The precise mechanisms by which beneficial responses to acute stress are transformed into long-term pathological effects of chronic stress are largely unknown. Western blot analyses revealed that members of the AP1 transcription factor family are differentially regulated by single and repeated stress in the rat adrenal medulla, suggesting distinct roles in establishing stress-induced patterns of gene expression in this tissue. The induction of c-fos was transient, whereas marked elevation of long-lasting Fos-related antigens, including Fra2, was observed after repeated immobilization. We investigated DNA protein interactions at the AP1-like promoter elements of two stress-responsive genes, tyrosine hydroxylase and dopamine β-hydroxylase. Increased DNA-binding activity was displayed in adrenomedullary extract from repeatedly stressed rats, which was predominantly composed of c-Jun- and Fra2-containing dimers. The induction of Fra2 and increased AP1-like binding activity was reflected in sustained transcriptional activation of tyrosine hydroxylase and dopamine β-hydroxylase genes after repeated episodes of stress. The functional link between Fra2 and regulation of tyrosine hydroxylase and dopamine β-hydroxylase transcription was confirmed in PC12 cells coexpressing this factor and the corresponding promoter–reporter gene constructs. These studies emphasize the potential importance of stress-evoked increases in the expression of the Fra2 gene for in vivo adaptations of the adrenal catecholamine producing system.

Key words: AP1-like factors; Fra2; repeated stress; adrenal; transcription; tyrosine hydroxylase; dopamine β-hydroxylase

Immunolocalization of immediate-early gene (IEG) products and especially c-fos is widely used as a functional marker to identify activated neurons and extended circuitries that are responsive to a variety of extracellular challenges (Morgan and Curran, 1995; Chaudhuri, 1997; Kovacs, 1998). Different types of acute stress increase the expression of c-fos and other IEGs in specific brain regions and in peripheral tissues (Palkovits et al., 1995; Senba and Ueyama, 1997; Del et al., 1998). The induction of c-fos expression is typically transient. In contrast, chronic glucocorticoid administration as well as repeated stress attenuate the subsequent acute immobilization stress-induced expression of c-fos, Fos B, Jun B, and Egr1 (Umemoto et al., 1997). These results suggest that other members of the AP1 family may mediate the effects of chronic stimuli. Examples of sustained elevations in the expression of other members of c-fos family in the brain provoked by chronic stimuli have been reported. These Fos-like proteins, termed chronic Fos-related antigens (Fras), are induced in a region-specific manner in response to several chronic perturbations, including: electroconvulsive seizures, administration of cocaine, morphone, and nicotine, and psychotropic drug treatments and lesions (Hope et al., 1994b; Nye et al., 1995; Penev and Hong, 1995; Bing et al., 1996; Doucet et al., 1996; Moratalla et al., 1996; Nye and Nestler, 1996; Pich et al., 1997; Atkins et al., 1999). Once induced, the chronic Fras, shown to be the truncated splice variant of the FosB gene–Delta FosB isoforms, accumulate for relatively long periods because of their high stability. They are attractive candidates for mediating some of the longer-lasting transcriptional changes involved in the regulation of the brain function (Chen et al., 1997; Nestler et al., 1999).

Many stress-responsive genes, including those encoding catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), contain AP1-like binding sites in their promoters, and AP1 factors could be important in establishing stress-induced patterns of gene expression in different tissues. We have shown previously that the transcriptional activation of TH and DBH in adrenal medulla of animals exposed to immobilization stress is correlated with increased binding of AP1 factors to oligonucleotides comprising their AP1-like promoter elements (Nankova et al., 1993, 1994, 1999). Nevertheless, our studies with c-fos knock-out mice revealed that c-fos is not essential for the induction of adrenal TH and DBH genes by repeated stress (Serova et al., 1998).

How the expression of different members of the extended Fos family of proteins is regulated by acute and chronic challenges in the adrenal medulla is important in understanding their role in mediating the stress response. The present study was undertaken to examine the expression patterns of c-fos- and Fos-related proteins in animals exposed to different stress paradigms. We found differential induction of c-fos and other Fos-related antigens, particularly Fra2, by single and repeated immobilization stress. The functional differences between the AP1 complexes induced by single and repeated stress can be attributed to differences in the composition of AP1 DNA-binding complexes formed in response to each challenge.

**MATERIALS AND METHODS**

**Immobilizations.** Male, murine pathogen-free, Sprague Dawley rats (280–320 gm) were obtained from Taconic (Germantown, NY). Immobilization stress was performed as previously described (McMahon et al., 1992; Nankova et al., 1996). For repeated stress, the animals were immobilized for 2 hr daily on consecutive days. Control groups were either not exposed to stress (absolute controls) or handled briefly on each day for the same number of days as the animals exposed to repeated immobilization stress (handled controls). The animals were killed by decapitation. The adrenal
medullae from 10 to 12 animals per experimental group were dissected and frozen immediately on liquid nitrogen.

**Electroconvulsive seizure.** Electroconvulsive shock (ECS) treatments were performed, as described earlier (Hope et al., 1994a), Briefly, male Sprague Dawley rats (150–250 gm) were connected to earclip electrodes. For chronic studies, animals received a single ECS (45 mA; 0.3 sec) daily for 10 d, and they were then killed 18 hr after the last treatment. The acute and control animals were also connected to the electrodes, but no current was applied. After pretreatment, the filters were treated with the acute effects of handling stress (Campeau et al., 1991; Sharp et al., 1991). On day 11, acute animals were given a single ECS and were killed 2 hr later. Dorsal parietal and prefrontal cortex were obtained and immediately frozen on liquid nitrogen.

**Nuclear extracts.** Nuclear protein extracts were prepared (Dignam et al., 1983) from frozen punches of adrenal medullae of control rats and rats exposed to repeated immobilization stress as described earlier (Nankova et al., 1993; Sabban et al., 1996).

**Electrophoretic mobility shift assays.** DNA–protein-binding reactions were performed at room temperature for 30 min in binding buffer (12% glycerol, 12 mM HEPES, 8 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, and 60 mM KCl). The reaction included: 1 µg of BSA, 1 µg of poly(dI-dC), 0.5 ng of 32P-end labeled double-stranded oligonucleotide (~30,000 cpm of radioactive probe) and nuclear extract (3–5 µg of protein) in a final volume of 15 µL. Competition was performed by adding a 100-fold molar excess of nonradioactive oligonucleotides to the reaction before the nuclear extract. For antibody supershift experiments, 1–2 µg of antisense were preincubated for 30 min at room temperature with the nuclear extract before adding the binding oligonucleotide directly or after heating for 1 hr at 90°C. The DNA–protein complexes were resolved on 6% polyacrylamide gels in 0.25× Tris-borate buffer. Subsequently, the gels were fixed, vacuum-dried, and autoradiographed using intensifying screens and Kodak XAR-5 film. Each binding assay was repeated at least three times, with extracts from independent experiments.

The following antibodies were used in this study: c-fos (epitope corresponding to N-terminal amino acids 3–16), Fra1 (epitope corresponding to N-terminal amino acids 3–22 of human Fra1, specific for Fra1), Fra2 (rabbit polyclonal antibody raised against a peptide corresponding to amino acids 3–22 mapping at the amino terminus of Fra2 of human origin), and FosB (epitope corresponding to amino acids 102–117, specific for FosB and delta FosB). All were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The cAMP response element modulator (CREM) family-specific antibody (recognizes all known CREM isoforms) was from Upstate Biotechnology (Lake Placid, NY), and N-terminal c-fos antibody (against amino acids 1–111, broadly reactive with c-fos, FosB, Fra1, and Fra2) was from Oncogene Science. Jun-family antibody was a gift from Dr. R. Bravo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). The antibody recognizing all family of the Fos-related antigens was a gift from Dr. M. Iadarola (National Institutes of Health, Bethesda, MD). The oligonucleotides were synthesized by BioServe Biotechnologies and are shown on the corresponding figures.

**Western blot analysis.** Total protein extracts from adrenal medullae of individual animals (four to eight rats from each experimental group) were prepared in total adrenomedullary homogenates from the corresponding control groups (Ac, absolute controls; Hc, handled controls) and from rats exposed to single or repeated (7×) immobilization stress by consecutively performing Northern analyses with the signal intensities determined using the Bradford assay. For immunoblots, equal amounts of protein were assayed for reporter gene activity. CAT activity was calculated by subtracting background signals hybridized to the null vector, pBluescript.

**Transfection.** Transient transfection experiments were performed with CMV-Fra2 expression vector or control parental vector (provided by Dr. S. H. Yuspa, National Institute of Arthritis and Musculoskeletal and Skin Disease, Bethesda, MD) and promoter constructs driving the expression of a reporter gene. TH promoter construct contained (~272+27) bp of the rat TH promoter fused to chloramphenicol acetyltransferase (CAT) reporter gene (a gift from Dr. D. Chikaraishi, Duke University Medical School, Durham, NC). DBH promoter construct had the first 248 bp of the rat DBH promoter (McMahon and Sabban, 1992) subcloned into a pGL3-Basic vector (Promega, Madison, WI) expressing firefly luciferase. PC12 cells were transfected using the Superfect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Renilla luciferase reporter pRL-TK (Promega) was also added to control for transfection efficiency. After 12, 24, and 48 hr the cells were harvested, and protein concentrations in total cell lysates were determined. Aliquots with equal amounts of protein were assayed for reporter gene activity. CAT activity was determined by a liquid scintillation method with [3H] chloramphenicol (Seed and Sheen, 1988). Comparisons between controls and Fra2-expressing cells at the indicated times after transfection, in triplicate, were from the same experiment, with the same promoter construct plasmid.
DNA. Sequential quantitation of both firefly and Renilla luciferase activities was performed using the Dual-Luciferase Reporter Assay System from Promega.

**RESULTS**

**Induction of Fos-related antigens in rat adrenal medulla by immobilization stress**

To characterize the AP1 family members induced in response to single and repeated immobilization stress in adrenal medulla, we performed consecutive Western blot analyses (Fig. 1). Although barely detectable in controls as previously reported (Sabban et al., 1995; lanes 3 and 4), c-fos protein was markedly induced with 2 hr single immobilization (lane 2). In contrast, after the last of seven consecutive episodes of stress, the levels of c-fos-immunoreactive protein were similar to controls. The same filters were probed using an antisera that recognizes all known Fos-like proteins (Young et al., 1991). Under basal conditions, low levels of Fras were observed. Immobilization stress resulted in robust induction of Fos-like proteins with $M_r$ in the range of 30–46 kDa in adrenal medulla (compare lanes 1 and 2 to lane 3). Similar patterns of Fra induction were observed in adrenal medulla after exposure to single (lane 2) and several times repeated (lane 1) immobilization. However, in contrast to c-fos, the levels after repeated stress were even higher than with a single exposure to immobilization.

Induction of Fos-related antigen-immunoreactive proteins in many different brain regions has been studied after different repeated or chronic treatments. FosB gene products were identified as transcription factors critical for long-term neural and behavioral plasticity to repeated stimuli (for review, see Nestler et al., 1999). To test if FosB or FosB-derived proteins are induced in adrenal medulla by immobilization stress, we used an N terminus-specific FosB antibody, which is able to recognize all FosB gene products. No significant changes were observed in the levels of immunoreactive Fos B ($M_r$, 46 kDa) or delta FosB proteins (with $M_r$ values of 37, 35, 33, 29, and 28 kDa) with stress (data not shown). Furthermore, the Fos-like proteins induced in adrenal medulla by immobilization stress differed in $M_r$ from the late Fras induced in frontal cortex after chronic electroconvulsive seizures (Fig. 1B).

The same filters were also probed with Fra2-specific antibody. Fra2 was detected as one of the Fra family members markedly induced by stress. Increased Fra2 immunoreactivity was evident after a single stress (Fig. 1A, lane 2) and especially after repeated stress (Fig. 1A, lane 1).

**Increased transcription of stress-responsive gene with functional AP1 promoter element**

Next, we examined whether the induction of c-fos and Fos-related antigens by immobilization stress is associated with increased transcription of stress-responsive genes, which harbor functional AP1 motifs in their promoters. Figure 2A shows a schematic diagram of the TH promoter and the sequence of the AP1 enhancer region. Nuclear run-on assays were used to evaluate changes in the relative rate of transcription of TH in response to single and repeated immobilization stress. Increased elongation of TH primary transcripts was found in nuclei from immobilized animals. Immobilization of the animals for 2 hr once (1×) or on each of 7 consecutive days (7×) resulted in a threefold to fourfold increase in TH gene transcription (Fig. 2B), as compared to the corresponding group of unstressed animals (C) or animals handled daily (Hc). In contrast, the relative rate of transcription of a stress-unresponsive gene, CPH, was unaffected by immobilization (Fig. 2B, insert).

**Variation and composition of AP1-binding activity after single and repeated stress**

To investigate which members of the Fos family of proteins may be involved in activation of TH gene transcription in adrenal medulla after exposure to stress, we performed gel shift assays. The oligonucleotide, spanning the AP1-like promoter element of the TH promoter (Fig. 2A), was labeled and incubated with nuclear extracts isolated from adrenal medulla of controls and animals exposed to stress. Two major DNA–protein complexes of low mobility were formed (Fig. 2C, lane 1). The formation of complex I (or complexes with similar mobility) was greatly induced by single immobilization stress, whereas complex II was barely changed (lane 2), as reported previously (Nankova et al., 1994). Both complexes were efficiently competed by addition of excess unlabeled AP1 oligonucleotide (lane 3), but not affected by addition of the same amount of nonrelated oligonucleotide (lane 4). The DNA–protein complex induced by single immobilization (complex I) contains...
mainly c-fos and c-Jun as revealed by disruption or supershift of the band after addition of specific antibodies (lanes 5 and 6). Repeated immobilizations also resulted in increased formation of a complex of similar mobility (Fig. 2D, lane 3), although c-fos is no longer present (Fig. 1, lane 2). Addition of Fra family-specific antibody efficiently supershifted this complex in extracts from handled controls and repeatedly immobilized rats (lanes 2 and 4), which indicates that some Fra family members are involved. Complex II was not significantly influenced by these treatments.

Next, we examined the binding to the AP1-like motif of another stress-responsive gene, DBH. The upstream DBH promoter region is shown on Figure 3A (McMahon and Sabban, 1992; Shaskus et al., 1992). An oligonucleotide spanning the multifunctional enhancer region DB1 in the promoter was radiolabeled and used in gel shifts. Incubation with nuclear extracts from adrenal medulla of control (Fig. 3B, lane 1) or immobilized (lane 2) animals resulted in formation of two major DNA–protein complexes. The specificity of complexes formed under these conditions was confirmed by competition with 100-fold excess of specific (DB1, lane 3) and lack of competition with nonrelated oligonucleotides (Fig. 3B, lane 4). In addition, replacement mutation of the AP1/CRE motif at position -168 to -162 rendered the DB1 oligonucleotide into a nonspecific competitor in the binding reaction (data not shown).

To identify the factors that bind to DB1 sequence, antibodies specific for Fos-, Jun-, and CREB families of proteins were included in the DNA-binding reaction. Fos and Jun family of proteins were identified as proteins that comprise the slower mobility complex I enhanced by immobilization stress, based on the ability of antibodies against these proteins to diminish the intensity of the binding complex or to form a supershifted band (lanes 5 and 6). In contrast, CREM-specific antisera supershifted the complex with highest mobility (complex II, arrow), that is not affected by immobilization stress (Fig. 3B, lane 7). CREB and ATF1 were not identified in the DNA–protein complexes formed with the DB1 oligonucleotide (data not shown).

To further examine the transcription factors involved in DNA–protein interactions at the DB1 promoter element, we performed supershift analyses with antibodies specific for individual Fos family members (Fig. 3C). A c-fos specific antibody was unable to change the migration pattern (data not shown), suggesting that c-fos may not be involved in this interaction. This finding is not surprising, because c-fos is not induced by repeated stress (Fig. 1). Addition of Fra-family specific antibody efficiently supershifted complex I (lane 6), which indicates the presence of a Fos family member. Antibodies against Fra1 or FosB did not alter the gel shift pattern (lanes 4 and 5). In contrast, Fra2-specific antisera supershifted complex I (lane 2). Taken together, our results suggest for the first time the importance of Fra2 in the transcriptional regulation of gene expression after repeated immobilization stress.

**TH and DBH: potential target genes for Fra2 induced in adrenal medulla by immobilization**

Given that repeated immobilization stress triggers persistent increases in the expression of TH and DBH genes (Kvetnansky and Sabban, 1998; Nankova and Sabban, 1999), we tested the possibility that long-lasting induction of Fra2 (Fig. 1) may correlate with the prolonged transcriptional activation of TH and DBH in response to chronic stress. Animals were exposed to repeated immobilizations on 7 consecutive days, and they were killed immediately or 1 or 2 d after the last episode of stress. Representative Western blots with Fra family or Fra2-specific antibodies are shown on Figure 4A. Compared to the handled controls (Fig. 4A, lane 1), repeated exposure to stress resulted in increased expression of Fos-related antigens in adrenal medulla (lane 2). The levels of Fra proteins, although no longer maximal, remained higher than in controls when examined 1 or 2 d after the last stress session (Fig. 4A, lanes 3 and 4). Marked induction of Fra2 by 7× Immo was also observed (Fig. 4B, compare lanes 1 and 2). One or two days after cessation of the stress, levels of Fra2 declined but remained higher than controls (Fig. 4B, lanes 3 and 4; see the summary data on the right panel).

The relative rate of TH and DBH transcription was also examined under these conditions (Fig. 4C). Repeated stress was found to evoke threefold to fourfold increases in the transcription of both genes, evident even 1 d after the last episode of stress. In contrast, the expression of the stress unresponsive gene CPH was not affected (see the insert). Taken together the observed pattern of induction and DNA-binding activities of Fra by stress suggest that they may participate in the sustained transcriptional activation of TH and DBH genes after repeated immobilization.

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**Fra2 increases transcription from TH and DBH promoters**

Having demonstrated that exposure to immobilization stress stimulates the expression of Fra2, including Fra2 in rat adrenal medulla, associated with increased AP1-like binding and stimulated transcription of AP1-harboring genes TH and DBH, our next aim was...
to evaluate whether Fra2 can directly affect their transcription. Therefore, we cotransfected PC12 cells with constructs in which a reporter activity is driven by a fragment of the rat TH or DBH promoter and with a Fra2 expression vector. Analysis of the reporter activities (Fig. 5) showed that over expression of Fra2 resulted in twofold to threefold, statistically significant upregulation of both TH and DBH promoter activity, when examined 24 hr after transfection.

**DISCUSSION**

This study implicates Fra2 in underlying the persistent activation of TH and DBH transcription in adrenal medulla in response to repeated immobilization stress. Fra2 was identified originally in growth-stimulated chicken embryo fibroblasts (Nishina et al., 1990) as a novel protein cross-reactive with antiserum to c-fos. Cloning and characterization of the mouse fra-2 gene (Foletta et al., 1994) revealed similar overall gene structure (four exons and three introns) between c-fos and fra-2. High Fra2 mRNA expression has been observed in a diverse range of adult mouse tissues (ovary, stomach, lung, intestine, brain, and heart; Foletta et al., 1994). Here we found detectable levels of Fra2 protein in total homogenates from adrenal medulla of control rats. Exposure to immobilization stress elicited robust induction of Fra2 and other Fos-related members of the Fos family of proteins, including Fra2. Various agents have been reported to cause induction of Fra2 mRNA and protein expression: serum stimulation, the phorbol ester TPA, cAMP, and calcium ionophores in different cell systems; and in vivo metrazole-induced seizures increase Fra2 expression in the rat hippocampus (for review, see Foletta, 1996).

Our work is the first to show activation of Fra2 expression by in vivo stress. Similar to other stimuli (for review, see Foletta, 1996), the pattern of induction of c-fos and Fra2 differed. Whereas c-fos immunoreactivity was rapidly but transiently induced by a single episode of stress, Fra2 expression was stimulated by both single and repeated immobilization. Multiple bands in the range of 30–50 kDa were visualized with Fra family-specific antibody, suggesting that other Fos-related antigens were also induced by stress. When directly compared, the pattern of Fos-related proteins induced in the adrenal medulla by repeated immobilization stress and in the frontal cortex by chronic electroconvulsive seizures differed. In contrast to the effect of chronic challenges in the brain (Hope et al., 1994a; Chen et al., 1997; Hiroi et al., 1998; Nestler et al., 1999), immobilization stress did not stimulate the expression of FosB and/or the truncated splice variants of gene–Delta FosB isoforms in adrenal medulla. These results are consistent with the hypothesis that different members of the extended Fos-family of proteins seem to be involved in central and peripheral responses to chronic stimuli.

It has been shown that stress-elicited increases in the expression of genes that encode catecholamine biosynthetic enzymes may involve transcriptional activation (Nankova et al., 1994, 1999; Osterhout et al., 1997). Depending on the duration and reiteration of the stress signal, different phases of transcriptional activation were observed (Nankova and Sabbah, 1999; Nankova et al., 1999). Both TH and DBH genes contain functional AP1-like sites in their promoters (Kumer and Vrana, 1996; Sabban, 1997; Swanson et al., 1997). Depending on the duration and reiteration of the stress signal, different phases of transcriptional activation were observed (Nankova and Sabbah, 1999; Nankova et al., 1999). Both TH and DBH genes contain functional AP1-like sites in their promoters (Kumer and Vrana, 1996; Sabban, 1997; Swanson et al., 1997).
ity parallel the transcriptional upregulation of TH and DBH. It has been shown that Fra2 displays similar dimerization and DNA-binding properties to that of c-fos protein, because it forms stable heterodimers with Jun proteins and binds specifically to AP1 sites or related sequences (Suzuki et al., 1991). Although both c-fos and Fra2 are stimulated by single immobilization in adrenal medulla, the complexes formed at the TH AP1-like site and elevated by single stress consisted mainly of c-fos/c-Jun dimers. In contrast, c-fos is unlikely to be involved in the transcriptional activation of DBH by single immobilization, as revealed by the results from gel shifts presented here and earlier photochemical cross-linking experiments (Nankova et al., 1994, Sabban et al., 1995). Thus, with a single stress signal different sets of transcription factors within the same cell can be triggered or not in response to extracellular mitogenic or other signals. TH and DBH gene expression in response to acute and chronic stress and how they would affect the activation of TH and DBH expression remains to be determined. Even without cotransfection of a Jun-expression vector, exogenous Fra2 expression triggered modest, but statistically significant, upregulation of both TH and DBH promoters.

The results of this study revealed differential induction of c-fos and Fos-related antigens in rat adrenal medulla in vivo induced by single and repeated immobilization stress. The robust stimulation of Fra2 expression paralleled the increased transcription of the stress-responsive genes TH and DBH. Fra2 may play an important role in mediating the stress response in the adrenal medulla and may contribute to the adaptation of the norepinephrine-producing system to in vivo challenges.

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