Decreased Expression of Myotonin–Protein Kinase Messenger RNA and Protein in Adult Form of Myotonic Dystrophy

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The myotonic dystrophy mutation has recently been identified; however, the molecular mechanism of the disease is still unknown. The sequence of the myotonin–protein kinase gene was determined, and messenger RNA spliced forms were identified in various tissues. Antisera were developed for analytical studies. Quantitative reverse transcription–polymerase chain reaction and radioimmunoassay were used to demonstrate that decreased levels of the messenger RNA and protein expression are associated with the adult form of myotonic dystrophy.

Myotonic muscular dystrophy (DM) is an autosomal dominant inherited human genetic disease with multisystemic effects, including myotonia and weakness, cardiac conduction defects, cataracts, and male baldness (*1*). Myotonic dystrophy has the genetic feature of anticipation, that is, increasing disease severity from generation to generation (*2*). The genetic basis of DM is now known to include mutational expansion of a repetitive trinucleotide sequence $(CTG)_n$ located in the 3′ untranslated portion of mRNA from a gene designated as myotonin–protein kinase (Mt-PK), because the gene product has extensive homology to protein kinase catalytic domains (*3–5*).

Two other diseases caused by triplet repeat expansions are Kennedy syndrome and Fragile X syndrome. In Kennedy syndrome, the repeat expansion occurs in the androgen receptor protein coding region and destroys the function of the protein (*6*). In Fragile X syndrome, the expanded triplet repeat is located at the 5′ untranslated region of the Fragile X mental retardation–1 (FMR-1) mRNA (*7*). The expansion of the repeat also causes the methylation of the 5′ region of the FMR-1 gene and consequently prevents its transcription. The effect of the DM-associated expansion on Mt-PK mRNA transcription, processing, and stability has not been previously delineated, and the pathophysiology of this complex disease is still not understood.

All previous information regarding the Mt-PK gene was based on its cDNA sequence. In order to understand the structure of the gene and to investigate any possible involvement of mRNA processing in the disease mechanism, we determined the sequence of the Mt-PK gene. The genomic sequence of Mt-PK (11,612 bp) was obtained from average size 1.5-kbp random subclones (*8*) of a cosmid (MDY1) (*3*) containing the Mt-PK gene. Features of the Mt-PK gene, which contains a minimum of 14 exons, are shown in Fig. 1. We identified the positions of exons by comparing the genomic sequences of Mt-PK with the sequences of cDNA clones and from the sequences of alternative splice forms of the mRNA identified by reverse transcription polymerase chain reaction (RTPCR).

Polymerase chain reaction primer pairs (*9*) that span the Mt-PK cDNA were used to scan for alternatively spliced forms of mRNA. We detected multiple alternatively spliced forms that differed primarily in the 5′ and 3′ regions of the mRNA (Fig. 1). The protein kinase sequence homology (located in exons 2 to 6) was preserved in all splice forms. Four alternatively spliced forms that differ in the 5′ region were identified. Form I was previously reported from a brain cDNA library (*3*). Its sequence is colinear with the nuclear gene sequence from nucleotide (nt) 1 to 826. Consensus donor and acceptor sequences are present at all splice junctions. The same splice acceptor site (nt 735) is used in forms II, III, and IV. Form IV, which is detected in heart and skeletal muscle, is complex in that it uses the splice form of III in addition to a splice donor position at nt 108 and acceptor at nt 222. Thus, in heart and muscle a plethora of alternative 5′ splice options are used. The first in-frame AUG at nt 545 only appears in form I. In forms II, III, and IV the first in-frame AUG occurs at nt 842. Forms I, II, and III were identified in all the tissues examined to date (brain, heart, liver, muscle, kidney, skin, ovary, thyroid, lung, and spleen) by RTPCR of mRNA.

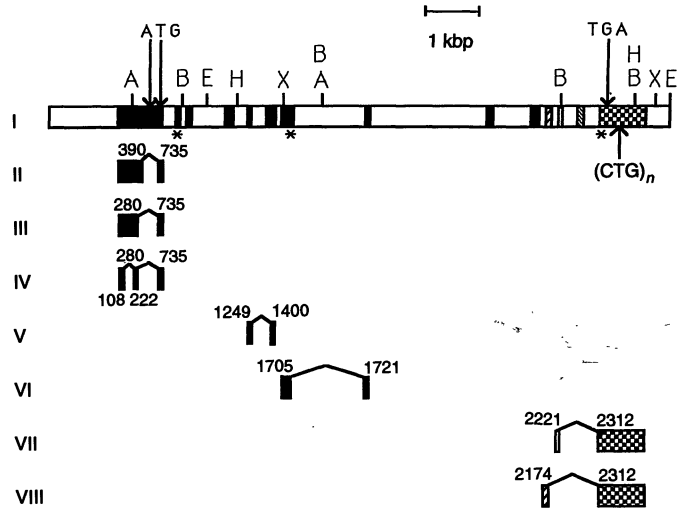

Fig. 1. Gene structure of Mt-PK and various spliced forms of Mt-PK mRNA. Filled and patterned boxes indicate the locations of exons. In the spliced forms II through VIII, only the exons involved in the changes are shown. Spliced forms II, III, and IV are alternatively spliced variants of form I. In each case the sequence of the RTPCR product was determined by DNA sequencing from a plasmid clone of the amplified product. Each spliced form was amplified from mRNA by means of primers selected to avoid amplification products from genomic DNA. The numbers indicating the positions of splice donor and acceptor sites are based on the sequence of cDNA clone (*3*). All products were assured to be reverse transcriptase–dependent. A 5′ extended cDNA clone isolated from a human fetal brain cDNA library was colinear up to nt 697 of genomic sequence, and its 3′ end terminates in the third exons. Restriction enzymes are as follows: A, Xba I; B, Bam HI; E, Eco RI; H, Hind III; and X, Xho I. The locations of synthetic peptides used to raise antisera are indicated as asterisks under the gene structure.

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Forms I and III are the major forms in all tissues except in fetal and newborn muscle, where form IV is the major spliced form.

In form V, alternative splicing results in a reduction of 50 amino acids from exon 6, whereas in form VI there is a deletion of five amino acids from exon 7. Three alternative forms of Mt-PK that differ in the 3′ region were also identified by RTPCR. Form VII uses the same splice donor site as form I at nt 2221 and an in-frame splice acceptor at nt 2312, the acceptor for coding exon 14. The effect of such splicing is to delete exon 13 (30 amino acids). Form VIII lacks both exons 12 and 13. This generates a termination codon immediately after the splice acceptor, which removes a predicted COOH-terminal transmembrane domain of the protein. We have identified independent cDNA clones from a cardiac cDNA library that confirm the three 3′ splice forms (type I, VII, and VIII). All these alternative 3′ splice forms were detected in various tissues examined to date (including brain, heart, muscle, skin, and liver) by RTPCR.

In order to understand Mt-PK protein expression, we developed and characterized antibodies directed against both synthetic peptides and purified Mt-PK protein expressed in *Escherichia coli*. We raised antibodies against synthetic peptide immunogens, using selected amino acid sequences of Mt-PK as designated in Fig. 1. Furthermore, we developed a specific and high-affinity antibody (10033), using as antigen a truncated Mt-PK protein produced with the prokaryotic expression vector pRSET (Invitrogen). This construct incorporated the spliced form VIII 3′ termination codon and lacked the Mt-PK putative membrane-spanning domain. In addition, the metal binding domain of the pRSET vector was fused in-frame at the NH_2-terminus of Mt-PK with the AUG of nt 842. This recombinant vector provided a chimeric peptide that was subsequently purified by nickel affinity chromatography. The prokaryotic expressed protein (molecular size, 55,000 daltons) exhibits a higher apparent mass (65,000 daltons) on SDS–polyacrylamide gel electrophoresis (PAGE), a common phenomenon previously observed for polyhistidine (metal binding site) fusion proteins (Fig. 2). After purification of the expressed protein on a nickel column, we carried out NH_2-terminal amino acid sequencing to verify the identity of the purified product. The sequence obtained corresponded to the nickel binding epitope, followed by four residues of the Mt-PK sequence initiating with methionine encoded at nt 842 (M-K-Q-T) (M, Met; K, Lys; Q, Gln; T, Thr), confirming the authenticity of the chimeric Mt-PK protein. The fusion protein was then used to validate the specificity of antibodies raised against peptides and the prokaryotically expressed antigen, as shown in Fig. 2. We used these antisera to detect endogenous Mt-PK protein in selected tissues by protein immunoblots. These antisera detected a protein of molecular size 55,000 daltons expressed in skeletal muscle, heart, and to a lesser extent in brain. These antisera were also used in histochemical studies. The Mt-PK protein detected by the antisera in sections of human muscle is restricted to muscle fibers and appears to be uniformly distributed within the cells (*10*). This finding is consistent with results of subcellular fractionation studies, which indicate that Mt-PK behaves as soluble antigen under a variety of disruption conditions (*10*). Antibody binding to the tissue antigen can be competed with by the addition of saturating amounts of the purified fusion protein to the primary antibody incubation, demonstrating the specific binding of the antibody to the endogenous Mt-PK protein.

In order to understand better the effect of CTG amplification on Mt-PK expression, we used specific analytical assays for Mt-PK mRNA and protein expression levels in normal and myotonic adult muscle. In order to determine whether the CTG repeat expansion altered the level of the mRNA in muscle tissues, we developed a quantitative RTPCR assay (*11*). The mRNA for the transferrin receptor (TFR), a stably expressed gene, was used as an internal quantitation standard. In adults with myotonic dystrophy the mRNA levels were found to be uniformly low (0.42 ± 0.163, mean ± SD) (Fig. 3). In contrast, Mt-PK mRNA levels in normal adult muscle show considerable variation (1.24 ± 0.75, mean ± SD). The average level of Mt-PK mRNA in normal adults is thus significantly greater than that of DM patients (*t* test, $P < 0.01$). However, the mRNA level in some normal adults is in the same range as in DM patients. The expla-

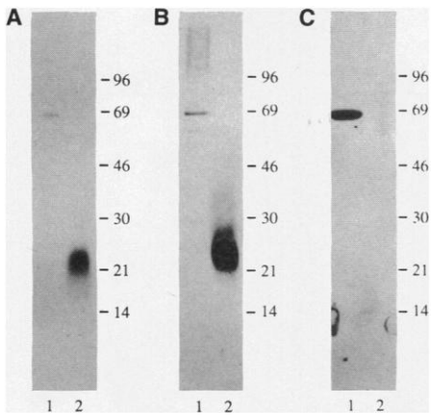

Fig. 2. Purified fusion protein (lane 1; 50 ng) and synthetic peptide 2 (lane 2; 100 ng) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antisera 254 (**B**) or 10033 (**C**). The left panel (**A**) shows the purified fusion protein (3 μg) and synthetic peptide (10 μg) stained with Coomassie blue after SDS-PAGE. Molecular size standards (in kilodaltons) are as indicated.

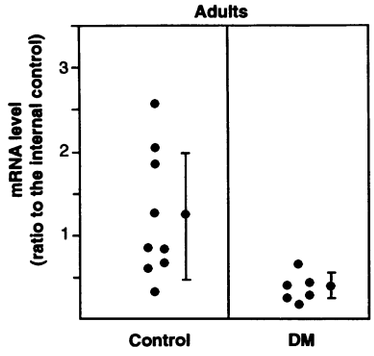

Fig. 3. Quantitation of mRNA levels for normal and DM adult patients by RTPCR. Only the results from muscle are shown. Each dot represents the average of nine determinations for each RNA sample. For each RNA sample, three independent reverse transcription experiments were carried out. Three independent PCR reactions were then performed on each of the reverse transcription products. The mean value for these nine experiments was then taken as the representative number for the particular RNA sample. The numbers shown here are the ratio of the Mt-PK RTPCR product to the RTPCR product of the human transferrin receptor. Values (mean ± SD) are as follows: 1.24 ± 0.75 for normal and 0.42 ± 0.163 for DM adult muscle ($P < 0.01$).

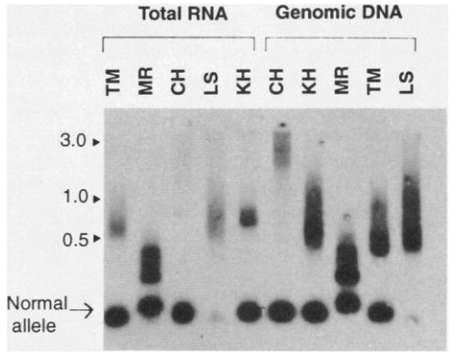

Fig. 4. Southern (DNA) blot for PCR products from genomic DNA and total RNA. Genomic DNA and total RNA were isolated from lymphoblastoid cell lines. RNA was treated with deoxyribonuclease I before RTPCR reaction. PCR primers flanking the CTG repeat were used, and PCR products were loaded onto a 2% agarose gel. Oligonucleotide of 21 residues in length was used as a probe for the Southern analysis. The number of repeats of the mutant alleles in each sample was as follows: 68 (MR), 120 (TM), 160 (LS), 205 (KH), and 800 (CH). Molecular sizes are indicated at left (in kilobase pairs).

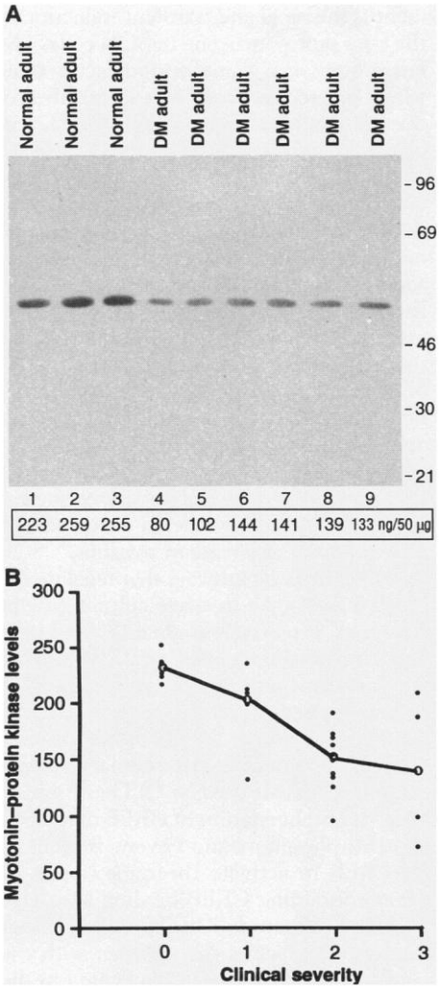

Fig. 5. Evaluation of Mt-PK protein expression in adult muscle. Skeletal muscle biopsies from normal individuals and DM patients were processed for protein immunoblots. Exactly 50 μg of total protein were loaded per lane. The numbers below the lanes show the amount of Mt-PK detected in the samples by RIA. Tissue extracts (1 to 10 μg) were incubated with ^{125}I-labeled Mt-PK fusion protein (7 μCi per microgram of protein) and antisera to Mt-PK (antiserum 254, at a final dilution of 1:1000) for 2 hours at room temperature. The immune complex was precipitated with protein A–agarose for 45 min. The precipitate was washed three times with buffer containing tris-HCl (pH 8.3), 1% NP-40, 3% bovine serum albumin, and 0.5 M NaCl. Values are the means from triplicate determinations and were expressed as nanograms per 50 μg of tissue protein. (**A**) The number of repeats of the mutant alleles for DM patients are (from left to right): 1300, 975, unclear, 975 and 1200, 440 and 1200, and 98 and 810. Levels of Mt-PK protein (mean ± SEM; $n = 3$) are as follows: Lane 1, 223 ± 20; lane 2, 259 ± 19; lane 3, 255 ± 25; lane 4, 80 ± 16; lane 5, 102 ± 18; lane 6, 144 ± 22; lane 7, 141 ± 24; lane 8, 139 ± 24; and lane 9, 133 ± 21. (**B**) Correlation between Mt-PK protein levels and disease severity of adult DM patients. Values (mean ± SD) for each group are given as follows: 0 (control), 239 ± 11 ($n = 7$); 1 (mild), 207 ± 36 ($n = 6$); 2 (moderate), 156 ± 27 ($n = 10$); and 3 (severe), 149 ± 66 ($n = 4$).

nation for these unusual cases is presently unknown. Moreover, these "normal" muscle samples are tissues collected from nonmyotonic adults with other diseases. Whether other disease states may affect the Mt-PK mRNA level is not clear at present. Furthermore, a greater sample size may help clarify this concern. Using primer pairs that permit a distinction between the two different alleles (primers flanking the CTG repeats), we found that the lower levels of mRNA detected by RTPCR in the patients was due to lack of coequal transcription of wild-type and mutant mRNA. We were able to detect the normal mRNA level from wild-type alleles, reduced mRNA levels from alleles with 100 to 200 CTG repeats, and an extremely low level of mRNA from mutant alleles with 800 repeats (Fig. 4). Genomic DNAs with the same number of repeats were used as the control for evaluation of PCR efficiency. Coequal expression of mRNA from two normal alleles was consistently observed in the normal control individuals. These data indicate that the decreased levels of mRNA for the adult myotonic patients is a result of defective synthesis or processing, or decreased stability of the mutant allele mRNA, and is inversely related to repeat length. The level of mRNA from alleles in the 100 to 200 repeat range decreases to <20% of that for normal alleles, whereas the mRNA level for alleles with >700 repeats is only ~1% or less of that for normal alleles. Thus, the dominant phenotype would appear to be a dosage-dependent event with the expression of mutant Mt-PK allele reduced to a lower level.

In order to confirm the results of these mRNA expression studies, we directly examined the level of Mt-PK protein by immunoblot and radioimmunoassay. We examined a total of 20 DM patients and 7 normal individuals. Decreased MtPK protein levels were found in 18 out of 20 adult patients. Representative results for three normal and six DM patients are shown in Fig. 5A. The decreased amount of Mt-PK protein is correlated, in general, with the severity of disease (Fig. 5B). Thus, by two independent methods, Mt-PK protein expression was shown to be decreased in adult DM muscle as compared with normal adult muscle. Furthermore, the quantitative measurement of Mt-PK protein appears to have a greater correlation to disease severity than to repeat size measurement. We speculate that this is due to the high degree of repeat mosaicism found in muscle, which makes it difficult to estimate the repeat number in the functional allele. Protein measurements appear to resolve this ambiguity.

The results of our studies on the level of Mt-PK expression in normal and DM adults suggest that there is modulation of the steady-state level of both Mt-PK mRNA and protein. In the case of adult DM, there are reduced levels of both mRNA and protein. We carried out postmortem examination of similar parameters on four congenital myotonic infants. Not surprisingly, the highly expanded mutant Mt-PK mRNA was not detectable. We found that Mt-PK levels in these infants and in a matched set of infants who died as a result of other forms of myotonia were markedly lower than in adults (approximately 10% or less of the values from normal adult muscle). Because only mRNA from the normal allele was detected at control levels for adult DM patients, we propose that the dominant character of the disease is likely to be dosage-dependent as a result of an abnormality in synthesis or processing of the transcript from the abnormal allele. Such an abnormality has been reported for the *doublesex* mutant of *Drosophila*, where a repeat sequence normally binds proteins involved in mRNA processing; in the mutant, however, mRNA processing is defective (*12*).

Adults with myotonic disease have reduced levels of mRNA and protein for myotonin protein kinase. In view of the central role of protein kinases in cell regulation, especially in developmental and physiological modulation of different channel proteins (*13–15*), we suggest that the reduction in myotonin kinase leads to the severity of the disease by disruption of signal transduction and amplification pathways. The results of this work provide new insight into the complex features of myotonic muscular dystrophy and suggest that the autosomal dominant character of the disease may be due to an Mt-PK dosage deficiency. This finding suggests that means of elevating Mt-PK level or activity should be explored for therapeutic intervention in adult patients.

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8. We obtained the sequence of the Mt-PK genomic region by using a shotgun strategy. The DNA fragment was sonicated and size-selected on an agarose gel. DNA fragments of the appropriate size were recovered from the gel by electroelution into a dialysis membrane, and the DNA ends were repaired by T4 DNA polymerase, Klenow fragment, and mung bean nuclease. DNA fragments were ligated into an M13 vector. DNA of white colonies from the X-gal color selection was isolated and sequenced with dideoxynucleotide termination reactions. The genomic sequence of Mt-PK was submitted to GenBank (accession number L00727). Sequencing reactions were prepared on a Biomek 1000 Automated Labstation according

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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16. All tissues were obtained with agreement for research usage accompanied by a written consent form. C.T.C. is an Investigator with the Howard Hughes Medical Institute. Supported by U.S. Department of Energy grant CD-FG05-88ER60692 (C.T.C.) and a Veterans Administration merit review award (T.A.). Automated sequencing was supported by NIH grant P30-HG00210 and the W. M. Keck Center for Computation Biology at Baylor College of Medicine and Rice University. A.P. is a Muscular Dystrophy fellow. We thank D. Armstrong and H. Vogel for supplying valuable tissues, L. Chen for technical assistance, R. Cook for amino acid sequencing and peptide synthesis, and R. Roberts for research support.

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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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