to a cycle sequencing protocol. Reactions were analyzed on an automated DNA sequencer (ABI 373) with fluorescently labeled oligonucleotide primers. Sequence ambiguities were then resolved by ABI dye terminators with the use of specific primers. Sequence information derived from 185 random M13 clones was assembled with Sequence Assembly Manager software (Molecular Biology Information Resource, Department of Cell Biology, Baylor College of Medicine).

9. Amplification of cDNA and genomic DNA was carried out as follows: Two microliters of the RT product or 100 ng of genomic DNA were mixed with 3 pmol of each primer in a total volume of 50 μl containing 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dTTP, and dGTP, and 0.75 U of AmpliTaq DNA polymerase. The reactions were heated to 95°C for 5 min, followed by 26 cycles of DNA reannealing (60°C, 30 s), elongation (72°C, 1 min), and denaturation (95°C, 45 s). The 5′ PCR primer used in the procedure for determination of 5′ alternative splice forms contained sequences from the first exon (nt 38 to 61), whereas the 3′ primer (nt 890 to 913) contained sequences spanning exons 2 and 3. The 5′ PCR primer used for determination of the 3′ alternative forms (nt 2049 to 2072) was placed in the tenth exon, whereas the 3′ PCR primer (nt 2466 to 2489) was placed in the last exon. The number of the primer sequence is given according to our previously reported cDNA sequences (*3*). All these alternative splice forms (I through VIII) have been detected by RTPCR in various tissues from several different sources.
10. Y.-H. Fu *et al.*, unpublished results.
11. Total RNA was extracted from various tissues by RNAzol. One microgram of total RNA was used for the reverse transcription reaction with Superscript RT (Bethesda Research Laboratories) and both oligo(dT) and random hexamer as primers. The reverse transcription reaction was heat-terminated and diluted 2.5× with water. The RT product (2 μl) was then used for the PCR reaction, which used two sets of primers (one set for the Mt-PK gene and one set for the human transferrin receptor gene (as internal control). The PCR products were analyzed on a 2% agarose gel and scanned by a Gene Scanner (ABI). The peak area of the Mt-PK divided by the peak area of the human TFR was used as the ratio of the particular sample. Primer sequences for RTPCR reactions are as follows: Mt-PK, 5′-CACCTCTCTCTGCCGCTGGTGGAC and 5′-CCTAGCGGCGCACCTTCCCGAATG; and transferrin receptor, 5′-CAGCTCCCTGAATAGTCCAAGTAG and 5′-GAATTGAACCTGGACTATGAGAGG.
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9 November 1992; accepted 23 February 1993

Regulation of CREB Phosphorylation in the Suprachiasmatic Nucleus by Light and a Circadian Clock

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Mammalian circadian rhythms are regulated by a pacemaker within the suprachiasmatic nuclei (SCN) of the hypothalamus. The molecular mechanisms controlling the synchronization of the circadian pacemaker are unknown; however, immediate early gene (IEG) expression in the SCN is tightly correlated with entrainment of SCN-regulated rhythms. Antibodies were isolated that recognize the activated, phosphorylated form of the transcription factor cyclic adenosine monophosphate response element binding protein (CREB). Within minutes after exposure of hamsters to light, CREB in the SCN became phosphorylated on the transcriptional regulatory site, Ser^{133}. CREB phosphorylation was dependent on circadian time: CREB became phosphorylated only at times during the circadian cycle when light induced IEG expression and caused phase shifts of circadian rhythms. These results implicate CREB in neuronal signaling in the hypothalamus and suggest that circadian clock gating of light-regulated molecular responses in the SCN occurs upstream of phosphorylation of CREB.

In mammals, light-dark cycles synchronize hormonal and behavioral circadian rhythms (*1, 2*). The primary biological clock, or pacemaker, regulating the periodicity of circadian rhythms resides within the SCN of the hypothalamus (*2, 3*). Neurons of the SCN receive direct input from ganglion cells of the retina. Impulses carried by these retinohypothalamic projections convey information to the SCN about light-dark cycles in the environment and thereby synchronize the pacemaker within the SCN (*2, 3*).

A molecular correlate of photic entrainment is the rapid and transient induction of the c-*fos* proto-oncogene in SCN cells. The Fos protein is a transcription factor that, when heterodimerized with a member of the Jun family of transcription factors, regulates the expression of late response genes containing AP-1 binding sites in their regulatory regions (*4*). The selective expression of such late response genes may determine long-term cellular responses, such as adaptive changes in mature neurons (*5*). In the SCN, expression of c-*fos* is increased by exposure of animals to light, but only at times when light also induces a phase shift of behavioral rhythms (*6–8*). Furthermore, light-induced phase shifting of the circadian rhythm and light-induced expression of c-*fos* have similar photic illumination thresholds (*7*). These observations suggest that Fos plays a key role in light-induced phase shifting of circadian rhythms.

The signaling pathways that regulate the expression of c-*fos* in tissue culture systems have been extensively studied (*5, 9*). In the pheochromocytoma cell line PC12, the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) mediates expression of c-*fos* in response to agents that increase intracellular concentrations of cAMP or Ca^{2+}. These signals trigger phosphorylation of CREB on Ser^{133}, and this phosphorylation event is required for CREB to activate the transcription of genes containing CREB binding sites (*10, 11*). In addition to CREB, several other transcription factors that interact with the c-*fos* promoter have been implicated in the regulation of c-*fos* transcription (*12*). To identify the signaling pathways that trigger the induction of c-*fos* transcription in the intact nervous system, we have generated antibodies to the activated, phosphorylated form of CREB. We have used these antibodies to show that light stimuli that phase shift circadian rhythms induce the phosphorylation of CREB at Ser^{133} in the nuclei of neurons of the SCN.

Antibodies (anti-PCREB) were obtained from rabbits immunized with a phosphopeptide corresponding to amino acids 123 to 136 of CREB (*13*). Anti-PCREB recognized CREB that was purified from cells infected with baculovirus and phos-

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phorylated in vitro on Ser[133] but failed to recognize CREB that was not phosphorylated on Ser[133] (Fig. 1A). Both the phosphorylated and unphosphorylated forms of CREB were detected with an antibody (anti-CREB) that was raised to a TrpE-CREB fusion protein (*14*).

Anti-PCREB specifically recognized the phosphorylated form of CREB present in whole cell lysates of various cell types. In PC12 cells, CREB becomes newly phosphorylated on Ser[133] when the cells are stimulated with forskolin to activate adenylate cyclase or after membrane depolarization, which stimulates Ca^{2+} influx (*10, 15*). Anti-CREB, which recognizes CREB regardless of the phosphorylation state of Ser[133], immunoprecipitated comparable amounts of the 43-kD CREB protein from extracts of ^{32}P-labeled PC12 cells prepared before or after stimulation with forskolin or increased concentrations of extracellular KCl (Fig. 1B). CREB can be detected as a phosphoprotein even in untreated cells because, although it is not phosphorylated on Ser[133] in these cells, it is phosphorylated on other sites (*16*). Anti-PCREB did not immunoprecipitate CREB from extracts of unstimulated cells but did specifically recognize CREB present in PC12 cells minutes after treatment with forskolin or KCl (Fig. 1B). In addition to recognizing CREB (*17*), anti-PCREB immunoprecipitated at least two other phosphoproteins from PC12 cell extracts. Because of their relative molecular sizes, we suspect that these proteins may be two members of the CREB-ATF family, ATF-1 and CREM, that are known to be similar in sequence to CREB in the region that includes Ser[133]. Thus, anti-PCREB should be useful in determining the phosphorylation state of CREB and other related proteins under different conditions of cell stimulation.

Anti-PCREB was used to examine whether CREB activation occurs during trans-synaptic signaling in neurons. In the central nervous system in general, and in the SCN in particular, excitatory trans-synaptic signaling is predominantly mediated by the neurotransmitter glutamate. In the SCN, stimulation of the NMDA subtype of glutamate receptor appears to be critical for induction of c-*fos* mRNA (*18*) and for behavioral phase shifting by light (*19*). To determine if the induction of phosphorylation of CREB on Ser[133] is correlated with glutamate-induced transcription of c-*fos*, anti-PCREB was used to assess the phosphorylation state of CREB in cultured hippocampal neurons before and after exposure to glutamate. Treatment of cultured hippocampal neurons with either glutamate (10 μM) or KCl (55 mM), conditions that lead to increased transcription of c-*fos* (*20*), led to an increase in the amount of phosphorylated CREB immunoprecipitated with anti-PCREB from extracts of [^{32}P]-labeled cells (Fig. 2A). The induction by glutamate, KCl, or forskolin of phosphorylation of CREB was also detected by immunoblot analysis of whole cell extracts of hippocampal cells (Fig. 2B). In these experiments, a doublet that migrated with the 43-kD molecular weight marker was detected by anti-PCREB but not by preimmune serum (*20*). The two inducible bands detected by immunoblot may be CREBα

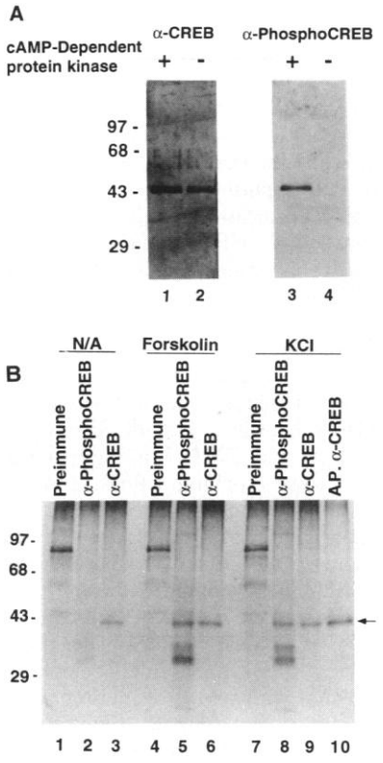

Fig. 1. Anti-phosphoCREB recognizes CREB phosphorylated on Ser[133]. (**A**) Baculovirus-expressed CREB was incubated with (lanes 1 and 3) or without (lanes 2 and 4) cAMP-dependent protein kinase and adenosine triphosphate to phosphorylate Ser[133]. CREB was resolved by SDS-PAGE and immunoblot analysis was carried out (*24*) with anti-CREB (α-CREB) (lanes 1 and 2) or anti-PCREB (α-PhosphoCREB) (lanes 3 and 4). Identical results were obtained when CREB was phosphorylated with Ca^{2+}/calmodulin–dependent protein kinase II, which also phosphorylates Ser[133] (*20*). Preimmune serum did not detect CREB (*20*). (**B**) Immunoprecipitation of CREB phosphorylated on Ser[133] from PC12 cell extracts with anti-PCREB. The ^{32}P-labeled PC12 cells were treated for 10 min as follows: no addition (N/A) (lanes 1 to 3), 10 μM forskolin (lanes 4 to 6), or 60 mM KCl (lanes 7 to 10). Extracts were incubated with preimmune serum (lanes 1, 4, and 7), anti-PCREB (lanes 2, 5, and 8), anti-CREB (lanes 3, 6, and 9), or affinity-purified anti-CREB (A.P. α-CREB) (lane 10). Immune complexes were collected, boiled, and resolved by SDS-PAGE as described (*25*). The arrow indicates CREB.

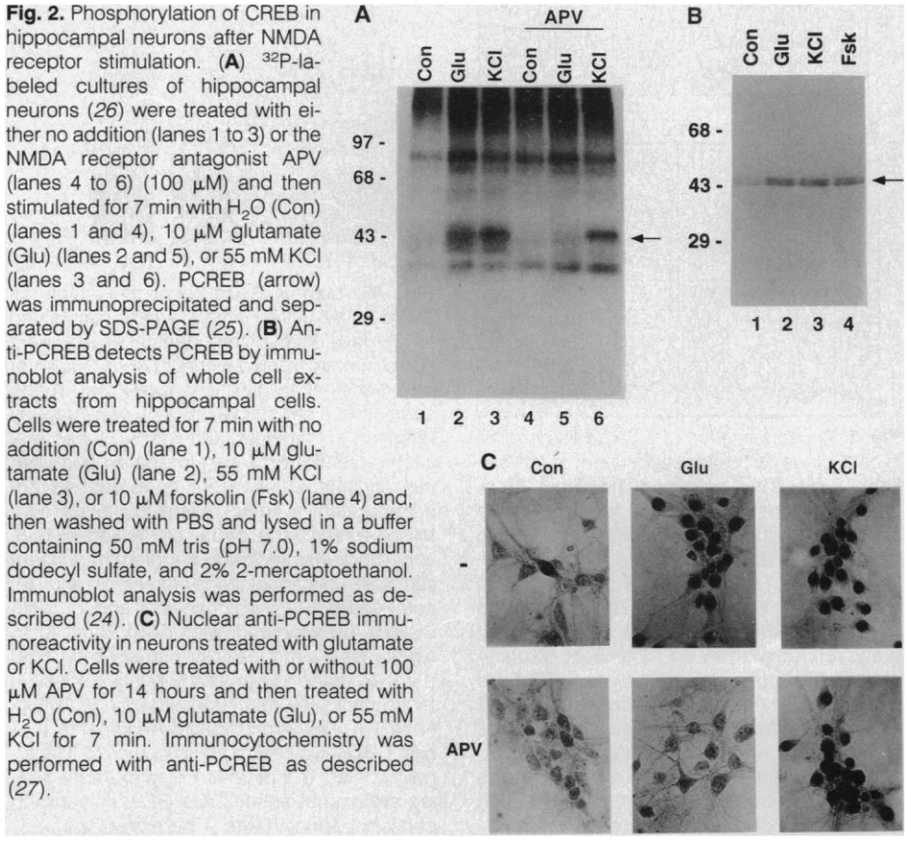

Fig. 2. Phosphorylation of CREB in hippocampal neurons after NMDA receptor stimulation. (**A**) ^{32}P-labeled cultures of hippocampal neurons (*26*) were treated with either no addition (lanes 1 to 3) or the NMDA receptor antagonist APV (lanes 4 to 6) (100 μM) and then stimulated for 7 min with H_2O (Con) (lanes 1 and 4), 10 μM glutamate (Glu) (lanes 2 and 5), or 55 mM KCl (lanes 3 and 6). PCREB (arrow) was immunoprecipitated and separated by SDS-PAGE (*25*). (**B**) Anti-PCREB detects PCREB by immunoblot analysis of whole cell extracts from hippocampal cells. Cells were treated for 7 min with no addition (Con) (lane 1), 10 μM glutamate (Glu) (lane 2), 55 mM KCl (lane 3), or 10 μM forskolin (Fsk) (lane 4) and, then washed with PBS and lysed in a buffer containing 50 mM tris (pH 7.0), 1% sodium dodecyl sulfate, and 2% 2-mercaptoethanol. Immunoblot analysis was performed as described (*24*). (**C**) Nuclear anti-PCREB immunoreactivity in neurons treated with glutamate or KCl. Cells were treated with or without 100 μM APV for 14 hours and then treated with H_2O (Con), 10 μM glutamate (Glu), or 55 mM KCl for 7 min. Immunocytochemistry was performed with anti-PCREB as described (*27*).

and CREBΔ, which differ in sequence by 14 amino acids. The increased phosphorylation of CREB on Ser^{133} appeared to result from activation of the NMDA subtype of glutamate receptor because it was blocked by the specific NMDA receptor antagonist D(−)-2-amino-5-phosphonovalerate (APV) (Fig. 2A). The effects of APV appeared to be specific to glutamate because this inhibitor did not block phosphorylation of CREB induced by membrane depolarization (Fig. 2A) or treatment of cells with forskolin (*20*). Glutamate also rapidly induced anti-PCREB immunoreactivity in the nuclei of hippocampal neurons in the absence but not the presence of the NMDA antagonist APV (Fig. 2C).

In the SCN, CREB was also newly phosphorylated at Ser^{133} at times when light triggers an induction of c-*fos* expression and a phase shift of the circadian rhythm. Hamsters were placed in constant darkness for 5 days and subsequently exposed to light for 5 min during their subjective night, and brain sections were stained with anti-PCREB (*21*). Exposure to light for 5 min during subjective night at circadian time (CT)19 (Fig. 3A) or CT14 (*20*) led to increased anti-PCREB immunoreactivity in cells of the SCN (Fig. 3A). The immunostaining appeared to be specific because it was not detected with anti-PCREB that was preadsorbed with a peptide containing phospho-Ser^{133}. Whereas the induction of anti-PCREB immunoreactivity was rapid (within 5 to 10 min) and robust, synthesis of CREB appeared not to change as a function of photic stimulation because anti-CREB immunoreactivity was similar in the absence or presence of light (Fig. 3A). These results demonstrate that exposure of hamsters to light during the subjective night leads to the phosphorylation of a CREB family member in neurons of the SCN.

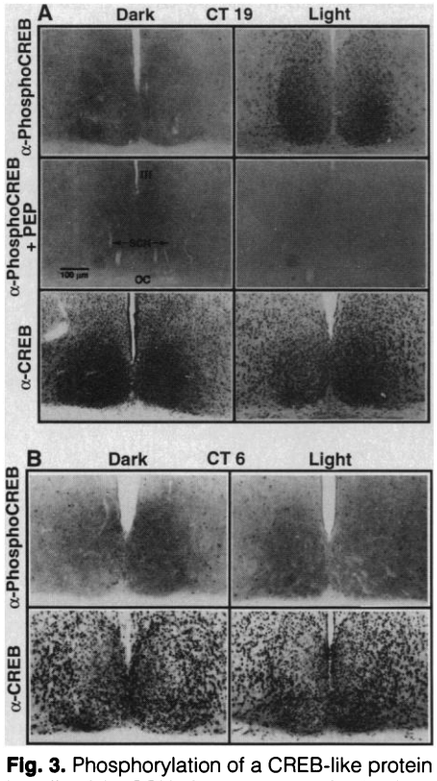

Fig. 3. Phosphorylation of a CREB-like protein in cells of the SCN after exposure of animals to light. Light-dark entrained hamsters were placed in constant darkness for 5 days and subsequently stimulated during (**A**) subjective night (CT 19) or (**B**) subjective day (circadian time 6) without or with light for 5 min and then killed. Brain sections were immunostained with anti-PCREB, anti-PCREB that had been preadsorbed with an equal amount of phosphopeptide (PEP) (1 μg/ul), or anti-CREB as described (*21*). The experiment shown in (A) has been performed four times with a total of nine animals in each group (light and dark). The experiment shown in (B) has been done three times with a total of six animals in each group; III, third ventricle; OC, optic chiasm.

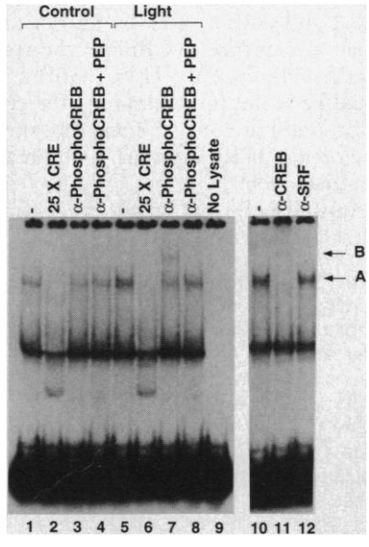

Fig. 4. Light-induced phosphorylation of CREB in the SCN. Hamsters were maintained for 5 days in constant darkness and were subsequently treated without (lanes 1 to 4) or with (lanes 5 to 12) light for 5 min during subjective night (CT19). Brains were rapidly removed in darkness and the SCN was dissected (*8*). Extracts were prepared and DNA mobility-shift assays were performed as described (*22*). Binding reactions (25 μl) contained extract and probe alone (lanes 1, 5, and 10) or also included 2.4 pmol of consensus CRE oligonucleotide (lanes 2 and 6), anti-PCREB (0.15 μg) (lanes 3 and 7), anti-PCREB preadsorbed with PCREBtide (0.15 μg) (lanes 4 and 8), affinity-purified anti-CREB (0.08 μg) (lane 11), or affinity-purified anti-SRF (antibody to serum response factor) (0.20 μg) (lane 12). The arrow labeled A indicates specific CREB–Ca-CRE binding complex. The arrow labeled B indicates complex formed by the addition of anti-PCREB. PEP, immunizing phosphopeptide. Lane 9, no extract.

To determine if light stimulation specifically leads to an increase in phosphorylation of CREB on Ser^{133}, rather than phosphorylation of another member of the CREB family, tissue extracts were prepared from the SCN and DNA mobility-shift assays were performed with the Ca^{2+}-cAMP response element (Ca-CRE; sequence TGACGTTT) of the c-*fos* promoter as a probe (*22*). A factor that binds to the Ca-CRE was detected in extracts from the SCN (Fig. 4). The binding of this factor to the Ca-CRE was abolished by including an excess of an oligonucleotide (25 times the amount of the Ca-CRE probe) containing a consensus CRE (TGACGTCA) in the binding reaction. The formation of this complex was also blocked by including in the binding reaction affinity-purified anti-CREB but was not blocked by an antibody to an unrelated transcription factor (Fig. 4). Because anti-CREB immunoprecipitates CREB, but not ATF-1 or CREM (Fig. 1B), these results suggest that CREB is the major Ca-CRE binding factor in the SCN.

To determine if CREB phosphorylated on Ser^{133} was present in SCN extracts and regulated by light, anti-PCREB was included in the DNA binding reactions. Rather than blocking CREB binding to the Ca-CRE, incubation of the SCN extracts with anti-PCREB reduced the electrophoretic mobility of a fraction of the Ca-CRE–CREB complex (Fig. 4). This shifted complex was not detected when anti-PCREB was incubated with the immunizing phosphopeptide before use. Consistent with results of the immunohistochemistry experiments (Fig. 3), the amount of the shifted complex was increased in animals that were exposed to light for 5 min at CT19. Thus, CREB in the SCN becomes newly phosphorylated in response to synaptic activity elicited by light at a transcriptional regulatory site, Ser^{133}.

To determine if circadian phase affects light-induced phosphorylation of CREB, anti-PCREB immunoreactivity was examined in animals stimulated with light during the subjective day phase of the circadian cycle. Light induces expression of c-*fos* and phase shifts behavioral rhythms during the subjective night period of the circadian cycle but not during the subjective day (*6*, *7*, *23*). Light also failed to induce anti-PCREB immunoreactivity if the stimulus was given during subjective day at CT6. The failure to detect anti-PCREB immunoreactivity was not due to a lack of CREB in these neurons during subjective day because at this time neurons within the SCN were positively stained with anti-CREB. Because there is

a correlation between the circadian phase–dependence of light-induced phosphorylation of CREB and light-induced transcription of c-*fos* and because CREB participates in the control of early gene expression in cultured cells, it is likely that CREB Ser^{133} phosphorylation plays a role in the control of transcription of c-*fos* in cells of the SCN. Regulation of gene expression in the SCN by CREB may be important for the entrainment of the pacemaker that orchestrates hormonal and behavioral rhythms. In addition, the components of the circadian clock that gate light-sensitive molecular responsiveness in the SCN may act upstream of phosphorylation of CREB.

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13. To generate anti-PCREB, 3.4 mg of CREBtide (Lys-Arg-Arg-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser-Tyr-Arg-Lys) was phosphorylated with catalytic subunit of cAMP-dependent protein kinase (250 U, Sigma) for 5 hours at 30°C. Another 250 U of catalytic subunit was then added and the mixture was incubated for 14 hours. The phosphopeptide was desalted and coupled to keyhole Limpet hemocyanin (KLH) by incubation with glutaraldehyde. Rabbits were injected with PCREBtide (200 μg) in complete Freunds adjuvant (FA) and boosted 28 and 56 days later with 200 μg of peptide in incomplete FA. Serum was collected 10 days after the last injection, and the immunoglobulin G (IgG) fraction was purified by protein A–Sepharose chromatography. To remove antibodies with high affinity to unphosphorylated CREB, the IgG fraction was applied to an affinity chromatography column containing unphosphorylated CREBtide. The flow-through from this column contained antibodies specific for CREB phosphorylated on Ser^{133}.
14. Anti-CREB was prepared against a TrpE-CREB fusion protein. CREB sequences were amplified by polymerase chain reaction as described (*11*). The amplification product was digested with Pst I and phosphorylated with polynucleotide kinase. The 5′ fragment containing coding sequences for the first 205 amino acids of CREB was gel-purified and ligated into the PATH11 vector (provided by T. J. Koerner and A. Tzagaloff, Columbia University) digested with Sma I and Pst I. The TrpE-CREB fusion protein was prepared as described (*28*) and injected into rabbits as described (*13*). Affinity-purified antibodies were prepared with a TrpE-CREB affinity column prepared as described (*29*). Anti-CREB recognized CREB but not other family members on immunoblots of PC12 cell extracts (*20*).
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17. The conclusion that the 43-kD protein present in the anti-PCREB immunoprecipitates is CREB is based on the findings that this protein comigrated with purified CREB during SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and that it has an identical tryptic peptide map to purified CREB phosphorylated on Ser^{133} in vitro (*20*).
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21. Animals were first entrained to a 14:10 light:dark schedule and subsequently maintained in darkness for 5 days. Animals were exposed to white light for 5 min and immediately anesthetized with sodium pentobarbital (160 mg/kg). Five minutes later, animals were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 to 5 min. Their brains were then removed and fixed for 2 hours at 4°C. Immunocytochemistry was done on frozen brain sections (50 μM) through the SCN. Anti-PCREB or anti-CREB was used at 0.73 μg/ml and 1:5000 dilutions, respectively, and specific immune complexes were visualized with an avidin biotin detection system (Vector Laboratories).
22. The Ca-CRE oligonucleotide (15) was labeled with [γ-^{32}P]ATP by polynucleotide kinase and subsequently purified on a 4% agarose gel. SCN gel shifts were done as described (*8*) except that SCN extraction buffer also included 1 mM EGTA, 5 μM microcystin, and 1 mM NaF. Antibodies were added immediately before adding the probe. The consensus CRE probe used for competition, 5′-AGAGATTGCC**TGACGTCA**GAGAGCTAG-3′, was purchased from Promega. The CRE is indicated in bold letters.
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24. Protein immunoblots were done with CREB purified from cells infected with baculovirus (Fig. 1) or whole cell extracts from hippocampal neurons (Fig. 2B). The purified protein was either unphosphorylated or phosphorylated with the catalytic subunit of PKA. Purified CREB or cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose, rinsed in TBST [10 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20], incubated with 3% BSA in TBST, and incubated with either anti-CREB (1:200 in TBST) or anti-PCREB (0.7 μg/ml in TBST) for 1 hour. Immunoblots of whole cell extracts were done with anti-PCREB (0.07 μg/ml in TBST) with a 2-hour incubation. Antibody-antigen complexes were detected with a goat antibody to rabbit IgG conjugated to alkaline phosphatase (Promega).
25. PC12 cells (2×10^6 to 3×10^6 cells per plate) or hippocampal cells (0.5×10^5 to 1×10^5 cells per plate) were incubated with 250 μCi [^{32}P]orthophosphate per plate, treated as described in the figure legends, and lysed in 200 μl of a solution containing 0.5% SDS, 50 mM tris (pH 8), trazalol (200 μg/ml), and 2% 2-mercaptoethanol heated to 100°C. The sample was then boiled for 5 min. Subsequently, lysates were diluted to 1 ml in RIPA [final concentrations: 50 mM tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 2 mM NaF, 1 mM ammonium molybdate, phenylmethanesulfonyl fluoride (20 μg/ml), aprotinin (40 μg/ml), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] and centrifuged at 100,000*g*. Immunoprecipitations were done from the supernatant from hippocampal extract (1 ml) or PC12 extract (0.2 ml). Extracts were incubated with either anti-PCREB (7.3 μg/ml) or anti-CREB (1:200 dilution) for 1 hour. Extracts were then incubated for 1 hour with Protein A–Sepharose, and immune complexes were collected by centrifugation and washed successively with RIPA; a buffer containing 0.5 M LiCl, 0.5% NP-40, and 50 mM tris (pH 7.4); a buffer containing 0.5 M LiCl and 50 mM tris (pH 7.4); and finally a buffer containing 10 mM tris (pH 7.4). Immune complexes were boiled in SDS sample loading buffer, and the immunoprecipitated proteins were separated by SDS-PAGE (10% gel) and visualized by autoradiography.
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27. Cells were treated as described in the figure legends, rinsed with PBS, and fixed with 4% paraformaldehyde in PBS for 30 min. Cells were permeabilized with 0.5% NP-40 in PBS and then incubated with 3% BSA in PBS for 1 hour at 22° C. Anti-PCREB immunoreactivity was detected with anti-PCREB [0.73 μg/ml in PBS containing BSA (3%) incubated for 16 hours at 4°C] and biotinylated antibody to rabbit IgG as described in instructions supplied with the Vectastain kit (Vector Laboratories). No nuclear immunoreactivity was detected when the primary antibody was preadsorbed with PCREBtide (*20*).
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30. We thank C. Miranti for baculovirus-expressed CREB, V. Rivera for affinity-purified anti-SRF antibody, and C. DeFranco and L. Rosen for critical reading of this manuscript. Supported by NIH grants NRSA F32 NS08764 (D.D.G.) and F31 MH10241 (J.M.K.); American Heart Association, Massachusetts affiliate, 13-446-912 (D.D.G.); NIH grant NS 28829 (M.E.G.); and grants from the NIH R37 MH39592 and MH49241 and the NSF Center for Biological Timing (J.S.T.). M.E.G. is a recipient of the American Cancer Society Faculty Research Award (FRA-379) and of a scholar's award from the McKnight Endowment Fund for Neuroscience.