

The Serum Response Factor and a Putative Novel Transcription Factor Regulate Expression of the Immediate-Early Gene *Arc/Arg3.1* in Neurons

Sean A. Pintchovski,^{1,6} Carol L. Peebles,^{1,6,7} Hong Joo Kim,¹ Eric Verdin,^{2,3} and Steven Finkbeiner^{1,4,5,6,7}

Gladstone Institutes of ¹Neurological Disease and ²Virology and Immunology, San Francisco, California 94158, and Departments of ³Medicine, ⁴Neurology, and ⁵Physiology, ⁶Neuroscience Graduate Program, and ⁷Medical Scientist Training Program, University of California, San Francisco, California 94143

The immediate-early effector gene *Arc/Arg3.1* is robustly upregulated by synaptic activity associated with learning and memory. Here we show in primary cortical neuron culture that diverse stimuli induce *Arc* expression through new transcription. Searching for regulatory regions important for *Arc* transcription, we found nine DNaseI-sensitive nucleosome-depleted sites at this genomic locus. A reporter gene encompassing these sites responded to synaptic activity in an NMDA receptor-dependent manner, consistent with endogenous *Arc* mRNA. Responsiveness mapped to two enhancer regions ~6.5 kb and ~1.4 kb upstream of *Arc*. We dissected these regions further and found that the proximal enhancer contains a functional and conserved “Zeste-like” response element that binds a putative novel nuclear protein in neurons. Therefore, activity regulates *Arc* transcription partly by a novel signaling pathway. We also found that the distal enhancer has a functional and highly conserved serum response element. This element binds serum response factor, which is recruited by synaptic activity to regulate *Arc*. Thus, *Arc* is the first target of serum response factor that functions at synapses to mediate plasticity.

Key words: plasticity; brain-derived neurotrophic factor; Zeste; serum response factor; Arc; gene transcription

Introduction

Activity-dependent, long-term changes in synaptic strength that mediate learning and memory require new gene expression (Kandel, 2001). Among genes induced during learning, the activity-regulated cytoskeletal immediate-early gene (IEG) *Arc/Arg3.1* (Link et al., 1995; Lyford et al., 1995) is essential for memory consolidation (Plath et al., 2006). Cellular models of plasticity depend on *Arc* too. If *Arc* is suppressed, long-term potentiation (LTP) is induced but not maintained (Plath et al., 2006; Messaoudi et al., 2007). *Arc* regulates AMPA receptor (AMPA) trafficking (Chowdhury et al., 2006) at synapses and actin polymerization in spines (Messaoudi et al., 2007). Thus, *Arc* offers a

unique opportunity to investigate relationships between synaptic activity, gene expression, and memory.

Arc induction may be tuned to specific signals from synaptic activity associated with learning and memory. Compared with two other activity-dependent IEGs, *zif268* and *c-fos*, *Arc* expression in the hippocampus correlates better with spatial task acquisition (Guzowski et al., 2006). Learning associated with novelty may produce activity-dependent signals that evoke *Arc*. In the hippocampus, *Arc* levels are significantly higher in newly trained rats than in pseudotrained or overtrained animals (Kelly and Deadwyler, 2002). Exposing rats to the same environment triggers neural activity, but *Arc* induction becomes uncoupled from that activity with repeated exposures (Guzowski et al., 2006). Similarly, patterns of visual experience-induced *Arc* expression in the occipital cortex depend on stimulus novelty (Wang et al., 2006). *In situ* detection of *Arc* mRNA is thus used to monitor behaviorally relevant synaptic activity and to map neuronal ensembles *in vivo* (Guzowski et al., 1999).

How extracellular stimuli regulate *Arc* production is incompletely understood. Behaviorally induced synaptic activity initiates new *Arc* transcription *in vivo* (Guzowski et al., 2006), but the transcription factors that mediate rapid *Arc* transcription remain unknown. *Arc* apparently lacks consensus binding sites for the well known Ca²⁺- and cAMP-response element binding protein (CREB) (Montminy and Bilezikjian, 1987; Sheng et al., 1990), suggesting that stimuli regulate *Arc* transcription through other signaling pathways (Waltereit et al., 2001). To discover how activity regulates *Arc* production, the effects of specific stimuli, including brain-derived neurotrophic factor (BDNF) (Rao et al.,

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Correspondence should be addressed to Steven Finkbeiner, Gladstone Institute of Neurological Disease, 1650 Owens Street, San Francisco, CA 94158. E-mail: sfinkbeiner@gladstone.ucsf.edu.

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2006), cAMP (Waltereit et al., 2001), and Ca^{2+} influx (Waltereit et al., 2001; Ichikawa et al., 2003), on *Arc* were examined. Yet it remains unclear which signaling pathways are specifically recruited by synaptic activity to regulate *Arc* expression and whether transcription initiation is a shared mechanism of *Arc* regulation for these pathways. *Arc* induction can be distinguished from that of at least some other IEGs and is closely correlated with learning, raising the intriguing possibility that *Arc* transcription is controlled by yet-to-be discovered activity-dependent signaling pathways and transcription factors.

We used DNaseI hypersensitivity assays to screen the endogenous chromatin structure flanking the *Arc* gene locus for discrete “open” nucleosome-depleted regions important for transcription. Our results provide important insights into the molecular mechanisms underlying *Arc* gene expression and have broad implications for how specific patterns of synaptic activity regulate neuronal gene expression to mediate long-term plasticity and memory.

Materials and Methods

Cell cultures. Neonatal pup cortices were dissected and treated with papain (10 units/ml, 30 min; Worthington Biochemical) and then with a trypsin inhibitor (10 mg/ml, 15 min; Sigma). After trituration, dissociated neurons were plated on plastic tissue-culture plates (3.4×10^5 cells/cm²) coated with laminin (BD Biosciences) and poly-D-lysine (Chemicon). After 2 h, cells were transferred into a neuronal culture medium (NCM) based on Basal Medium Eagle (Invitrogen) (Rao et al., 2006). Undifferentiated rat pheochromocytoma (PC12) cells were maintained in standard growth medium consisting of Dulbecco's modified Eagle medium with 4.5 g/L glucose and 25 mM HEPES (Mediatech), supplemented with 5% calf serum, 5% horse serum (Hyclone), 1% penicillin-streptomycin (10 mg/ml), and 1% 200 mM L-glutamine (Invitrogen).

Pharmacology. BDNF was from Amgen. D-2-amino-5-phosphonovaleric acid (APV) and UO126 were from Tocris. Tetrodotoxin (TTX) was from Calbiochem. H89, forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma. NGF was from Austral Biologicals.

Quantitative fluorogenic reverse-transcription PCR. Cortical neurons (11 d *in vitro* [DIV]) were transferred to serum-free medium (SFM, otherwise identical to NCM described above). Cortical neurons challenged with the TTX-withdrawal paradigm were transferred into SFM supplemented with TTX (2 μ M). At 45–48 h after transfer (13 DIV), neurons were stimulated either with drugs or by TTX washout for 6 h, lysates were collected, and RNA was isolated (QIAshredder Kit, RNeasy Plus Kit; Qiagen). Reverse transcription (TaqMan Reverse Transcription Reagents; Applied Biosystems) and qPCR were performed with SYBR Green PCR Master Mix (Applied Biosystems) and an ABI Prism 7700 sequence detector (Applied Biosystems). All qPCR primers have been described, including those for GAPDH (internal control gene for normalization) (Rao et al., 2006). Genomic DNA background was excluded by testing control samples that were not reverse transcribed. “No template” controls were also tested to exclude background signal from primer dimers.

In silico analyses. To identify the orthologous regions of the human and mouse genomes flanking *Arc*, we used the University of California, Santa Cruz, Genome Bioinformatics website to query the Blast-like alignment tool (BLAT) (Kent, 2002) with the highly conserved coding region of rat *Arc*. These upstream and downstream sequence sets were then entered into the online EVOPRINTER software (Odenwald et al., 2005) to identify nucleotides conserved among these three species for both sequence and relative genomic position. The BLAT and EVOPRINTER analyses were performed with the default program settings (<http://evoprinter.ninds.nih.gov/evopoverview.htm>). The short conserved nucleotide stretches identified by this approach were then used to query the Transcription Element Search System (TESS) (Overton and Schug, 1997) for transcription factor DNA binding elements. The *Arc* locus conservation map was generated using default settings for human BLAT analysis (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>).

DNaseI hypersensitivity assays. DNaseI hypersensitivity assays (DHAs) were performed as described (Verdin, 1991), with the following exceptions. PC12 cells (~90% confluent) and cortical neurons (11 DIV) were transferred to SFM, and the next-day stimulations were performed for 3 h. Nuclei were isolated in ice-cold buffer to preserve *in vivo* chromatin architecture, and aliquots were subjected to a log_{1.3} concentration gradient of DNaseI (New England Biolabs): 0, 2, 2.6, 3.3, 4.4, 5.7, 7.4, 9.6, 12.5, and 16.25 units/~ 3.25×10^6 nuclei. Genomic DNA (~30 μ g) was isolated from each DNaseI-treated aliquot of nuclei and digested overnight with *SspI* (New England Biolabs), a restriction enzyme with cut sites throughout the *Arc* locus: ~13.2 kilobases (kb) upstream of, 925 base pairs (bp) downstream of, and ~19.3 kb downstream of the *Arc* transcription start site (TSS). DNA samples were then run on a 1.2% agarose gel at 40 V for 16 h and transferred to Hybond-N+ membrane (Amersham Biosciences). Southern blot probes (see Fig. 3A) were amplified by PCR from bacterial artificial chromosome (BAC) CH230–208M7 (Children's Hospital Oakland Research Institute, Oakland, CA) with the following primers: 5'-gagagctgaaagggtgacag-3' and 5'-ttccaggtgctcaggaactc-3' for the upstream probe (500 bp), 5'-tccagatccagaaccatga-3' and 5'-cctagttgacaaaagccacagac-3' for the downstream probe (537 bp). The upstream DNA ladder was produced by digesting BAC 208M7 with *SspI* and then performing secondary digests with *AflII* (5137 bp), *BamHI* (4296 bp), *EcoRI* (3544 bp), *XhoI* (3058 bp), *HindIII* (1830 bp), *DraIII* (1667 bp), *BssHII* (1149 bp), *SgrAI* (941 bp), or *SacII* (657 bp). The downstream ladder was created by using many of the same secondary enzymes: *EcoRI* (9992 bp), *HindIII* (5834 bp), *XhoI* (5338 bp), *SgrAI* (3852 bp), *BamHI* (2747 bp), *DraIII* (2162 bp), or *AflII* (1996 bp).

Plasmids. For the new full-length *Arc* promoter reporter gene, BAC 208M7 was used to PCR amplify the genomic regions referred to as A (560 bp; primers: 5'-taagctctgtgtgtagcctcc-3' and 5'-ctggtccaactccagatgg-3'), B (653 bp; primers: 5'-tgggtgtggcaggctctgc-3' and 5'-caggatcaggagacgaattgagg-3'), C (521 bp; primers: 5'-tccagctccattgtgtgtgg-3' and 5'-tctctctgagccacaagaacatg-3'), H (483 bp; primers: 5'-agcaggctagccaggaatg-3' and 5'-gaaacacagacatgtctcaatc-3'), and I (308 bp; primers: 5'-aacagctctcaggatggagtg-3' and 5'-cccgactctgctcacaagg-3') with a high-fidelity proof-reading DNA polymerase (PfuUltra; Stratagene). These amplicons were subcloned into TOPO vectors (Invitrogen), sequence verified, and subcloned into a 2.6-kb *Arc* promoter reporter gene described previously (Ichikawa et al., 2003). Plasmid pSRE-Luc (*SRE-Luciferase*), used to measure serum response factor (SRF) activity, has five tandem serum response elements (SREs) driving firefly *luciferase* (Stratagene). Plasmid *pRL0-Renilla*, used to control for transfection efficiency and sample handling in the reporter assays, was constructed by removing promoter elements from *pRL-SV40* (Promega). The wild-type (pCS2⁺/hSRF-VP16) and mutant (SRFAM-VP16) SRF-VP16 constructs have been described (Schratt et al., 2002), as have the anti-SRF siRNAs (Yin et al., 2006).

Transient transfections and reporter gene assays. Cortical neurons (11 DIV) were cotransfected with one of the *Arc* reporter gene plasmids and *pRL0-Renilla* (1:1 molar ratio) with Lipofectamine 2000 (Invitrogen) and transferred into SFM. At 20–24 h after transfection, neurons were stimulated with drugs for 8 h, and lysates were collected and assayed with a Dual Luciferase Kit (Promega) and a luminometer (Thermo Electron). Cortical neurons challenged by TTX withdrawal were transferred into SFM supplemented with TTX (2 μ M) after transfection. At 40–42 h after transfer (13 DIV), neurons were stimulated by TTX washout, and lysates were harvested after 8 h. Each condition was tested in triplicate, and measured values were averaged. To ensure that reporter gene activity accurately reflects transcriptional activity, the DNA quality of every construct was tested for degradation and supercoiling, which affect transfection efficiency. Furthermore, two separate plasmid maxipreps were tested for each construct to ensure consistent reporter activities.

Chromatin immunoprecipitations. These experiments were performed with a commercial kit (Active Motif) and the manufacturer's protocol. Primary cultured rat cortical neurons (11 DIV) (~ 5×10^6 cells yielding ~30 μ g of genomic DNA) were cross-linked with 1% formaldehyde. Chromatin was enzymatically sheared to a final size of 500–1500 bp and incubated with 10 μ g of rabbit anti-SRF antibody (Santa Cruz Biotech, s.c.-335X) or IgG control antibody. The following primer pairs were used for semi-quantitative PCR: *Arc* SRE1 (forward, 5'-gaagctctctgctgctg-

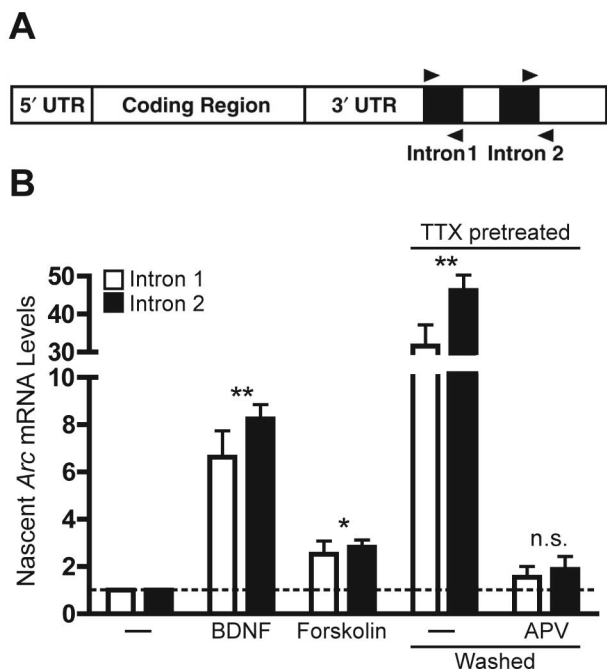


Figure 1. Diverse stimuli induce new *Arc* transcription. **A**, Diagram of *Arc* pre-mRNA and the qfRT-PCR primer pairs (arrowheads) used to detect its levels. **B**, Induction of nascent *Arc* pre-mRNA was measured by qfRT-PCR after 6 h stimulations with either BDNF (100 ng/ml), the adenylyl cyclase activator forskolin (50 μ M) and the phosphodiesterase inhibitor IBMX (100 μ M), or 6 h after washout of chronic TTX (2 μ M, 48 h; TTX pretreated). This induction was inhibited by APV (100 μ M) added after washout. Values are mean \pm SEM. $N = 3$ distinct cDNA pools, each amplified from a separate neuronal culture prep. Paired t test: ** $p < 0.01$, * $p < 0.05$ versus control; n.s., not statistically significant.

catgg-3'; reverse, 5'-gtttctcgtctgttctctgcaattcc-3'; 130 bp amplicon), *c-fos* SRE (forward, 5'-tccccctgctgcgctgcaccctcaga-3'; reverse, 5'-caacagggaccg-gcgtggaacct-3'; 130 bp amplicon), and *GAPDH* promoter (forward, 5'-aaacaagtccaccatgtgaaa-3'; reverse, 5'-ccagggtgacaaaggtgagtt-3'; 180 bp amplicon). PCR products were separated on 1.5% agarose gels.

Western blots. These assays were performed as described (Rao et al., 2006) with the following antibodies: phospho-p44/42 MAPK (Cell Signaling, 9101; 1:2000), p44/42 MAPK (Cell Signaling, 9102; 1:2000), β -actin (Sigma, clone AC15; 1:10,000), *GAPDH* (Abcam, ab9485; 1:5000), and SRF (Santa Cruz Biotech, s.c.-335X; 1:1000); Recombinant Arc protein was expressed and purified as described (Lyford et al., 1995), and rabbit immunization and antibody purification was performed through Invitrogen (1:5000). AMAXA nucleofections for SRF-VP16 overexpression were performed as described (Gärtner et al., 2006). Briefly, $5\text{--}6 \times 10^6$ cortical neurons and 3 μ g of DNA were used for each nucleofection reaction using program O-004. Recombinant lentivirus was generated by cotransfecting the transfer vector (FUGW) with two helper plasmids, delta8.9 (packaging vector) and VSV-G (envelope vector), into 293T cells (American Type Culture Collection). Supernatant was collected and virus was precipitated with PEG-it (System Biosciences). Cortical neurons were infected at 4 DIV with lentivirus expressing control or anti-SRF siRNA. At 6 d postinfection, the neurons were stimulated with BDNF, and lysates were harvested after 4 h.

Electrophoretic mobility shift assays. Nuclear extracts from cortical neurons were prepared as described (Tao et al., 1998). The binding reaction was performed in a 16- μ l-mixture containing 3.75 mM HEPES, pH 7.8, 3.75 mM MgCl₂, 37.5 mM KCl, 0.375 mM DTT, 7.5% glycerol, 8 μ g of poly(dI-dC), 2 μ g of salmon sperm DNA, ~ 10 μ g of nuclear extract protein, and 3–5 fmol of ³²P-labeled probe. Probe Z1 was synthesized as complementary oligodeoxynucleotide strands (22 bp; 5'-agaggccgtgagtgggggca-3' and 5'-tgcccccaactcacggcctct-3') and labeled with ³²P-ATP with T4 polynucleotide kinase, according to standard procedures. Probe mZ1 was synthesized as complementary oligodeoxynucleotide strands (22 bp; 5'-agaggccgttatatgggggca-3' and 5'-

tgcccccatatacggcctct-3'). After a labeled probe was added, the mixture was incubated for 20 min at 25°C. Fifteen microliters of the binding reaction mixture was then loaded into a 5% TBE Criterion precast gel (Bio-Rad) and electrophoresed in 0.5 \times TBE buffer at 85 V for 1.75 h. After electrophoresis, the gels were dried and exposed to film (BioMax MS Film; Kodak) at -80°C in a cassette with enhancer screens.

Statistical analyses. Student's paired t tests and one-way ANOVA with *post hoc* Tukey's t tests were performed with Prism (GraphPad Software).

Results

Diverse stimuli induce new *Arc* transcription

To determine whether distinct physiologically relevant stimuli induce new *Arc* mRNA transcription in neurons, we used intronic quantitative fluorogenic reverse-transcription PCR (qfRT-PCR) to detect nascent *Arc* pre-mRNA. The rat *Arc* gene contains two introns within the 3' untranslated region (UTR) that are spliced out of *Arc* pre-mRNA to form the mature transcript (Giorgi et al., 2007). We designed and validated two qfRT-PCR primer pairs that anneal entirely within intron 1 or across the intron 2–exon 3 boundary to detect *Arc* pre-mRNA (Rao et al., 2006) (Fig. 1A).

We first confirmed that synaptic activity directly regulates *Arc* mRNA synthesis *in vitro*, as seen *in vivo* (Guzowski et al., 2006). To evoke synaptic activity *in vitro*, we used a method that triggers rebound synaptic activity after a period of synaptic inhibition with TTX (Turrigiano et al., 1998; Riegle and Meyer, 2007). This widely used paradigm has been especially useful for studying mechanisms of synaptic homeostasis, which may be particularly relevant to *Arc* function (Shepherd et al., 2006). Briefly, synaptic activity in mature cultures was suppressed for ~ 2 d by bath application of TTX, a sodium-channel antagonist. Treated neurons were washed into TTX-free medium, producing a rebound of synaptic activity and an induction (~ 40 -fold) of nascent *Arc* mRNA transcripts (Fig. 1B). *In vivo*, addition of the NMDA receptor (NMDAR) antagonist APV blocked increases in *Arc* levels induced by synaptic activity (Steward et al., 1998). Here, we found that addition of APV after TTX washout blocked new *Arc* transcription, indicating that synaptic activity drives *Arc* transcription initiation in an NMDAR-dependent manner *in vitro*. We then tested whether BDNF and cAMP induce *Arc* expression (Waltereit et al., 2001; Wibbrand et al., 2006) by initiating new *Arc* transcription. Indeed, the synthesis of nascent *Arc* transcripts was increased approximately sevenfold by BDNF and approximately threefold by forskolin (an adenylyl cyclase activator that produces cAMP) (Fig. 1B).

In silico analysis identifies conserved regions flanking *Arc*

To help identify conserved DNA response elements in the *Arc* promoter that might mediate this new transcription, we used the University of California, Santa Cruz (UCSC) BLAT server (Kent, 2002) and EVOPRINTER freeware (Odenwald et al., 2005) to align the orthologous regions of the human, mouse, and rat genomes flanking *Arc*. The output was used to identify nucleotides throughout these alignments whose sequence and relative position in the genome were conserved. We found seven discrete clusters (Fig. 2, regions A–G) of short conserved nucleotide stretches dispersed throughout the ~ 13 -kb genomic region upstream and two clusters (Fig. 2, regions H, I) within the ~ 16 -kb genomic region downstream of *Arc*. In contrast, genomic sequences between these clusters largely lacked sequence and position conservation. The high degree of conservation of regions A–I suggests a physiologically important role in *Arc* transcription.

Biochemical identification of putative regulatory regions flanking *Arc*

We next sought to determine whether the putative regulatory sites identified *in silico* correspond to open genomic regions with chromatin structure accessible to transcription factors (Elgin, 1988). The DHA identifies gene regulatory regions throughout large genomic expanses by screening for discrete open nucleosome-depleted regions that are hypersensitive to DNaseI cleavage (Pipkin and Lichtenheld, 2006) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Since regulatory regions have been found 5' and 3' to sites of transcription initiation (Kikuchi et al., 1992), we used DHAs to examine the upstream and downstream genomic domains flanking *Arc* (Fig. 3A).

We first performed DHAs on undifferentiated PC12 cell cultures, which upregulate *Arc* mRNA in response to multiple stimuli, including nerve growth factor (NGF) (Lyford et al., 1995). PC12 cell cultures were stimulated with NGF (100 ng/ml), genomic DNA was isolated, and DNaseI hypersensitive sites (HSs) were identified. The ~13-kb region upstream of *Arc* contained six discrete open DNaseI HSs (Fig. 3B, bands A–D, F, G). The more distal sites, referred to as A, B, and C, reside ~9.3 kb, ~6.5 kb, and ~4.5 kb (respectively) 5' of the *Arc* transcription start site (TSS). These sites are upstream of regions examined in previous studies of *Arc* transcription (Waltereit et al., 2001; Ichikawa et al., 2003). Two additional DNaseI HSs (Fig. 3B, bands H, I) were found in the ~16-kb region downstream of *Arc*. A ~200-bp tract of accessible genomic DNA occurred at the *Arc* TSS.

To rule out sample handling as a source of these DHA sites, we tested whether chromatin that had been stripped of all DNA-binding proteins ("naked" DNA), but was otherwise handled in the same manner, would yield similar DNaseI cleavage sites. Bands A and G persisted in the naked DNA control (Fig. 3B), indicating that they might be artifacts or produced by DNaseI cleavage at atypical HSs (Bode et al., 2003). None of the other previously identified HSs were present, indicating that most of the HSs we identified result from DNaseI cleavage at hypersensitive open domains within the native chromatin structure.

To determine whether the same chromatin architecture is present in a more physiological system, DHAs were performed on rat cortical neuron cultures. Neurons were stimulated or not with BDNF (100 ng/ml). We found upstream HSs in locations similar to those in PC12 cells (Fig. 3B), but we did not identify any downstream HSs (data not shown). Since the intensity of DHA bands may vary with the extent to which a bound protein factor affects DNaseI accessibility, the presence rather than the intensity of a band is the critical indicator of an open genomic region (Gross and Garrard, 1988) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The open sites we identified were present under both basal and BDNF-stimulated conditions (and after chronic TTX washout, data not shown), indicating that they are static and suggesting that stimulus-induced *Arc* transcrip-

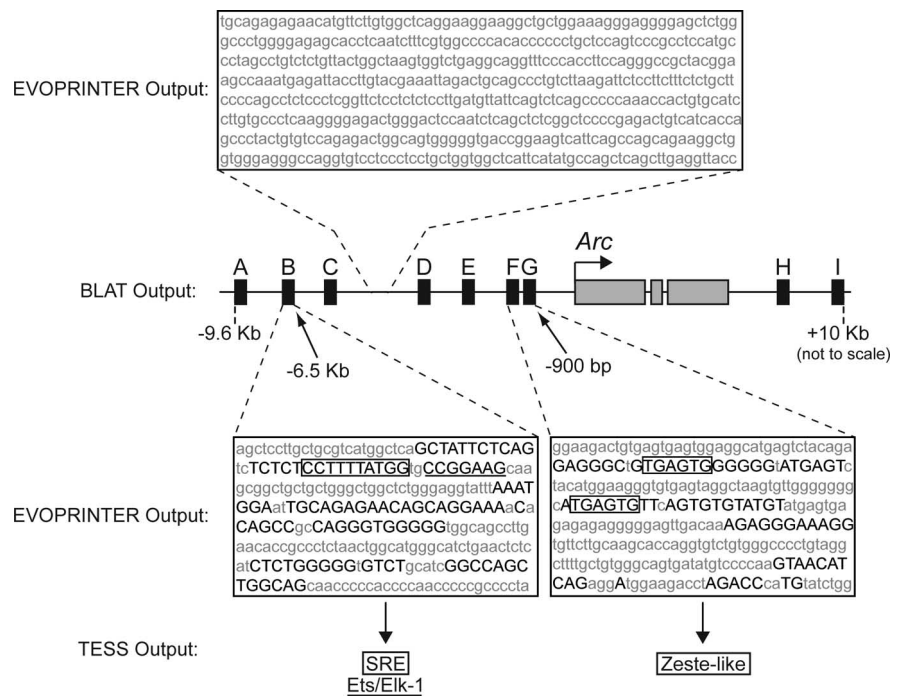


Figure 2. *In silico* identification of putative regulatory regions containing conserved transcription factor binding sites. The online UCSC BLAT and the online EVOPRINTER analysis tools were used to identify seven upstream (A–G) and two downstream (H, I) regions (black rectangles) flanking *Arc* (gray rectangles) that are enriched in evolutionarily conserved sequences. Nucleotides within region B and regions F/G that are conserved in both sequence and relative genomic position are shown in black capital letters. In contrast, a portion of the genomic region between regions C and D demonstrates the relative lack of such conservation in the surrounding areas (see supplemental Fig. 3, available at www.jneurosci.org as supplemental material, for additional analysis). TESS was used to screen these conserved sequence elements for consensus binding sites, revealing a putative SRE (boxed) and an adjacent putative Ets/Elk-1 element (underlined) in region B as well as two putative Zeste-like binding sites (boxed) in regions F/G. All distances are from the *Arc* TSS.

tion does not result from changes in chromatin accessibility. We conclude that neurons and PC12 cells contain a common set of open genomic regions at the *Arc* locus. The positions of these regions correlated well with those of the conserved nucleotide clusters predicted by *in silico* analysis (Fig. 4A). These observations strongly suggest that these regions are physiologically important and likely contain response elements that regulate *Arc* transcription.

In silico identification of putative regulatory elements within DHA regions

Open genomic regions are often enriched in relevant transcription factor binding sites (Gilbert et al., 2004). We analyzed the short conserved nucleotide stretches in regions A–I above for consensus transcription factor binding sites with the online TESS query database (Overton and Schug, 1997). Region B contained many conserved binding sites, including adjacent sites for SRF and Elk-1, which together form a ternary complex factor (TCF) involved in gene regulation (Norman et al., 1988; Buchwalter et al., 2004) (Fig. 2; supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The other regions (A, C–I) contained putative binding sites for factors implicated in neuronal plasticity, including nuclear factor of activated T cells (Groth and Mermelstein, 2003), nuclear factor κ B (Meffert et al., 2003), and myocyte-specific enhancement factor 2 (Flavell et al., 2006) (data not shown). Putative binding sites were found for other factors, including Nkx-2.5 (Moskowitz et al., 2007), N-Oct-3 (Blaud et al., 2004), and Zeste (Biggin et al., 1988) (data not shown). Although putative CREB binding sites were found, they did not

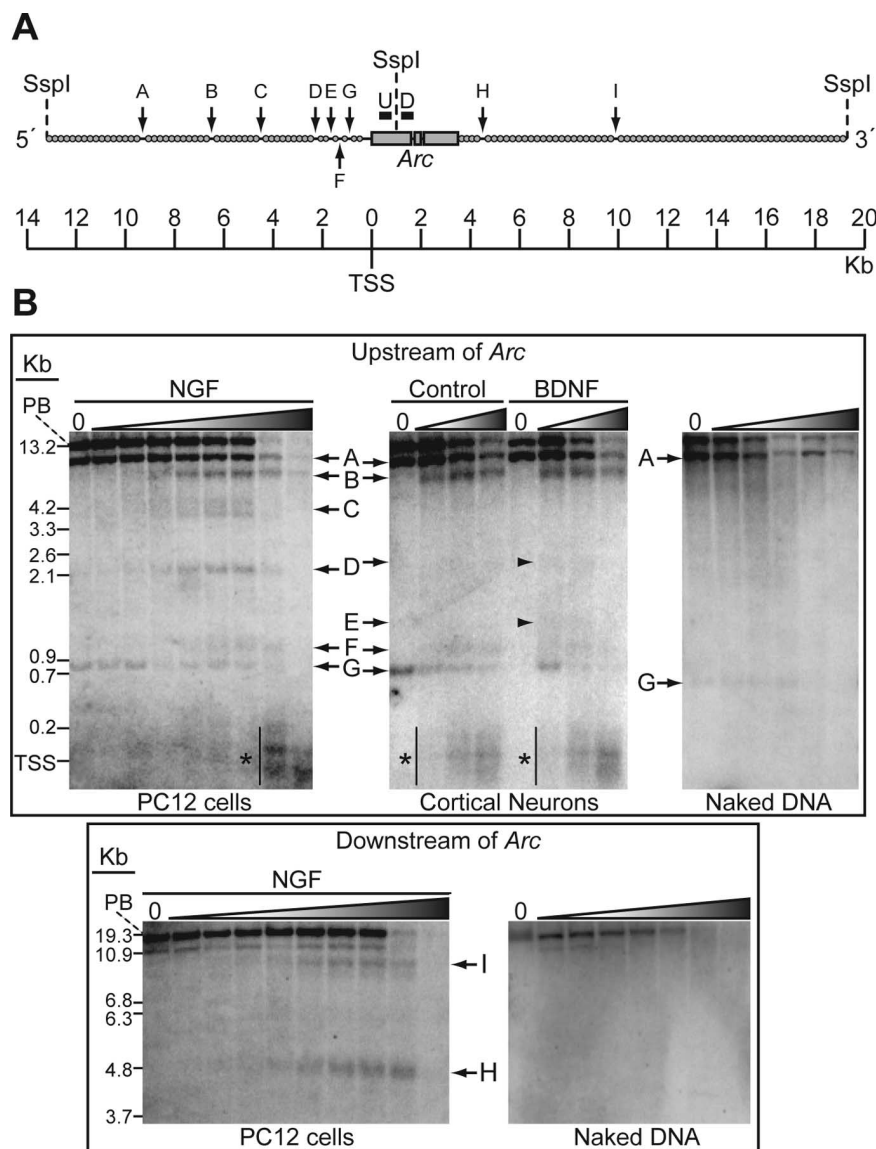


Figure 3. DHAs identify nucleosome-depleted regions flanking *Arc*. **A**, Probes used to map hypersensitive sites at the *Arc* locus. Genomic DNA fragments were detected by Southern blot probes that annealed either immediately upstream (“U”; black bar) or downstream (“D”; black bar) of the *Ssp*I restriction site in *Arc*. Arrows indicate the relative positions of the nine DNase I HSs (A–I) identified at the *Arc* locus; gray circles represent DNase I-inaccessible nucleosome-packaged regions. **B**, Top, DHAs identify seven discrete open regions (bands A–G; arrows) upstream of *Arc*. Some are present in both rat cortical neurons (RCNs) and PC12 cells (bands A, B, D, F, G); others are visible only in RCNs (band E) or only in PC12 cells (band C). There is a larger open smear (asterisks) at the TSS. The “naked” DNA negative control, performed using both PC12 and RCN DNA, exhibits only bands A and G, suggesting that they are either DHA artifacts or atypical sites and that the remaining bands and smear reflect the native chromatin structure upstream of *Arc*. Bottom, DHAs identify two discrete open regions (bands H, I) downstream of *Arc* in PC12 cells, but not in RCNs (data not shown). The naked DNA control demonstrates that both bands represent native chromatin structure. Blot contrasts were chosen to best represent all observed bands. Bands D and E (arrows and arrowheads) are more clearly visible in higher-contrast images (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). All Southern blot images are representative of at least three experiments. Distances are in kb from the *Arc* TSS. The DNase I concentration gradient, indicated above each blot, increases from left to right and includes a “no DNase I” negative control in lane “0” (see Materials and Methods) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). PB: primary band resulting from *Ssp*I restriction cuts.

meet the stringent cross-species conservation criteria of this *in silico* method.

Identification of novel distal and proximal enhancer regions

Previous reporter gene studies of *Arc* have been inconclusive (Waltereit et al., 2001; Ichikawa et al., 2003), perhaps because they focused on proximal regions of the 5' *Arc* promoter that lacked many of the HSs we found. To assess the functional role of

these putative regulatory regions, we made a new *Arc* reporter gene containing all nine DNase I HSs (A–I). We began construction with a 2.6-kb genomic fragment of the proximal *Arc* promoter, which contains four of the HSs (D–G) (Ichikawa et al., 2003). We appended the remaining upstream HSs (A–C) in a tandem array immediately upstream of the 2.6-kb fragment, and the remaining downstream HSs (H, I) immediately downstream of the firefly *luciferase* reporter gene to maintain their relative positions *in vivo* (Fig. 4A). This reporter design likely mimics how genomic DNA normally flexes to bring distal and proximal regulatory regions together, enabling protein binding factors to interact and regulate transcription (West and Fraser, 2005).

In cortical neurons, this new reporter gene responded to synaptic activity in an NMDAR- and ERK-dependent manner (Fig. 4B), like endogenous *Arc* *in vitro* (Fig. 1B) and *in vivo* (Lyford et al., 1995; Ying et al., 2002). The reporter also responded to neuronal stimulation with BDNF (~10-fold relative to control) and forskolin (~fourfold relative to control) (Fig. 4C, right) in a manner that closely paralleled the stimulus-mediated inductions of endogenous *Arc* mRNA (Fig. 1B). Thus, this new *Arc* reporter gene, whose construction was based on the structure of chromatin at the *Arc* locus *in vivo*, faithfully recapitulates important features of the induction of endogenous *Arc* mRNA. These findings also indicate that one or more of these promoter regions contain DNA response element(s) that mediate stimulus-dependent *Arc* transcription.

To assess the role of each regulatory region, we created a set of reporter constructs with systematic deletions of DHA regions A–I and tested them in neurons. Region B and regions F/G mediated responses to synaptic activity (Fig. 4C, left). Region B was also a major mediator of both BDNF- and forskolin-induced reporter gene responses (Fig. 4C, right). Furthermore, regions F/G were required to mediate the reporter's full response to BDNF but not to forskolin (Fig. 4C, right). Although regions F and G appear as distinct DHA bands (Fig. 3B, top), they are physically too close to be clearly separated and were considered together here and below.

Identification of a distal functional SRE

Region B of the *Arc* promoter is required to mediate responses induced by synaptic activity, BDNF, and forskolin, but is it sufficient? To answer this question, we determined whether region B can enhance the transcription of a distinct promoter. Indeed, when inserted ectopically into a luciferase vector driven by the

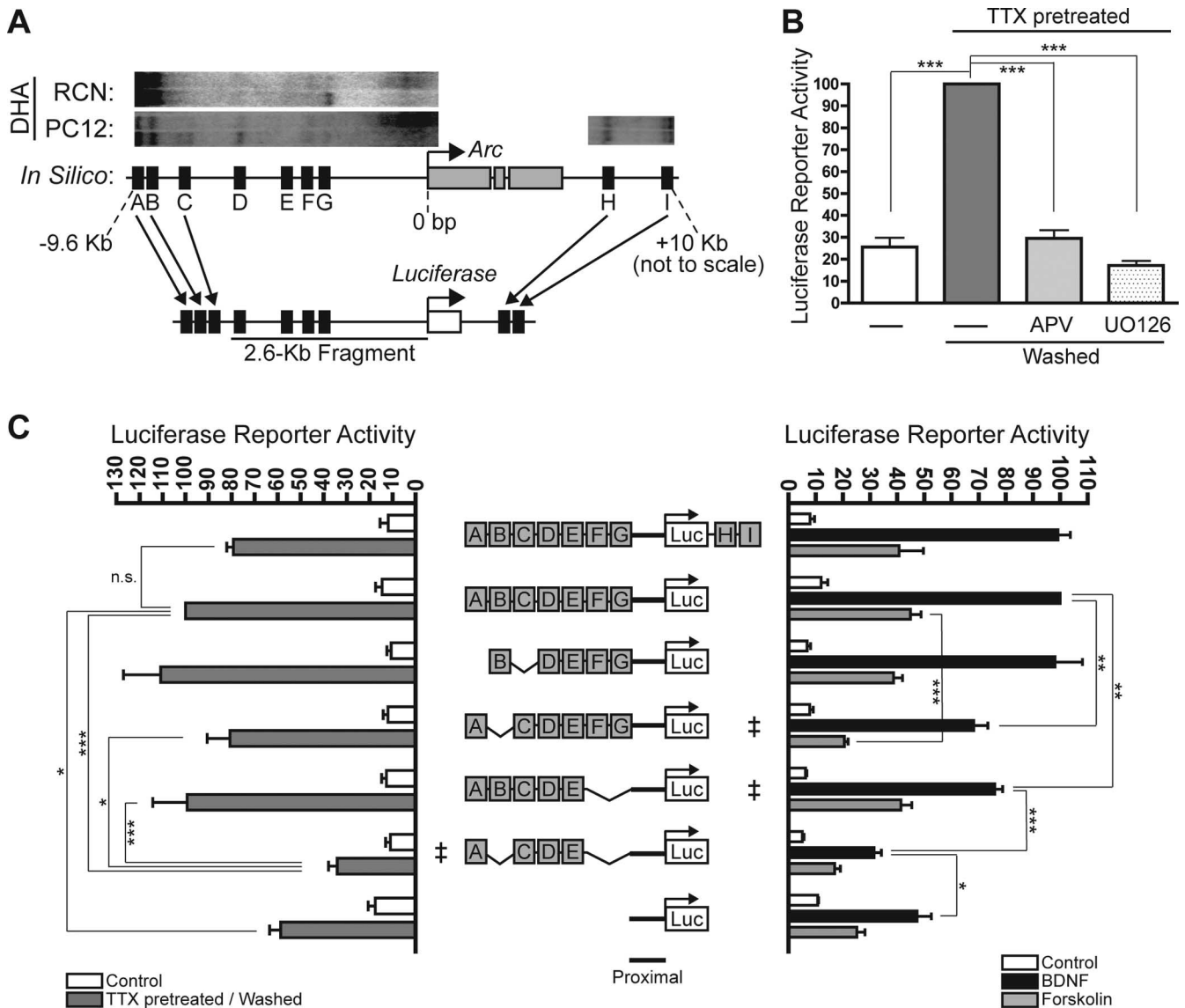


Figure 4. Stimulus-inducible distal and proximal enhancer regions. **A**, A strong correlation was observed between the genomic positions of the DHA Hs and the evolutionarily conserved regions, suggesting potential involvement in *Arc* transcription. A reporter with a 2.6-kb genomic fragment containing the proximal *Arc* promoter, which already contained four of the nine putative regulatory sites (D–G), was supplemented with the remaining upstream (A–C) and downstream (H, I) Hs. **B**, The new full-length *Arc* reporter is induced by synaptic activity. In agreement with activity-regulated expression of endogenous *Arc* mRNA, this reporter response is completely blocked by the NMDAR inhibitor APV or the ERK activation inhibitor UO126. **C**, A systematic deletion analysis of the new full-length *Arc* reporter. Left, “#” indicates the reporter construct with the minimal deletions (ΔB and $\Delta F/G$ together) that result in a reduced response to synaptic activity. “Proximal” indicates the ~900-bp basal promoter region immediately upstream of *Arc*. All values are percentages (mean \pm SEM) of the maximum measurement for reporter ABCDEFG. $N = 4$, one-way ANOVA and *post hoc* Tukey’s *t* tests: *** $p < 0.001$, * $p < 0.05$; n.s., not statistically significant. Right, “#” indicates the reporter constructs with the minimal deletions (ΔB or $\Delta F/G$ individually) that result in a reduced stimulus-mediated response. This side-by-side construct comparison highlights how these two regions differentially contribute to BDNF- and forskolin-induced reporter activity. All values are percentages (mean \pm SEM) of the maximum BDNF measurement for reporter ABCDEFG. $N = 5$, one-way ANOVA and *post hoc* Tukey’s *t* tests: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

SV40 promoter (pGL3-Promoter; Promega), region B increased the promoter’s response to synaptic activity; the enhancement was completely blocked by the NMDAR inhibitor APV or the PKA inhibitor H89 (Fig. 5A), suggesting that Ca^{2+} influx and PKA activation are required for synaptic activity to induce region B. Region B also enhanced the reporter’s response to forskolin (Fig. 5B), confirming that PKA induces region B. Furthermore, region B enhanced reporter responses to BDNF. Thus, it may be sensitive to ERK signaling. In contrast, region D, which is similar in length to region B, failed to enhance stimulus-induced activity of the SV40 reporter gene (Fig. 5B). Thus, region B is a *bona fide* enhancer that contains one or more functional DNA elements.

We reviewed the list of conserved transcription factor binding

sites in region B (Fig. 2) and focused on the consensus SRE (referred to as SRE1). ERK activation is required for *Arc* induction (Rao et al., 2006), and SRE can mediate responses to ERK through bound TCF and SRF. However, ERK activates many transcription factors in addition to TCF and SRF (Johnson and Lapadat, 2002), so a direct role for SRE/SRF in *Arc* regulation remains to be established. *Arc* levels in the brains of SRF-null mice were normal in one study (Etkin et al., 2006) but significantly reduced in another (Ramanan et al., 2005). Since SRF was absent during development, it is hard to tell from these studies whether SRF regulates *Arc* directly or indirectly or whether compensatory changes have obscured its role. Previous efforts to directly identify a functional SRE within the *Arc* promoter have not been successful (Walthereit et al., 2001).

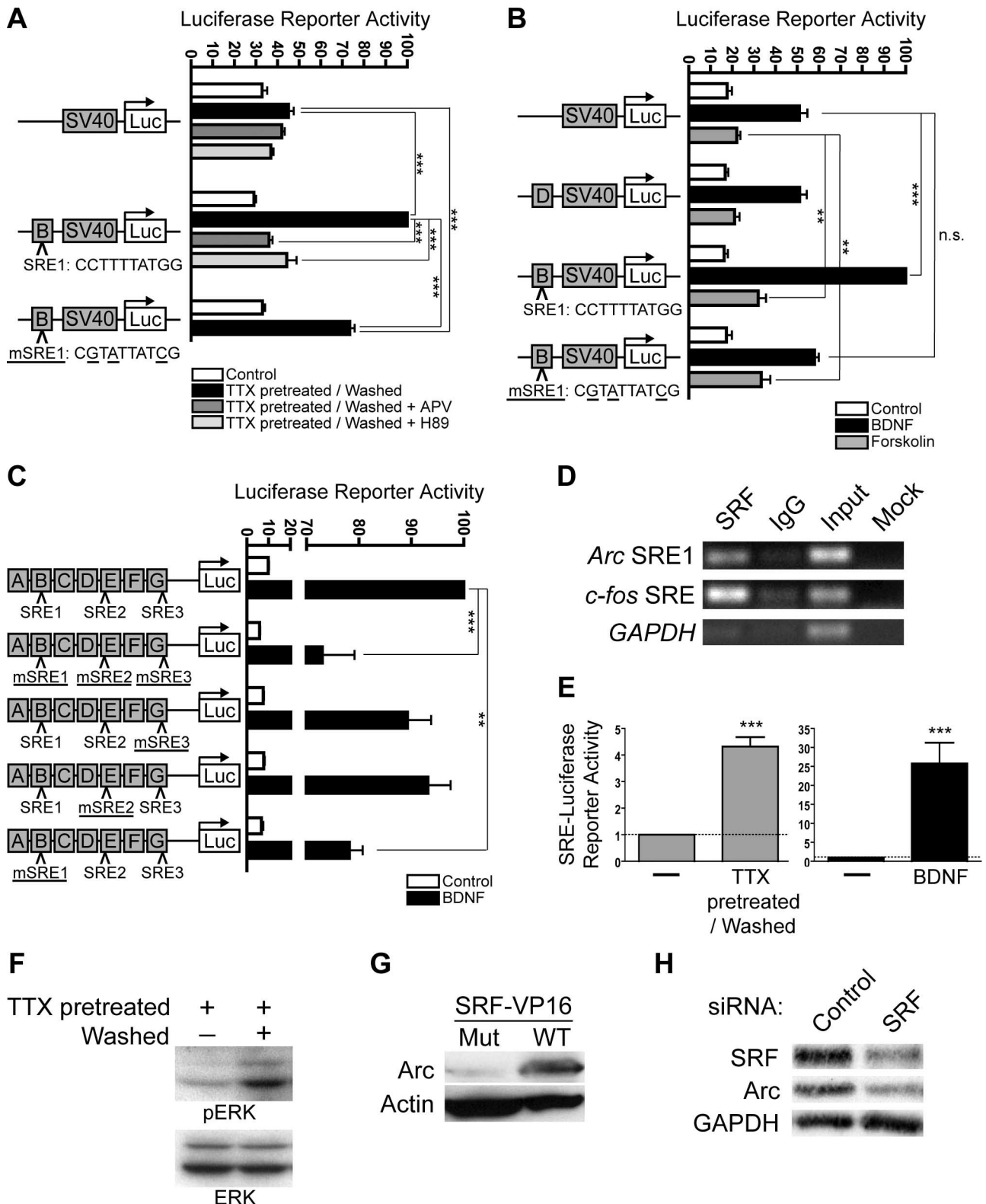


Figure 5. SRF mediates enhancer activity of distal region B. **A**, Region B is sufficient to enhance SV40 promoter activity in response to synaptic activity, and inactivating point mutations of SRE1 (*mSRE1*, with mutated base pairs underlined) reduce this response by ~50%. This enhancer activity is completely blocked by the NMDAR inhibitor APV or the PKA inhibitor H89. All values are percentages (mean ± SEM) of the maximum induction for B. *N* = 4, one-way ANOVA and *post hoc* Tukey's *t* tests: ****p* < 0.001. **B**, Region B is sufficient to enhance SV40 promoter activity in response to both BDNF and forskolin (consistent with Fig. 4C, right). SRE1 is necessary to mediate the response to BDNF but is not important for the response to forskolin. In contrast, region D has no effect on BDNF- or forskolin-induced SV40 promoter activity. All values are percentages (mean ± SEM) of the maximum for B. *N* = 5, one-way ANOVA and *post hoc* Tukey's *t* tests: ****p* < 0.001, ***p* < 0.01; n.s., not statistically significant. **C**, Three putative SREs identified *in silico* were systematically inactivated by subtle point mutations (*mSRE*) in the context of the full-length *Arc* reporter. SRE1, located within region B, mediates ~20% of the response to BDNF and is the only functional element identified. All values are percentages (mean ± SEM) (Figure legend continues.)

Therefore, to test the functional significance of SRE1 in *Arc* expression, we introduced inactivating point mutations. Mutating SRE1 reduced the response to synaptic activity by ~50% (Fig. 5A) and completely blocked BDNF-mediated, but not forskolin-mediated, enhancement of SV40 reporter activity by region B (Fig. 5B). TESS analysis also identified a conserved SRE in region E (SRE2) and one in region G (SRE3) (Fig. 5C). To further test the functional significance of these SREs in the context of the *Arc* promoter, we created a panel of full-length *Arc* reporters with inactivating point mutations of SRE1–3, either individually or all together. SRE2 and SRE3 were dispensable for BDNF-induced reporter activity (Fig. 5C), as expected from our deletion analysis (Fig. 4C, right) and previous findings (Waltereit et al., 2001). In contrast, inactivation of SRE1 reduced BDNF-induced reporter activity to nearly the same extent as deletion of region B from the full-length reporter (Fig. 4C, right). Thus, SRE1 is a functional transcription enhancer element that responds to synaptic activity in an NMDAR-, PKA-, and ERK-dependent manner (Figs. 4B, 5A). However, these data also suggest a separate component of region B enhancer activity that is dependent on synaptic activity and PKA.

If SRE1 functions as a response element for *Arc in vivo*, then its cognate factor, SRF, should be bound at this genomic region. To determine whether SRF is bound to SRE1 at the endogenous *Arc* promoter, we performed chromatin immunoprecipitations (ChIPs). An SRF-specific antibody immunoprecipitated genomic DNA fragments from neurons that contain *Arc* SRE1, as shown by semi-quantitative PCR (Fig. 5D). The ChIP was specific, as the SRF-specific antibody also immunoprecipitated the genomic region with a known SRE from the *c-fos* promoter (Norman et al., 1988), but did not enrich for promoter regions of *GAPDH*, which lacks an SRE. These results show SRF binds SRE1 *in vivo*, further establishing SRE1 as a functional response element that is physiologically relevant for *Arc* expression.

To demonstrate that SRF functionally links stimulation to SRE1-mediated *Arc* expression, we tested whether SRF itself is regulated by synaptic activity or BDNF. Both significantly induced an SRE-driven firefly luciferase reporter gene (*SRE-Luciferase*) that directly measures SRF activity (Fig. 5E). Synaptic activity also led to the phosphorylation of ERK (Fig. 5F), a kinase that activates SRF-containing promoter complexes and SRE-dependent gene transcription and is required for *Arc* induction

←

(Figure legend continued.) of the maximum for ABCDEFG (top construct). $N = 3$, one-way ANOVA and *post hoc* Tukey's *t* tests. $***p < 0.001$, $**p < 0.01$. **D**, ChIPs demonstrate that SRF physically associates *in vivo* with SRE1. Semi-quantitative PCR shows that an SRF-specific antibody enriches for genomic region B compared with a nonspecific control IgG. "Input" refers to the genomic input DNA used for the immunoprecipitation. "Mock" refers to a no-template control PCR. Gel images are representative of three experiments. **E**, Stimulation paradigms that induce *Arc* also activate SRF. BDNF treatment or chronic TTX pretreatment and washout induce *SRE-Luciferase* reporter gene activity, indicating that SRF mediates transcriptional responses to both stimuli. Values are mean \pm SEM. $N = 2$, unpaired *t* test. $***p < 0.001$. **F**, Synaptic activity induced by chronic TTX washout ("Washed") leads to the phosphorylation of ERK1/2 ("pERK"), an upstream regulator of SRF-containing SRE-bound complexes. Total ERK1/2 expression levels are unaffected ("ERK"). All Western blot images are representative of three experiments. **G**, Constitutively active SRF (SRF-VP16) is sufficient to induce endogenous *Arc* protein expression. Wild-type SRF-VP16 ("WT") robustly induces *Arc* expression, while a SRF-VP16 mutant ("Mut") lacking the SRE DNA-binding domain of SRF does not. Actin served as a loading control. All Western blot images are representative of two experiments. **H**, SRF is important for the expression of endogenous *Arc*. Specific knockdown of SRF expression causes a corresponding decrease in BDNF-induced *Arc* levels, compared with a negative control siRNA that has no effect on SRF levels or BDNF-induced *Arc* expression. *GAPDH* served as a loading control. All Western blot images are representative of two biological experiments performed in triplicate.

(Rao et al., 2006). These data show that SRF mediates transcription in response to patterns of synaptic activity or BDNF stimulation that induce endogenous *Arc* expression (Fig. 1B).

We therefore tested whether SRF is necessary and whether activated SRF is sufficient for *Arc* transcription. In neuronal culture, transient transfection of a constitutively active version of SRF fused to the VP16 transactivation domain (SRF-VP16) drove robust expression of endogenous *Arc* protein even in the absence of stimulation. In contrast, a mutant version of SRF-VP16 lacking the SRE DNA-binding domain of SRF did not induce *Arc* (Fig. 5G). Since active SRF was sufficient to induce *Arc* expression, we next tested whether SRF is required for stimulus-dependent *Arc* expression. Transduction of cultured neurons with anti-SRF siRNA led to a specific and reproducible reduction in SRF levels, which correlated with a reproducible and significant reduction in BDNF-dependent *Arc* induction. A negative control siRNA had no effect on SRF levels or BDNF-induced *Arc* expression (Fig. 5H). The incomplete knockdown of SRF might explain why *Arc* induction was not blocked completely; however, an independent contribution from the Zeste-like response element (described below) could also explain, at least in part, the remaining *Arc* expression. Together, the above findings clearly link synaptic activity, SRF activity, SRE1, and *Arc* expression.

A putative novel neuronal transcription factor regulates *Arc*

Next, we investigated the contribution of regions F/G to synaptic activity- and BDNF-induced *Arc* transcription. When subcloned into the SV40 promoter reporter, regions F/G enhanced reporter gene responses to synaptic activity (Fig. 6A). APV or H89 blocked this enhanced response (Fig. 6A), indicating that NMDAR and PKA activation are required for synaptic activity to induce regions F/G. Regions F/G also enhanced the reporter's response to BDNF but not to forskolin (Fig. 6B), suggesting regions F/G respond to ERK-mediated signaling. Together with our deletion analysis (Fig. 4C, right), these findings establish that regions F/G contain one or more DNA elements that selectively mediate responses to synaptic activity and BDNF but not to forskolin.

Bioinformatic analysis of regions F/G identified two conserved and closely spaced consensus binding sites for Zeste (Fig. 2). Efficient Zeste binding and function often require multiple binding sites in close proximity (Mansukhani et al., 1988), suggesting that the sites we found in *Arc* might be important. However, Zeste is an invertebrate transcription factor without a known mammalian ortholog (Levine et al., 2002), and it has not been implicated in plasticity-related neuronal gene expression (Biggin et al., 1988). We hypothesized that a novel "Zeste-like" mammalian factor binds to these sites and is important for *Arc* expression.

To investigate the functional significance of the Zeste-like sites, we first took a mutagenesis-based approach. We made point mutations in the upstream site (Zeste1) in the *Arc* promoter that would be expected from studies in *Drosophila* (Moses et al., 2006) to disrupt Zeste binding. If a mammalian Zeste-like factor binds that site with similar specificity, these inactivating point mutations should block the enhancer activity of regions F/G. Indeed, a subtle 3-bp point mutation of Zeste1 eliminated the enhancer activity of this ~500-bp region in response to synaptic activity (Fig. 6A) and BDNF (Fig. 6B). With the data above, these findings indicate that the novel Zeste1 site mediates transcriptional responses to synaptic activity in an NMDAR-, PKA-, and ERK-dependent manner (Figs. 4B, 6A).

To further investigate how these sites contribute to the transcriptional responses conferred by regions F/G, we deleted an

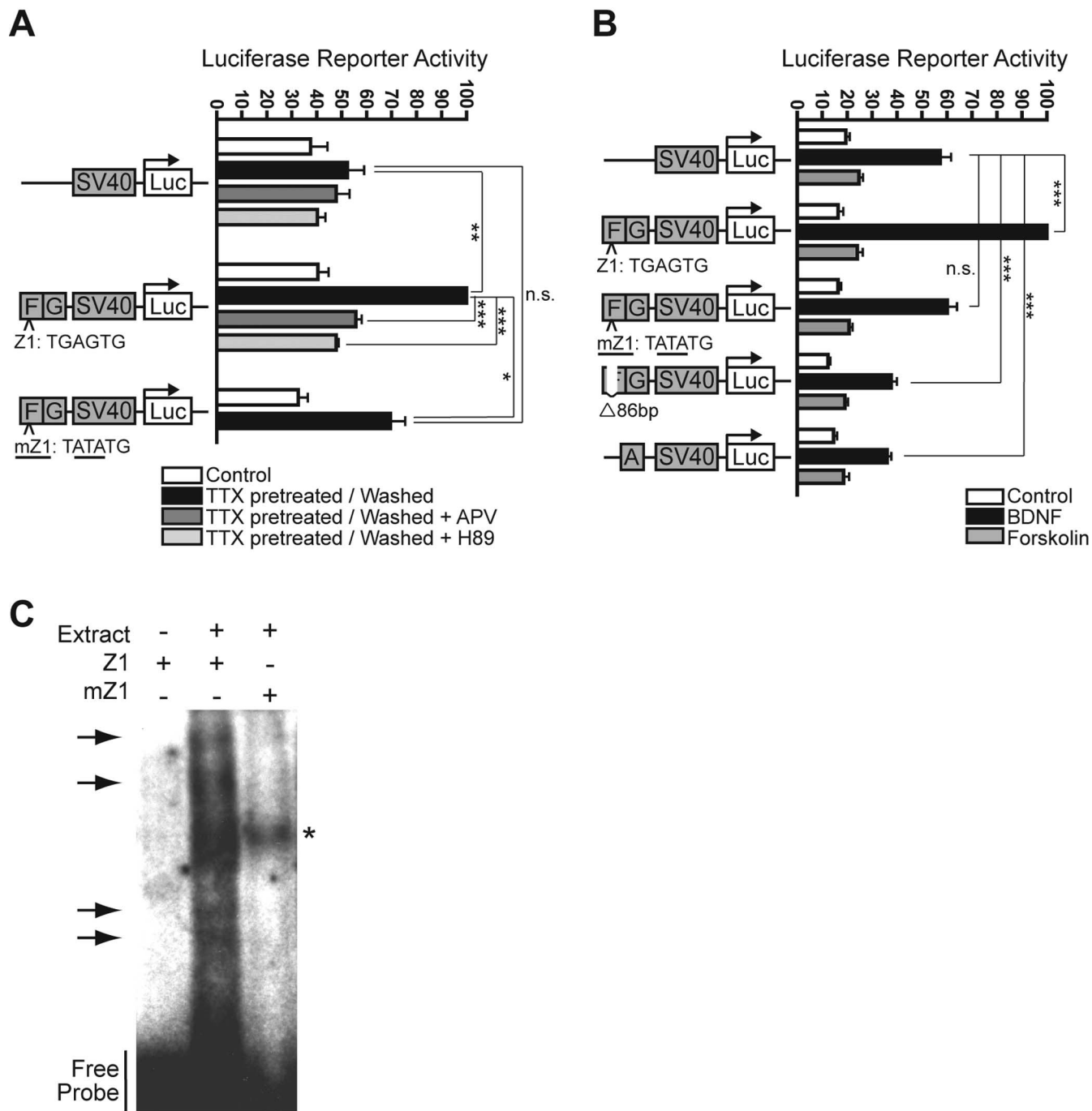


Figure 6. A putative novel factor regulates proximal enhancer regions F/G. **A**, Regions F/G are sufficient to enhance SV40 promoter activity in response to synaptic activity. This enhancer activity is blocked by the NMDAR inhibitor APV or the PKA inhibitor H89. Inactivating point mutations of Zeste1 (*mZ1*, with mutated base pairs underlined) demonstrate that this site is necessary to mediate this enhanced response. All values are percentages (mean ± SEM) of the maximum induction for regions F/G. *N* = 3, one-way ANOVA and *post hoc* Tukey's *t* tests: ****p* < 0.001, ***p* < 0.01, **p* < 0.05; n.s., not statistically significant. **B**, Regions F/G are sufficient to enhance SV40 promoter activity in response to BDNF but not to forskolin (consistent with Fig. 4C, right). Inactivating point mutations of Zeste1 demonstrate that this site is necessary to mediate the enhanced response to BDNF. Deletion of an 86-bp stretch containing the Zeste1 and Zeste2 elements (Δ86bp) reduces BDNF-induced SV40 promoter activity below baseline, suggesting the presence of possible repressor elements in the remaining region. Region A also suppresses BDNF-induced SV40 promoter activity. All values are percentages (mean ± SEM) of the maximum induction for regions F/G. *N* = 5, one-way ANOVA and *post hoc* Tukey's *t* tests: ****p* < 0.001; n.s., not statistically significant. **C**, EMSAs demonstrate that a nuclear protein factor or factors physically associate with the Zeste1 element. Several distinct mobility shifts (arrows) are observed when the wild-type Zeste1 probe (Z1) is pre-incubated with nuclear protein extracts ("Extract"), while the mutant Zeste1 probe (*mZ1*), containing the same point mutations that eliminate enhancer activity) did not show these corresponding shifts. The single shift observed for *mZ1* (indicated by an asterisk) might represent a nonspecific binding event. Blot images are representative of three experiments.

86-bp stretch that contains both of the putative Zeste-like binding sites. This deletion eliminated the BDNF-induced enhancement of SV40 promoter activity by regions F/G (Fig. 6B), further supporting the notion that this 86-bp stretch contains at least one

binding site for a transcriptional enhancer. The deletion also reduced reporter gene activity to a level below that produced by the SV40 promoter alone, indicating that the portions of regions F/G surrounding the Zeste-like binding sites contain a transcriptional

repressor element or elements. Region A also repressed BDNF-induced SV40 promoter activity, consistent with the observation that deleting both region B and regions F/G from the full-length reporter (but retaining region A) led to lower BDNF-induced levels of activity than were obtained with the ~900-bp proximal *Arc* promoter alone (Fig. 4C, right).

If Zeste-like elements mediate synaptic activity- and BDNF-induced *Arc* transcription through a protein-bound factor, the corresponding DNA sequence should bind one or more nuclear proteins in neurons. Since this putative transcription factor is unknown, we cannot perform ChIPs, so we performed electrophoretic mobility shift assays (EMSA). Short radiolabeled oligonucleotides encoding the wild-type Zeste1 site (probe Z1) or a version with point mutations (probe mZ1) that eliminate the enhancer activity of Zeste1 (Figs. 6A, B) were incubated with nuclear lysates from rat cortical neurons and analyzed by electrophoresis. The mobility of probe Z1 was significantly retarded by the formation of several distinct higher-molecular-weight species between probe Z1 and neuronal nuclear factor(s) (Fig. 6C). If the factor(s) from rat cortical neurons that binds Z1 behaves similarly to Zeste from *Drosophila*, these results might be expected. In *Drosophila*, Zeste forms large oligomeric protein complexes *in vitro* (Chen and Pirrotta, 1993), and the availability of excess binding sites in the EMSA reaction might favor the formation of different oligomeric species. Importantly, mutations in the Zeste1 element (probe mZ1) that disrupt its ability to mediate transcription induced by synaptic activity and BDNF also eliminate nearly all binding of the neuronal nuclear factor(s) to the probe. The one apparent higher-molecular-weight band seen for probe mZ1 might represent a nonspecific binding event.

Discussion

Whether signaling pathways triggered by synaptic activity, such as Ca^{2+} , cAMP, or receptor tyrosine kinase activation, induce *Arc* chiefly through new transcription has been debated (Waltereit et al., 2001; Ichikawa et al., 2003; Giorgi et al., 2007). Our results show that these diverse stimuli induce the synthesis of nascent *Arc* mRNA. *In silico* analysis and DHAs of genomic DNA from PC12 cells and neurons revealed nine conserved nucleosome-depleted regions at the *Arc* locus. A reporter gene controlled by these nine promoter regions recapitulated patterns of endogenous *Arc* induction; two of the regions contained enhancer activity, and two contained repressor activity. The region located ~6.5 kb upstream of *Arc* enhanced transcription induced by synaptic activity, BDNF, and forskolin. This region contains an SRE required to mediate responses to synaptic activity and BDNF, but not to forskolin, and it binds SRF *in vivo*. In turn, SRF is recruited

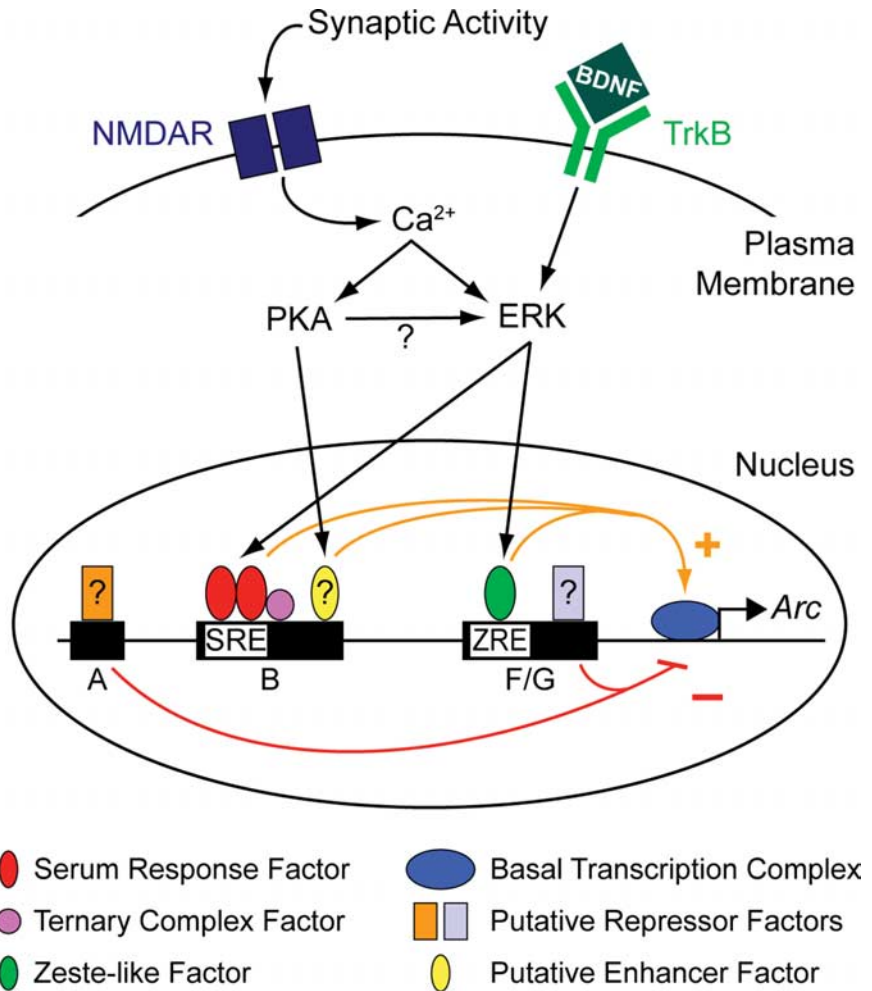


Figure 7. A model of *Arc* transcriptional regulation, showing how the activity-dependent PKA and ERK signaling pathways contribute to *Arc* transcription. Synaptic activity drives *Arc* transcription by inducing SRF/TCF activity in an NMDAR-, PKA-, and ERK-dependent manner (Figs. 4B, 5A, 5B, 5E–H). SRF binds to a conserved SRE in distal open region B (Figs. 3, 5D), and a conserved TCF binding site is found immediately downstream (Fig. 2). An unidentified enhancer factor likely also binds to region B to mediate the remaining response to synaptic activity and PKA (Figs. 5A, 5B). Synaptic activity also induces *Arc* transcription through a Zeste-like response element (ZRE) in proximal open regions F/G (Figs. 3, 6C) in an NMDAR-, PKA-, and ERK-dependent manner (Figs. 4B, 6A, 6B). Tyrosine receptor kinase B (TrkB) activation by BDNF induces *Arc* transcription via SRE and ZRE in an ERK-dependent manner (Figs. 4B, 5B, 6B). Some unidentified repressor factors likely bind to region A (Figs. 4C, 6B) and to regions F/G (Fig. 6B) to regulate stimulus-induced inhibition of reporter activity. The role of these putative repressors in *Arc* expression remains unclear.

by synaptic activity to directly regulate *Arc* transcription. The region located ~1.4 kb upstream of *Arc* enhances synaptic activity- and BDNF- but not forskolin-induced reporter gene activity. The region contains two conserved Zeste-like elements that must be present to mediate such responses. Although there is no known mammalian ortholog of Zeste, subtle mutations that would be predicted to disrupt Zeste binding blocked the ability of the element to mediate stimulus-induced transcription in neurons and the binding of a putative novel neuronal nuclear protein *in vitro*.

The discovery that SRF/SRE is a critical regulator of *Arc* transcription suggests a signaling pathway that links behavior, synaptic activity, and gene expression. Mice lacking SRF show significant but selective defects in learning and memory (Etkin et al., 2006) and alterations in the expression of many genes (Ramanan et al., 2005). It is unknown which genes with altered expression in SRF^{-/-} mice are direct SRF targets, and which of those are responsible for the memory defects. SREs have been found in sev-

eral other genes that are induced by synaptic activity, including *c-fos* and *zif268* (Rivera et al., 1990; DeFranco et al., 1993). However, *Arc* appears to be the first gene regulated directly by SRE/SRF that functions at synapses and is required for learning and memory. SRE-dependent transcription can be activated by stimuli such as BDNF and synaptic activity (Fig. 5E) through the Ras/ERK pathway. ERK1/2 activates Elk-1, a transcription cofactor that forms a ternary complex with a dimer of SRF (Buchwalter et al., 2004), which recruits and activates the basal transcriptional machinery. An Elk-1 binding site is located 3 bp downstream of SRE1, positioned to mediate SRE1-dependent *Arc* transcription. Such a mechanism is consistent with the observations that synaptic activity induces phosphorylation of ERK1/2 (Fig. 5F) and that inhibitors of ERK1/2 activation block stimulus-induced *Arc* expression (Rao et al., 2006). SRF activity can also be regulated independently of Ras by MAL, a member of the myocardin-related transcription factor family (Tabuchi et al., 2005). It is unknown whether MAL is important for *Arc* regulation. How region B of the *Arc* promoter mediates PKA-dependent transcription is also unclear. Mutations in SRE1 that disrupt SRF binding did not eliminate the response of region B to forskolin or to synaptic activity, suggesting that other response elements must exist within region B.

We also discovered novel Zeste-like elements within the *Arc* promoter that contribute almost as much as SRE1 to synaptic activity- and BDNF-induced *Arc* transcription. They are identical to the cognate binding sequence of the *Drosophila* transcription factor Zeste (Biggin et al., 1988), but no mammalian ortholog is known (Levine et al., 2002). Although several bioinformatic screens failed to find a mammalian candidate (data not shown), our data show that a nuclear factor from rat cortical neurons binds to this element. Thus, *Arc* transcription may be controlled partly by a novel Zeste-like mammalian neuronal transcription factor bound to this element. Such a factor may play a wide role in neuronal gene expression and function. Three Zeste-like elements reside within a known enhancer region of human *BACE1* (Sambamurti et al., 2004), a molecule implicated in Alzheimer's disease (Rossner et al., 2006). *BACE1* also regulates amyloid precursor protein cleavage under conditions associated with learning, memory, and synaptic plasticity (Ma et al., 2007). We also found Zeste-like elements in the promoter of *Homer1a* (data not shown), which plays a key regulatory role in synaptic plasticity (Van Keuren-Jensen and Cline, 2006). *Arc* and *Homer1a* are expressed in the same neuronal subpopulations (Vazdarjanova et al., 2002); they may be part of a program of activity-dependent gene expression that specifically elicits long-term adaptive responses through activity-dependent structural and functional synaptic modifications (Park et al., 2008; Ronesi and Huber, 2008; Waung et al., 2008). This scenario would imply that these genes (and perhaps others) are regulated at least in part by a specific shared set of transcription factors that may include a Zeste-like factor.

Identifying the first functional response elements required for rapid *Arc* transcription made it possible to elucidate the specific signaling pathways that impinge upon them to induce *Arc* expression in response to synaptic activity, BDNF, and forskolin (Fig. 7). This study revealed that synaptic activity must induce both the PKA and ERK pathways to regulate region B. PKA directly regulates some yet unidentified transcription factor (or factors) in region B. It remains unclear, however, whether ERK signaling regulates SRF-dependent transcription in parallel with or downstream of PKA signaling. Although the ERK activation inhibitor UO126 completely blocked the full reporter's response

to synaptic activity (Fig. 4B), it was not possible to further assess how ERK directly regulates region B, as UO126 also blocked the basal activity of the SV40 promoter (data not shown). We also show that synaptic activity regulates Zeste-like factor-dependent transcription in regions F/G by recruiting both the PKA and ERK pathways. Curiously, the PKA inhibitor H89 blocked the enhancer activity of F/G (Fig. 6A), but forskolin alone did not induce a response from F/G (Fig. 6B) [in contrast to region B, where forskolin drove transcription (Fig. 5B)]. This could reflect a requirement for PKA activity in basal promoter activity or for cross-talk between ERK and PKA signaling for proper regulation of regions F/G (Impey et al., 1998). The insights gathered here will help answer important questions regarding how synaptic activity regulates specific sets of neuronal genes.

The combined use of DHAs, *in silico* analyses, and reporter genes in primary neurons was critical to identify regulatory elements in *Arc*. Previous studies of *Arc* focused on proximal 5' promoter regions because it is difficult to incorporate large genomic stretches into reporter genes. However, *cis*-acting regulatory regions can be located 5' and 3' to, as well as long distances from, a basal promoter (West and Fraser, 2005). Although this study appears to be the first to apply DHAs to neuronal IEG expression, they have been used for over 25 years to find candidate regulatory regions of genes within large genomic expanses (Pipkin and Lichtenheld, 2006). In addition, DHAs can be applied to native chromatin structure found *in vivo* in neurons, increasing the chances of finding physiologically relevant regulatory regions. Our DHAs and *in silico* analyses defined region B in the *Arc* promoter and led to the discovery of SRE1. SRE1 is located farther upstream than sequences examined in previous studies of *Arc*, and farther upstream than any element known to regulate the transcription of a plasticity-related IEG. Our DHAs and *in silico* analyses also identified regions F/G in the *Arc* promoter, which led to the discovery of a novel synaptic activity- and BDNF-responsive Zeste-like element. More widespread use of these techniques in neuroscience may help provide critical new insights into the broader mechanisms underlying neuronal gene expression.

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