Control of the Calcitonin Gene-related Peptide Enhancer by Upstream Stimulatory Factor in Trigeminal Ganglion Neurons*

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The neuropeptide calcitonin gene-related peptide (CGRP) is a key player in migraine. However, the transcription factors controlling CGRP expression in the migraine-relevant trigeminal ganglion neurons are unknown. Previous in vitro studies demonstrated that upstream stimulatory factor (USF) 1 and USF2 bind to the CGRP neuroendocrine-specific 18-bp enhancer, yet discrepant overexpression results in cell lines, and the ubiquitous nature of the USF cast doubts about its role. To test the functional role of USF, we first demonstrated that small interfering RNAs directed against USF1 and USF2 reduced endogenous CGRP RNA and preferentially targeted the USF binding site at the 18-bp enhancer in the neuronal-like CA77 cell line. In cultured rat trigeminal ganglion neurons, knockdown of either USF1 or USF2 reduced CGRP promoter activity. Conversely, overexpression of USF1 or USF2 increased promoter activity. The activation was even greater upon cotransfection with an upstream activator of mitogen-activated protein kinases and was synergistic in a heterologous cell line. To begin to address the paradox of how ubiquitous USF proteins might direct neuronal-specific activity, we examined USF expression and used a series of adenoviral reporters in the cultured ganglia. Unexpectedly, there was more intense USF immunostaining in neurons than nonneuronal cells. Importantly, the 18-bp USF enhancer driving a minimal promoter was sufficient for neuronal-specific activity. Furthermore, the 18-bp enhancer is active only in this non-neuronal cell line that does not express the endogenous CGRP gene. In contrast, in another non-neuronal cell line (COS7) and in the neuronal-like CA77 thyroid C cell line, USF overexpression failed to stimulate promoter activity. Furthermore, the 18-bp enhancer is active only in neuroendocrine thyroid C cell lines (11), yet USF is ubiquitously expressed. These discrepancies raised the need to demonstrate whether USF is indeed a regulator of the CGRP 18-bp enhancer in neurons.

USF was initially identified as a cellular transcription factor for the adenovirus-2 major late gene (14, 15). Because of this initial finding, USF has been identified as a transcription factor for many genes involved in a range of cellular processes, including proliferation (16), stress responses (17), and metabolism (18). The two USF proteins, USF1 and USF2, share 44% identity overall and 70% identity within the C-terminal region, which includes basic-helix-loop-helix and leucine zipper domains (13). The two proteins can form homodimers, although the heterodimer is usually the most abundant form (13, 19, 20). USF1 and USF2 are ubiquitously expressed, including in the nervous system (13). A paradox is that several helix-loop-helix proteins

Calcitonin gene-related peptide (CGRP) is a potent vasodilatory neuropeptide (1) that has been implicated in the pathophysiology of migraine (2–4). Although the mechanisms underlying migraine remain controversial, there is a growing acceptance of the involvement of the trigeminal ganglion neurons, which express CGRP and relay nociceptive signals from the vasculature and dura to the brainstem (5, 6). Most notably, systemic administration of CGRP induces migraine-like symptoms among migraineurs (7), and a CGRP receptor antagonist can attenuate migraine (8). The possibility that CGRP synthesis is elevated during migraine is suggested by elevation of serum CGRP levels during spontaneous migraine (9, 10). Given the generally long duration of migraine, it seems reasonable that these elevated CGRP levels might be sustained by increased transcription. Hence, an understanding of CGRP regulation in trigeminal neurons may provide clues regarding the pathophysiology of migraine.

We have previously reported that a heterodimer of the transcription factor USF1 and USF2 binds to the 18-bp enhancer of the CGRP gene in vitro (11). In addition to the binding data, USF overexpression increased CGRP promoter activity in a lung carcinoma cell line (12). However, the activation by USF was only observed in this non-neuronal cell line that does not express the endogenous CGRP gene. In contrast, in another non-neuronal cell line (COS7) and in the neuronal-like CA77 thyroid C cell line, USF overexpression failed to stimulate promoter activity. Furthermore, the 18-bp enhancer is active only in neuroendocrine thyroid C cell lines (11), yet USF is ubiquitously expressed. These discrepancies raised the need to demonstrate whether USF is indeed a regulator of the CGRP 18-bp enhancer in neurons.

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that are ubiquitously expressed, including USF, can also be involved in cell-specific expression (21, 22).

The USF proteins can be regulated by phosphorylation. In vitro kinase assays have shown that p38 MAP kinase, but not Jun N-terminal kinase (JNK), phosphorylates threonine 153 of USF1 (17). Phosphorylation of USF1 by p38 MAP kinase is necessary for transcriptional activation of the tyrosine kinase promoter (17). A physical interaction between phosphorylated extracellular signal-regulated kinase (ERK) and USF1 has been suggested (23). Additionally, ERK MAP kinase appears to act through USF to stimulate the Cox-2 promoter (24).

Luciferase activity using reagents from Promega. NCI-H460 cells 48–96 h after cotransfection, cell lysates were assayed for luciferase activity using the pStec1. The resultant plasmid pStec1-luc was linearized by digestion with PstI, treated with mung bean nuclease, then ligated with Gateway® Reading Frame Cassette C.1 (Invitrogen). pENTR-hCGRP, pDestination C-Luc was generated by subcloning the β-globin/IgG chimeric intron from pCI (Promega) into BamHI and PstI sites of pGEM®-4Z (Promega) to make pStec1 and firefly luciferase from pGL3-Basic (Promega) into XhoI and XbaI sites of pStec1. The resultant plasmid was transfected into CA77 cells and tested for their effects on CGRP promoter activity. Two USF1 siRNA duplexes decreased promoter activity, whereas the other duplex did not affect activity. The most potent duplex was used for further studies. Only one USF2 siRNA duplex decreased promoter activity. Rat USF1 siRNA is 5’-CCAACGCUACGUAGCUUCGGA-3’; rat USF2 siRNA is 5’-GCAUCCUGCACCCGGCUGAUAU-3’. Stealth™ RNAi Negative Control Medium GC Duplex (Invitrogen) was used as the nonspecific control siRNA duplex.

Reverse Transcription (RT) and Quantitative PCR (qPCR)—Transfection of siRNA duplexes was performed as described above. A plasmid encoding cytomegalovirus (CMV) promoter-driven green fluorescent protein (GFP) was cotransfected with either 5 nM nonspecific control siRNA or USF siRNA (2.5 nM USF1 siRNA and 2.5 nM USF2 siRNA) duplexes. After 72 h, GFP-positive cells were collected by flow cytometry, and RNA was extracted using a QiAshredder column and RNeasy Mini kit (Qiagen). For each sample, about 500 ng of DNase I-treated RNA was applied per RT reaction using a random primer as recommended (Applied Biosystems). One-tenth of the cDNA was subjected to real-time qPCR using SYBR Green as described (31) with 50 nM CGRP primers or 333 nM 18 S rRNA primers. For each sample qPCR was performed in triplicate. The PCR protocol was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of denaturing at 95 °C for 15 s, annealing at 60.7 °C for 30 s, and extension at 72 °C for 1 min. PCR primers were: rat CGRP (GenBank M11597), sense, 5’-AACCTTAGAAG-CAGCCCCAGGCTAT-3’, and antisense, 5’-GTGGGCAAAAGTTGTCCTTCACCA-3'; rat 18 S rRNA (GenBank V0127), sense, 5’-ATTGCCGTTCTTAGTTGTG-3’, and antisense, 5’-AACGCCACTTGTCCCTCTAATA-3’. Relative quantification of CGRP mRNA level was determined using the ΔΔCt method (32).

Isolation and Culture of Neurons from Rat Trigeminal Ganglia—Ganglia were removed from Sprague-Dawley rat pups (2–4 days old) and cultured as previously described with some modifications (31). Four ganglia were used per sample. Cells were resuspended in complete medium (10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml mouse 2.5 S nerve growth factor (Alomone Labs), L-15 medium (Leibovitz)) and plated onto laminin (Roche Applied Science)-coated coverslips placed in a 6-well dish. The laminin-coated coverslips were prepared by loading 4 mg/ml of phosphate-buffered saline (PBS) onto each 4-cm² coverslip and subsequent overnight incubation at 4 °C.

Transfection of Rat Trigeminal Ganglia Cultures—The 2.24-kb-human CGRP promoter-luciferase plasmid (hCGRP-luc) was generated by homologous recombination of pDestination C-Luc and pENTR-hCGRP. pDestination C-Luc was generated by subcloning the β-globin/IgG chimeric intron from pCI (Promega) into BamHI and PstI sites of pGEM®-4Z (Promega) to make pStec1 and firefly luciferase from pGL3-Basic (Promega) into XhoI and XbaI sites of pStec1. The resultant plasmid (pStec1-luc) was linearized by digestion with PstI, treated with mung bean nuclease, then ligated with Gateway® Reading Frame Cassette C.1 (Invitrogen). pENTR-hCGRP was generated by subcloning a PCR fragment of the 2.24-kb hCGRP promoter into pGEM-T Easy (Promega) then into the EcoRI site of Gateway® pENTR™11 vector (Invitrogen). Human USF1 and mouse USF2 expression vectors have been described (12). The T153A and T153E mutant USF1 vectors were generated from the USF1 vector using the QuikChange® site-directed mutagenesis kit (Stratagene). The MEKK (amino acids 380–672) plasmid from Stratagene has been described (30).
Within 24 h of culturing the dissociated cells in each well of a 6-well dish (Falcon) were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Plasmids were mixed with Lipofectamine 2000 (ratio, 1 µg to 1 or 2 µl) in warm L-15 medium. After incubation with transfecting solutions, cultured cells were scraped with 1 ml of PBS and transferred to Eppendorf tubes. Cells were collected by centrifugation at 14,000 rpm for 3 min. Cell lysates were prepared with 50 µl of 1× reporter lysis buffer (Promega) and subjected to freeze-thawing to aid lysis. For luciferase activity assays, 20 µl of lysate was mixed with reagents from Promega. Transfections of siRNA duplexes involved procedures similar to the plasmid transfections. After 48–72 h, the cells were lysed and assayed for luciferase activity and Western blotting.

Adenoviral Infections of Trigeminal Ganglia Cultures—AdrCGRP-luc, an adenovirus containing the 1.25-rCGRP fused with firefly luciferase in pGL3 has been described (28). The AdrCGRP-Bam-luc adenoviral vector has a BamHI linker inserted into the 1.25-rCGRP. The 1.25-kb rCGRP-Bam mutant promoter fragment was obtained by digestion of the 1.25-kb rCGRP-Bam-luc plasmid (12) with XbaI and SacI, then subcloned into the Nhe and SacI sites of the pGL3 luciferase vector (Promega) and transferred as an XbaI-KpnI fragment into pacAd5K-Npa adenoviral shuttle vector. Adenoviruses were generated and purified by the University of Iowa Gene Transfer Core Facility. An adenoviral vector containing a minimal TK promoter with three copies of the 18-bp enhancer and the β-galactosidase reporter gene (Ad18-bp-TK-lacZ) was generated from a lacZ shuttle plasmid and the previously described 18-bp-TK-luciferase plasmid (29). The AdCMV-β-galactosidase (AdCMV-lacZ) adenoviral vector has been described (33).

Trigeminal ganglia cultures were infected with adenovirus 24 h after plating. Cultures were incubated with 200 µl of L-15 media containing 1.1 × 10⁶ plaque-forming units of AdrCGRP-luc or AdrCGRP-Bam-luc per sample for 4 h at 37 °C and ambient CO₂. Then 2 ml of the complete medium described above was added. After 24 h of incubation, cultures were subjected to immunocytochemistry. For infections of Ad18-bp-TK-lacZ and AdCMV-lacZ, 2 × 10⁶ or 2.4 × 10⁷ plaque-forming units, respectively, were used. After 36 h of incubation immunocytochemistry was performed.

Immunocytochemistry—Cultures were rinsed in PBS and fixed in cold methanol for 10 min at −20 °C. After washing with PBS, the cells were incubated with 10% bovine serum albumin in PBS for 30 min. This was followed by 1 h of incubation with primary antibodies, a monoclonal mouse anti-β-tubulin III antibody (1:800 dilution, Sigma), and a polyclonal rabbit anti-β-galactosidase antibody (1:100 dilution, Santa Cruz Biotechnology). The primary antibodies were diluted in 1.5% bovine serum albumin containing PBS. After washing with PBS, the cells were incubated in 10% bovine serum albumin containing PBS for 30 min. Then rhodamine anti-rabbit IgG and fluorescein isothiocyanate-anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch Laboratories) were added to the cells. After washing with PBS, the cells were incubated with ToPro3 (1:1000 diluted in dimethyl sulfoxide, Molecular Probes) for 5 min. For USF1 and USF2 immunocytochemistry, a similar process was followed. Primary antibodies were rabbit IgG anti-USF1 (sc-229) and anti-USF2 (sc-861) used at a dilution of 1:50. For immunocytochemistry of NCI-H460 cells, cells were transfected with 20 µg of pCMV-GFP and 20 µg of USF expression vector. After 3 days of incubation, a similar process was performed using a mouse monoclonal anti-GFP antibody (1:800 dilution, G 6539, Sigma) and rabbit IgG anti-USF antibody (1:50–1:500 dilution).

For luciferase immunocytochemistry, infected rat trigeminal ganglia cultures were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were incubated with 1:1 (v/v) acetone:water for 3 min at 4 °C followed by acetone for 5 min at 4 °C. Then cells were incubated with 1:1 (v/v) acetone:water for 3 min at room temperature. After rinsing with PBS for 3 min, samples were blocked with 1% fetal bovine serum (diluted in PBS) for 15 min. Samples were incubated with a goat anti-luciferase antibody (1:50 dilution, Promega) and a mouse anti-β-tubulin III antibody (1:800 dilution) in 0.1% fetal bovine serum for 1 h. After washing 3 × 5 min with PBS, samples were incubated with fluorescein isothiocyanate-anti-goat IgG and rhodamine-anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch Laboratories) for 30 min. After 3 × 5 min washes with PBS, ToPro3 was added for 5 min. Images were taken by using confocal microscope (Zeiss). For analysis of nuclear versus cytoplasmic staining, USF1 and USF2 images were analyzed at different focal planes with z-stack program then compiled to generate one image for the figure. USF staining results were confirmed by blind analyses done by a second individual.

Western Blotting—Cell lysates were analyzed as described (12), except that transfers were done at 45 V for 2 h at 4 °C. Primary antibodies were used at 1:1000 dilutions overnight at 4 °C, and secondary antibodies were diluted 1:5,000–10,000 for 0.5–1-h incubations. The membrane was stripped and reprobed with new primary antibody after blocking. The rabbit IgG anti-USF1 (sc-229) and anti-USF2 (sc-861), goat anti-glycerol-dehyde-3-phosphate dehydrogenase (GAPDH, sc-20357), and donkey anti-goat horseradish peroxidase secondary antibodies (used to detect the anti-GAPDH antibodies) were all from Santa Cruz Biotechnology. To detect the anti-USF antibodies, donkey anti-rabbit horseradish peroxidase secondary antibodies (GE Healthcare) were used. The mean value from the histogram analysis performed using the NIH ImageJ software was used for the quantification of protein band intensity.

RESULTS

USF Knockdown Decreases CGRP mRNA Levels in CA77 Cells—Our first test was to determine whether USF proteins regulate the endogenous CGRP gene. This was particularly important because our previous evidence for USF activation of the CGRP promoter (12) was not observed in the neuronal-like CA77 thyroid C cell line, which expresses CGRP. To resolve this issue, we used siRNA-mediated knockdown of USF proteins followed by RT–qPCR measurement of CGRP mRNA. The use of the CA77 cells was necessary because initial attempts to reduce endogenous CGRP RNA by USF1 and USF2 siRNAs in trigeminal ganglia cultures were not successful (data not shown).

This may have been due to a more stable pool of CGRP mRNA in neurons than reported in a cell line (half-life about
Neuronal USF Regulation of CGRP

**A**

![Graph](image)

**B**

![Western blots](image)

17 h) (34), which has been observed for the mRNAs of heavy and mid-sized neurofilament subunits (35). Another possible reason could have been the cellular heterogeneity of the cultures if siRNA duplexes were taken up by non-neuronal cells more easily than by neurons. To resolve these technical problems, we turned to the homogenous CA77 cell line to examine the endogenous CGRP gene.

A combination of siRNAs targeting USF1 and USF2 was transfected into CA77 cells. The pCMV–GFP reporter plasmid was included to allow for the selection of transfected cells by flow cytometry before RNA extraction. CGRP mRNA levels in the samples were measured by RT-qPCR and were normalized to 18 S ribosomal RNA levels in the same samples. The data were then compared with the signal obtained after treatment of the cells with nonspecific control siRNA duplexes. We found that the combined transfection of siRNAs targeting USF1 and USF2 decreased the level of the endogenous CGRP mRNA by about 60% that in controls (Fig. 1A). Likewise, the protein levels of USF1 and USF2 were reduced to 42 and 81%, respectively, that in samples transfected with the control siRNA (Fig. 1B). As a loading and specificity control, GAPDH levels were not affected (96–104% of the levels after transfection with control siRNA) (Fig. 1B). Overall, these results indicate that expression of the endogenous CGRP gene in CA77 cells requires USF proteins.

**Specificity of USF siRNA Duplexes**—Given that the USF proteins regulate many genes, the siRNA-mediated knockdown of USF could potentially indirectly reduce CGRP gene expression. To address this possibility, we tested reporter plasmids with various promoters: a minimal TK promoter, TK promoter plus 3 copies of the 18-bp enhancer (18-bp–TK), wild-type rat CGRP 1.25-kb promoter (1.25–rCGRP), and a mutant 1.25-kb rCGRP promoter in which a BamHI linker interrupts the USF binding site of the 18-bp enhancer (1.25–rCGRP Bam mut). These experiments were performed in CA77 cells, because the activity of the minimal TK promoter with or without the 18-bp enhancer was too low for reliable detection in the primary culture cells, probably due to the low transfection efficiency of neurons.

The activity of the 18-bp–TK promoter was significantly reduced by transfection with USF2 siRNA (Fig. 2A), whereas TK promoter activity was not reduced. As expected, the activity of the 18-bp–TK promoter was much higher than the TK promoter, consistent with previous reports (11, 12). The TK promoter activity was sufficiently high (usually about 5000 light units above background) to have been able to detect a decrease in activity. Activity of the 1.25–rCGRP was reduced by USF2 siRNA to 30% that of control (Fig. 2B). In the case of the 1.25–rCGRP Bam mut, the activity was also decreased by USF2 knockdown, although to a lesser degree than wild-type activity. The decrease in activity of the 1.25–rCGRP Bam mut may be due to other potential USF sites within the promoter region.

Notably, USF2 knockdown was not sufficient to abolish the activity of the 18-bp enhancer or the 1.25–rCGRP. A possible reason for this is the presence of USF1 and residual USF2. To address this point, we attempted to simultaneously knock down both USF1 and USF2 using both USF1 and USF2 siRNA duplexes. In addition, the concentration of the combined siRNA duplexes was decreased 4-fold from previous experiments (2.5 nm of each USF1 and USF2 siRNAs instead of 20 nm USF2 siRNA). The activity of the wild-type 1.25–rCGRP was reduced by 1.25–rCGRP to that of the mutant by the combination of USF1 and USF2 siRNAs (Fig. 2C). Furthermore, simultaneous knockdown of USF1 and USF2 did not affect the 1.25–rCGRP Bam mut activity, which suggests that the 18-bp site is the major site of USF activity. Overall, these results support the conclusion that siRNA-mediated knockdown of USF1 and USF2 is directly responsible for reducing CGRP promoter activity via the 18-bp element.

**No Compensation by Knockdown of Individual USF Genes**—A previous report had shown that USF2 is up-regulated in USF1 knock-out mice, whereas USF1 is down-regulated in USF2 knock-out mice (20). Therefore, we tested whether the USF1 siRNA duplexes affected USF2 protein level and vice versa. For these studies we turned to the cultured rat trigeminal ganglia neurons. Transfection of USF1 and USF2 siRNA duplexes reduced only USF1 and USF2 protein levels, respectively (Fig. 3). This indicates that transient knockdown of one USF gene does not affect the expression of the other in this culture system.
USF Knockdown Reduced CGRP Promoter Activity in Rat Trigeminal Ganglia Cultures—To extend the promoter regulation results from the CA77 cell line to neurons, we tested the effects of a USF knockdown in rat trigeminal ganglia primary cultures. The cells were transfected with both USF siRNAs and hCGRP-luc. The human promoter was used because this reporter vector gave a higher activity than the rat CGRP promoter vector, which allowed more reliable measurements given the low transfection efficiency of primary cultures. Knockdown of USF1 decreased CGRP promoter activity to about 30% that of control (Fig. 4A). Similarly, knockdown of USF2 reduced CGRP promoter activity to about 25% that of control (Fig. 4B). Western blots using lysates from at least two independent experiments confirmed that the siRNA treatments decreased USF protein levels. After treatment with USF1 siRNA, the ratio of USF1 to the internal GAPDH control was decreased to 74% that of the nonspecific siRNA control lysates (Fig. 4C). Transfection of USF2 siRNA decreased the USF2 to GAPDH ratio to 56% that of the nonspecific siRNA control (Fig. 4D). These knockdown data indicate that USF proteins are needed for CGRP promoter activity in cultured trigeminal ganglia.

USF Overexpression Increased CGRP Promoter Activity in Neuronal Cultures—To complement the knockdown approach, we performed the converse overexpression experiments. Vectors containing USF1 or USF2 were transfected with the hCGRP promoter reporter plasmid into trigeminal ganglia cultures. Overexpression of each USF protein caused a dose-dependent increase in luciferase activity. The 4-μg USF1 vector increased activity by 2.6-fold (Fig. 5A), and the 4-μg USF2 expression vector increased activity by 6.4-fold (Fig. 5B). Combined transfection of 2-μg USF1 and 2-μg USF2 yielded a similar activation as seen after separate transfection of 4-μg USF1 or USF2 (data not shown). These overexpression data show

2.5 nM USF2 siRNA duplexes (USF1 + 2-si). Values are relative to the luciferase activity of 1.25-rCGRP plus Con-si. For all panels, the mean and S.E. are shown from three independent experiments (each in triplicate) with Student’s t test used for statistical analyses between the indicated pairs.
that USF can activate the CGRP promoter in trigeminal ganglia cells.

**MAP Kinase Regulation of USF in Trigeminal Ganglia Cultures**—The relationship between USF proteins and the MAP kinase pathway was examined using an expression vector encoding a truncated MEKK protein that can activate all three of the major MAP kinases. To avoid potential competition between plasmid and siRNA duplexes, we performed the transfections in series. The hCGRP-luc reporter and MEKK expression plasmids were cotransfected into the cultures 24 h after mock or siRNA duplex treatments. In this experimental setup, MEKK activation of the hCGRP promoter was observed with either mock or nonspecific siRNA treatments (Fig. 6A). The degree of activation was consistent with previous reports in which MEK1 was transfected to trigeminal ganglia cultures (27, 28). When cells were treated with USF1 siRNA, MEKK activation was compromised by about 50%. These data suggest that USF1 and USF2 act downstream of the MAP kinases that stimulate the CGRP promoter.
As a complementary approach, USF and MEKK were overexpressed both separately and in combination. Transfection of USF1 caused a 4.5-fold activation, and MEKK caused a 2.4-fold activation in trigeminal cultures (Fig. 7A). The combined overexpression of USF1 and MEKK increased promoter activity by 9-fold. A similar pattern was observed in the case of USF2. Transfection of USF2 or MEKK increased promoter activity by 2.7- or 3-fold, respectively (Fig. 7B). The combined overexpression of USF2 and MEKK increased promoter activity by 5.4-fold. We then tested the effect of USF and MAP kinase activation in the NCI-H460 lung carcinoma cell line because these cells are known to have a low level of endogenous USF1 and USF2 proteins (36). Separate overexpression of USF2 or the upstream activator of the ERK MAP kinase, MEK1 (30), increased CGRP promoter activity by 5-fold. In contrast, combined overexpression of USF2 and MEK1 yielded a synergistic 20-fold increase in promoter activity (Fig. 7C). This synergistic activation further supports the prediction that MAP kinases stimulate the CGRP promoter through USF proteins.

We then tested the one known MAP kinase phosphorylation site on USF proteins, threonine 153 in USF1. Because USF1 threonine 153 was reported to be phosphorylated by p38 MAP kinase in melanoma cells (17), we created a threonine 153 to alanine mutant (T153A) to abolish the phosphorylation site and a threonine 153 to glutamic acid mutant (T153E) to mimic phosphorylated threonine. USF T153A was previously shown to be unable to activate the tyrosinase promoter even under MAP kinase stimulation, whereas T153E USF1 increased basal activity of the tyrosinase promoter (17). However, both mutants yielded the same activation as seen with wild-type USF1 (Fig. 7D). Furthermore, T153A USF1 had the same effect as wild-type USF1 when cotransfected with MEKK (data not shown). These results suggest that either MAP kinase activation does not involve direct USF phosphorylation or that phosphorylation at another site(s) is involved.

Neuronal Cell-specific Activity of the 18-bp Enhancer—We first confirmed that USF proteins were expressed in both neurons and non-neuronal cells under our culture conditions. As expected, both USF1 and USF2 were detected in both cell types (Fig. 8, A and B). Immunostaining of the neuronal-specific β-tubulin III protein was used to identify neurons, and the DNA dye ToPro3 was used to visualize nuclei of all cells. A
small population of the non-neuronal cells had glial fibrillary acidic protein immunoreactivity (data not shown), which indicates the presence of Schwann and/or satellite cells (37). The identity of the remaining non-neuronal cells is not known.

Almost all the neurons had USF immunoreactivity; 60 of 61 cells were USF1-positive, 99 of 99 cells were USF2-positive. Likewise, >95% of the neurons are CGRP-positive under these conditions (33). Interestingly, USF staining generally appeared more intense in neurons than non-neuronal cells by >2-fold (Fig. 8C). Furthermore, in non-neuronal cells the USF staining was predominantly in the nuclei, yet in neuronal cells the staining was detected in both the cytoplasm and nuclei. Within the neuronal population, the subcellular localization of USF1 differed from USF2. In many neurons (33 of 60) there was a greater USF1 signal in the nucleus than the cytoplasm, whereas this pattern was not seen for USF2. On the contrary, almost half of the neurons (44 of 99) had USF2 predominantly in the cytoplasm (Fig. 8D). The implications of these expression patterns on CGRP expression are considered under “Discussion.”

The specificity of the antibodies against USF was verified using NCI-H460 cells, which have a low level of endogenous USF proteins. NCI-H460 cells were transfected with USF1 or -2 expression vectors along with pCMV-GFP to identify transfected cells. Most cells having GFP immunoreactivity were also intensely stained with USF1 or -2 antibodies when the cells were cotransfected with USF1 or -2 vectors, respectively (data not shown). Cells without GFP immunoreactivity were not stained with USF antibodies above the background level. Furthermore, immunoreactivity of both USF1 and -2 in transfected NCI-H460 cells was observed exclusively in the nucleus.

To examine whether the USF binding site is sufficient to dictate neuronal-specific activity of the CGRP promoter, we infected trigeminal ganglia cultures with adenovirus carrying β-galactosidase under the regulation of 3 copies of the 18-bp enhancer. The β-galactosidase reporter was expressed only in neuronal cells, even though many non-neuronal cells were present in the culture (Fig. 9A). Among 122 cells with β-galactosidase signal, 111 cells (91%) were neuronal despite the fact that only 315 of 2489 cells in the culture were neurons (13%) (Table 1). As a control to confirm that the adenoviral vector was capable of infecting the non-neuronal cells in culture, we used the cytomegalovirus promoter, which is not neuronal-specific. The β-galactosidase signal was detected both in neurons and non-neuronal cells (Fig. 9B). Of 208 β-galactosidase-positive cells, only 37 (18%) were neurons, yielding a percentage close to that representing the neuronal cells in the culture (17%) (Table 1). This demonstrates that the neuronal-specific expression

antibodies against USF2 were used in place of USF1. In both panels nuclei were detected by post-staining with the dye ToPro3. A merged image is shown. Arrows indicate neurons, and arrowheads indicate non-neuronal cells. Magnification bars are 20 μm. C, intensity of USF immunoreactivity in neuronal cell bodies and non-neuronal cells was analyzed with ImageJ histogram. The mean signal intensity (total pixel intensity/number of pixels) and S.E. of the cells (n = number of cells) are shown. Student’s t tests are shown for comparisons with the neurons. D, subcellular localization of USF1 and USF2 in neurons was categorized as follows; distributed evenly in cytoplasm and nucleus (Cyt+Nuc), mainly in the cytoplasm (Cyt-enriched), and mainly in the nucleus (Nuc-enriched).

FIGURE 8. Localization of endogenous USF proteins in cultured rat trigeminal ganglia. A, cells were double-stained with antibodies against the neuronal-specific β-tubulin III and USF1. B, same as in panel A except
exhibited by the 18-bp enhancer reporter is not a feature intrinsic to the adenoviral reporter.

The converse experiment was then performed to test whether the USF site is required for neuronal-specific expression. The cultures were infected with an adenoviral vector containing the wild-type and a mutant 1.25-rCGRP with a mutation in the USF site used in earlier experiments (Fig. 2B). The wild-type promoter linked to the β-galactosidase reporter gene was previously shown to direct neuronal specific expression (33). With both the wild-type and mutant promoters, luciferase was detected predominantly in neurons (Fig. 9, C and D). This suggests that other sites in addition to the 18-bp enhancer within the 1.25-rCGRP can also direct neuronal expression.

**DISCUSSION**

We have investigated the regulation of neuronal CGRP promoter activity by the transcription factors USF1 and USF2. The contribution of USF proteins was suggested by previous in vitro DNA binding studies (11). In this report we have used siRNA treatments of a neuronal-like cell line and rat trigeminal ganglia cultures to demonstrate that USF1 and USF2 enhance expression of the endogenous CGRP gene and CGRP promoter activity. siRNA-mediated repression of both rat and human CGRP promoters was observed. As a complement to the knockdown approach, overexpression of USF1 and USF2 increased CGRP promoter activity in the cultured neurons. The ability of either USF1 or USF2 to activate the promoter and the reduction of activity by siRNAs against either USF1 or USF2 suggest that the CGRP promoter is controlled by a heterodimer of USF1 and USF2. A similar conclusion was reached using in vitro DNA binding data (11). This finding is in agreement with other systems, where USF acts predominantly as a heterodimer (19, 38, 39).

The ERK MAP kinase has been shown to regulate the CGRP 18-bp enhancer in trigeminal neurons (27). In addition to ERK, JNK and p38 are also important in CGRP promoter activity (28). However, the downstream target of MAP kinases was not known. In this study we have shown that knockdown of USF compromises MAP kinase stimulation of CGRP promoter activity. In addition, overexpression of USF1 or USF2 with upstream activators of MAP kinases increased activation of the CGRP promoter. The additive stimulation of the CGRP promoter by USF2 and MEKK is consistent with either MEKK acting on USF2 or independent mechanisms. However, in the NCI-H460 cell line, which has reduced levels of USF1 and USF2 (36), co-expression of USF2 and MEK1 led to synergistic activation of the CGRP promoter. These data suggest that USF might be a downstream target of MAP kinases that activate the CGRP promoter. Whether USF1 or USF2 is directly phosphorylated by MAP kinases in our system remains to be determined, although the mutant studies rule out phosphorylation at threonine 153 on USF1 as a key residue.

We found that the 18-bp element is sufficient to direct neuronal-specific expression. This raises a paradox because the USF proteins are ubiquitously expressed. One possible explanation would be if USF levels and/or activity are higher in neurons than non-neuronal cells. It is intriguing that USF1 and USF2 immunoreactive signals were greater in neurons and that cytoplasmic staining was only seen in neurons. Meanwhile, USF was predominantly stained at the nuclei of non-neuronal cells in trigeminal ganglia cultures and the NCI-H460 cell line, which do not express CGRP (data not shown). These observations suggest that there are increased USF levels in neurons, and there might be an “extra pool” of neuronal USF that could be recruited to the nucleus. Especially, ~50% of USF2 positive neurons had USF2 immunoreactivity mainly in the cytoplasm, suggesting that USF2 might be recruited to the nucleus by upstream stimulation. Although in most cases USF1 and USF2 are localized in the nucleus (40, 41), in mast cells USF2 is in the cytoplasm and translocates to the nucleus after IL-3 stimulation (42). We speculate that the CGRP gene may be especially susceptible to the levels of USF based on our previous biochemical studies showing that the intrinsic binding affinity of the USF binding site in the 18-bp enhancer is suboptimal (11). Furthermore, the possibility that USF activity may be greater in

**TABLE 1**

| Promoter | β-Gal* cells | β-Tubulin* cells | β-Gal* and β-tubulin* cells | Total cells (ToPro3) |
|----------|--------------|-----------------|---------------------------|---------------------|
| Ad18-bp-TK-β-gal | 122 | 315 | 111 | 2489 |
| AdCMV-β-gal | 208 | 62 | 37 | 373 |
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Neurons is suggested by findings that USF can be activated by Ca$^{2+}$ influx after depolarization (43) and by nerve growth factor stimulation (44). Taken together these findings suggest that neuronal-specificity of CGRP expression may be supported by elevated neuronal USF levels and activity.

An alternative, but not mutually exclusive possibility is that the 18-bp enhancer is bound by a neuronal-specific accessory factor or a USF co-activator. There is precedence for such mechanisms (38, 45, 46). Most notably, the CGRP 18-bp enhancer is controlled by USF and the cell-specific FoxA2 protein in thyroid C-cell lines (12). Similar partnerships with other factors have been reported for USF (41, 47), including in the nervous system (48, 49). Although we cannot rule out an unknown accessory factor or co-activator, the FoxA2 protein is not a candidate because mutation of the FoxA2 site did not decrease reporter activity in cultured neurons (27), and neither FoxA2 RNA nor protein could be detected in rat trigeminal ganglia by RT-PCR or Western blots (data not shown). Another possibility is that there might be neuronal-specific binding of USF to the 18-bp enhancer. In support of this possibility, CpG methylation at the USF binding site established tissue-specific binding of USF to hibernation-specific gene promoters (50). Finally, although we are focused on the 18-bp enhancer, the observed neuronal-specific expression of the mutant 1.25-kb promoter suggests that in the context of the entire gene there are other sites that contribute to neuronal-specific expression. Future studies will be required to address these and other possibilities.

In summary, we have identified USF1 and USF2 as activators of the neuronal-specific enhancer of the CGRP gene in the trigeminal ganglion. The reported ability of USF to respond to nerve activation (43) and to MAP kinases (17, 24), which can be activated in trigeminal neurons by at least one cytokine implicated in migraine (28), provides a potential mechanism by which events during migraine may elevate CGRP synthesis. It is tempting to speculate that activation of USF in trigeminal neurons may contribute to elevated CGRP synthesis and, hence, the prolonged nature of migraine.

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