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Induction of tyrosine hydroxylase gene expression by a nonneuronal nonpituitary-mediated mechanism in immobilization stress

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ABSTRACT **Stress stimulates the sympathoadrenal system, causing activation of the catecholamine biosynthetic enzymes. Here we examine the changes of gene expression of tyrosine hydroxylase (TH; EC 1.14.16.2), the initial enzyme of catecholamine biosynthesis, with stress. A single immobilization of rats led to a large transient elevation in TH mRNA and a small elevation in TH immunoreactive protein and activity. Repeated daily immobilizations triggered more sustained changes in TH mRNA levels. After two immobilizations, the levels remained elevated even 3 days later. The rise in TH mRNA was followed by increased immunoreactive protein but only a small elevation in activity. With seven repeated immobilizations, the animals did not appear to adapt and still manifested a further rise in TH mRNA. TH activity was markedly elevated and returned to control levels 7 days after the immobilization. The rise in TH mRNA with a single immobilization occurred even in adrenals of hypophysectomized rats that underwent splanchnic nerve section. Immobilization for 30 min was sufficient to increase TH mRNA. The effect was abolished by the transcriptional inhibitor actinomycin D. Mobility gel-shift assays revealed increased binding of c-Fos and c-Jun to the AP-1 transcription factor site after a single immobilization, and the binding was not further elevated with repeated stress. This study shows that a single immobilization can activate TH gene expression by a nonneuronal nonpituitary-mediated pathway associated with increased binding of AP-1 transcription factors.**

Chronic stress is considered one of the most important precipitating factors in mental disorders. During stress catecholamine biosynthesis and secretion are markedly activated (1–3). Since the catecholamines are important mediators in the maintenance of internal homeostasis, aberrations in catecholamine neurotransmission are implicated in a variety of neuropsychiatric disorders, including schizophrenia and depression, and also in cardiovascular disorders.

The highly elevated synthesis of catecholamines in repeated stress is accompanied by activation of tyrosine hydroxylase (TH; tyrosine 3-monooxygenase, EC 1.14.16.2), the first and major rate-limiting enzyme in catecholamine biosynthesis, and of other enzymes of the pathway (reviewed in ref. 4). The increase in adrenal TH activity after repeated and chronic stress involves increased TH mRNA levels (5–8). Recently, activation of TH gene expression was found with a single immobilization stress (7, 8).

The regulation of the TH gene has been studied in cultured cells in response to a variety of stimuli, including various neuronal and hormonal factors, growth factors, and cell density (e.g., refs. 9–14). A number of genomic regulatory elements have been found in the promoter region of the TH gene, including an AP-1 site, a cAMP regulatory element, and putative AP-2, SP1 and POU/Oct, heptamer, and E2A/MyoD (E box) sites (15–18). The CRE region, involved in regulation by elevated cAMP (11), has been recently shown to be sufficient for the regulated expression of the TH gene by depolarization and by nicotine treatment (13, 19). The AP-1 site has been shown to be involved in cell-specific expression, as well as in the induction of TH by nerve growth factor, phorbol esters, and angiotensin (10, 14, 17, 20–22). On the basis of nuclear protein binding studies, the AP-1 site of the TH gene has recently been implicated in the regulation of TH by cold stress (23).

In this study we analyzed the changes in TH mRNA, protein, and activity levels after a single and repeated immobilizations. Our results show that a single immobilization stress can cause transcriptional activation of the TH gene by a nonneuronal, nonpituitary-mediated mechanism. The increased binding of AP-1 factors to the TH promoter region observed under these conditions suggests that c-Fos and c-Jun are among the factors mediating the effect of immobilization stress on TH gene expression. Further exposure to immobilization (2 and 7 repeated treatments) led to increased steady-state levels of TH mRNA, protein, and activity. The results have important implications for the long-term dangers to health manifested with repeated stress.

METHODS

Animals. Male pathogen-free Sprague–Dawley rats (280–320 g) were obtained from Taconic Farms. In some experiments, hypophysectomized and unilateral adrenal-denervated rats were used 9 days after the operation.

Immobilization Stress. Immobilization stress, as administered under this protocol, was approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke. Immobilization by taping all four limbs of the rat to metal mounts attached to a board was previously described (7, 24). Each group contained 6–12 animals.

Isolation of mRNA and Analysis of mRNA Levels. Isolation of mRNA and Northern blot analysis were as previously described (7, 25). TH mRNA levels were expressed relative to controls on the same Northern blot. Statistical differences between experimental groups and controls were determined by one-way ANOVA.

Measurement of Immunoreactive TH Protein Levels. Adrenals were homogenized in 0.05 M potassium phosphate, pH 6.65/0.2% Triton X-100 and centrifuged at 10,000 × *g* for 20 min at 4°C. Supernatant proteins were fractionated by 10%

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Abbreviation: TH, tyrosine hydroxylase.

polyacrylamide/SDS gel electrophoresis and electrotransferred to nitrocellulose membranes. The levels of immunoreactive protein were measured by using a monoclonal antibody to rat TH (Boehringer Mannheim), visualized by using the Western light chemiluminescent detection system (Tropix, Bedford, MA), and analyzed by densitometry.

Assay of TH Activity. Adrenal homogenates were assayed for TH activity by a modification of the method of Waymire *et al.* (26), using 6-methyltetrahydropterin, ferrous ammonium sulfate, and ascorbic acid (27).

Preparation of Nuclear Extracts and Mobility Gel-Shift Assays. The isolation of adrenomedullary nuclear extracts was as previously described (28). The following oligonucleotides and their complements were synthesized with an Applied Biosystems model 380A Cyclone DNA synthesizer:

TH AP-1 site (32-mer, positions −217 to −185)

5′-CGGGCTGAGGGTGATTCAGAGGCAGGTGCCTG-3′
AP-1 E box

and

Nonspecific competitor for AP-1 site (mutated AP-1 site)

5′-TGGGGGACCCAGAGGGGCTTGGACGTCAGCCT-3′
AP-1

The labeling of the oligonucleotides and the binding reactions were as previously described (25). In some experiments the nuclear extracts were preincubated with the antibodies to c-Fos (N-terminal domain) and to c-Jun (DNA-binding domain) from Oncogene Science for 30 min at 20°C before addition of the labeled oligonucleotide. After another 30 min with the DNA, the extracts were analyzed by electrophoresis and autoradiography.

In some experiments 5 μg of total adrenomedullary nuclear proteins was incubated with the labeled or unlabeled oligonucleotides in a standard gel-shift reaction, then irradiated with short-wavelength (260–280 nm) UV light with an intensity of 600 $\mu W/cm^2$ for 20 min at 4°C (29). After boiling for 5 min in loading buffer, complexes were analyzed on 15% polyacrylamide/SDS gel electrophoresis and were either dried and exposed to autoradiography or transferred to reinforced nitrocellulose membranes and probed with specific antibodies.

RESULTS

Time Course of the Changes in TH Gene Expression in Response to Single or Repeated Immobilization. Kinetic analysis of the changes in rat adrenal TH mRNA, immunoreactive protein, and activity levels after exposure of rats to a single 2-hr immobilization or to a daily 2-hr immobilization repeated two or seven times is shown in Fig. 1. A single 2-hr immobilization stress elicited a large rise in adrenal TH mRNA immediately after the immobilization. There was a further rise that peaked 3 hr after removal of the animals from the immobilization. The elevation of TH mRNA with this treatment, while among the largest effects observed on TH mRNA levels, was transient. Six hours after the immobilization the levels were declining, and they approached baseline values 24 hr after the immobilization. A single immobilization caused a slight, although not statistically significant, rise in TH protein that was maximal 6 hr after the immobilization (Fig. 1*B*). The changes in TH activity were also small, and they were statistically significant ($P < 0.05$) only at 12 and 24 hr after immobilization (Fig. 1*C*).

The elevation in TH mRNA after the second immobilization was more sustained. Again, TH mRNA levels increased immediately after the second immobilization and were maximal 3 hr later. After this interval TH mRNA levels declined gradually, but they remained higher than controls even 3 days

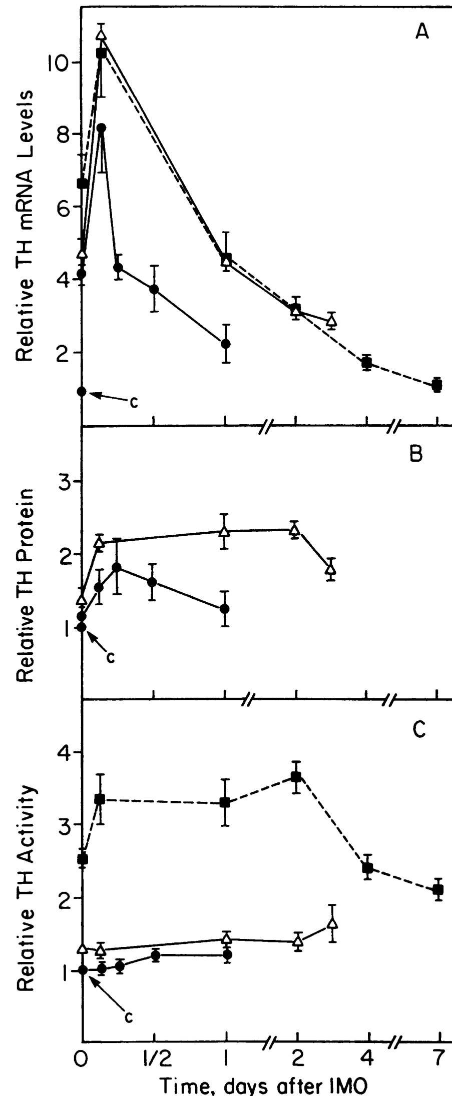

FIG. 1. Effects of single or repeated immobilization on TH mRNA, protein, and activity levels. Rats were immobilized once for 2 hr (●), twice for 2 hr daily (△), or seven times for 2 hr daily (■) and euthanized immediately or at various times after the last immobilization (IMO). One adrenal was taken to determine mRNA levels (*A*) and the second to determine TH immunoreactive protein (*B*) or TH activity (*C*). The TH mRNA levels are in arbitrary densitometric units relative to nonstressed control animals (c).

after the second immobilization (Fig. 1*A*). The TH immunoreactive protein in the adrenals rose after two immobilizations and remained at near maximal levels for 2 days after the last immobilization (Fig. 1*B*). The protein levels were maximal at times when the TH mRNA levels had already declined. The increase in TH enzymatic activity was small, but statistically above control values ($P < 0.01$), at 1–3 days after the second immobilization. Three days after the immobilization, TH enzymatic activity was still maximally elevated (Fig. 1*C*).

After seven repeated immobilizations there was still a further rise in TH mRNA 3 hr after the seventh immobilization. The effect was persistent, and 4 days after the seventh immobilization the levels were still 2 times higher than the control levels (Fig. 1*A*). The TH activity was elevated at times when the mRNA had already declined, remaining maximally increased for about 2 days after the last immobilization (Fig. 1*C*).

Mechanisms of Increased TH Gene Expression with a Single Immobilization Stress. Neuronal input has been shown to be

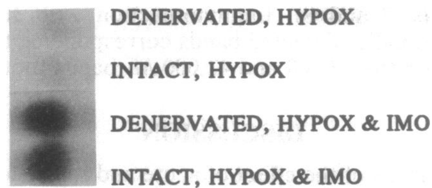

FIG. 2. Elevation of TH mRNA in unilaterally splanchnictomized (denervated) hypophysectomized (hypox) rats. A representative Northern blot of TH mRNA in the innervated or denervated adrenal from the same control animals or animals 3 hr after a 2-hr immobilization (IMO) is shown. Overall there was no significant difference in the increase in TH mRNA between the innervated and denervated adrenals (n = 8 per group).

a major factor in mediating the increase in adrenal TH activity by different types of stress. Immobilization stress rapidly raises circulating glucocorticoids. Therefore we examined the role of neuronal input and intact pituitary–adrenal axis in the large rise in TH mRNA with a single immobilization. Unilaterally splanchnicotomized hypophysectomized rats were subjected to a single immobilization. As shown in the representative Northern blot in Fig. 2, TH mRNA levels were elevated by immobilization in both innervated and denervated adrenals of hypophysectomized rats. Similar results were obtained in rats subjected to hypophysectomy alone or to splanchnicotomy alone (data not shown). These results indicated that a nonneuronal nonpituitary-mediated mechanism is primarily involved in the rise of TH mRNA levels in response to a single immobilization.

Since large increases in adrenal TH mRNA were observed with a single 2-hr immobilization stress, we examined what is the minimal time of immobilization required to elevate adrenal TH mRNA. Rats were subjected to immobilization for shorter times. As shown on Fig. 3, a continuous immobilization for 60 min or less did not change adrenal TH mRNA levels immediately after the treatment. However, an immobilization for 30 min elicited as large a rise in TH mRNA as an immobilization for 2 hr, if the rats were not euthanized until 2 hr from the beginning of immobilization (90 min after return to the home cages). Immobilization for 5 and 15 min, which leads to elevated plasma catecholamines and metabolites (30, 31), had no effect on adrenal TH mRNA levels, even when examined after 2 hr from initiation of stress. Since the time required to trigger the increase in TH mRNA levels in response to a single immobilization is so narrow (between 15 and 30 min of immobilization), compared with the reported half-life of TH mRNA of 6–9 hr (32), it is probable that the

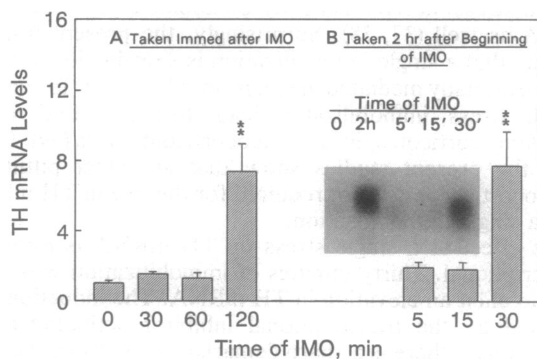

FIG. 3. Effect of duration of a single immobilization on TH mRNA levels. Rats were immobilized for the times indicated and were euthanized either immediately from the immobilization boards (*A*) or at 2 hr from the beginning of the immobilization (after return to the home cages; *B*), and adrenal mRNA levels were measured. The summary data are shown by the bar graph and a representative Northern blot is inset. **, $P < 0.001$ versus nonstressed controls.

increase occurs through predominantly transcriptional mechanisms. Therefore rats were treated with the transcriptional inhibitor actinomycin D. Pretreatment of rats with actinomycin D alone had no effect on adrenal TH mRNA levels, whereas it prevented the rise in TH mRNA levels in response to a single immobilization (not shown).

To further examine the mechanism of activation of the TH gene in response to immobilization stress, nuclear protein extracts were prepared from adrenal medullae of control rats and of rats exposed for various times to immobilization stress. The binding of the nuclear proteins to the AP-1 region of the TH promoter was examined by gel mobility-shift assay (Fig. 4). With adrenomedullary nuclear extracts from control rats, gel mobility-shift assays show one specific shifted band (lane 1, shown by filled arrow). Thirty minutes (lane 2) or shorter term (5 min, 15 min; not shown) immobilization did not show increased binding to the TH AP-1 site. However, increased formation of the AP-1 complex was observed at 120 min of immobilization, either for a single immobilization (lane 4, over 3-fold as revealed by scanning the autoradiograms) or for a three times repeated immobilization (lane 3, over 2-fold increase compared with the controls). The binding was

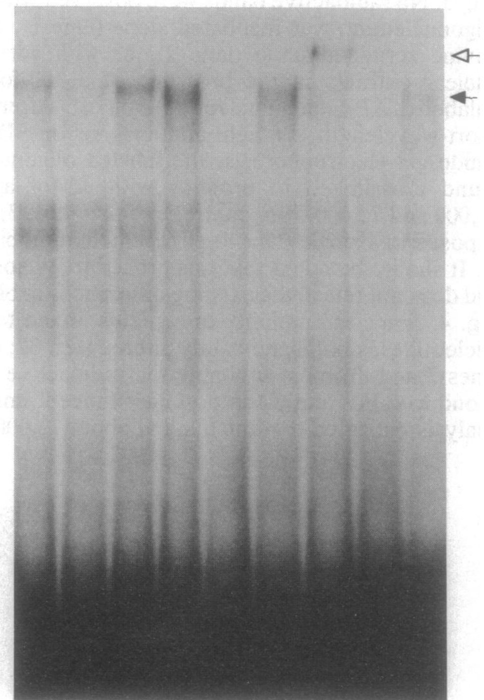

FIG. 4. Effect of immobilization stress on AP-1 specific DNA/protein binding. Mobility gel-shift assays were carried out with labeled AP-1 oligonucleotide and nuclear protein extracts from untreated rats (lane 1) or rats immobilized for 30 min (lane 2), 120 min (lanes 4–9), or three times for 120 min daily (lane 3). Where indicated in the "Compete" line a 100-fold excess of unlabeled synthetic oligonucleotides was used for specific (AP-1 oligonucleotide, Sp, lane 5) and nonspecific (mutated Ap-1 oligonucleotide, Nsp, lane 6) competition. In the remaining reactions the nuclear extract from adrenal medullary cells of rats exposed to a single 120-min immobilization was preincubated with antibodies to c-Fos or c-Jun (Oncogene Science; lanes 7–9). The AP-1 specific protein/DNA complex and the supershifted band with c-Fos antibody are marked by a filled arrow and an open arrow, respectively.

abolished by addition of 100-fold excess of unlabeled AP-1 to the reaction mixture (lane 5), but not by addition of the same amount of a nonspecific oligonucleotide competitor, containing a mutated AP-1 site (lane 6; see *Methods*), confirming that, under these conditions, sequence-specific DNA/protein complexes were formed. To show the involvement of c-Fos/c-Jun-related proteins, adrenomedullary nuclear extracts from controls and 2-hr-immobilized rats were pretreated with antibodies raised against the N-terminal domain of c-Fos, which should cause an additional shift in the mobility of the complex, or against the DNA-binding domain of c-Jun, which should prevent complex formation. As expected, preincubation of the nuclear extract with antiserum to c-Fos (30 min at 20°C) elicits a supershift of the AP-1 complexes in both experimental groups (shown for 120-min immobilization, lane 7, open arrow). The addition of c-Jun antibodies reduced the DNA/protein complex formation below the levels in the control group (lane 9). The effect of addition of the antiserum could be prevented by boiling it or by preincubation with the cognate antigenic peptide (data not shown), confirming the specificity of their interaction with the adrenal medullary complexes. Our results show that there is a marked increase in binding of c-Fos- and c-Jun-like factors to the AP-1 site of the TH promoter in adrenomedullary nuclear extracts from rats, exposed to a single 2-hr immobilization stress.

To further identify the proteins forming sequence-specific complexes with the TH AP-1 fragment, we used photochemical crosslinking procedures. Typical results are shown in Fig. 5. No radioactive bands were observed when the labeled oligonucleotide was incubated alone (lane 1), with 10 μg of bovine serum albumin (lane 2), or with adrenomedullary nuclear extracts in the presence of a 100-fold excess of unlabeled AP-1 oligonucleotide (lane 6). After exposure to short-wavelength UV light and analysis on SDS/polyacrylamide gel electrophoresis, the labeled oligonucleotide was found crosslinked to proteins with M_r of approximately 42,000 and 72,000 from control extracts (lane 3) and extracts exposed to a single (lane 4) or repeated immobilization (lane 5). It should be noted that this procedure is not quantitative and does not reveal the existing differences in binding seen in Fig. 4. Since the mobility of proteins linked to short oligonucleotides is not significantly altered (ref. 33; our results in lanes 7 and 8), most probably the radioactive bands correspond to c-Fos and c-Jun proteins. Indeed, immunoblotting analysis indicated that the band at about 72,000 M_r contains c-Fos (lanes 7 and 8). Immunoreaction with Jun-family-specific antibodies detected bands corresponding to the mobility of both the 72,000 and 42,000 M_r bands (not shown).

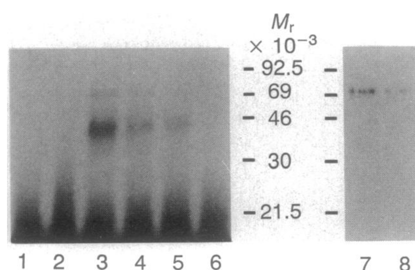

FIG. 5. Direct identification of AP-1-binding proteins by UV crosslinking. Typical gel-shift reactions were carried out with adrenomedullary nuclear extracts and the TH AP-1 oligonucleotide (labeled, lanes 1–6, or unlabeled, lanes 7 and 8) followed by UV irradiation. After separation on an SDS/polyacrylamide gel, proteins covalently bound to the DNA were visualized by autoradiography or by immunoblotting the same gel after interaction with c-Fos-specific antibody 2G9C3 (Santa Cruz). The samples contained the following: no protein added (lane 1); bovine serum albumin (lane 2); control nuclear extract (lane 3); nuclear extract after a single 2-hr immobilization (lanes 5–8); and after repeated immobilization (lane 4). In lane 6, 100-fold excess of unlabeled AP-1 oligonucleotide was added to the gel-shift reactions. The sample in lane 7 was not UV-irradiated.

DISCUSSION

Kinetic analysis of the effect of acute and repeated immobilization stress on TH gene expression indicates that exposure to a single immobilization triggers a large rise in adrenal TH mRNA. This rise is transient and is reflected in small increases in TH immunoreactive protein and enzymatic activity, consistent with earlier studies (33). It is rather surprising that a rise of about 8-fold in TH mRNA levels results in only a small rise in TH protein levels. There are several possible explanations for this finding. One possibility is that the mRNA is not efficiently translated, due to either translational regulation or a delay in exiting from the nucleus. Alternatively, the pool of TH protein may be so large compared with the mRNA pool, and its turnover is such that elevation of mRNA for several hours is not sufficient to markedly alter TH immunoreactive protein and hence activity levels. In this regard, continuous cold stress increased adrenal TH mRNA much before the rise in TH protein, which occurs predominantly after 24 hr of cold (34). The effect of a single immobilization would be negligible in terms of changes in adrenal TH, were it not followed by exposure to a second stress. Its major physiological importance is that it sensitizes or primes the system to respond very differently to further episodes of stress.

With second repeated stress, there is a further, more persistent rise in TH mRNA. TH immunoreactive protein was elevated 2- to 3-fold over control values. However, there were only small elevations in TH activity. A similar dissociation between immunoreactive protein and TH activity was previously noted for cold stress (34).

Repeated daily immobilizations for up to 7 times give a further peak in TH mRNA levels, indicating that the animals have not adapted to the stress and still respond. TH activity after 7 daily immobilizations was persistently elevated, consistent with previous studies (35, 36). After cessation of the daily treatment (after the seventh immobilization), TH activity decreased toward preimmobilization levels with a turnover time of about 3 days (35). The results reported here indicate that TH is regulated by immobilization stress at both the pre- and post-translational levels.

Neuronal input to the adrenal is generally considered to be crucial for regulation of TH. For example, splanchnicotomy prevents the rise in adrenal TH activity in response to reserpine, cold, or immobilization stress (reviewed in ref. 3). In cold stress, adrenal denervation abolished the rise in TH mRNA as well (37, 38). Surprisingly, the present findings indicate that a single immobilization is exerting its effect by a nonneuronally mediated mechanism. Moreover, in contrast to cold stress, immobilization leads to a manyfold rise in circulating corticotropin and glucocorticoid levels (39). However, the present studies show that an intact pituitary–adrenocortical axis is not required for the rise in TH mRNA after a single immobilization.

The effect of a single stress on TH mRNA is primarily transcriptional. Thirty minutes of immobilization was sufficient to elicit an elevation in TH mRNA. The induction was inhibited by the transcriptional inhibitor actinomycin D. Interestingly, there is a sharp demarkation between the time of immobilization (15 min) that did not cause significant changes in TH mRNA levels, and presumably would not have any long-term consequences, and the time (30 min) that elicited the complete effect on TH mRNA levels. In contrast, plasma levels of norepinephrine and epinephrine are elevated very rapidly during immobilization stress and are maximal by 5–15 min (29, 30). Thus there appears to be a dissociation

between the secretion of catecholamines and the elevation of adrenal TH gene expression. Our data are consistent with adrenal medullary epinephrine levels, which are significantly reduced during immobilization as a consequence of massive release and a slow resynthesis (24, 40).

Immobilization stress induces increased binding of adrenomedullary nuclear proteins to the TH AP-1 site. To our knowledge, this is the first study examining binding of transcription factors to the TH gene in response to immobilization stress. Despite the differences in the mechanism of activation of TH, alterations in the DNA/protein complexes formed at the AP-1 site of the TH gene have been reported in adrenal medulla of rats exposed to cold stress (23) and after reserpine treatment (14). The cold stress-elicited rise in AP-1 binding was abolished in denervated adrenals (23).

Repeated immobilization did not further increase formation of AP-1 complexes, suggesting that other mechanisms may be involved in the sustained rise in TH mRNA under these conditions.

The results show that the protein/AP-1 complexes from control and immobilized rats are very similar. The major binding factors were identified as being c-Fos and c-Jun, or Jun-related protein. It has been suggested that in the adrenal gland AP-1 binding is regulated mainly at the post-translational level, perhaps through dephosphorylation (41).

The present studies suggest that the increased binding of c-Fos and c-Jun to the AP-1 site of the TH promoter may be one of the regulatory mechanisms involved in mediating the response to immobilization stress. It is interesting to note that transgenic mice lacking a functional *c-fos* gene were found to have altered behavior and failed to respond in a typical fashion to stress, exhibiting no visible response to disturbances to the local environment (42). However, direct evidence for the role of c-Fos and c-Jun in the regulation of TH gene expression can be provided only by further experiments.

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