truncations at 20 to 30-bp intervals in the two regions of interest in the ICP0 promoter (Fig. 3; upstream of −669 TSS and between −231 TSS and −95 TSS) by PCR and tested them for heat shock-induced activity in the luciferase assay as before.

(a) Truncations upstream of −669 TSS. Basal activity of the ICP0 promoter fine truncation mutants decreased ca. 40% relative to the full-length construct with the deletion of sequences between −708 TSS and −686 TSS (Fig. 5A, one-way ANOVA [$P = 0.01$]). A more pronounced reduction (~2-fold) in heat shock-induced activity occurred with the deletion of that same region (Fig. 5B, one-way ANOVA [$P < 0.001$]), as measured by luciferase activity, suggesting that elements located within this region are necessary for efficient heat shock-induced activity of the promoter. The

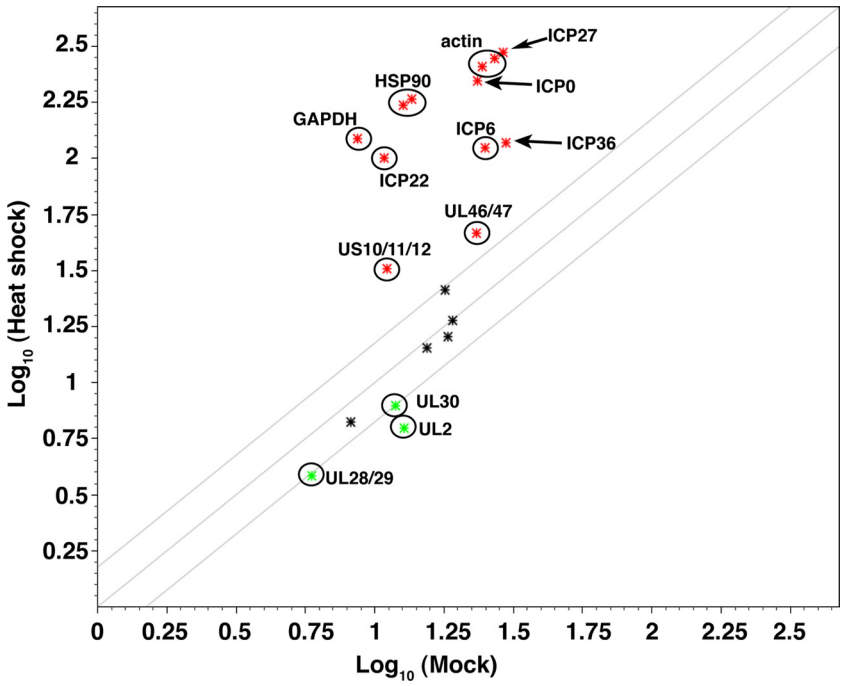

FIG. 2. Effect of heat shock on global HSV-1 transcription during lytic infection. Vero cells were infected with HSV-1 KOS at an MOI of 10 PFU/cell for 30 min, heat shocked for 1 h at 43°C or maintained at 37°C (mock heat shocked), and allowed to recover at 37°C for 1 h. Total RNA was then harvested, labeled, and hybridized to HSV-1 microarrays. Arrays were visualized by chemiluminescence, and images were captured with a charge-coupled device camera and processed with GEArray Expression Analysis Suite 2.0 (SABiosciences). The microarrays were standardized by using the interquartile method. The upper line represents a 1.5-fold increase, the middle line represents no change in expression, and the lower line represents a 1.5-fold decrease relative to the mock-treated control. Red stars indicate data points upregulated by >1.5-fold with treatment, black stars indicate data points not significantly affected by treatment, and green stars indicate data points downregulated by >1.5-fold compared to control. Upregulated genes of interest are indicated by circles and arrows. The figure represents three independent experiments.

elements potentially involved in ICP0 promoter heat shock-induction are discussed below.

(b) Truncations between −231 TSS and −95 TSS. Truncations introduced between −231 TSS and −95 TSS of the ICP0 promoter are diagrammed in Fig. 3. Basal activity of the ICP0 promoter fine truncation mutants located between −231 TSS and −95 TSS decreased relative to the full-length construct as previously published (8) (Fig. 5C, one-way ANOVA [$P < 0.0001$]). The series of truncations in this region of the ICP0 promoter presented a complicated profile of increases and decreases in the heat shock-induced activity of the promoter (Fig. 5D), suggesting a complex pattern of binding sites for both activating and inhibitory transcription factors. A reproducible and statistically significant decrease in the heat shock-induced activity of the ICP0 promoter was seen with the deletion of sequences between −107 TSS and −95 TSS (Fig. 5D, one-way ANOVA [$P = 0.0001$]), suggesting that sequences necessary for efficient heat shock-induced activity of the promoter are located in this region. Basal promoter activity increased with the deletion of sequences between −107 TSS and −95 TSS while the heat shock-induced activity decreased, suggesting that the observed phenotype is real and the sequences contained in this region of the promoter are involved in the response of the ICP0 promoter to heat shock.

Identification and characterization of cellular transcription factors that bind between −708 TSS and −686 TSS in the ICP0 promoter. We first analyzed the sequences between −708 TSS and −686 TSS of the ICP0 promoter using transcription factor binding site prediction software, Genomatix MatInspector (Fig. 3). The analysis yielded five predicted binding sites for four cellular transcription factors. The five sites included two binding sites for nuclear respiratory factor 1, involved in the expression of respiratory genes, and one site each for PAX-5, a B-cell-specific activating protein; Sp1, a well-characterized GC-box binding factor important for basal expression of viral and cellular genes (24, 34); and NF-Y, a CCAAT box binding factor. Of the four transcription factors listed, NF-Y is the most likely to be involved in upregulating promoter activity following heat shock due to of its well-defined role in the stress response and ubiquitous expression. For these reasons, we chose to assay the role of NF-Y in ICP0 promoter heat shock-induced activation.

NF-Y associates with the ICP0 promoter in Vero cell extracts. To determine whether NF-Y associates with the ICP0 promoter at −708 TSS in Vero cells, gel shifts with consensus NF-Y, wild-type, and mutant ICP0 probes were carried out using Vero cell nuclear extracts. A complex formed on the wild-type ICP0 promoter probe with the same electrophoretic mobility as that formed on consensus NF-Y binding site probe (Fig. 6A, lanes 1 and 3). The addition of an anti-NF-YA antibody to both the consensus NF-Y and wild-type ICP0 probes led to a complete supershift of the protein-DNA complex, indicating that it contained NF-Y (Fig. 6A, lanes 2 and 4). Complex formation was ablated by the

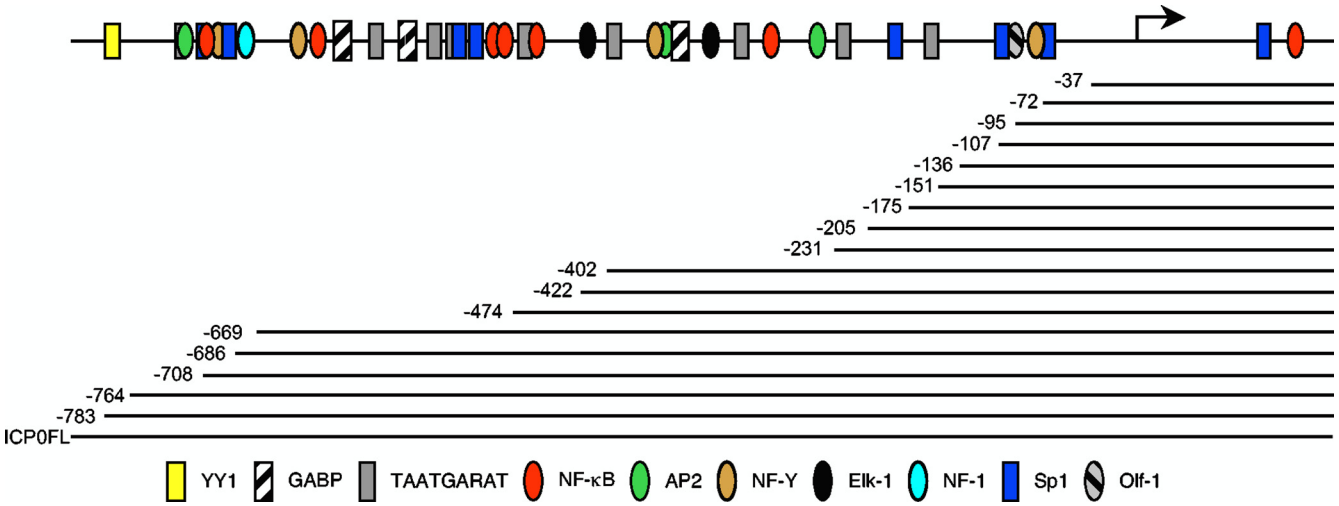

FIG. 3. Diagram of the wild-type ICP0 promoter and truncations used in the present study. Truncations of the ICP0 promoter were generated by PCR and named for the location of the truncation relative to the TSS. The full-length promoter construct is shown as ICP0FL. Predicted transcription factor binding sites are shown and the TSS is indicated by an arrow. ICP0FL represents the full-length ICP0 promoter, spanning from −800 to +150 bp relative to the TSS.

addition of specific cold competitor (lane 8) and failed to form on ICP0 NF-Y mutant probes containing mutations previously shown to abrogate NF-Y association (−708NFYmut1 and −2, lanes 5 and 6, mutations described in Materials and Methods) (22, 28). These observations suggest that complex association with the ICP0 promoter probe is specific for the NF-Y binding site and that the mutations are sufficient to abrogate binding of NF-Y to the ICP0 promoter at that site. Protein-DNA complexes with similar mobilities and characteristics were also seen in gel shifts with PC12 cell nuclear extracts (data not shown), suggesting that NF-Y binding to the ICP0 promoter is not cell type-specific and can occur in cells of neuronal origin.

It is interesting that although the introduction of both mut1 and mut2 mutations into the ICP0 promoter probe abrogated NF-Y binding, only mut2 was impaired for heat shock-induced activity in the luciferase assay. The mut2 set of point mutations removes the core CCAAT box sequence entirely (CCAAT is changed to AACGC), while the mut1 set of mutations alters only 2 bp (CCAAT changed to CGAGT). Although it is commonly accepted that all 5 bp in the CCAAT box are required for NF-Y binding (4, 30), it is possible that the mut1 mutation significantly decreased, but did not abolish NF-Y association. NF-Y binding may have been below the level of detection of the EMSA but was sufficient to drive ICP0 promoter activity.

Role of NF-Y in ICP0 promoter heat shock-induced activity. In order to determine the role of NF-Y binding in ICP0 promoter heat shock-induced activity, the same point mutations used in the gel shift probes were introduced into the predicted NF-Y binding site in the ICP0 promoter by using the pAlter-1 mutagenesis system (the construction of the mutations is described in Materials and Methods) (22, 28). The pAlter-1 mutagenic approach required the generation of a new wild-type ICP0 plasmid, ICP0pAlter (in the pGL3 backbone), which differs from the ICP0FL construct in its flanking multiple cloning site sequence. Changes in the vector containing the wild-type ICP0 promoter did not significantly affect heat shock-induced activity of the promoter (data not shown). The mutations were verified by sequencing and the mutant constructs were tested for basal and heat shock-induced activity in the luciferase assay system.

The basal activity of −708NFYmut1 and −2 was not significantly affected compared to the wild-type construct (ICP0pAlter; Fig. 6B). Heat shock-induced activity of NF-Ymut1 was modestly reduced compared to the wild-type construct (ICP0pAlter), while that of NF-Ymut2 was moderately impaired (Fig. 6C, one-way ANOVA [$P = 0.0005$]). These data suggest that the NF-Y site located in the 5′ end of the ICP0 promoter is required specifically for heat shock-induced, but not basal activity. Since heat shock induction is reduced compared to the −686 construct, but not abolished by mutation of the NF-Y site, it is likely that NF-Y cooperates with factors that interact with this region to regulate ICP0 promoter activity following heat shock.

Identification and characterization of cellular transcription factors binding between −107 TSS and −95 TSS in the ICP0 promoter. A second decrease in ICP0 promoter heat shock-induced activity was observed with the deletion of sequences between −107 TSS and −95 TSS. The 12 bases deleted between −107 TSS and −95 TSS have been shown previously to contain an olfactory neuron-specific transcription factor, Olf-1, binding site (11). In our experimental system, gel shifts testing Olf-1 binding to the ICP0 promoter proved inconclusive, whereas mutation of the Olf-1 site did not affect either basal or heat-induced ICP0 promoter activity (data not shown). Thus, Olf-1 binding is not likely to be involved in the upregulation of ICP0 promoter activity after heat shock. Interestingly, transcription factor binding site predictions pointed to the presence of an inverted NF-Y site in close proximity to the Olf-1 site (between −71 TSS and −75 TSS), previously published by O'Rourke and O'Hare (33).

Having identified NF-Y as potentially involved in ICP0 promoter heat shock-induced activity, we extended our study of

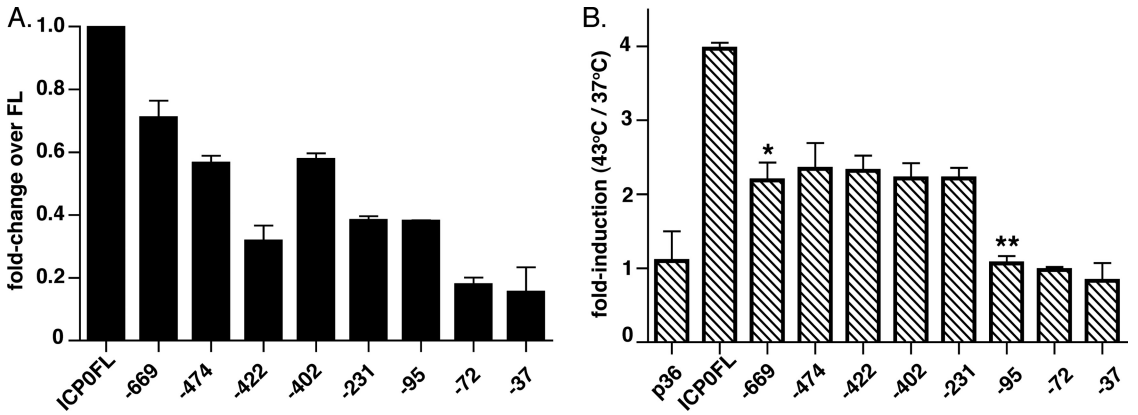

FIG. 4. Two regions within the ICP0 promoter are important for efficient upregulation after heat shock. Truncations of the ICP0 promoter were generated by PCR. Vero cells were transiently transfected with the full-length ICP0 promoter (ICP0FL) or truncated constructs in duplicate, heat shocked or mock heat shocked, and allowed to recover. Lysates were prepared, and the luciferase activity was quantitated. (A) Basal activity of the truncated promoters relative to the full-length construct. (B) Heat-induced activity in the fold change versus the mock heat-shocked control (43°C/37°C) ± the SEM ($n = 3$; one-way ANOVA [$P < 0.0001$]; Bonferroni's multiple comparison post-test [*, $P < 0.001$; **, $P < 0.01$]; HSE induction, 242.8 ± 46.45-fold).

the role of NF-Y in promoter activation to this second region of interest. NF-Y association with −75 TSS of the ICP0 promoter was demonstrated by gel shift analysis using Vero cell nuclear extracts. A complex formed on the wild-type −75 ICP0 promoter probe with the same electrophoretic mobility as that formed on consensus NF-Y binding site probe (Fig. 7A, lanes 1 and 3). The addition of an anti-NF-YA antibody, but not nonspecific immunoglobulin G (data not shown) led to complete supershifts of the protein-DNA complexes formed on both the consensus NF-Y and wild-type ICP0 probes, indicating that they contained NF-Y components (Fig. 7A, lanes 2 and 4, and data not shown). Complex formation was ablated by the addition of specific cold competitor (lane 8) and failed to form on ICP0 NF-Y mutant probes containing mutations previously shown to abrogate NF-Y association (−75NFYmut1 and −2, lanes 5 and 6) (22, 28), suggesting that the complex is specific for NF-Y binding and that the mutations are sufficient to abrogate binding of NF-Y to the ICP0 promoter at −75 TSS. A complex migrating below the nonspecific band does not bind the consensus NF-Y probe but does specifically associate with the wild-type ICP0 probe (Fig. 7A, lanes 1 to 4). The potential identity and role of this complex in ICP0 promoter activity are noted in the Discussion.

The same two mutations that abrogated NF-Y association in the gel shifts were introduced into the ICP0 promoter at −75 TSS and tested for basal and heat shock-induced activity in the luciferase assay as described previously. Mutation of the NF-Y site at −75 TSS did not affect either the basal or heat-induced activity of the ICP0 promoter (Fig. 7B and C, one-way ANOVA [basal, $P = 0.31$; induced, $P = 0.14$]). This suggests that either the NF-Y binding site at −75 TSS does not play a role in the heat shock-induced activity of the promoter or that other transcription factors binding in the near vicinity can compensate for the loss of NF-Y function. Mutation of both NF-Y sites in the same ICP0 promoter-luciferase construct did not yield a further decrease in induced activity (data not shown), suggesting that the NF-Y site at −708 TSS, but not at −75 TSS, is important for ICP0 promoter response to heat shock in Vero cells.

Role of NF-Y in the ICP0 promoter response to heat shock in the presence of other viral factors. To address the role of NF-Y in heat shock-induced ICP0 promoter activity in the context of a viral infection, we performed quantitative RT-PCR analysis with RNA from cells expressing wild-type (YA) or dominant-negative (YAm) forms of NF-YA (20) infected with HSV-1. Cells were transduced with Ad.GFP, Ad.YA, and Ad.YAm adenoviral vectors, and the expression of NF-YA was verified by Western blotting (data not shown).

Vero cells were superinfected with HSV-1 KOS, heat shocked, and allowed to recover for 1 h at 37°C. Total RNA was then harvested, reverse transcribed (RT^+) or mock reverse transcribed (RT^-) and used in real-time RT-PCRs with primers specific for ICP0, ICP22, and cellular β-actin as an internal control.

No signal was detected in RT^- samples, confirming the absence of genomic DNA contamination (data not shown). A twofold decrease in heat shock-induced transcription of the ICP0 promoter was seen in the presence of DN NF-YA (Fig. 8A, two-tailed t test [$P = 0.033$]). This decrease was not observed for ICP22 transcripts (Fig. 8B). Since heat shock leads to a transient shutdown of cellular transcription (3), the fold induction of promoter activity after heat shock was less than one. Therefore, cells expressing basal or endogenous wild-type NF-YA were better able to recover from or overcome the block in transcription to direct synthesis of ICP0 transcripts, suggesting that NF-Y activity is important for ICP0 promoter activation after heat shock (3). Although we cannot definitively establish that the dominant-negative NF-Y has a direct or indirect effect on ICP0 promoter activity, the results of the RT-PCR are consistent with data presented in Fig. 6, which suggest that NF-Y is likely necessary for efficient ICP0 promoter activity following heat shock.

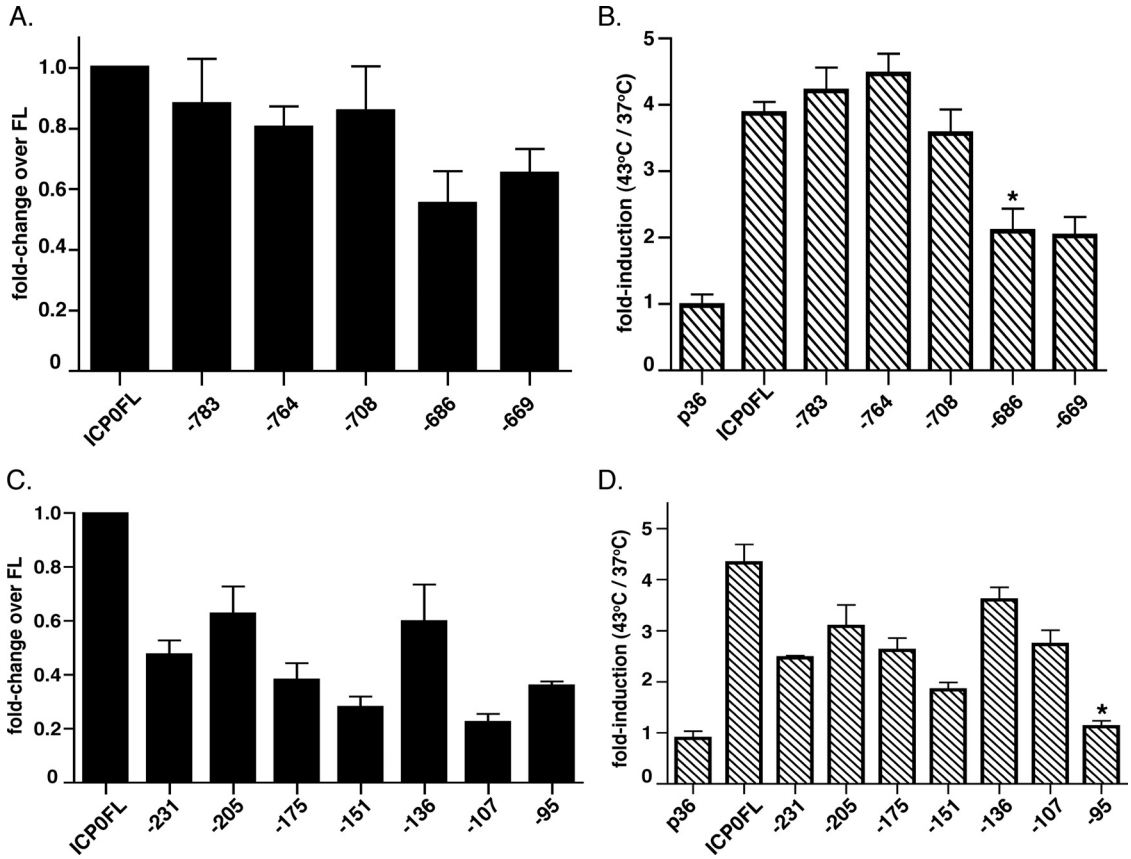

FIG. 5. Fine mapping of the ICP0 promoter to identify elements important for upregulation after heat shock. Truncations in the 5′- and TSS-proximal regions of the ICP0 promoter were generated by PCR. Vero cells were transiently transfected with ICP0FL or truncated constructs in duplicate, heat shocked or mock heat shocked, and allowed to recover. Lysates were prepared, and luciferase activity was quantitated. 5′ truncations are indicated as follows: basal activity of the promoters relative to the full-length construct (A) and heat-induced activity in the fold change versus the mock heat-shocked control (43 or 37°C) ± the SEM (B) ($n = 3$; one-way ANOVA [$P = 0.0002$]; Bonferroni's multiple comparison post-test [*, $P = 0.05$]; HSE induction, 257.5 ± 19.27-fold). TSS-proximal truncations are indicated as follows: basal activity of the promoters relative to the full-length construct (C) and Heat-induced activity in the fold change versus the mock heat-shocked control (43 and 37°C) ± the SEM (D) ($n = 3$; one-way ANOVA [$P = 0.0001$]; Bonferroni's multiple comparison post-test [*, $P < 0.01$]; HSE induction, 328.1 ± 32.17-fold).

DISCUSSION

In the present study, we set out to define which HSV-1 promoters are responsive to cellular stress induced by heat shock in order to determine (i) which viral genes are likely to be the first transcribed following the reactivation-inducing stimulus, and (ii) which cellular factors are responsible for their activation. We used heat shock, a well-characterized inducer of HSV-1 reactivation both in vivo and in vitro, as a model cellular stress to test the activity of a panel of viral promoter-reporter constructs. We found that the IE ICP0 and ICP22 promoters were the most strongly induced in the absence of other viral factors following heat shock in the luciferase system. Microarray analysis of HSV-1 transcription after heat shock in a lytic infection confirmed the upregulation of ICP0 and ICP22 activity in the presence of all other viral and cellular proteins in Vero cells.

Although ICP22 is an important viral regulatory protein that plays a key role in replication in vivo (32), we chose to focus our studies first on ICP0, an IE transactivator that has been implicated in reactivation in a number of studies (17, 46). We used mutagenesis to identify regions and elements within the ICP0 promoter important for the response to heat shock. From our studies, an NF-Y binding site at −708 TSS was identified as a determinant of the heat shock-induced activity of the ICP0 promoter in the absence of other viral factors. RT-PCR analysis of infected cells expressing a dominant-negative form of NF-YA confirmed the requirement for NF-Y activity for ICP0 promoter heat shock-induced upregulation. Taken together, these findings suggest that (i) members of the IE gene class are most potently upregulated in the absence of other viral factors after heat shock (as determined by the luciferase assay) and that (ii) NF-Y plays a role in the upregulation of at least one member of the IE gene class, ICP0, and (iii) members of the IE gene class may be among the first to be induced early in reactivation.

Role of NF-Y in heat shock-induced ICP0 promoter activity. Mutation of the NF-Y site at −708 TSS decreased ICP0 promoter induction by heat shock, but did not abolish it entirely, suggesting that other cellular factors are necessary for efficient promoter upregulation. It is possible, and likely, that NF-Y

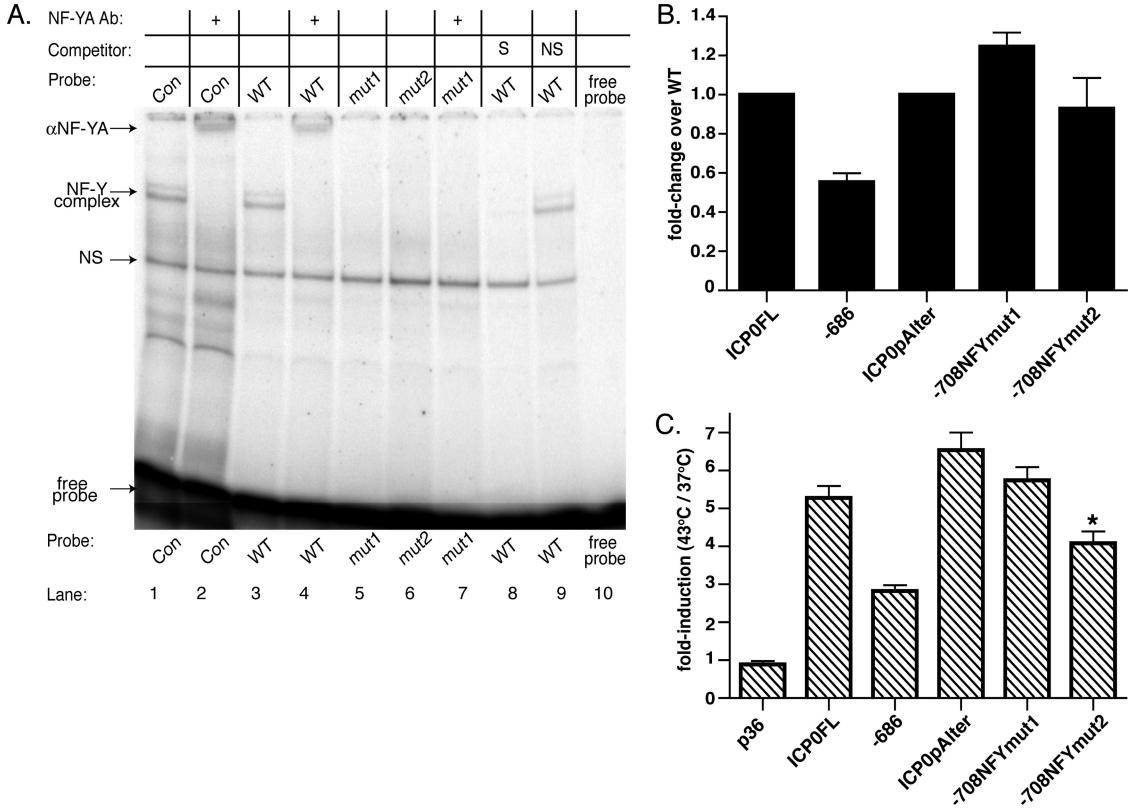

FIG. 6. NF-Y associates with the ICP0 promoter at −708 and is important for efficient promoter upregulation after heat shock. (A) Gel shift analysis of NF-Y association with the ICP0 promoter. Vero cell nuclear extracts were incubated with αNF-YA antibody (Santa Cruz) for 1 h on ice. Either wild-type (WT) or mutant ^{32}P-labeled probe was then added, and the binding reaction was allowed to proceed for 30 min on ice. Protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE. The locations of the NF-Y and supershifted complexes and of the unbound probe are indicated by arrows. Lanes: 1, consensus NF-Y probe (Con); 2, consensus probe with NF-YA antibody; 3, wild-type −708 ICP0 probe; 4, wild-type −708 ICP0 probe with NF-YA antibody; 5, −708NFYmut1 probe; 6, −708NFYmut2 probe; 7, −708mut1 probe with NF-YA antibody; 8, specific (S) cold competitor; 9, nonspecific (NS) cold competitor; 10, no nuclear extract; NS, nonspecific band. (B and C) Luciferase assays for ICP0 promoter activity. Vero cells were transiently transfected with the wild-type (ICP0FL and ICP0pAlter) or mutated constructs in duplicate, heat shocked or mock heat-shocked, and allowed to recover. Lysates were prepared, and the luciferase activity was quantitated. (B) Basal activity of the promoters relative to the wild-type construct. (C) Heat-induced activity in the fold change versus the mock heat-shocked control (43 and 37°C) ± the SEM (n = 3; *, one-way ANOVA [P = 0.0005]; HSE induction, 543.7 ± 59.77-fold).

cooperates with transcription factors binding at proximal sites to regulate ICP0 promoter activity. One potential cellular factor is Oct-1. 5′-TAATGARAT-3′ elements located throughout the ICP0 promoter contain degenerate Oct-1 recognition sequences that can bind Oct-1 independently of the VP16/HCF complex (43). In addition to mediating HSV-1 IE gene expression, Oct-1, a cellular POU domain transcription factor, has been shown to be involved in the cellular stress response. Specifically, Oct-1 was shown to act in concert with NF-YA to upregulate transcription of Gadd45 following DNA damage (21). Intact Oct-1 and NF-Y binding sites were necessary for optimal upregulation, suggesting that the functions of both NF-Y and Oct-1 are necessary for efficient stress-induced transcription of Gadd45. Therefore, it is possible that complete inhibition of ICP0 promoter stress-induced activity requires mutation of both Oct-1 and NF-Y sites.

Likewise, mutation of both the Olf-1 and −75 TSS NF-Y site could have a greater effect on promoter activity than mutation of the Olf-1 or NF-Y sites alone. The study by O'Rourke and O'Hare that originally published the NF-Y site at −75 TSS in the ICP0 promoter noted the binding of an additional, unidentified factor (termed F2) to the same probe as that bound by NF-Y (33). It is possible that F2 plays a role in heat shock-induced ICP0 activity and may be present in our gel shifts for NF-Y binding at −75 TSS as a complex migrating below a nonspecific band with the wild-type ICP0 probe (Fig. 7A, lanes 1 to 4). Abrogating F2 but not NF-Y binding to the ICP0 promoter by mutagenesis and testing the effect of these mutations on ICP0 promoter basal and heat shock-induced activity should give a clearer picture of the role of NF-Y and neighboring factors in heat-induced and basal activity of the ICP0 promoter.

Effects of heat shock on global HSV-1 transcription. Expression of ICP0 and ICP22 increased after heat shock, confirming data from the luciferase assay. However, microarray analysis revealed additional viral genes upregulated by heat shock, such as ICP27 and ICP6, which were not upregulated when tested in the luciferase assay system. There are a number of possible explanations for this discrepancy. The luciferase assay tests promoter induction in the absence of other viral factors,

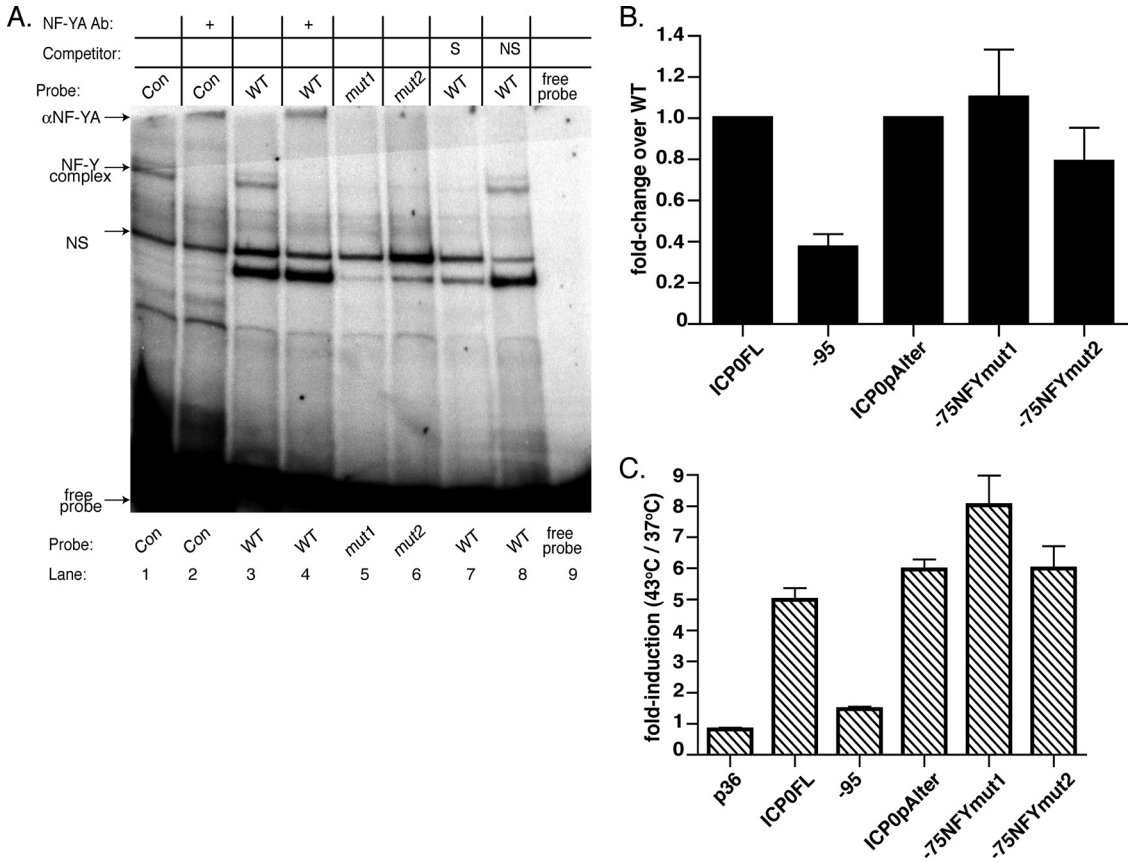

FIG. 7. NF-Y associates with the ICP0 promoter at −75 but is not required for efficient promoter upregulation after heat shock. (A) Gel shift analysis of NF-Y association with the ICP0 promoter. Vero cell nuclear extracts were incubated with αNF-YA antibody (Santa Cruz) for 1 h on ice. Either wild-type (WT) or mutant ICP0 ^{32}P-labeled probe was then added to the binding reaction. Binding was allowed to proceed for 30 min on ice. Protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE. The locations of the NF-Y and supershifted complexes are indicated by arrows. Lanes: 1, consensus NF-Y probe (Con); 2, consensus probe with NF-YA antibody; 3, wild-type −75 ICP0 probe; 4, wild-type −75 ICP0 probe with NF-YA antibody; 5, −75NFYmut1 probe; 6, −75NFYmut2 probe; 7, specific (S) cold competitor; 8, nonspecific (NS) cold competitor; 9, no nuclear extract; NS, nonspecific band. (B and C) Luciferase assays for ICP0 promoter activity. Vero cells were transiently transfected with the wild-type (ICP0FL and ICP0pAlter) or mutated constructs in duplicate, heat shocked or mock heat shocked, and allowed to recover. Lysates were prepared, and the luciferase activity was quantitated. (B) Basal activity of the promoters relative to the wild-type construct. (C) Heat-induced activity in the fold change over mock heat-shocked control (43 and 37°C) ± the SEM (HSE induction, 514 ± 51.66-fold; $n = 4$).

whereas microarray analysis includes all viral gene products that are normally expressed in the first 2 h of infection. Notably, ICP0 has been shown to be a potent inducer of the ICP6 promoter (10), which likely explains why ICP6 transcripts were readily detected in our experiments. In addition, the promoters used in the luciferase assay were defined in previous studies, primarily by deletion mutagenesis, and do not necessarily reflect the boundaries of the promoter found in the viral genome. Enhancer elements located kilobases away from the TSS could also affect the expression of the gene of interest, with or without heat shock, but cannot be included and tested in the luciferase reporter constructs.

Gene expression during reactivation. Our findings suggest that IE genes ICP0 and ICP22 are responsive to a representative cellular stress (heat shock) and may be among the first viral genes activated during reactivation. Over the years, studies have both supported and refuted the possibility that IE genes are the first to be expressed. One study used transgenic mice expressing reporter genes driven by HSV-1 promoters to characterize the in vivo response of viral promoters to stress in the absence of other viral factors (29). The findings in that study demonstrated that the activity of the ICP0, but not ICP27 or ICP4 promoters, was upregulated in the TG by UV irradiation and transient hyperthermia, suggesting that the ICP0 promoter is responsive to cellular stress. Conversely, a study using RT-PCR analysis of explanted, latently infected TG found that E and DE genes (TK, ICP6, and VP5) were the first to be expressed following explantation (44). Although the latter study was carefully controlled and executed, the issue of differential primer sensitivities is one that is difficult to reconcile.

A recent study took a very similar approach to our own in order to define the heat shock response of HSV-1 promoters (7). The study utilized quiescently infected differentiated PC12 cells that nonetheless expressed readily detectable levels of productive cycle genes, to assay the response of HSV-1 promoter-luciferase reporters to heat shock by the dual luciferase assay. This experimental approach poses a number of problems

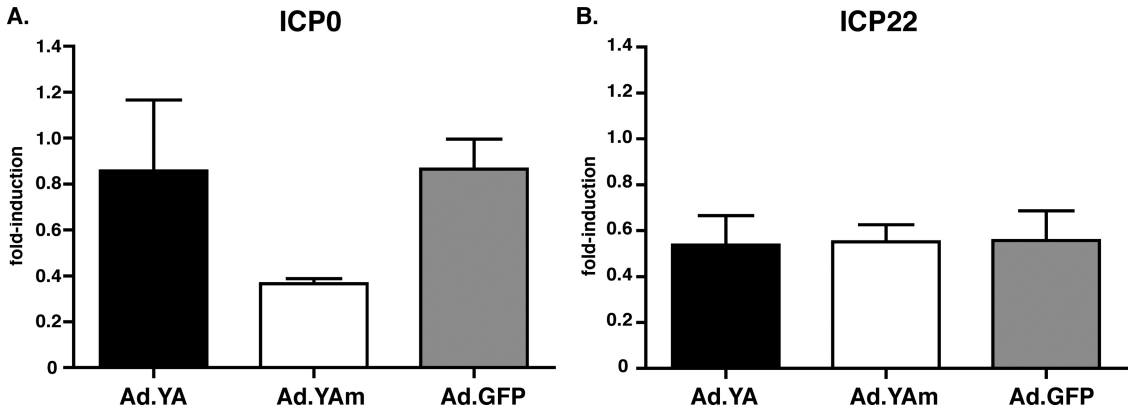

FIG. 8. NF-Y activity is necessary for induction of the ICP0 promoter after heat shock during lytic infection. Vero cells were infected with wild-type, DN NF-YA, or GFP adenoviruses at an MOI of 25 PFU/cell and superinfected with HSV-1 KOS at an MOI of 1 PFU/cell for 30 min, heat shocked for 1 h at 43°C, and allowed to recover for 1 h at 37°C, and the RNA was harvested for RT-PCR. PCRs were performed in triplicate with primers specific for ICP0, ICP22, and β-actin. Reactions were standardized to β-actin. ICP0 (A) and ICP22 (B) transcript levels. The results are presented as the fold induction of transcription in response to heat shock (43 or 37°C) ± the SEM ($n = 3$).

and concerns. This model does not accurately mimic latency, in the course of which very few viral transcripts can be detected at exceedingly low levels. In addition, no recovery time was permitted the promoter-luciferase transfected cells following the heat shock, raising the question of whether the promoters were given adequate time to drive the expression of detectable levels of luciferase after the generalized host cell transcription shut down following heat shock (3). The authors of that study concluded that HSV-1 promoters failed to respond to cellular stress as tested by the response to heat shock and forskolin treatment; however, based on the design of the assays, these conclusions should be interpreted with care.

Our study does not rule out the possibility that a reactivation-inducing stimulus acts to initiate translation of the low levels of viral transcripts (ICP0, ICP4, and TK) found in latently infected neurons (25, 35, 46). Further work is necessary to uncover whether translation of existing transcripts takes place immediately following a reactivation-inducing stimulus and how it contributes to the reactivation program. Importantly, the transcription of viral genes from latent genomes and translation of ICP0, ICP4, or TK from existing transcripts are not mutually exclusive pathways. They could, hypothetically, work in concert to push the virus toward lytic replication.

A previous study examined the activation of the ICP0 promoter by heat shock during reactivation. Thompson and Sawtell (46) found that a mutant HSV-1 containing a 350 base pair internal deletion of the ICP0 promoter (ΔTfi) (9) was mildly impaired for lytic replication but was unable to reactivate or to efficiently do so in vivo after heat shock. The ΔTfi mutant ICP0 promoter contains the 5′ NF-Y site at −708 but is deleted of the NF-Y site at −75. These results suggest that the region around −75 may play a pivotal role in activating ICP0 transcription by heat shock-induced reactivation. Although it would appear that the NF-Y site at −708 is not required for heat shock-induced reactivation, its closer proximity and stereotopology to downstream ICP0 promoter elements in ΔTfi may impair its ability to stimulate ICP0 transcription. A previous study that examined the role of the cyclic AMP-response element of the HSV-1 latency-associated transcript promoter supports this possibility (1). Clearly, additional genetic studies will have to be performed to establish the importance of this region and the NF-Y sites in reactivation.

The Thompson and Sawtell study also found that ICP0-null viruses supported protein expression following heat shock-induced reactivation in vivo but not virus production, leading these authors to conclude that ICP0 is not necessary for initiating reactivation but simply for entry into the lytic phase of replication (46). A more recent study by the same group demonstrates that the VP16 promoter is upregulated after heat shock of latently infected ganglia (45). These findings suggest that VP16 may be the first gene expressed after reactivation and that it could in turn activate the expression of ICP0 via 5′-TAATGARAT-3′ elements in its promoter. It is clear that VP16 is important for efficient reactivation from latency; however, it is not likely to be the only viral protein required for this critical process. Our study identifies a pathway in which the promoter of ICP0, an IE gene previously implicated in reactivation, interfaces with at least one key stress-responsive cellular transcription factor and could work in concert with VP16 to promote efficient reactivation.

Potential mechanism of ICP0 promoter upregulation. ICP0 and ICP22 appeared to be upregulated after heat shock both in the presence and in the absence of other viral factors (in lytically infected Vero cells assayed by microarray and in luciferase assays, respectively). This suggests that the IE ICP0 and ICP22 genes may be among the first genes to be transcribed following a reactivation-inducing stimulus, such as heat shock, in latently infected cells, and NF-Y is involved in the regulation of the ICP0 promoter specifically after stress.

The involvement of NF-Y with HATs is an attractive model for the mechanism of promoter activation following a reactivation-inducing stimulus. It is possible that sites within selected HSV-1 promoters, such as ICP0, are maintained in a transcriptionally permissive state during latency through their association with NF-Y, as is seen with multiple cellular stress-inducible promoters (hsp70, Grp78, and XBP-1) (12, 19). The IE gene promoter can thereby be quickly and efficiently upregu-

lated in response to a stressful stimulus, leading to the production of IE proteins and the initiation of HSV-1 lytic replication.

Future experiments to define the heat shock response of viral promoters in neurons will be necessary to corroborate our data in Vero cells. Furthermore, additional studies to define the mechanism of NF-Y induction of the ICP0 promoter will offer a clearer picture of the early events involved in HSV-1 reactivation.

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