Adrenocortical Function and Regulation of the Steroid 21-Hydroxylase Gene in NGFI-B-Deficient Mice

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The immediate-early gene *NGFI-B* encodes an orphan nuclear receptor that binds DNA as a monomer and activates transcription through a canonical response element (NBRE). *NGFI-B* is expressed under basal conditions and in response to external stimuli in many mammalian tissues. In particular, *NGFI-B* expression is dramatically elevated in the adrenal cortex in response to stress and in Y1 adrenocortical cells in response to adrenocorticotropin. *NGFI-B* activates transcription through an NBRE of the gene encoding 21-hydroxylase (*P450_{c21}*) in Y1 cells. Steroidogenic factor 1 (SF-1), a homolog of *NGFI-B*, also activates the *P450_{c21}* promoter. To examine the influence of these factors on *P450_{c21}* expression in vivo and the function of the hypothalamic-pituitary-adrenocortical axis as a whole, we generated *NGFI-B* (–/–) mice. These mice thrive and reproduce normally and maintain normal basal adrenocorticotropin, corticosterone, and *P450_{c21}* mRNA levels. In response to increases in adrenocorticotropin, *NGFI-B* (–/–) and wild-type mice demonstrated equivalent increases in serum corticosterone levels. Furthermore, and in contrast to in vitro results, no increases in *P450_{c21}* mRNA levels were observed in response to increases in adrenocorticotropin in *NGFI-B* (–/–) or wild-type mice. While SF-1 mRNA levels were not increased with increased steroidogenic demand, adrenal expression of *Nurr1*, a close homolog of *NGFI-B*, was induced to a greater extent by lipopolysaccharide in *NGFI-B* (–/–) mice than in wild-type mice. Finally, when the administration of dexamethasone for suppression was stopped, *P450_{c21}* mRNA and serum corticosterone levels recovered at the same rate in wild-type and *NGFI-B* (–/–) mice. Thus, while *NGFI-B* appears poised to affect the structure and function of the adrenal gland, the gland functions normally in its absence, suggesting that other factors, including *Nurr1* and SF-1, are sufficient to drive *P450_{c21}* expression in mice and maintain normal steroidogenesis.

NGFI-B (also known as Nur77) is a transcription factor encoded by an immediate-early gene, which is inducible by a variety of stimuli (9, 20). It is expressed in many tissues, where it is presumed to mediate transcriptional changes necessary to alter the cellular phenotype appropriate to the inducing stimulus. For example, its transcription is markedly amplified in the adrenal cortex in response to stress in mice and rats, as well as in Y1 adrenocortical cells treated with adrenocorticotropin (ACTH) or cyclic AMP (cAMP) (4, 5, 36). In addition, stress induces *NGFI-B* expression in the paraventricular nucleus of the hypothalamus, where arginine vasopressin and corticotropin-releasing factor are synthesized (1, 23). Thus, *NGFI-B* may play a dynamic role in the function of the entire hypothalamic-pituitary-adrenocortical (HPA) axis.

NGFI-B is a member of the steroid-thyroid-retinoid superfamily of receptors, but because no ligand has been shown to regulate its transcriptional activity, it is referred to as an orphan receptor. The DNA binding properties of the molecule have been well characterized (34, 35, 37). *NGFI-B* binds as a monomer to a recognition element (NBRE) which consists of the classical estrogen receptor half-site preceded by two adenines (5'-AA AGGTCA). A homolog of *NGFI-B*, the orphan receptor steroidogenic factor 1 (SF-1), is constitutively expressed in the adrenal cortex (11, 13) and binds to an element similar to but distinct from the NBRE, 5'-CA AGGTCA (35).

Another important distinction between the DNA binding properties of these two orphan receptors is that SF-1 binds more promiscuously than *NGFI-B*, which is highly selective for the NBRE.

Several of the genes encoding adrenocortical steroidogenic enzymes exhibit elements which potentially bind nuclear receptors such as *NGFI-B* and SF-1 (for a review, see reference 22). The murine gene encoding steroid 21-hydroxylase (*P450_{c21}*, CYP21) has three such elements, at –210, –140, and –65 relative to the transcription initiation site. The first two elements bind SF-1 but not *NGFI-B*; the last element is an NBRE and binds *NGFI-B* as well as SF-1 (11, 22, 36). Deletion of any of these three elements results in a dramatic reduction in transcription of a reporter gene in Y1 cells (24) and in mice (4).

In primary cultures of bovine adrenocortical cells, transcription of the gene encoding *P450_{c21}*, as well as that of other steroidogenic enzyme-encoding genes, is inducible by the addition of ACTH or cAMP to the culture medium (for a review, see reference 31). Because this induction is inhibited by cycloheximide and does not proceed within the time frame characteristic of activation by the cAMP response element-binding protein, CREB (27), it appears that de novo synthesis of transcription factors, such as *NGFI-B* or SF-1, is required for *P450_{c21}* induction. Both SF-1 and ACTH-induced *NGFI-B* activate transcription of *P450_{c21}* through the –65 element in Y1 cells, although SF-1 binds the site with much less affinity than *NGFI-B* (11, 35, 36). It is possible that, under basal adrenocortical stimulation, when *NGFI-B* expression is low, *P450_{c21}* transcription is mediated by the constitutive SF-1, but

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upon stimulation by ACTH, the induction of NGFI-B results in a further activation of *P450_{c21}* gene transcription to enhance steroidogenesis.

To test the role of NGFI-B in HPA axis function, we examined mice which carried a targeted mutation in the *NGFI-B* gene. We report that the basal regulation of hypothalamic and pituitary functions as well as adrenocortical steroidogenesis in NGFI-B ($-/-$) mice is normal, as demonstrated by normal levels of ACTH, corticosterone, and *P450_{c21}* mRNA. Moreover, despite strong increases in *NGFI-B* expression in the adrenal glands of wild-type mice, secondary to the administration of metyrapone or lipopolysaccharide (LPS), there is no difference in *P450_{c21}* mRNA levels between wild-type and NGFI-B-deficient mice subjected to these stresses; in fact, *P450_{c21}* mRNA levels do not increase in response to increases in ACTH in mice. Corticosterone levels were equally increased by LPS in mice of both genotypes. The administration of dexamethasone, which suppresses corticosterone as well as *P450_{c21}* and SF-1 mRNA levels, revealed that these levels are restored at the same rate in NGFI-B-deficient and wild-type mice when dexamethasone is removed. These findings suggest that NGFI-B-related orphan receptors, including SF-1, may be sufficient to regulate *P450_{c21}* gene transcription and adrenocortical function and demonstrate that NGFI-B is not required for HPA axis function.

MATERIALS AND METHODS

Generation of NGFI-B ($-/-$) animals. NGFI-B ($-/-$) mice were generated by homologous recombination by using a targeting vector which contained a neomycin resistance gene insertion in the region encoding the amino-terminal domain of NGFI-B. Homologous recombinants were screened from murine strain 129/SvJ embryonic stem cells and implanted into murine C57BL/6 blastocysts (15).

Tissue processing and histology. For histological preparations, male, postpubescent wild-type and NGFI-B ($-/-$) mice were rapidly killed by halothane overdose. The adrenal glands were quickly dissected, submersion fixed overnight in 4% paraformaldehyde, washed in 70% ethanol, mounted in epoxy resin, and cut into 1- μ m-thick sections. Samples were stained with toluidine blue for 2 s and washed briefly with water.

Physiologic manipulations. Mice (strain C3H or C57BL/6 for the wild type) were housed under standard laboratory conditions (12 h of light and 12 h of dark; lights on at 0700 h) and were fed laboratory chow and water ad libitum. Metyrapone (catalog no. M 1259; Sigma Chemical Company, St. Louis, Mo.) was injected subcutaneously into postpubescent wild-type and NGFI-B ($-/-$) male mice at 200 mg/kg of body weight in 0.15 M NaCl (in suspension). A single injection of LPS (endotoxin) from *Escherichia coli* O157:H7 (Sigma catalog no. L 3756) was administered intraperitoneally to both postpubescent male and female (as indicated in the figure legend) wild-type and NGFI-B ($-/-$) mice at 10 mg/kg ($\sim$ 250 μ g per mouse) in 0.15 M NaCl. Dexamethasone (Sigma catalog no. D 1756) was dissolved at 100 μ g/ml in drinking water and supplied ad libitum to postpubescent male wild-type and NGFI-B ($-/-$) mice for 80 h, after which normal tap water was supplied. The animals were rapidly killed by halothane overdose.

Hormone assays. Blood was promptly collected from freshly killed mice via needle aspiration from the right ventricle. Samples were spun to isolate serum for hormone assays and stored at -20°C . For corticosterone assays, serum from each mouse was extracted twice with a 1:1 mixture of ether-ethyl acetate and measured by radioimmunoassay with a rabbit anti-corticosterone antibody (Sigma catalog no. C 8784), using tritiated corticosterone (catalog no. NET-399; DuPont-NEN, Boston, Mass.) as a competitor. For ACTH measurements, a sandwich assay (catalog no. 60-4175; Nichols Institute, San Juan Capistrano, Calif.) was used according to the manufacturer's recommendations. For statistical comparisons, unpaired Student's *t* tests were used with significance set at a *P* value of <0.05 .

Gene expression analysis. The adrenal glands were rapidly dissected from the retroperitoneum, cleaned of fat, quick-frozen on dry ice, and stored at -70°C . Total RNA was isolated by the method of Chomczynski and Sacchi (2) and electrophoresed on 1% agarose-1.5% formaldehyde gels. RNAs were transferred to hybridization membranes (catalog no. S4056-C; Oncor). Probed membranes were washed with $0.2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min at 65°C and exposed to Kodak film at -70°C . Quantitation of *Nurr1* expression was performed by using a PhosphorImager (Molecular Dynamics) and the ImageQuant software package. Message levels were determined relative to the level of cyclophilin. For probes, the following were used: for NGFI-B, a fragment spanning nucleotides 1889 to 2300 of the murine cDNA; for *Nurr1*, a fragment spanning nucleotides 1 to 1044 of the murine cDNA; for SF-1,

a fragment spanning nucleotides 1 to 850 of the murine cDNA; for *P450_{c21}*, an $\sim$ 200-nucleotide fragment amplified from the first exon by PCR; for *P450_{SCC}*, the entire rat cDNA; and for cyclophilin, an $\sim$ 200-nucleotide fragment amplified by PCR.

RESULTS

NGFI-B ($-/-$) mice develop, reproduce, and function normally. NGFI-B ($-/-$) mice were generated by standard targeted disruption techniques (for details, see reference 15). Normal 1:1 ratios of males to females and 1:2:1 ratios of $+/+$, $+/-$, and $-/-$ pups were obtained from heterozygote matings, indicating no interference with sex determination and no embryonic or neonatal lethality. The animals thrived, reproduced, and appeared to be normal. Because NGFI-B is normally expressed in the adrenal cortex and hypothalamus, we analyzed the structural and functional integrity of these tissues. Adrenal glands were examined histologically and found to be structurally indistinguishable from those of wild-type animals, composed of distinct zonae glomerulosae, fasciculatae, and reticulares and an adrenal medulla (Fig. 1A). The hypothalamic paraventricular nucleus of NGFI-B ($-/-$) animals is also structurally equivalent to that of wild-type animals (data not shown). To assess HPA axis function, we measured basal serum corticosterone and ACTH concentrations and found no significant differences in these concentrations between wild-type and NGFI-B ($-/-$) mice (Fig. 1B). Also, basal expression of the gene encoding *P450_{c21}* is the same in wild-type and NGFI-B ($-/-$) mice (Fig. 1C). Taken together, these data indicate that in NGFI-B ($-/-$) mice, basal function of the HPA axis is intact.

NGFI-B ($-/-$) mice respond normally to changes in HPA axis stimulation. NGFI-B is encoded by an immediate-early gene induced in the adrenal gland by ACTH and regulates the expression of the gene encoding *P450_{c21}* in vitro. To determine whether NGFI-B ($-/-$) mice harbor any deficits in gene expression or hormone production, we challenged the adrenal gland by manipulating serum ACTH levels. We used three paradigms (metyrapone, dexamethasone, and LPS administration) to alter HPA axis hormone levels. Because these methods influence the hypothalamic and pituitary levels of the HPA axis as well as the adrenal gland, they provide robust, endogenous, and quantifiable ACTH inductions to the adrenal cortex.

To evaluate the requirement for NGFI-B at the hypothalamic and pituitary levels of the HPA axis, we administered metyrapone to wild-type and NGFI-B ($-/-$) mice. This agent is an inhibitor of the adrenal gland's 11β -hydroxylation reaction, which prevents the formation of corticosterone, and thus stimulates unabated pituitary secretion of ACTH (8). In addition, metyrapone induces corticotropin-releasing factor synthesis in the hypothalamus (10). Administration of metyrapone substantially increased ACTH levels in both wild-type and NGFI-B ($-/-$) mice (Fig. 2A). This demonstrates that NGFI-B is not required for hypothalamic-pituitary induction of ACTH in response to glucocorticoid deficiency, despite NGFI-B-specific expression in the hypothalamus during stress (1, 23).

To examine the effects of increased ACTH levels on *NGFI-B* expression in the adrenal gland, we also performed Northern blots on adrenal gland RNA from mice treated with metyrapone. Consistent with its behavior as an immediate-early gene we found that *NGFI-B* expression is rapidly but transiently induced in the adrenal gland of metyrapone-treated wild-type mice, suggesting that it may play a role in the function of the stressed adrenal cortex (Fig. 2B). As expected, no transcript was seen from the NGFI-B ($-/-$) mice.

To test whether induced NGFI-B is required to respond to massive stress, we administered LPS (bacterial endotoxin),

FIG. 1. The basal function of the HPA axis in NGFI-B ($-/-$) mice is normal. (A) Histological analysis of adrenal glands was performed as described in Materials and Methods. (B) Basal ACTH and corticosterone hormone assays were performed as described in Materials and Methods. For the ACTH assay, 11 and 5 ($P = 0.13$) wild-type and NGFI-B ($-/-$) mice respectively, were tested; for the corticosterone assay, 11 and 9 ($P = 0.48$) wild-type and NGFI-B ($-/-$) mice, respectively, were tested. (C) Basal $P450_{c21}$ mRNA levels were assessed by using total adrenal gland RNA and a probe against the $P450_{c21}$ cDNA. The membrane was probed with a cyclophilin cDNA fragment to control for loading and transfer. +/+, wild-type; $-/-$, NGFI-B ($-/-$).

which mimics septic shock. LPS is known to stimulate the hypothalamus and pituitary very strongly and lead to stably elevated serum corticosterone levels (26). As expected, administration of LPS strongly induced ACTH levels (above 1,600 pg/ml for both genotypes [data not shown]) and NGFI-B expression in the adrenal gland of wild-type animals (Fig. 3A).

To determine if this increase in NGFI-B and increased demand for adrenocortical steroidogenesis resulted in elevations of $P450_{c21}$ expression, RNA samples from the adrenal glands of LPS-treated wild-type and NGFI-B ($-/-$) mice were examined by Northern blot analysis (Fig. 3B). No change in $P450_{c21}$ expression was observed in mice of either genotype under these conditions; thus, increases in steroidogenic demand do not elevate $P450_{c21}$ transcription, and NGFI-B is not required for the maintenance of $P450_{c21}$ expression under basal or stimulated conditions. As for $P450_{c21}$, the levels of $P450_{SCC}$ mRNA, which encodes the rate-limiting steroidogenic enzyme, were unchanged in both sets of mice in response to increased adrenocortical stimulation (Fig. 3B).

Because NGFI-B is clearly not required to maintain $P450_{c21}$ expression, we investigated the expression of *SF-1*, which activates the $P450_{c21}$ and $P450_{SCC}$ promoters in cell transfections (3, 11). As observed for $P450_{c21}$ and $P450_{SCC}$, we found that LPS administration did not induce *SF-1* expression above basal

levels in wild-type or NGFI-B ($-/-$) mice (Fig. 3C). Thus, *SF-1* mRNA levels appear to be regulated in parallel to those of $P450_{c21}$ and $P450_{SCC}$. We also examined the expression of the immediate-early gene *Nurr1*, whose DNA- and ligand-binding domains are very similar to those of NGFI-B (14, 19, 30), and found that its expression was induced threefold higher in the NGFI-B ($-/-$) mice than in wild-type mice (Fig. 3C), suggesting that it may compensate for the loss of NGFI-B.

When we measured the hormonal response to LPS in wild-type and NGFI-B ($-/-$) mice, we found no differences in the extent or duration of the LPS-induced corticosterone increase. Mice from both genotypes showed dramatically increased levels of corticosterone within 1 h of treatment; levels peaked 2 h after treatment [449 ± 104 ng/ml for wild-type mice and 591 ± 62 ng/ml for NGFI-B ($-/-$) mice] and remained very high for more than 24 h after treatment (Fig. 4). Moreover, NGFI-B ($-/-$) and wild-type mice exhibited equally severe symptoms of septic shock. Taken together, these results indicate that NGFI-B ($-/-$) mice possess a normally functioning HPA axis and physiologic response.

To determine if NGFI-B is required for the recovery of adrenocortical function from suppression, we gave dexamethasone orally for 3 days to suppress HPA axis function and measured $P450_{c21}$ mRNA levels in both wild-type and NGFI-B

FIG. 2. Response of HPA axis to metyrapone. Metyrapone (200 mg/kg) was administered to mice as described in Materials and Methods. This experiment was repeated four independent times, and each trial gave results consistent with those displayed here. In this particular trial, for each time point and genotype, two mice were tested. (A) Metyrapone induction of ACTH levels in wild-type and NGFI-B (-/-) mice. At 1 h after injection ($P < 10^{-6}$ and $< 10^{-5}$, respectively, compared with untreated samples). No Rx, no treatment; veh, saline vehicle; met, metyrapone. (B) Total adrenal gland RNA from animals assessed for ACTH levels was pooled and hybridized with an NGFI-B 32 P-labeled probe. +/+, wild-type; -/-, NGFI-B (-/-). The membrane was probed with a cyclophilin cDNA fragment to control for loading and transfer.

(-/-) animals after discontinuation of the treatment. The administration of dexamethasone resulted in a dramatic reduction of $P450_{c21}$ mRNA levels, but within 6 days after the administration of dexamethasone was stopped, these levels recovered significantly in both wild-type and NGFI-B (-/-) mice (Fig. 5), indicating that NGFI-B is not essential for the restoration of $P450_{c21}$ expression after suppression by dexamethasone.

To investigate which transcription factor(s) might be responsible for these changes in $P450_{c21}$ mRNA levels, we examined SF-1 mRNA levels and found that suppression by dexamethasone also reduced expression of *SF-1*. After suppression was discontinued, *SF-1* expression rose but less rapidly than that of $P450_{c21}$ (Fig. 5), indicating that factors in addition to SF-1 may serve as critical regulators of $P450_{c21}$ expression.

We also measured serum corticosterone concentrations in mice treated with dexamethasone and found that the level of corticosterone was reduced to almost zero in both wild-type and NGFI-B (-/-) animals (Fig. 6). After cessation of treatment, we found that recovery of the adrenal gland began rapidly; within 2 days after dexamethasone administration was stopped, corticosterone concentrations began to increase in +/+ and -/- animals, and by 6 days, most mice (both +/+ and -/-) had regained normal levels of corticosterone in serum, indicating that NGFI-B is not required for the recovery of the adrenal cortex from inactivity. These results also indicate that the hypothalamus and pituitary recover at the same rates in wild-type and NGFI-B (-/-) mice after cessation of suppression by dexamethasone.

DISCUSSION

NGFI-B encodes an orphan nuclear receptor expressed in the adrenal gland which has been shown in vitro to specifically

FIG. 3. Effect of LPS on adrenal gland gene expression in wild-type and NGFI-B (-/-) mice. LPS (10 mg/kg) was administered as described in Materials and Methods. This experiment was repeated three independent times, using either all male or all female mice. The results were indistinguishable between the sexes and among experiments. In this trial, three female mice were tested for each time point and genotype. Adrenal glands were pooled, total RNA was purified, and Northern blots were performed. (A) NGFI-B mRNA levels; (B) $P450_{c21}$ and $P450_{scc}$ mRNA levels; (C) SF-1 (note that a band corresponding to *Nurr1* mRNA also appears in the lanes labeled 1, as this membrane had previously been probed for *Nurr1*) and *Nurr1* mRNA levels. The membranes were probed with a cyclophilin cDNA fragment to control for loading and transfer.

bind and transactivate the $P450_{c21}$ gene promoter (36). As such, NGFI-B appears poised to influence adrenocortical steroidogenesis; however, an examination of NGFI-B-deficient mice shows no deficits in adrenocortical or overall HPA axis structure or function at basal levels.

Metyrapone- and LPS-induced rises in ACTH increase expression of NGFI-B in the adrenal cortex of wild-type mice. In addition, NGFI-B transcription is known to be inducible in the paraventricular nucleus of the hypothalamus (1, 23). Therefore, to evaluate the requirement of NGFI-B in vivo, we attempted to expose potential deficits harbored by the NGFI-B (-/-) mouse by using paradigms that would test all levels of HPA axis function during stress. After administering metyrapone and LPS to NGFI-B (-/-) mice, we concluded that NGFI-B is required neither for HPA axis function at the hypothalamic-pituitary level nor for acute or chronic phases of ACTH-induced adrenocortical steroidogenesis, including the maintenance of $P450_{c21}$ expression. Even during recovery of $P450_{c21}$ expression from suppression by dexamethasone, NGFI-B is not required to usher $P450_{c21}$ expression back to baseline. One explanation for this phenotype is that functionally redundant transcription factors, such as SF-1 and Nurr1, may compensate for the loss of NGFI-B.

Nurr1, as mentioned above, encodes a product which is highly homologous to NGFI-B and modulates transcription through the NBRE (35). Interestingly, we found that the LPS-induced expression of the *Nurr1* gene was threefold higher in

FIG. 4. Response to LPS in wild-type and NGFI-B ($-/-$) mice. Corticosterone radioimmunoassays were performed with the sera of mice treated with LPS in the experiments described in the legend to Fig. 3. For the 0, 1, 2, 5, 24, and 48 h time points, 11, 3, 4, 6, 9, and 3 wild-type mice, respectively, and 9, 3, 4, 5, 9, and 3 NGFI-B ($-/-$) mice, respectively, were tested. Two hours after injection, both wild-type and NGFI-B ($-/-$) mice had elevated corticosterone levels ($P < 10^{-5}$ and $P < 10^{-7}$, respectively) and maintained significantly high levels 24 h after injection ($P < 0.001$ and $P < 0.004$, respectively).

NGFI-B ($-/-$) animals than in wild-type animals. This enhanced expression of *Nurr1* potentially contributes to the NGFI-B ($-/-$) animal's compensation for the loss of NGFI-B.

Several additional *cis* elements in the $P450_{c21}$ promoter between -330 and $+5$ act in concert with the -65 element to direct its expression. It is possible that the transcription factors that bind these closely apposed elements act together by heterodimerizing to create an activating complex (6, 21). NGFI-B may interact through these mechanisms, but receptors such as *Nurr1* may be able to compensate in that role.

Functional redundancy of proteins appears to be a common theme among members of gene families (for a review, see reference 32) and could explain the lack of defects in NGFI-B null mice. For example, despite the work of two teams of investigators showing *in vitro* that NGFI-B (Nur77) function is required for T-cell receptor-mediated T-cell apoptosis (17, 38), NGFI-B ($-/-$) mice undergo completely normal thymocyte and peripheral T-cell deletion (15). It is known that *Nurr1* is also expressed in peripheral T cells stimulated by agents which trigger the T-cell receptor (19). Thus, one could hypothesize that the function of NGFI-B and its close relatives is responsible for permitting T-cell apoptosis to occur and not that of NGFI-B alone. The same relationships may be true in the regulation of $P450_{c21}$ and other adrenocortical functions.

In Y1 cells, the promoter region between -330 and $+5$ of the murine gene encoding $P450_{c21}$ proves to be sufficient both to drive minimal transcription and to provide ACTH or cAMP inducibility (7). Despite these findings, we were not able to demonstrate inducibility of the gene encoding $P450_{c21}$ above baseline levels in the mouse, even with serum ACTH levels as high as 1,600 pg/ml. When adrenocortical cells are maintained in the absence of ACTH *in vitro*, steroidogenic enzyme gene expression is artificially low; the ACTH-enhanced transcription of those genes brings expression levels up to those that would normally be maintained *in vivo*. We propose that the continuous presence of at least baseline ACTH stimulation of the adrenal cortex in mice maximally drives expression of $P450_{c21}$, $P450_{SCC}$, and, potentially, all of the steroid hydroxylase genes. Further demand for steroidogenesis does not require increased mRNA levels from these genes, and therefore dramatic increases in glucocorticoid production proceed with-

FIG. 5. Adrenal gland gene expression in wild-type and NGFI-B ($-/-$) mice during and after dexamethasone administration. Dexamethasone (Dex) was administered to 18 mice from each genotype for 3 days as described in Materials and Methods. For each genotype, the adrenal glands of three mice were harvested and pooled at the following times: on the day that dexamethasone administration was stopped (day 0) and 1, 2, and 4 days later. Between 6 and 10 days after treatment was stopped, the adrenal glands of six animals from each genotype were harvested and pooled. Total RNA was purified from these sets of adrenal glands, and Northern blots were hybridized with the indicated probes. B, baseline.

out their enhanced transcriptional activation, as shown by Le-Houx and colleagues (16) for $P450_{c21}$ and Sander and colleagues (29) for $P450_{SCC}$.

Despite the lack of inducibility of the genes encoding $P450_{c21}$ and $P450_{SCC}$, the transcription of these genes can be modulated *in vivo*. In experiments on suppression by dexamethasone, we showed that the mRNA levels decrease coordinately with corticosterone concentrations upon suppression but recover in parallel with corticosterone concentrations after the treatment is suspended (data not shown for $P450_{SCC}$). Direct glucocorticoid suppression of steroidogenic enzyme gene transcription in adrenocortical cells has been observed by Trzeciak and colleagues (33), but an additional mechanism through which dexamethasone suppression may abrogate $P450_{c21}$ expression was demonstrated by Imai and colleagues (12). They noted in hypophysectomized rats a complete loss of $P450_{c21}$ gene expression, which could be restored with exogenous ACTH. Because ACTH levels were dramatically reduced by dexamethasone (data not shown), both mechanisms may be operant in our experiments.

FIG. 6. Serum corticosterone levels during and after dexamethasone (Dex) administration. Corticosterone radioimmunoassays were performed with the sera of the same mice used for adrenal gland RNA analysis described in the legend to Fig. 5. Dexamethasone significantly reduced corticosterone levels [$P < 0.05$ for wild-type and $P < 0.01$ for NGFI-B ($-/-$)]. Corticosterone levels recovered to within normal limits by 6 days after cessation of dexamethasone administration.

As mentioned above, SF-1 may bind as many as three elements in the *P450_{c21}* promoter, and it activates the gene encoding *P450_{c21}* in Y1 cells. During recovery from suppression by dexamethasone, however, *SF-1* transcription is restored later than that of *P450_{c21}*, suggesting that other activators may be more important for *P450_{c21}* expression. Furthermore, it will be difficult to determine the role of SF-1 in the regulation of the genes encoding steroidogenic enzymes in vivo, since animals deficient in SF-1 lack adrenal glands (18, 28).

Thus, while in vitro experiments demonstrated that NGFI-B has an important influence on expression of *P450_{c21}* through the -65 element, it appears that its function can be replaced by other transcription factors in vivo. If *P450_{c21}* expression depends strongly on Nurr1 in the absence of NGFI-B, then a dramatic reduction in *P450_{c21}* expression may be seen in animals lacking both NGFI-B and Nurr1 functions. Alternatively, significant disruption of *P450_{c21}* expression may require the removal of other factors, such as COUP-TF, which may bind the -210 half-site on the *P450_{c21}* 5'-flanking region (25). The regulation of steroidogenic enzyme gene expression in vivo occurs through complex mechanisms that most likely do not rely on one transcription factor but instead depend on a multitude of *cis* elements and *trans*-activating factors.

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