Circadian Expression of Transcription Factor Fra-2 in the Rat Pineal Gland*

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Physiological changes in Fos-like immunoreactivity in the rat pineal gland are shown here to be due primarily to changes in a 42kDa Fos-related antigen (Fra). Studies are presented that indicate this 42kDa Fra is Fra-2, a poorly understood member of the Fos family of transcription factors. Both Fra-2 mRNA and protein are absent during the day and increase robustly at night on a circadian basis; organ culture studies indicate that regulation is mediated by an adrenergic → cyclic AMP mechanism. AP-1 binding activity changes in parallel to changes in the level of Fra-2 protein.

Circadian1 changes in mammalian pineal function are driven by norepinephrine (NE),2 which is released from terminals in the gland in response to signals originating in the master circadian oscillator in the suprachiasmatic nucleus (SCN; Refs. 1 and 2). NE acts through [Ca2+]i and cyclic AMP to control pineal function (3–5), including the circadian output of melatonin. Large nocturnal increases in melatonin production are mediated by an adrenergic mechanism. AP-1 binding activity changes in parallel to changes in cyclic AMP (5, 6). The molecular basis of signal transduction involved in regulating the increase in NAT activity is not well understood, but available data indicate that the increase in NAT activity and the abundance of other pineal proteins requires de novo transcription (1, 5, 7–10).

An interesting series of investigations on transcriptional products of pineal gland tissues have pointed to the involvement of an unidentified member of the Fos family of transcription factors in pineal signal transduction (11, 12). These and subsequent reports indicated drug was added. After treatment, glands were removed, placed in a 1.5-mL microtube in solid CO2, or placed in organ culture. Cultured glands were incubated (37 °C; 95% O2, 5% CO2) in chemically defined culture medium (BGJb, (Life Technologies, Inc.; 2 glands/well, 200 µl/well) in a 24-well plate (Costar, Cambridge, MA) for 48 h prior to treatment. The medium was changed every 24 h. After 48 h, each gland was placed in fresh medium for 1 h and then the indicated drug was added. After treatment, glands were removed, placed in a 1.5-mL microtube in solid CO2, and stored (−70 °C).

Gel Mobility Shift Assay—Glands that had been quick-frozen on dry ice (2-microtube) were homogenized in a 30-µl sample of ice-cold buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 0.42 mM NaCl, 0.2 mM EDTA, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM sodium fluoride, 5 µM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 25% glycerol) with a tight-fitting pestle (20 strokes). Homogenates were centrifuged (15,000 × g, 10 s) vortexed, refrozen on dry ice for 5 min, incubated in ice water for 15 min, and centrifuged (15,000 × g, 15 min, 4 °C). This procedure extracted 40–50 µg of soluble protein/pineal gland (Bio-Rad protein assay). Gel mobility shift assays were done using a 32P-radiolabeled, double-stranded digoxigenin-labeled probe containing a consensus (nucleotide) AP-1 binding element (5′-CGCTGATGAGTCAGGGCAAG-3′) and 3 µl of total extract (27).

Western Blot and V8 Peptide Analysis—Extracts were electrophoresed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and proteins were transferred to Immobilon-P (Millipore, Bedford, MA). The following anti-peptide sera were used: anti-c-Fos Ser25–31 serum (F2P1; Ref. 26; generously provided by M. Iadarola, NIH); anti-c-Fos Ser9–16, a rabbit affinity-purified polyclonal anti-human c-Fos (SC-52, Santa Cruz Biotechnology, Santa Cruz, CA); and anti-c-Fos Ser128–135 mouse monoclonal anti-human c-Fos (Ab-1 PC06, Oncogene Science, Uniondale, NY). Signals were visualized by enhanced chemiluminescence (Amersham Corp.). Densitometric analysis of the Western blot data was performed with the Quantity ONE™ software package (Protein Database Inc., Huntington Station, NY) on a Sun Sparc workstation (Sun Microsystems, Inc. Mountain View, CA). Blots were stripped for reprobing by incubation in 62.5 mM Tris, pH 6.7, 2% SDS, 10 mM 2-mercaptoethanol (70 °C, 60 min).

For Cleveland Blot (V8), immunoprecipitated Fra-42/46 and in vitro translated Fra-2 were first resolved by SDS-PAGE (12% gel). Fra-containing bands were excised and placed in the wells of a second 18% PAGE gel. Proteins were treated with V8 protease (0.5–5000 ng/ml, 30 min) in the stacking gel (29). The partial proteolytic products were detected using anti-c-Fos Ser25–31 antisera and visualized by enhanced chemiluminescence.

Metabolic Labeling—For metabolic labeling studies, pineal glands

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1 The term circadian refers to biological rhythms with a period of circa 24 h.

2 The abbreviations used are: NE, norepinephrine; SCN, suprachiasmatic nucleus; NAT, N-acetyltransferase activity; RIPA, radiimmunoprecipitation buffer; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; DMEM, Dulbecco’s modified Eagle’s medium; β-AR, β-adrenergic receptor; bp, base pair(s); kb, kilobase(s); ISO, isoproterenol; CRE, cyclic AMP-responsive element; SCGX, superior cervical ganglionectomy.

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were incubated for 48 h and then transferred to phosphate-free B27. Thirty minutes later \(^{32}P\)-labeled NaO
\(_4\)PO
\(_4\) (2 mCi/ml, specific activity 1 Ci/mmol) was added together with the indicated drugs. Glands were incubated (3–5 h; 37°C), rinsed and quick-frozen on solid CO
\(_2\). For immunoprecipitation, groups of 6 frozen glands were sonicated for 10 s in 180 \(\mu\)l of RIPA (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% Deoxycholate, and 0.1% SDS; the resulting homogenate was incubated (10 min, 4°C) and centrifuged (15,000 \(\times\) g, 15 min, 4°C). The clear supernatants were incubated with 4 \(\mu\)l of anti-c-Fos(29–153) antiserum. Immune complexes were collected on RIPA-washed Protein A-agarose beads (Life Technologies, Inc.) and washed seven times with ice-cold RIPA; to prepare the samples for analysis, a 100- \(\mu\)l volume of Laemmli buffer (30) was added to the Protein A-agarose beads and the mixture was boiled for 10 min. Proteins were resolved by SDS-PAGE (12% gel), transferred to blotting membrane, and detected by autoradiography and anti-c-Fos(29–153) immunoblotting.

RNA Analysis—Northern blots (Hyb-d to Amersham) containing 15 \(\mu\)g of total pineal RNA were prepared. The probes were: a randomly labeled PCR product generated by amplifying fra-2 sequences between nucleotides 459 and 981 (Fig. 3A); a full-length cDNA fragment derived from a RAT-c-fos construct, pG-fos (generously provided by M. Iadarola), as a derivative of SP65-c-fos (31); and a full-length cDNA fragment derived from a human glycerolaldehyde-3-phosphate dehydrogenase vector (Clontech, Palo Alto, CA). After autoradiography, the blots were stripped by two brief washes in 0.1% SDS in distilled water (97°C).

For the RNase protection assays, pCRII ARF2 (antisense version of pCRIIFRA) was linearized with Accl or BamHI to generate partial or full riboprobes. T7 polymerase (Promega, Madison, WI) was used for transcription in the presence of [\(^{32}\)P]UTP (Amersham; 500 Ci/mmol) and products were gel-purified. After hybridization (18 h, 45°C) with 10 \(\mu\)g of total pineal RNA, RNA:RNA hybrids were digested for 1 h with a mixture of RNase A and T1 (Boehringer Mannheim). Protected fragments were resolved using a denaturing PAGE (6% urea gel) and visualized using a PhosphorImager.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)–Total RNA was extracted using Trizol (Life Technologies, Inc.). One \(\mu\)g of total RNA was used for cDNA synthesis (avian myeloblastosis virus reverse transcriptase, Promega) in the presence of random hexamers (Pharmacia). The primers used were APEP (5'-ATGGCCAGCAGAGTGGG-3') and QPEP (5'-CAGAGCCAGCAGAGTGGG-3'); the incubation was for 30 cycles (denaturation at 94°C, annealing at 60°C, and extension at 72°C, each for 1 min). RT-PCR products were resolved using a Novex gel system (Novex, San Diego, CA). The 980-bp product was subcloned into pCRII and pCR3 vectors (Invitrogen, San Diego, CA). Total RNA was used for cDNA synthesis (avian myeloblastosis virus reverse transcriptase, Promega) in the presence of random hexamers (Pharmacia). The primers used were APEP (5'-ATGGCCAGCAGAGTGGG-3') and QPEP (5'-CAGAGCCAGCAGAGTGGG-3'); the incubation was for 30 cycles (denaturation at 94°C, annealing at 60°C, and extension at 72°C, each for 1 min). RT-PCR products were resolved using a Novex gel system (Novex, San Diego, CA). The 980-bp product was subcloned into pCRII and pCR3 vectors (Invitrogen, San Diego, CA) following the manufacturer's protocol and sequenced using the dideoxy-chain termination method (Sequenase, United States Biochemical Corp.). Clones with inserts in the sense orientation relative to the T7 or cytomegalovirus promoters were isolated and referred to as pCRII/FR A and pCR3/FRA, respectively. For transfection studies DNA was purified using the Nucleobond AX kit (Macherey-Nagel-Duren, Switzerland).

Transfections and in Vitro Translaction—Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, Inc.). Transfections were done routinely using 2.5 \(\times\) 10
\(^5\) Cos-7 cells that had been plated 18 h earlier with 1 ml of serum-free DMEM containing 1 \(\mu\)g of DNA previously mixed with 9 \(\mu\)l of Lipofectamine reagent (Life Technologies, Inc.) in serum-free DMEM. For Western blot analysis of cellular proteins, cells were harvested 40 h post-transfection. For the cell-free synthesis of Fra-2 protein, transcription and translation reactions were done with T7 polymerase and unsupplemented rabbit reticulocyte lysis/extracts (Promega), respectively, following the manufacturer's recommendations.

Statistics—Data are presented as the mean of the results of analysis of at least two pools (2–4 glands/pool). When quantitation is presented, experiments were carried out at least four times with essentially identical results. Qualitative data are representative of at least two independent experiments, the results of which were fundamentally similar.

RESULTS

A 42–46-kDa Fra Is under Circadian Regulation in the Rat Pineal Gland—This study was started by monitoring the daily pattern of expression of pineal Fra's through a light/dark cycle of 14:10 h (Fig. 1A). Several c-Fos antisera were used. One, termed anti-c-Fos(29–153), is similar to the serum used in cytochemical reports (11); it detects a peptide sequence found in c-Fos and related proteins (28). This reagent revealed a dramatic nocturnal increase in a 42–46-kDa Fra (Fra(42–46) and in a 35-kDa Fra (Fra(35), but failed to detect c-Fos in this tissue. \(^4\) These observations confirm cytochemical reports of a nocturnal increase in Fos-like immunoreactivity in the pineal gland (11), and indicate this is associated with an increase in Fra(42–46).

The second serum used, anti-c-Fos(29–36), detects c-Fos at lower levels and in a selective manner. It failed to detect Fra(42–46), but detected c-Fos (62 kDa) at relatively constant levels at all times of the day; this was confirmed using a mouse monoclonal anti-c-Fos(29–153). \(^4\) These observations indicate that changes in c-Fos do not seem to contribute to the increase in Fos-like immunoreactivity seen in cytochemical studies (11). To obtain an independent indication of the pineal function, NAT enzyme activity was also measured in the same protein extracts. NAT activity increased at night in parallel with the increase in Fra(42–46) (Fig. 1A, bottom panel).

To determine if the increase in Fra(42–46) was circadian in nature, as is true of the increase in NAT activity, both were measured during a 24-h period after animals had been housed for 4 days in constant darkness (dark/dark) (Fig. 1B). Fra(42–46) increased during the subjective night, the period that corresponds to the night period of the preceding 14:10 lighting cycle. The increase in Fra(42–46) under these circumstances is clear evidence that the day/night rhythm in Fra(42–46) is truly circadian.

It is likely that the circadian rhythm in Fra(42–46) is generated by the SCN oscillator, because the increase in Fra(42–46) was blocked by two treatments which block SCN→pineal stimulation, i.e. continuous light at night (L→L) or superior cervical ganglionection (SCGX, Fig. 1C). The lack of an increase in Fra(42–46) did not reflect tissue refractoriness because it was possible to increase Fra(42–46) in SCGX or in L→L animals by treatment with the \(\beta\)-adrenergic receptor (\(\beta\)-AR) agonist isoproterenol (ISO; Fig. 1C and data not shown).

The robust circadian increase in Fra(42–46) parallels an equally robust increase in AP-1 DNA binding activity (Fig. 1B; Ref. 32).

Fra(42–46) Expression Is Regulated by a \(\beta\)-AR→Cyclic AMP Mechanism—Organ culture was used to study first and second messenger regulation of Fra(42–46) expression (Fig. 2). NE treatment increased Fra(42–46) expression (Fig. 2A). The largest increase was seen with 0.1 \(\mu\)M NE; distinctly smaller responses were obtained at both higher and lower concentrations. The peak response occurred at 6 h, which is similar to the time course of the responses of other Fra proteins (33–36) and of NAT activity (Fig. 2A, bottom panels).

The 62-kDa c-Fos signal was not increased by adrenergic treatment (data not shown). The 55-kDa c-Fos signal, a less modified form of c-Fos (37), was detected by anti-c-Fos(29–153) (Fig. 2A) and anti-c-Fos(29–153) (data not presented); this signal did not increase in response to treatment with 0.1 \(\mu\)M NE. However, a higher concentration of NE (1.0 and 10 \(\mu\)M) caused a 20-fold increase in 55-kDa c-Fos, with a peak at 3 h; this confirms previous findings in organ culture (14).

It is of interest to compare in vivo changes in expression of 42–46-Fra and c-Fos (Fig. 1A) with those seen in vitro due to NE treatment (Fig. 2A). This indicates that the physiological profile seems to be reproduced by treatment with 0.1 \(\mu\)M NE.

\(^3\) The terms 42/46 Fra and Fra-2 refer to the same 42/46-kDa proteins. However, the term 42/46-Fra is used in the text until data presented establish that this protein is Fra-2.

\(^{4}\) R. Balcer and D. C. Klein, unpublished results.
Glands were removed and rapidly frozen on solid CO₂ following a 2.5-h treatment with phenylephrine (10 mM) in vivo. Higher doses produce large changes in c-Fos protein not seen by treating glands with a cyclic GMP analog (dibutyryl cyclic GMP, 1 mM), while [Ca²⁺]i in whole pineal extracts were subjected to immunoblotting with anti-c-Fos129–153 antiserum (top panel). The second antiserum was horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin. The blot was stripped and re-exposed to an anti-c-Fos129–153 antiserum (second from top panel), and processed as above. Each lane represents a pool of two glands. The third panel is a densitometric quantitation of Western blot data (solid circles, Fra-42/46; open circles, c-Fos). The fourth panel presents NAT activity in the extracts (46). Analysis of whole glands from animals housed in a 14:10 light/dark lighting cycle (L:D) or in constant darkness for 4 days (D:D). Pineal extracts were analyzed (from top to bottom) for anti-c-Fos129–153 and anti-c-Fos3–16 immunoreactivities, AP-1 DNA binding activity (free probe is not shown), and NAT activity (open circles). C, effect of continuous light exposure at night and SCGX on Fra-42/46. Groups of 4 pineal glands were harvested at the indicated time of day, and whole pineal protein extracts were analyzed by anti-c-Fos129–153 immunoblotting. Animals were exposed to darkness (L→D) or to light (L→L) at night. Animals that had been SCGXed 2 weeks earlier were housed in a 14:10 light/dark lighting cycle (L:D). One group of SCGX animals was injected with ISO (1 mg/kg) 4 h before death. In these and following figures, the migration of prestained protein molecular size standards (Amersham Corp.) is indicated on the right of each blot (kDa). Further technical details are given under “Experimental Procedures.”

Higher doses produce large changes in c-Fos protein not seen in vivo.

NE is known to act on the pineal gland through α- and β-adrenergic receptors (ARs). To determine whether Fra-42/46 was regulated by NE by one or the other receptor we used relatively selective AR agonists. Treatment with the selective β-AR agonist isoproterenol increased 42/46-kDa Fra; however, the α-AR agonist phenylephrine was without effect (Fig. 2B). Evidence of β-adrenoreceptor regulation points to cyclic AMP as the second messenger controlling Fra-42/46. This was supported by the finding that treatment with dibutylryl cyclic AMP (Bt₂cAMP; Fig. 2B) or forskolin (10 μM; data not shown) increased Fra-42/46. The response to Bt₂cAMP was not inhibited by propranolol, providing further evidence that propranolol blocked effects of NE through selective interaction with the β-AR.

The involvement of other second messengers was examined by treating glands with a cyclic GMP analog (dibutylryl cyclic GMP, 1 mM), with [Ca²⁺]i, elevating agents (10 μM ionomycin or 20 μM A23187) or with an activator of protein kinase C (100 μM phorbol 12-myristate 13-acetate). None elevated Fra-42/46 (data not shown). Accordingly, it appears that cyclic AMP is the essential second messenger mediating the effects of NE on Fra-42/46.

Examination of the Western blots in Figs. 1 and 2 indicates that during the period Fra-42/46 abundance is increased, there is a subtle upward shift in the dominant component of this broad signal, as seen with other Fra systems (34–38). This appears to be associated with phosphorylation because (a) treatment with alkaline phosphatase shifted the 46-kDa component of the signal toward the 42-kDa component (Fig. 2C), and (b) the 46-kDa and, to a lesser extent, the 42-kDa component became labeled in glands incubated with Bt₂cAMP and 32P-labeled NaO₃PO₄ (Fig. 2D). Phosphorylation of c-Fos was also observed (Fig. 2D, NE).

Evidence that Fra-2 mRNA Encodes Fra-42/46—We found that NE stimulation of Fra-42/46 was blocked by either actinomycin D or cycloheximide, demonstrating that these responses rely upon de novo transcription and translation (Fig. 2B, third panel). This pointed to the possibility that we could determine the identity of Fra-42/46 by identifying an mRNA that exhibited similar dramatic circadian changes. An RT-PCR approach was used with cDNA from control and NE treated glands; the primers selected bracket the full coding sequence of c-fos, fra-1, fra-2, and fra-3.
and fra-2 (15, 18, 19). A robust ~980-bp RT-PCR product was generated from template derived from NE-treated but not control glands (Fig. 3A). Sequence analysis indicated this product encodes a protein which contains the peptide sequence recognized by anti-c-Fos129–153 and is 98%, 97%, and 93% homologous to mouse, human, and chick Fra-2, respectively (19–21), indicating that it is the rat homolog of Fra-2 (Fig. 3B). All known Fra-2 transcripts encode ~35–36-kDa proteins with multiple candidate phosphorylation sites.

A prominent ~6.7-kb Fra-2 transcript was strongly expressed during a short period of a light/dark cycle starting ~1.5 h after lights off (Fig. 3C, upper panel, lane 4) and was induced in glands from rats injected with the β-AR agonist ISO but not in glands from animals injected with the α-AR agonist PE (Fig. 3C, upper panel, lanes 12 and 13). The Fra-2 transcript was increased during subjective night in animals maintained in constant darkness for 4 days (data not shown). Changes in the abundance of this transcript were paralleled by changes in the strength of a ~2.5-kb signal. The above observations are consistent with the hypothesis that the ~6.7-kb fra-2 transcript encodes either Fra-35 or Fra 42/46, or both.

c-fos mRNA was also analyzed (Fig. 3C, middle panel). The 2.4-kb c-fos transcript was expressed throughout the same 24-h sampling period, and was 2–3-fold higher ~1.5 h after the onset of darkness, as previously reported (13, 14). We also were able to confirm that the strength of this signal increased transiently ~10-fold following a 1-h treatment with 10 μM NE (not shown; Ref. 14). In contrast, treatment with a lower concentration of NE (0.1 μM) in vitro or with the β-AR agonist ISO (1 mg/kg) in vivo did not alter the 2.4-kb c-fos signal, whereas the 2.5- and 6.7-kb Fra-2 signals did increase (Fig. 3C). This establishes that the 2.5-kb Fra-2 signal (Fig. 3C) does not reflect nonspecific detection of c-fos; and, that fra-2 and c-fos expression are regulated independently.

We confirmed that fra-2 mRNA is regulated in the pineal gland using an RNase protection assay (Fig. 4A). Fully protected fragments were present in samples from Bt2cAMP-treated glands, but not control glands. This essentially eliminates the possibility that the regulated Northern blot signal...
Fig. 3. Isolation of a candidate cDNA encoding Fra-35 and/or Fra-42/46. A, RT-PCR was performed on cDNA from control (CO) or stimulated (NE, 0.1 μM; 3 h) pineal glands; primers were APEP and QPEP (see “Experimental Procedures”). The NE-induced 980-bp PCR product is indicated by an arrow. DNA size standards (in kilobase pairs; BioVentures, Murfreesboro, TN) were run on the right lane (Std). B, the 980-bp PCR fragment was subcloned and the resulting plasmid pCR1/FRA was sequenced. The deduced amino acid sequence of the NE-induced transcript is aligned with the mouse, human, and chicken Fra-2 proteins. Only deviations from the consensus amino acid sequence are shown. The underlined residues contain the epitope(s) recognized by the anti-c-Fos(129–153) antibody. Leucine residues involved in leucine-zipper interactions are indicated by the diamond symbols. C, a Northern blot of total pineal RNA was prepared from groups of 2 animals sacrificed at times (h): 12, 18, 19:30, 21, 22:30, 24, 1:30, 3, 4:30, and 6 (lanes 1–10) and from animals injected (subcutaneously) with saline (lane 11), ISO (1 mg/kg, lane 12), or PE (1 mg/kg, lane 13) at 16 h (2 h before death). The blot was probed with a radiolabeled fra-2-derived PCR fragment (nucleotides 495–981). The horizontal bar above the lanes represents the lighting cycle (dark phase 1900 to 0500 hours). The same blot was stripped and sequentially exposed to radiolabeled rat c-fos and human glyceraldehyde-3-phosphate dehydrogenase probes (G3PDH, middle and bottom panels in C). The position of RNA molecular weight standards (Life Technologies, Inc.) is indicated on the right of the blot. Further technical details are given under “Experimental Procedures.”

(Fig. 3C) reflected artifactual detection due to nonspecific or partial hybridization and confirms that fra-2 mRNA is increased by cyclic AMP. Similarly, fully protected fragments were detected in samples from glands obtained at night, but not in day glands (data not shown). The detection of a single RNase protected product suggests that the −6.7- and −2.5-kb transcripts (Fig. 3C, upper frame) are the result of differential posttranscriptional processing of a unique fra-2 mRNA species.

Fra-2 Expression Studies—The above studies provide strong evidence that Fra-42/46 is Fra-2. To test this conclusion, we expressed rat Fra-2 and compared it to Fra-42/46. Cos7 cells transfected with rat Fra-2 (Fig. 4B) synthesized immunoreactive −35- and −42/46-kDa proteins (Fig. 4B, lane 3). Similarly, a rabbit reticulocyte lysate incubated with fra-2 mRNA also synthesized a prominent immunoreactive 46-kDa protein (Fig. 4B, lane 5). V8 protease cleavage of this product and of native Fra-42/46 (Fig. 4B, lane 2) generated nearly identical patterns of immunoreactive cleavage products (Fig. 4C). Similar results were obtained when Fra-35 was analyzed in this fashion (data not shown). The results of these expression studies are consistent with the conclusion that fra-2 encodes multiple Fra-2 isoforms in the pineal gland, reflecting differential processing.

DISCUSSION

A Working Hypothesis for Fra-2 Regulation—These studies indicate that fra-2 gene expression increases in the pinealocyte
FIG. 4. Evidence that Fra-2 encodes Fra-42/46. A, an RNase protection assay was performed using 10 μg of total RNA extracted from Bt2cAMP-stimulated pineal glands. Full and partial length riboprobes were generated form pCRII/FRA2 (equivalent to pCRII/FRA1, except for the orientation of the insert) by linearization with BamHI or Accl respectively, and T7 RNA polymerase-driven transcription (right panel). Fra-2 coding region is represented by a shaded bar. Control hybridization reactions contained 10 μg of transfer (t) RNA. The migration of the undigested probes is presented in the last two lanes on the right. Radiolabeled HindIII-digested λ-DNA fragments (New England Biolabs, Beverly, MA) were run in parallel to estimate the molecular weight of the RNase digestion products (Std). B, in vivo and in vitro expression of the 980-bp Fra-2 open reading frame monitored by anti-c-Fos antiserum (Life Technologies, Inc.; lane 3). Mock-transfected cells were run for comparison. C, V8 protease partial digestion patterns of endogenous and recombinant Fra proteins. Further technical details are given under “Experimental Procedures.”

at night and that this is due to SCN stimulation of release of NE in the pineal gland. It appears that NE acts transsynaptically through β-ARs to increase cyclic AMP and initiate fra-2 gene expression. Cyclic AMP could act via phosphorylation of the CRE-binding protein (39), if the rat fra-2 promoter contains a cyclic AMP-responsive-like element (CRE), as does the mouse gene (21, 40).

Expression of the fra-2 gene results in accumulation of 6.7- and 2.5-kb fra-2 mRNA transcripts. The predicted size of the protein encoded by fra-2 is ~35 kDa. We suspect that a 35-kDa protein is a precursor that is subject to extensive posttranslational modification, including phosphorylation; such modification results in the appearance of multiple isoforms. This is in agreement with the findings that expression of human fra-2 in 208F cells generates a ~43-kDa protein (41), that there is similar posttranslational processing of c-Fos (31, 37), mouse Fra-1, and mouse Fra-2 (38), and with our transfection studies. Based on this, the terms Fra-2a, Fra-2d, and Fra-2e will be used in the future to identify Fra-2 isoforms with masses of 35, 42, and 46 kDa, respectively. It is possible that each isoform has distinctly different functional characteristics, based on current thinking about the functional importance of transcription factor phosphorylation and the observation that phosphorylation of Fra-2 appears to correlate with enhanced AP-1 DNA binding activity in another system (38).

It is interesting that changes in Fra-2 isoforms appear to be associated with changes in AP-1 DNA binding activity (Fig. 1B). The possibility that the increase in Fra-2 in turn increases AP-1 binding provides an interesting hypothetical mechanism through which Fra-2 could influence gene expression, i.e., through interaction with AP-1 or related (e.g., CRE-like) regulatory elements.

The precise function of Fra-2 in transcription has not been established in other systems. Whereas FosB, v-fos, and c-Fos are strong transcriptional activators, Fra-1, Fra-2, and FosB2 are not. This difference resides in functionally different transcription domains (41). In the case of Fra-2, efficient AP-1 binding combined with weak activation points to a possible negative role. This negative role is consistent with the report that Fra-2 can suppress c-jun un (42). However, c-jun D is not suppressed by Fra-2. Accordingly, it is possible that Fra-2 isoforms may have a complex role in the pineal gland because c-jun un and jun D display remarkably different patterns of nocturnal expression in this tissue (14).

If a Fra-2 isoform plays a negative role in the pineal gland, one can envision a dual negative feedback system controlling transcription under the regulation of cyclic AMP. Cyclic AMP-dependent induction of the inducible cyclic AMP early repressor (10) and of Fra-2 could coordinately inhibit expression of both CRE- and AP-1-regulated genes. Alternatively, it is possible that cross-talk exists between these systems, because of the similarity of the consensus binding sites (AP-1 site, TGAGTCA; CRE site, TGACGTTCA; Refs. 43–45).

Fra-2 and c-Fos in the Pineal Gland—Our findings and published reports indicate that different mechanisms regulate the expression of Fra-2 and c-Fos in the pineal gland. First, in vivo studies indicate that there is a robust rhythm in Fra-2 protein in animals in a light/dark 14:10 lighting cycle but not in c-Fos, which appears not to exhibit a circadian pattern of expression. Second, in vitro studies indicate that Fra-2 is induced by a concentration of NE (0.1 μM), which does not alter c-Fos. Third, the maximal adrenergic elevation of c-Fos protein is short-lived as compared to the maximal stimulation of Fra-2. Fourth, Fra-2 appears to be regulated by a β-AR → cyclic AMP mechanism, whereas c-Fos is regulated by an α-AR → Ca2⁺ mechanism (14).

Direction of Future Studies—These studies indicate that regulation of Fra-2 and NAT are remarkably similar. This supports the conclusion that expression of genes encoding these proteins is controlled by the same mechanism. This hypothetical proposal will be productively evaluated once the promoters for both genes are identified. The similar time courses of the increases in Fra-2 and NAT raise the issue of whether a causative relationship exists. It seems unlikely that Fra-2 is required for the increase in NAT activity, because both increase in a parallel manner. A more attractive hypothesis is that Fra-2 plays a negative role in the increase in NAT activity. This is consistent with evidence indicating that Fra-2 is a potential negative regulator of transcription (41, 42). However, the role of Fra-2 in regulating expression of the NAT gene will remain highly speculative until this issue is investigated using the NAT promoter.

The discovery that Fra-2 expression can be physiologically regulated by a transmitter → cyclic AMP mechanism has broad implications because of the central role cyclic AMP plays in cellular regulation. Accordingly, the results of studies of Fra-2 isoforms in the pineal gland should be helpful in understanding their physiological function in neural and non-neural tissues.

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