The Aplysia genes encoding for cAMP-response element-binding protein 1 (CREB1), CREB2, and ubiquitin C-terminal hydrolase (Ap-uch) have been implicated in the formation of long term memory. However, nothing is known about the promoter regions of these genes or the transcription factors that regulate them. We cloned the promoter regions of creb1, creb2, and Ap-uch and identified a canonical cAMP-response element (CRE) in the promoter region of creb1. Variants of the canonical CRE were identified in all three promoters. TATA boxes and C/EBP-binding motifs are also present in the promoter regions of these genes. Promoter immunoprecipitation assays and chromatin immunoprecipitation assays indicated that CREB1 and CREB2 bind to the promoter regions of creb1 and creb2, suggesting that feedback loops modulate the formation of long term memory. In a positive feedback loop, phosphorylated CREB1 might induce its own gene via CREs. In support of this suggestion, treatment with serotonin enhanced binding of CREB1 to its promoter region and increased mRNA levels of creb1. Levels of Ap-uch mRNA also increased in response to serotonin; however, binding of CREB1 or CREB2 to the promoter region of Ap-uch was not detected. The finding that the promoter region of creb2 has a CRE raises the intriguing possibility that its expression is regulated by CREB1 and/or CREB2. CREB2 may repress its own gene, forming a negative feedback loop, and CREB2 up-regulation via CREB1 may limit the activity of the CREB1-mediated positive feedback loop.

Memory storage has at least two distinct forms, short term and long term. Whereas short term memory lasts for only several minutes, and involves covalent modifications of preexisting proteins, long term memory (LTM) lasts for at least 24 h and involves the synthesis of new mRNAs and proteins (1). Characterization of the genes involved in the induction and consolidation of LTM is essential for elucidating the mechanisms of memory formation. The gene family of the cAMP-response element-binding protein (CREB) transcription factors is important for LTM in both vertebrates and invertebrates (2–6). Immediate-early genes coding for CCAAT enhancer-binding protein (C/EBP) and Aplysia ubiquitin C-terminal hydrolase (Ap-uch) are also essential for LTM (7–11).

The marine mollusc Aplysia has served for more than 35 years as a model organism for studying learning and memory (12). The simple nervous system of Aplysia has facilitated the study of changes in specific synapses and has allowed their correlation with particular learned behaviors (13, 14). Long term sensitization requires gene induction by cAMP (2) and depends on long term strengthening (facilitation) of sensory-motor synapses. These synaptic changes depend on a balance of gene induction and repression regulated by transcription factors such as the CREB family (15–17).

In Aplysia, two CREBs have been identified, CREB1 and CREB2. CREB1, which is homologous to mammalian CREB, is a transcription activator necessary for long term facilitation (LTF) (18, 19). Bartsch et al. (19) found that creb1 mRNA was increased in the CNS after exposure of the whole animal to serotonin (5-HT), a transmitter thought to mediate LTF. However, the induction mechanism of creb1 is not well understood, and its promoter region has not been characterized. Aplysia CREB2, which is homologous to vertebrate CREB2 (ATF4) (20), functions as a transcriptional repressor that may pose inhibitory constraints on memory formation (17, 21). This constraint can be regulated through phosphorylation (17, 22). However, the regulation of expression of creb2 has not yet been investigated previously, and the promoter region has not been characterized.

The canonical CRE motif, 5′-TGACGTCA-3′, is required for gene induction by CREB (23–26). CREB binds to the canonical CRE or to close variants. Most interestingly, mammalian creb has CREs in its promoter region (27). It is not known whether Aplysia creb1 has CREs in its promoter region. If so, then CREB1 could enhance its own transcription, forming a positive feedback loop that might help to maintain gene activation essential for consolidation of LTF. It is also not known whether Aplysia creb2 has CREs in its promoter region. CREs have been identified in the promoter region of cebp (7, 17), which encodes a transcription factor regulated by 5-HT and is necessary for LTF.
Ap-uch is induced by 5-HT and is necessary for LTF (8). Increased Ap-uch levels enhance hydrolysis and disassembly of multiubiquitin conjugates, increasing protein degradation via the ubiquitin-proteasome pathway (28). In particular, the degradation of the regulatory (R) subunit of PKA is enhanced, resulting in increased levels of free, autonomously active catalytic (C) subunits (29–31). This increased PKA activity could, in turn, maintain phosphorylation of CREB1 (32) and further induction of Ap-uch. However, the promoter region of Ap-uch has not yet been characterized. Regulation of Ap-uch by CREB1 or CREB2 has not been examined, and the plausibility of the Ap-uch feedback loop has not been assessed.

We report the cloning and sequencing of the promoter regions of creb1, creb2, and Ap-uch in Aplysia. We have identified putative CRE sequences, TATA boxes and C/EBP-binding motifs within each promoter region. We showed in a promoter immunoprecipitation assay that CREB1 and CREB2 proteins bind to their own gene promoter regions but not to the Ap-uch promoter region. These findings were confirmed and extended using in vivo chromatin immunoprecipitation (CHIP) assays. Finally, we report the induction of creb1 and Ap-uch in pleural ganglia following 5-HT treatment.

**MATERIALS AND METHODS**

**Aplysia Genome Walking**

Aplysia genomic DNA was isolated, digested with PvuII and Stul (Promega), and ligated with the GenomeWalker adaptor DNA (Universal GenomeWalker kit, Clontech). The genomic fragments were used as templates for PCR with an adaptor primer (AP1, 5'-GTAATA- CGACTCACTATAGGGC-3') and a gene-specific primer (see below). The PCR product (1 kb) was subjected to electrophoresis, extracted from agarose gel, and subcloned into pCR 2.1 TOPO TA vector (Invitrogen). The cloned fragment was sequenced by the automated DNA sequence analyzer (ABI model 7000) at the DNA Core Facility, Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, using M13 universal primers. The following gene-specific primers were used in the genome walks: for creb1, first genomic walk, 5'-GGCTGCAATC- GGATGTGATAAATTTCC-3' (based on Ref. 19); for the second genomic walk, 5'-AGAACATTTGTTCATGGCAGGATGTG-3' (based on the sequence from the first genomic walk) (see supplemental Fig. S1). This primer was also used as the gene-specific primer for creb1 in the PCR step of the promoter immunoprecipitation assay. The following sequences were used: for creb1, 5'-CCAGTGTTAAAGAATCTTGGCCTCAAGGCAGGTTGTTCTTGTACTGCTCCGGGCAAGAGTTG-3' (based on Ref. 21), GenBank accession number U40851 (21), SMART RACE cDNA amplification kit (Clontech) to obtain the remaining portion of the 5' UTR to examine the effect of the 5' UTR on transcription.

**Creb2**

The amplicons were subcloned into pCR 2.1 TOPO vector and sequenced to confirm their identity. The inserts were then separately cloned into the pGEK-4T-1 expression vector (which codes for glutathione S-transferase (GST)) at the EcoRI site (Amersham Biosciences). Recombinant fusion proteins were produced in Escherichia coli BL21 following standard procedures (37) and purified through GST affinity chromatography. The GST moiety was cleaved from the fusion proteins using thrombin (20 units per ml of PBS, incubated for 1 h at 22 °C) (Amersham Biosciences).

**Affinity-purified Antibodies against CREB Proteins**

The CREB1 and CREB2 peptides and the antibodies were raised by a commercial vendor (Genemed Synthesis, Inc., South San Francisco, CA).

**Antibodies for CREB1—Rabbit polyclonal antibodies were raised against the Ser152-phosphorylated (underlined) and the unphosphorylated versions of a CREB1 hybrid peptide constructed to juxtapose the sequences immediately surrounding two putative MAPK phosphorylation sites. The peptide sequence is SPDPDEQPGPSSPET, with phospho groups chemically added on Ser152 (1st underline) and Ser237 (2nd underline) for the phospho-peptide. Purification of the two antibodies was performed following the same procedure as for CREB1 antibodies.**

**Cloning, Expression, and Purification of CREB Proteins**

The cDNAs for creb1 and creb2 were cloned from total RNA extracted from Aplysia pleural ganglia, using RT-PCR with primers made according to the published sequences: for creb1 (based on Ref. 19), 5'-ATGG- CAGAAGGCATGCTCCGGC-3', 5'-CTGATATGGCGCCATGGTGTG-3'; for creb2 (based on Ref. 21), 5'-ATGGAGCGCTGCTTTGGA- 3', GenBank accession number U40851 (21), 5'-GGCTGCAATC- GGATGTGATAAATTTCC-3'.

**Promoter Immunoprecipitation Assay**

Cloned promoters and either recombinant CREB or CNS lysate were used in an assay modified from Guan et al. (17). The promoter fragments were isolated from the pCR 2.1 vector by digestion with restriction enzymes and separated by electrophoresis. These cloned fragments included the 5'UTRs of creb2 and Ap-uch, creb1 promoter fragments were constructed with or without the 5' UTR to examine the effect of the...
canonical CRE within the 5′ UTR (Fig. 1A2) on the binding of CREB proteins. Approximately 10 ng of DNA was incubated for 3 h at 22 °C with 5 μg of purified recombinant CREB protein (or 200 μg of protein from EXS) in 100 μl of PBST (PBS containing 0.05% Tween 20). At the end of incubation, 5 μl of total CREB1 antibody and 5 μl of phospho-CREB1 antibody (or total CREB2 and phospho-CREB2 antibody) were added together to the reaction mixture, to maximize the immunoprecipitation, and incubated for 18 h at 4 °C. In control experiments, 10 μl of preimmune rabbit sera replaced the specific antibodies. Protein A-Sepharose (50% slurry in PBST, 25 μl) was added to the mixture, which was then incubated for at least 2 h at 4 °C, followed by centrifugation at 5000 × g for 5 min. The pellet was washed in PBST to remove any unbound material and was resuspended in 25 μl of PBST. Two μl of this washed material was used for PCR (30 cycles) using gene-specific primers and Adaptor Primer 2 (see Aplysia genome walking section).

Protein amounts were estimated by a modified Lowry method (DC protein assay, Bio-Rad). Total RNA was estimated by measuring the absorbance at 260 nm using a Beckman DU 530 spectrophotometer.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were as described in Weinmann et al. (38). Briefly, pleural-pedal ganglia were isolated and treated with 5 pulses of 5-HT or vehicle, as described above. Immediately after the treatment, the ganglia were treated with 1% formaldehyde for 30 min at room temperature with rotation to cross-link proteins to DNA. The reaction was quenched by the addition of glycine (final concentration, 0.125 M). Following cell lysis (in 400 μl of lysis buffer: 10 μl of HEPES, 85 μl of KC1, 0.5% Nonidet P-40) in the presence of protease inhibitors, nuclei were recovered by low speed centrifugation, resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors, and sonicated six times for 10 s each time on ice to shear the genomic DNA to lengths of 0.3–1.3 kb. The lysates were then diluted with CHiP dilution buffer (0.01% SDS, 1.1% Trition X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, and protease inhibitors, pH 8.1). A portion of diluted lysate was kept for input control. Subsequently, the lysate was precleared with salmon sperm DNA/protein A-agarose beads for 1 h at 4 °C, followed by brief centrifugation to pull down the beads. Two μg of anti-tCREB1 (or anti-tCREB2) were added to the supernatant fraction and incubated overnight at 4 °C with rotation. For the negative control, preimmune serum replaced the specific antibody. Immunocomplexes were eluted by two 15-min incubations with 150 μl of elution buffer (10 mM Tris-HCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors, and Total Forms of CREB1 and CREB2—Development of Antibodies Specific for the Phosphorylated and Total Forms of CREB1 and CREB2—Polyclonal antibodies were raised against Aplysia CREB1 and CREB2 peptides. Two CREB1 antibodies were produced. The first term anti-tCREB1 was raised against a CREB1 peptide with the target residue for protein kinase A (PKA), Ser85, phosphorylated (see “Materials and Methods”). The second antibody, term anti-tCREB1, was raised against this peptide with unphosphorylated Ser85. Anti-tCREB1 recognized protein bands at 33 and 66 kDa in pleural ganglia extracts (Fig. 2A, arrowheads). The 33-kDa protein band was somewhat higher than the calculated molecular mass of CREB1, which is 29 kDa. To investigate this discrepancy, we cloned, expressed, and purified recombinant full-length Aplysia CREB1 protein (rec-CREB1). Western blot analysis revealed that rec-CREB1 also showed an apparent molecular mass of 33 kDa (Fig. 2A), suggesting that the 33-kDa protein band in the pleural ganglia extracts was the native CREB1 monomer. The minor shift in electrophoretic mobility might be due to post-translational modifications (e.g. phospho-

RESULTS

The CREB1, CREB2, and Ap-uch Promoter Regions Each Contain Variant CRE Sequences, and creb1 Contains a Canonical CRE—We cloned and sequenced 736 bp upstream of exon I using a gene-specific primer in the middle of the published sequence of exon I (19) (see supplemental Fig. S1; GenBank™ accession number DQ028783). Two rounds of genome walking were used to obtain the sequence of the creb1 promoter region were followed by 5′ RACE analysis to establish the putative TSS. Analysis of the genomic sequence of creb1 upstream of the TSS by the TESS program (see “Materials and Methods”) revealed three variant CREs: TGATGTCA at −79 bp, TGA GTCA at −465 bp, and TGCCCCGTG at −606 bp. Downstream of the TSS, a canonical CRE, TGAGCTCA, was identified at +141 bp as well as a variant CRE, TGACCTTG, at +265 bp in exon I. Variant CREs have asymmetrical or partial sequences that differ from the canonical sequence (5′-TGACCTCA-3′) by single or multiple nucleotide deletions or substitutions (39, 40) and commonly suffice for induction by cAMP (41). Putative TATA boxes were identified at −190 bp (Figs. 1A and supplemental S1) and at other locations further upstream. Putative C/EBP-binding motifs were identified throughout the promoter region (not shown).

By employing the same strategy, we cloned the promoter regions of creb2 and Ap-uch, using primers downstream of the Met initiation codons. The creb2 promoter region has a single variant CRE, TGCAGCAC, at −331 bp, and a TATA box at −243 bp (Figs. 1B and supplemental S2; GenBank™ accession number DQ028784). The Ap-uch promoter region has a variant CRE, TGATGTCA, at −717 bp and a TATA box at −226 bp (Figs. 1C and supplemental S3; GenBank™ accession number DQ028785). Both promoter regions have putative TATA boxes further upstream and putative C/EBP-binding motifs (not shown).

The nearest TATA box for creb1 is at −190 bp from the putative TSS. Whether this TATA box is functional is not clear at this time, as most mammalian genes have TATA boxes at around −30 bp. However, lower eukaryotic genes can have TATA boxes farther away than −30 bp (42). For creb2 and Ap-uch, we could not successfully sequence the 5′ RACE product to identify accurately the TSS, possibly because of complex DNA secondary structure. Therefore, we numbered the promoter regions of these genes starting from the translation initiation site (+1 for A in ATG). However, based on the size of the 5′ RACE product (200 bp), the TSS for creb2 and Ap-uch probably lie within −200 bp of the translation start sites (data not shown). Therefore, the TATA boxes at −243 bp for creb2 and at −226 bp for Ap-uch may be appropriately placed to regulate transcription initiation. CREs and C/EBP-binding motifs can control the transcription of various genes regardless of their position in the promoter region (43). A comparison of the cloned sequences of the promoter regions of creb1, creb2, and Ap-uch using the BLAST2 program from the National Center for Biotechnology Information (NCBI; URL: www.ncbi.nlm.nih) showed that there is no significant similarity among them.

Development of Antibodies Specific for the Phosphorylated and Total Forms of CREB1 and CREB2—Polyclonal antibodies were raised against Aplysia CREB1 and CREB2 peptides. Two CREB1 antibodies were produced. The first term anti-tCREB1 was raised against a CREB1 peptide with the target residue for protein kinase A (PKA), Ser85, phosphorylated (see “Materials and Methods”). The second antibody, term anti-tCREB1, was raised against this peptide with unphosphorylated Ser85. Anti-tCREB1 recognized protein bands at 33 and 66 kDa in pleural ganglia extracts (Fig. 2A, arrowheads). The 33-kDa protein band was somewhat higher than the calculated molecular mass of CREB1, which is 29 kDa. To investigate this discrepancy, we cloned, expressed, and purified recombinant full-length Aplysia CREB1 protein (rec-CREB1). Western blot analysis revealed that rec-CREB1 also showed an apparent molecular mass of 33 kDa (Fig. 2A), suggesting that the 33-kDa protein band in the pleural ganglia extracts was the native CREB1 monomer. The minor shift in electrophoretic mobility might be due to post-translational modifications (e.g. phospho-

The effect of 5′ RACE...
The 66-kDa protein band is most likely a dimer of CREB1 that persisted despite the denaturing conditions of the SDS gel. CREB1 readily forms homo- and heterodimers in vivo (21, 19, 39), and some protein complexes, such as the SNAREs, have been reported not to dissolve in the presence of denaturing agents (44, 45).

To investigate the specificity of the anti-tCREB1 antibody, we performed preabsorption experiments. Preincubation of anti-tCREB1 with rec-CREB1 protein blocked the signal of both the 33- and the 66-kDa protein bands in pleural ganglia extracts (Fig. 2A), suggesting that this antibody reacts with CREB1 and supporting the hypothesis that the 66-kDa protein band is a CREB1 dimer. The anti-pCREB1 antibody also recognized two protein bands in pleural ganglia extracts (Fig. 2C, arrowheads). To test the specificity of anti-pCREB1, the effect of treatment of recombinant GST-CREB1 with protein phosphatase on anti-pCREB1 immunoreactivity was investigated (Fig. 2D). Recombinant proteins produced in bacteria can undergo extensive phosphorylation (46). By taking advantage of this finding, GST-CREB1 was analyzed by SDS-PAGE, transblotted to nitrocellulose membranes, and treated with λ-protein phosphatase (2000 units/ml; New England Biolabs, Beverly, MA). The membranes were then incubated with anti-pCREB1, which was visualized through ECL (Amersham Biosciences). Subsequently, membranes were stripped and reprobed with anti-tCREB1, followed by ECL. Treatment with phosphatase diminished immunoreactivity of anti-pCREB1 (Fig. 2D), suggesting that this antibody binds preferentially to phospho-CREB1. However, the immunoreactivity of anti-tCREB1 was unaffected by phosphatase treatment, suggesting that this antibody recognizes both nonphosphorylated and phosphorylated CREB1.

Two CREB2 antibodies were similarly produced. The first, termed anti-pCREB2, was raised against a CREB2 hybrid peptide comprised of sequences surrounding two putative mitogen-activated protein kinase (MAPK) target residues, Ser152 and Ser237, with both residues phosphorylated (see “Materials and Methods”). The second antibody, termed anti-tCREB2, was raised against this same peptide with unphosphorylated serines. Anti-tCREB2 recognized a single protein band with apparent molecular mass of 50 kDa in Western blot analyses of extract of pleural ganglia (Fig. 2B), similar to the reported molecular mass for CREB2 (21). The position of this band corresponded well with the position of purified rec-CREB2. Preabsorption of anti-tCREB2 with rec-CREB2 protein blocked the 50-kDa band in pleural ganglia extracts, providing further evidence that the antibody reacts with CREB2 (Fig. 2B). The anti-pCREB2 antibody also recognized a 50-kDa band (Fig. 2C). Treatment with phosphatase, in the same manner as in Fig. 2D, diminished the immunoreactivity of anti-pCREB2, whereas the immunoreactivity of anti-tCREB2 was unaffected (Fig. 2E). These results suggest that anti-pCREB2 binds to

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**Fig. 1.** Schematic organization of cis-regulatory elements in the promoter regions of *creb1*, *creb2*, and *Ap-uch*, deduced from sequences of the genomic clones isolated from *Aplysia*. A1, the position and sequence of putative CREs and a TATA box (the one closest to the TSS) identified in the promoter region of *creb1*. A2, schematic representation of the promoter region and 5’UTR of *creb1*. B1, same as in A1 but for *creb2*. B2, same as in A2 but for *creb2*. C1, same as in A1 but for *Ap-uch*. C2, same as in A2 but for *Ap-uch*. The gray boxes represent the translated regions. For *creb1*, the numbering of regulatory elements is with reference to the TSS (+1). For *creb2* and *Ap-uch*, the numbering of regulatory elements is with reference to the translation start site (ATG, +1).
CREB proteins (Fig. 3, A) may be regulated by CREB proteins. By using immunoprecipitation, we made a truncated fragment of the creb1 promoter region, extending from −736 bp to −44 bp. This fragment contained all the putative regulatory elements noted above except for the canonical CRE and one variant CRE. CREB1 binds to this fragment, but CREB2 did not (Fig. 3E). These observations suggest that CREB2 may bind to the CREs in the 5’UTR of creb1, whereas CREB1 binds to one or more of the variant CREs upstream from the TSS. CREB1 also binds to the CREs in the 5’UTR of creb1 (see below). Putative CREs located downstream of the TSS have also been identified for other genes (47).

CREB1 and CREB2 Each Bind to the Promoter Regions of creb1 and creb2, but Not Ap-uch, in Vivo—The binding of CREB1 and CREB2 to the creb1, creb2, and Ap-uch promoter regions in vivo was investigated using ChIP assays. Pleural-pedal ganglia from *Aplysia* were treated with five pulses of 5-HT or vehicle, followed by formaldehyde treatment to crosslink the DNA–protein complexes. After sonication, chromatin complexes were immunoprecipitated with our anti-tCREB1 and anti-pCREB2 antibodies. The immunoprecipitated DNA was recovered and amplified through PCR, using primers specific for creb1, creb2, and Ap-uch promoter regions containing CREs. To study the binding of CREB1 to the creb1 promoter region, the primer set fp1-rp1, which covers the canonical CRE site in the 5’UTR (see “Materials and Methods” for details; Fig. 1A), was used. Similar results (not shown) were obtained with the other two primer sets (fp2-rp2 and fp3-rp3), which cover other variant CREs in the creb1 promoter region. To study the binding of CREB2 to the creb1 promoter region, only the fp1-rp1 primer set was used, based on our finding that CREB2 did not bind to the truncated creb1 promoter region lacking the 5’UTR CREs (see in vitro promoter immunoprecipitation assays above; Fig. 3E).

Under basal conditions, both anti-tCREB1 and anti-pCREB2

phospho-CREB2 and that anti-tCREB2 recognizes both nonphosphorylated and phosphorylated CREB2.

CREB1 and CREB2 Proteins Each Bind to the Cloned Promoter Regions of creb1 and creb2, but not of Ap-uch, in Vitro—The presence of CREs in the promoter regions of creb1, creb2, and Ap-uch raised the intriguing possibility that these genes may be regulated by CREB proteins. By using immunoprecipitation assays, we investigated whether CREB1 and/or CREB2 could bind to the cloned promoter regions. We used the cloned fragment of the creb1 promoter region that extended from −736 bp to +313 bp, which included variant CREs as well as the canonical CRE at +141 bp (Fig. 1, A1 and A2). For creb2, the cloned fragment that was used extended from −682 bp to +37 bp (Fig. 1, B1 and B2) and contained a variant CRE only. For Ap-uch, the cloned fragment that was used extended from −937 bp to +33 bp (Fig. 1, C1 and C2) and contained a single variant CRE. The antibodies against the total CREB and phospho-CREB peptides were used together to maximally immunoprecipitate complexes of CREB1 and/or CREB2 with the cloned promoter regions. Following immunoprecipitation, interaction of CREB proteins with the promoter regions was detected via PCR of any immunoprecipitated DNA, using gene-specific primer and adaptor primer 2 (see “Materials and Methods”).

Separation of PCR products by electrophoresis indicated that CREB1 and CREB2 proteins each bound to both the creb1 and creb2 promoter regions (Fig. 3, A and B). However, neither CREB1 nor CREB2 bound to the Ap-uch promoter region (Fig. 3, A and B). This result suggests that the interaction of CREBs with the creb1 and creb2 promoter regions is not because of nonspecific DNA binding of CREB proteins. Similar results were obtained when total protein extract (200 μg of protein per 100 μl) from *Aplysia* CNS was used in place of recombinant CREB proteins (Fig. 3, C and D). To investigate further the specificity of this promoter immunoprecipitation assay, the requirement for protein-promoter complex formation was examined by omitting the recombinant protein. In the absence of CREB protein, the antibodies did not immunoprecipitate any DNA, as indicated by the lack of PCR product (data not shown).

The presence of CREs in the 5’UTR of creb1 raised the issue whether CREB proteins can bind to this region. To address this issue, we made a truncated fragment of the creb1 promoter region, extending from −736 bp to −44 bp. This fragment contained all the putative regulatory elements noted above except for the canonical CRE and one variant CRE. CREB1 bound to this fragment, but CREB2 did not (Fig. 3E). These observations suggest that CREB2 may bind to the CREs in the 5’UTR of creb1, whereas CREB1 binds to one or more of the variant CREs upstream from the TSS. CREB1 also binds to the CREs in the 5’UTR of creb1 (see below). Putative CREs located downstream of the TSS have also been identified for other genes (47).
CREB1 and CREB2 bind to the creb1 and creb2 promoter regions but not to the Ap-uch promoter region. Results were replicated at least once for each experiment. A, promoter immunoprecipitation assays using recombinant CREB1 protein. rec-CREB1 was incubated with the promoter region of creb1, creb2, or Ap-uch. Addition of phospho-CREB1 and total CREB1 antibodies followed by immunoprecipitation and PCR (see “Materials and Methods” for details) showed that rec-CREB1 specifically bound to the promoter regions of creb1 and creb2 but not to the Ap-uch promoter region. Addition of preimmune sera in lieu of CREB1 antibodies failed to pull down the CREB1-promoter region complexes. Note that for creb1, the gene-specific primer used in the PCR step following immunoprecipitation started at −44 bp (see supplemental Fig. S1); therefore, the resulting product was 693 bp and not 1049 bp. B, immunoprecipitation assays using recombinant CREB2 protein. rec-CREB2 was incubated with the promoter regions of creb1, creb2, or Ap-uch. Phospho-CREB1 and total CREB2 antibodies were added, followed by immunoprecipitation and PCR. rec-CREB2 specifically bound to the promoter regions of creb1 and creb2 but not to the Ap-uch promoter region. Preimmune sera failed to pull down the CREB2-promoter region complexes. C, same as A except CNS extract replaced rec-CREB1. D, same as B except CNS extract replaced rec-CREB2. E, promoter immunoprecipitation assay using rec-CREB1 or rec-CREB2 and the truncated promoter fragment of CREB1 (CREB1trunc, −736 to −44 bp) that does not include the canonical CRE. Protein-DNA complexes were immunoprecipitated with total and phospho-CREB1 antibodies or total and phospho-CREB2 antibodies, followed by PCR. rec-CREB1 co-immunoprecipitated with CREB1trunc, which indicates that CREB1 protein bound to at least one of the variant CREs in creb1. However, rec-CREB2 did not interact with CREB1trunc, indicating that CREB2 bound to the region downstream of the TSS that contains the canonical CRE and a variant CRE. Preimmune sera failed to pull down any complexes.

imunoprecipitated creb1 promoter fragments (Fig. 4, A and B), suggesting that both CREB1 and CREB2 bind to the creb1 promoter region. The specificity of the immunoprecipitation was demonstrated by replacing the primary antibodies with preimmune sera, in which case no chromatin complexes were immunoprecipitated (as indicated by lack of detectable PCR product; data not shown). Immediately following 5-HT treatment, the CREB1 antibody immunoprecipitated more creb1 promoter fragments compared with vehicle-treated controls, suggesting that more CREB1 was recruited to the creb1 promoter region. In contrast, the CREB2 antibody did not immunoprecipitate any creb1 promoter fragments following exposure to 5-HT, suggesting that 5-HT may down-regulate the affinity of CREB2 for the creb1 promoter region.

Binding of CREB1 and CREB2 to the creb2 promoter region was also observed in the ChIP assay under basal conditions (Fig. 4, A and B). These interactions did not appear to change after 5-HT treatment, because no detectable difference was observed in the amount of chromatin complexes immunoprecipitated by our antibodies following 5-HT treatment.

The Ap-uch promoter region has a variant CRE site (Fig. 1C). However, neither CREB1 nor CREB2 was observed to bind to this promoter region under basal conditions, confirming our in vitro results (see above). Moreover, no detectable binding was observed immediately after 5-HT treatment (Fig. 4, A and B). creb1 and Ap-uch Are Induced by Exposure to Spaced 5-HT Pulses, but creb2 Does Not Appear to Be Induced—To test the hypothesis that 5-HT protocols that induce LTF also regulate creb1, creb2, and Ap-uch, paired ganglia were exposed to five 5-min pulses of either 5-HT or vehicle (L15:ASW). This 5-HT treatment, which is commonly used to induce LTF (48, 49), was predicted to increase expression of creb1, creb2, and Ap-uch, given that all these genes have CREs in their promoter regions. Following treatment, ganglia were collected and frozen either immediately or 1, 2, or 5 h after the last 5-HT pulse. The tissue was processed for QRT-PCR analysis of mRNA levels. Five hours after the last pulse of 5-HT, creb1 and Ap-uch mRNA levels in pleural ganglia increased nearly 2-fold compared with untreated controls (creb1 at 5 h, 183.88 ± 21.98% (mean ± S.E.), t6 = −2.7, p < 0.05; Ap-uch at 5 h, 166.28 ± 28.1%, t6 = −3.148, p < 0.05) (Fig. 5). For creb1 and Ap-uch, there were no significant differences in mRNA levels between 5-HT-treated and control-treated samples at any other time point examined (immediately (imm), 1 and 2 h) (creb1, pimm = 0.2, p1 = 0.38, p2 = 0.29; Ap-uch, pimm = 0.32, p1 = 0.63, p2 = 0.1; n = 7 for all
5-HT Treatment Enhances Recruitment of CREB1, but Abolishes the Binding of CREB2, to the creb1 Promoter Region—Our results from the ChIP assays suggested that 5-HT promotes the recruitment of CREB1 to the creb1 promoter region (Fig. 4) and induces creb1 transcription (Fig. 5). Therefore, it is plausible that CREB1 can induce its own transcription. We also found that 5-HT treatment decreased binding of CREB2 to the creb1 promoter region (Fig. 4). Relief of transcriptional repression because of removal of CREB2 may play an important role in the induction of creb1 in response to 5-HT.

Although CREB1 and CREB2 bind to the creb2 promoter region under basal conditions, 5-HT exposure did not appear to modulate this binding (Fig. 4). Levels of creb2 mRNA also do not appear to be significantly affected by 5-HT (Fig. 5). These results suggest that the induction of creb2 is not regulated by CREB1 or CREB2 in response to 5-HT. Nonetheless, it is plausible that creb2 induction is modulated at later time points (beyond 5 h).

Although Ap-uch has a CRE in its promoter region and is known to be up-regulated in response to 5-HT, no detectable binding of CREB1 and CREB2 was observed in vivo, either under basal conditions or immediately after the end of 5-HT exposure (Figs. 3 and 4). One possibility is that binding takes place at a time point later than we assayed, although the lack of basal binding would argue against this. Another possibility is that the 5-HT-induced regulation of Ap-uch may involve transcription factors other than CREB1 and CREB2. C/EBP is a potential candidate regulating Ap-uch expression in response to 5-HT. Indeed, the promoter region of Ap-uch contains consensus binding motifs for this transcription factor. However, basal levels of C/EBP are very low and therefore unlikely to contribute significantly to 5-HT-induced gene regulation (7). Following 5-HT treatment, levels of C/EBP mRNA and protein are increased (7), but the newly synthesized C/EBP probably does not regulate the 5-HT-induced up-regulation of Ap-uch because this up-regulation does not depend on new protein synthesis (8).

CRE Sequences May Generate Feedback Loops That Regulate Formation of LTF and LTM—The presence of CRE motifs in the creb1 promoter region suggests a positive feedback mech-

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**DISCUSSION**

Phosphorylation of transcription factors of the CREB family, and induction of genes with CREs in their promoter regions, are critical events for the formation of LTM in organisms as diverse as Aplysia (21, 2), Drosophila (50, 51), and mammals (3, 6, 16, 26, 52–56). Vertebrate genes that have CREs in their promoter regions, and that are up-regulated during LTM formation, include cebp (9), c-fos (57), BDNF (58, 59), and zif268 (60). Each of these genes is important for the induction of some forms of vertebrate LTM or long term synaptic potentiation (BDNF [61]; zif268 [62]; cebp [10]; c-fos [63]). We therefore examined whether CREs are present in Aplysia creb1, creb2, and Ap-uch, three genes necessary for LTF and LTM.

**Putative CRE Sequences in the Promoter Regions of creb1, creb2, and Ap-uch**—We sequenced the promoter regions of creb1, creb2, and Ap-uch (Fig. 1 and supplemental Figs. S1–S3). A comparison of these sequences revealed no significant homology among them, suggesting that the promoter regions are gene-specific. The promoter region of creb1 has several variant CREs, one canonical CRE, and several putative TATA boxes and C/EBP-binding motifs. The promoter region of creb2 has one variant CRE as well as several putative TATA boxes and C/EBP-binding motifs; and the promoter region of Ap-uch has one variant CRE as well as numerous putative TATA boxes and C/EBP-binding motifs.

Promoter immunoprecipitation assays using our specific antibodies to CREB1 and CREB2 peptides revealed that recombinant Aplysia CREB1 and CREB2 each bound to the cloned creb1 promoter region (Fig. 3). In vivo binding of CREB1 and CREB2 to the creb1 promoter region was confirmed by ChIP assays (Fig. 4). These results suggest that the CREs in this promoter region can function in vivo. The finding that CREB1 bound to the truncated promoter region of creb1, which contained only variant CREs, suggests that these variant CREs may be functional in vivo (Fig. 3E). Most interestingly, CREB2 did not bind to the region of the creb1 promoter that contains the variant CREs, but it did bind to a region downstream of the TSS that contains a canonical CRE (at +141 bp) and a variant CRE (at +265 bp) (Fig. 3A). Binding of CREB2 to this region is likely to modulate the dynamics of induction of creb1. CREB2 bound to the CREs in the 5' UTR could repress creb1 expression by hindering the progression of RNA polymerase II through this region. Activation of kinases to phosphorylate CREB2 may be required to promote dissociation of CREB2 from these CREs, thus facilitating the activation of creb1 by phosphorylated CREB1 protein.

The promoter region of human creb also has three variant CREs, spread over nearly 800 bp downstream (25), and that of mouse creb has one variant CRE (64). Neither region has a canonical CRE. Unlike human and mouse creb genes, which have no TATA box, Aplysia creb1 has more than one TATA box, as well as many putative C/EBP-binding motifs (Fig. 1A). We have not examined the functionality of these motifs.

The promoter immunoprecipitation assay also showed that both CREB1 and CREB2 bound to the promoter region of creb2. In vivo binding of CREB1 and CREB2 was confirmed by ChIP assays (Fig. 4), suggesting that the creb2 CRE at −331 bp may be functional. Similarly, human creb2 (ATF4) has a variant CRE (but no TATA box).²

Promoter immunoprecipitation assays did not show binding of rec-CREB1 or rec-CREB2 protein to the region in the Ap-uch promoter that contains a variant CRE (Fig. 3, A and B). Endogenous CREB proteins also did not bind to the promoter region of Ap-uch when CNS lysate was used instead of the recombinant proteins (Fig. 3, C and D). Implications of this observation are discussed below.

² K. Barlow, GenBank accession number AL022312, unpublished observations.
anism that may facilitate the formation of LTM (Fig. 6). Binding of phospho-CREB1 to the creb1 CREs, and consequent creb1 induction, would tend to increase levels of both total CREB1 protein and, by mass action, phospho-CREB1. This increase would further induce creb1, creating a positive feedback loop. Consistent with this hypothesis, we found that creb1 mRNA levels in pleural ganglia were increased nearly 2-fold 5 h after 5-HT treatment (Fig. 5). A similar increase in creb1 expression was observed previously when the whole animal was exposed to 5-HT, and the total CNS was analyzed for creb1 mRNA and protein (19). However, that study did not determine whether the 5-HT exposure induced LTF or LTM and whether creb1 mRNA was increased in the sensory neuron-containing pleural ganglia specifically, which are important sites of plasticity in Aplysia.

Positive feedback loops in biochemical or genetic pathways create the potential for multistability (i.e., multiple stable states of biochemical concentrations and reaction rates) (65, 66). Therefore, the putative positive feedback loop involving creb1 could be important in allowing a stimulus protocol of limited duration (such as repeated 5-HT applications) to induce a state of enhanced gene expression that lasts for many hours. Such an increase could be important for inducing genes involved in the consolidation of late phases of LTF and LTM.

Another positive feedback loop that has also been suggested to be important for LTF involves Ap-uch. Exposure to 5-HT results in increased levels of Ap-uch (8), which promotes degradation of the regulatory subunit of PKA via the ubiquitin-proteasome pathway. This action of Ap-uch results in increased levels of free, autonomously active catalytic subunits (30). This increased PKA activity could, in turn, maintain phosphorylation of CREB1 and further induction of Ap-uch. Prolonged CREB1 phosphorylation might be necessary for expression of late phases of LTF. This putative feedback loop has been noted previously (15, 32). However, our promoter immunoprecipitation and ChIP assays did not detect any binding of CREB1 or CREB2 to the Ap-uch promoter region. This result would suggest that Ap-uch induction is regulated by yet unidentified transcription factors, and therefore the putative CREB-Ap-uch feedback loop may not be operative in vivo.

Although variant CRE and C/EBP-binding motifs are present in the promoter region of creb2, this gene was not induced within 5 h after 5-HT treatment (Fig. 5). However, if creb2 was induced at a time later than we measured, accumulation of CREB2 could repress expression of creb1, cebp, and possibly other targets important for LTF via interaction with their CREs. Such repression could help terminate any positive feedback loops relying on induction of creb1 or cebp. Furthermore, the variant CREs in the creb1 promoter region are likely to have lower affinities for CREB1 than would a canonical CRE. For mammalian creb, Meyer et al. (27) speculated that a low affinity of variant CREs for CREB helps to limit the strength and duration of positive feedback. It is plausible that this constraint also limits the induction of Aplysia creb1. The induction of creb2, in turn, may be limited by a negative feedback loop (Fig. 6). In this loop, CREB2 accumulation would result in increased binding of CREB2 to the variant CRE in the creb2 promoter region, repressing creb2 expression.

Because the mouse and human creb promoters have variant CREs, a positive feedback loop in which CREB induces its own gene might facilitate synaptic strengthening and LTM consolidation in mammals. Further analysis of feedback loops involved in the regulation of key molecules, such as transcription factors, is likely to be important for understanding the induction and consolidation of LTM.

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