MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 861–868
0270-7306/93/020861-08$02.00/0

Vol. 13, No. 2

The Orphan Nuclear Receptor NGFI-B Regulates Expression of the Gene Encoding Steroid 21-Hydroxylase

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Received 20 July 1992/Returned for modification 31 August 1992/Accepted 11 November 1992

As part of its trophic action to maintain the steroidogenic capacity of adrenocortical cells, corticotropin (ACTH) increases the transcription of the cytochrome P-450 steroid hydroxylase genes, including the gene encoding steroid 21-hydroxylase (21-OHase). We previously identified several promoter elements that regulate 21-OHase gene expression in mouse Y1 adrenocortical tumor cells. One of these elements, located at nucleotide −65, closely resembles the recognition sequence of the orphan nuclear receptor NGFI-B, suggesting that NGFI-B regulates this essential steroidogenic enzyme. To explore this possibility, we first used in situ hybridization to demonstrate high levels of NGFI-B transcripts in the adrenal cortex of the adult rat. In cultured mouse Y1 adrenocortical cells, treatment with ACTH, the major regulator of 21-OHase transcription, rapidly increased NGFI-B expression. Gel mobility shift and DNase I footprinting experiments showed that recombinantly expressed NGFI-B interacts specifically with the 21-OHase −65 element and identified one complex formed by Y1 extracts and the 21-OHase −65 element that contains NGFI-B. Expression of NGFI-B significantly augmented the activity of the intact 21-OHase promoter, while mutations of the −65 element that abolish NGFI-B binding markedly diminished NGFI-B-mediated transcriptional activation. Specific mutations of NGFI-B shown previously to impair either DNA binding or transcriptional activation diminished the effect of NGFI-B coexpression on 21-OHase expression. Finally, an oligonucleotide containing the NGFI-B response element conferred ACTH response to a core promoter from the prolactin gene, showing that this element is sufficient for ACTH induction. Collectively, these results identify a cellular promoter element that is regulated by NGFI-B and implicate NGFI-B in the transcriptional induction of 21-OHase by ACTH.

NGFI-B (also called nur77) is a nuclear receptor encoded by an early-response gene that is rapidly induced in cells stimulated with growth factors and/or other extracellular ligands (7, 11). As with many other early-response (or immediate-early) genes that encode transcription factors (e.g., c-Fos, c-Jun, and NGFI-A), the NGFI-B protein presumably regulates the expression of other genes, ultimately culminating in phenotypic changes (reviewed in reference 8). To begin to understand the contribution of these proteins to cellular responses, we must identify target gene(s) whose expression is regulated by the early-response proteins. Toward this end, we recently used a genetic selection approach to identify the nucleotide sequence bound by NGFI-B: AAAGGTCA. This NGFI-B response element (NBRE) contains an estrogen receptor half-site (AGGTCA) preceded by two adenines (21, 22). The identification of this site allowed us to establish that NGFI-B activates transcription of reporter genes in a variety of mammalian cell lines in the absence of exogenously added ligand (13, 21).

Steroid 21-hydroxylase (21-OHase, Cyp21) is one of a group of related cytochrome P-450 enzymes that are required for steroid hormone biosynthesis (reviewed in reference 12). It is expressed only in the adrenal cortex, where it is essential for the production of both glucocorticoids and mineralocorticoids. Within adrenocortical cells, the transcription of 21-OHase is primarily regulated by corticotropin (ACTH), the predominant regulator of glucocorticoid biosynthesis. This ACTH-dependent increase in 21-OHase transcription is mimicked by treatment of adrenocortical cells with cyclic AMP (cAMP) analogs, leading to the hypothesis that cAMP mediates this transcriptional induction (9). In contrast to most other cAMP-regulated genes (17), the ACTH-mediated increase in 21-OHase transcription is manifest only after several hours and requires ongoing protein synthesis, suggesting that ACTH induces early-response genes which then increase 21-OHase transcription (9, 19).

Previous analysis of the promoter region of the mouse 21-OHase gene established that only 330 nucleotides (nt) of the 5′-flanking region were required for cell-specific and hormonally inducible expression (6). Consistent with the distinctive characteristics of 21-OHase induction by cAMP, none of the regulatory elements that were subsequently identified closely matched the consensus of the cAMP-responsive element found in most other cAMP-responsive genes (15, 17). However, essential elements at −210 and −65 contain nuclear receptor half-sites (4), implicating nuclear hormone receptor proteins in 21-OHase gene regulation. Specifically, the element at nt −65 contains 8 nt that correspond to the recognition sequence bound by NGFI-B (NBRE [21]).

In this study, we examine the possibility that NGFI-B regulates the 21-OHase gene. Using in situ hybridization, we showed high levels of NGFI-B mRNA in the zonae fasciculata/reticularis of the adrenal cortex. In mouse Y1 adrenocortical tumor cells, a useful cell culture model of adrenocortical function, ACTH rapidly and markedly increased NGFI-B expression, providing additional support for a role of NGFI-B in adrenocortical function. As predicted by our

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previous definition of the NBRE (21, 22), NGFI-B interacted with the 21-OHase −65 element in gel mobility shift and DNase I footprinting assays. By using a monoclonal antibody to NGFI-B, we demonstrated its presence in one complex that Y1 nuclear extracts form with the 21-OHase −65 element. Cotransfection experiments in Y1 adrenocortical cells showed that NGFI-B increases activity of the 21-OHase promoter in an NBRE-dependent manner. Finally, addition of the NBRE conferred ACTH responsiveness to a heterologous promoter. Collectively, these results establish that NGFI-B regulates 21-OHase, thereby identifying a physiological target for this orphan member of the nuclear receptor family.

MATERIALS AND METHODS

Materials. Restriction and modification enzymes and protease inhibitors were purchased from Boehringer Mannheim. Reagents for cell culture were from GIBCO. Radionuclides were purchased from New England Nuclear-E. I. DuPont. Oligonucleotides were synthesized by the solid-phase phosphoramidite technique by using an Applied Biosystems 380B synthesizer. Reagents for electrophoresis were obtained from Bio-Rad. Levels of human growth hormone (hGH) were measured by radioimmunoassay, using a kit purchased from Nichols Diagnostics. ACTH 1-24 (Cortrosyn) was obtained from Organon, Inc. (West Orange, N.J.). 8-Bromo-cAMP (8-Br-cAMP) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Plasmids. The plasmids used in this study included p21-OHaseGH, which contains 6.4 kb of 5′-flanking sequences from the mouse 21-OHase gene placed upstream of an hGH reporter gene; p21-OHaseGHmTT, a variant of p21-OHase GH in which positions −70 and −69 of the −65 element were changed from AA to TT; p-40GH, which contains the core promoter of the aldosterone synthase gene upstream of an hGH reporter gene (2); p-40GH(−65), which contains two copies of the 21-OHase −65 element (−95 to −58) cloned into the *Hin*dIII site of p-40GH; and p-40GH(−65M), which contains two copies of the 21-OHase −65 element with bases −70 to −66 changed from AGGTC to CTTGA. The NGFI-B expression plasmids were constructed by cloning the NGFI-B cDNA or mutated derivatives thereof into the mammalian expression plasmid pCMV. These plasmids include NGFI-Bwt (21); BΔ25-195, a deletion mutant that lacks residues 25 to 195 (13); and two DNA-binding-deficient mutants, BmC283A, in which Cys-283 was converted to Ala, and BmR345S,L348A, which was produced by converting Arg-345 to Ser and Leu-348 to Ala. The vector Pro36-luc contains the firefly luciferase coding region under the control of a minimal prolactin promoter (1). Plasmid NBRE-luc contains eight copies of the NBRE-containing B1a oligonucleotide upstream of the prolactin promoter in Pro36-luc (21). Plasmid p-65-luc contains five copies of the 21-OHase −65 element upstream of the prolactin promoter in this same vector.

Gel mobility shift and DNase I footprinting assays. Gel mobility shift assays were performed essentially as described previously (2), with 4 μg of poly(dI · dC-dI · dC) as the nonspecific competitor and 5 to 10 μg of crude nuclear extract. DNase I footprinting experiments were performed as described elsewhere (15), using an end-labeled probe containing 21-OHase sequences from −330 to +8. Nuclear extracts from Y1 adrenocortical cells were prepared by the method of Shapiro et al. (18). Whole cell extracts were prepared from hsB-CHO cells (which contain multiple copies of a plasmid in which NGFI-B expression is under control of the heat shock protein 70 promoter) that were either maintained at 37°C or heat shocked at 42°C for 1 h and then incubated for 1 h at 37°C (21). The effect of an anti-NGFI-B monoclonal antibody on the gel mobility shift interactions was assessed as previously described (5).

Cell culture. In experiments with the hGH reporter gene, Y1 adrenocortical cells were cultured and transfected by the $CaPO_4$ precipitation technique as previously described (15), using 2.5 μg of the reporter gene plasmid and 5 μg of the various mixtures of the pCMV and NGFI-B expression plasmids. Levels of hGH expression were determined 48 h after transfection. In experiments with the luciferase reporter genes, Y1 cells were transfected with 2 μg of the luciferase promoter plasmid and 4 μg of carrier DNA. Forty-eight hours after transfection, cells were treated with ACTH (25 mU/ml) or 8-Br-cAMP (1 mM) for an additional 7 h. Following hormone treatments, cell lysates were prepared and assayed as previously described (21). For the time course of ACTH induction, cells were treated with ACTH (10 mU/ml) for the indicated times before RNA was harvested and analyzed in Northern (RNA) blotting experiments. Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

In situ hybridization analysis. The adrenal glands were removed from an adult Sprague-Dawley rat and fixed in 4% paraformaldehyde. In situ hybridization was performed by using frozen sections as previously described (20). The NGFI-B antisense (nt 2112 to 1883) and sense (nt 1538 to 1920) probes (11) were synthesized by using [α-^{35}S]UTP (Amersham) and riboprobe transcription reagents (Promega) as instructed by the manufacturers.

RESULTS

NGFI-B is expressed at high levels in the adrenal cortex. Because NGFI-B was initially isolated from a rat pheochromocytoma cDNA library (11), we anticipated that NGFI-B mRNA present in the adrenal gland reflected expression in the neural crest-derived chromaffin cells of the adrenal medulla. Surprisingly, analysis of NGFI-B expression in the adrenal gland by in situ hybridization showed that NGFI-B was predominantly expressed in the adrenal cortex (Fig. 1). These studies demonstrated high levels of NGFI-B mRNA in the zonae fasciculata/reticularis, the predominant site of glucocorticoid production, with lower expression in the zona glomerulosa and negligible expression in the adrenal medulla. No signal above background was observed with the sense probe under the hybridization conditions used (11a). Similar results were obtained in immunohistochemical studies, indicating that NGFI-B protein is also expressed in the adrenal cortex (11a). These results thus suggest a role of NGFI-B in adrenocortical function.

ACTH rapidly induces levels of NGFI-B mRNA in Y1 adrenocortical cells. In the adrenal cortex, ACTH regulates both glucocorticoid production and transcription of the enzymes involved in glucocorticoid synthesis (12). Because of these key roles of ACTH and because NGFI-B is induced by a variety of growth factors and hormones (5, 7, 8), we next examined the effect of ACTH on NGFI-B expression in Y1 adrenocortical cells. ACTH increased steady-state levels of NGFI-B mRNA within 30 min after administration, and peak levels were attained 1 to 2 h later (Fig. 2). A comparable induction of NGFI-B expression followed treatment of Y1 cells with 8-Br-cAMP (12a). The rapid induction of

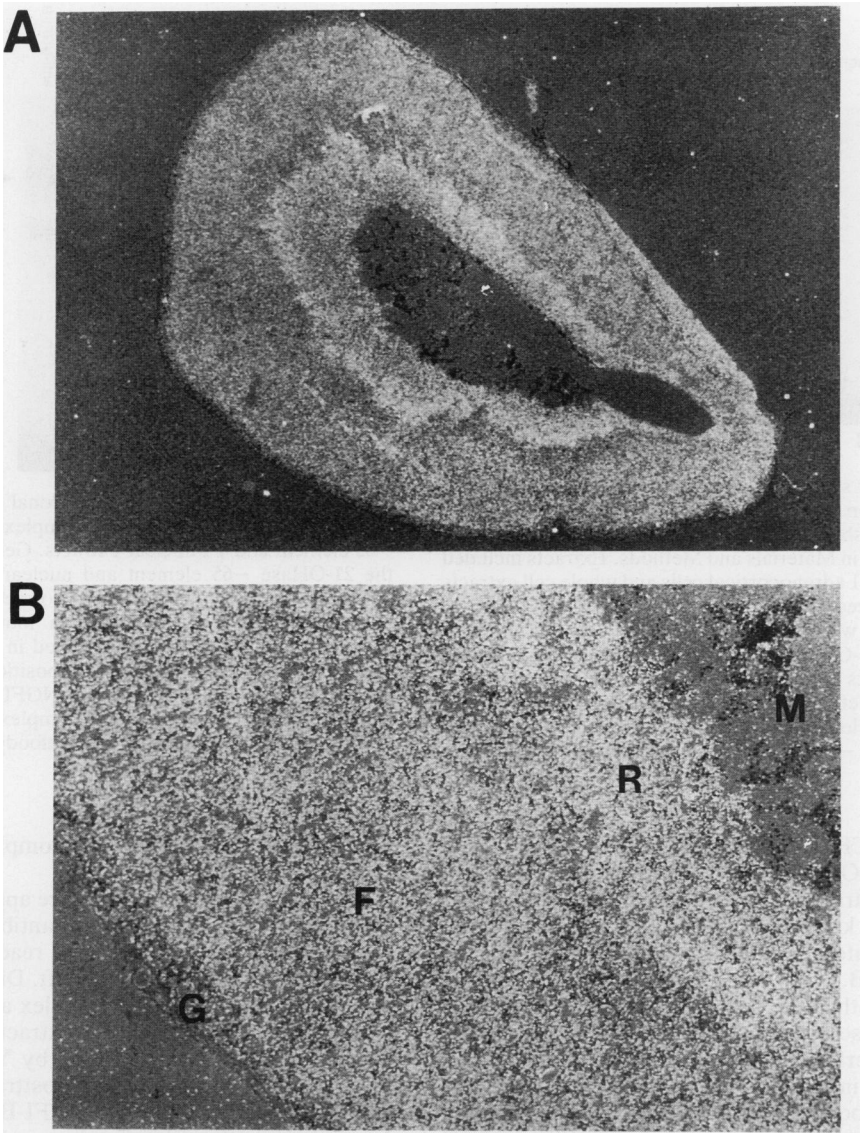

FIG. 1. High-level expression of NGFI-B in adrenocortical cells. In situ hybridization analysis of NGFI-B in the adrenal cortex was performed as described in Materials and Methods. (A) Dark-field examination at low-power magnification; (B) dark-field examination at high-power magnification. M, medulla; R, zona reticularis; F, zona fasciculata; G, zona glomerulosa.

NGFI-B by ACTH in Y1 cells clearly precedes that of the steroidogenic cytochrome P-450 enzymes, which in ACTH-treated Y1 cells do not achieve maximal steady-state mRNA levels until 9 to 24 h (3, 14), suggesting that NGFI-B may be an intermediary in ACTH action.

NGFI-B binds directly to the 21-OHase −65 promoter element. Previous analyses of the transcriptional regulation of the genes encoding adrenal steroidogenic enzymes identified a number of promoter elements that contained variations of a nuclear receptor half-site (i.e., AGGTCA) (16). In particular, the sequence of the mouse 21-OHase −65 element contains an NBRE (AAAGGTCA), the recognition sequence for the nuclear receptor NGFI-B (21). To test whether NGFI-B interacts directly with this element, we performed gel mobility shift assays with the 21-OHase −65 element, using whole cell extracts from hsB-CHO cells, which have low endogenous levels of NGFI-B but express high levels of NGFI-B in response to heat shock (21).

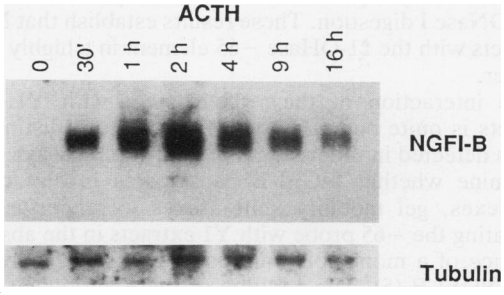

FIG. 2. Evidence that ACTH treatment of Y1 adrenocortical cells rapidly increases NGFI-B mRNA levels. Mouse Y1 adrenocortical tumor cells were treated with ACTH (10 mU/ml) for the indicated times, and total cellular RNA was prepared as described in Materials and Methods. Northern blotting analyses were performed by using 15 μg of each RNA sample and a mouse NGFI-B cDNA probe. The same filter was hybridized with a probe for α-tubulin to control for RNA loading and transfer.

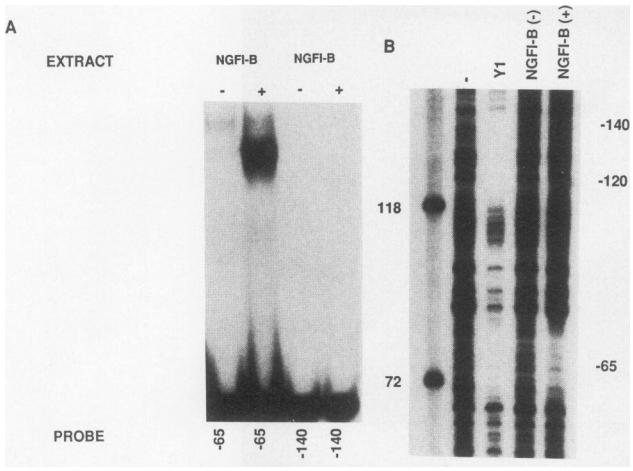

FIG. 3. Gel mobility shift and DNase I footprinting assays demonstrating an interaction between NGFI-B and the 21-OHase −65 element. Gel mobility shift and DNase I footprinting assays were performed as described in Materials and Methods. Extracts included nuclear extracts from Y1 adrenocortical cells and whole cell extracts prepared from uninduced (NGFI-B −) and induced (NGFI-B +) CHO cells transfected with pHSP-NGFI-B (21). (A) Gel mobility shift assays with the 21-OHase −65 and −140 probes; (B) DNase I footprinting experiments with a −330/+8 21-OHase promoter fragment. Numbers at the left indicate the positions of size markers of the indicated sizes (nucleotides). Positions of the footprints involving the 21-OHase −65, −120, and −140 elements are indicated.

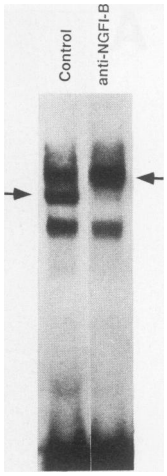

FIG. 4. Evidence that a monoclonal antibody against NGFI-B diminishes the intensity of one complex formed by the 21-OHase −65 element and Y1 nuclear extracts. Gel mobility shift assays with the 21-OHase −65 element and nuclear extracts from mouse Y1 adrenocortical tumor cells were performed as described in Materials and Methods. Where indicated, a mouse monoclonal antibody specific for NGFI-B (5) was included in the binding reaction. The arrow on the left indicates the position of a complex that is specifically diminished by the anti-NGFI-B antibody; the arrow on the right shows the position of a complex whose apparent intensity is increased by the anti-NGFI-B antibody.

Extracts from hsB-CHO cells formed a prominent shifted complex with the 21-OHase −65 element, while no complex was detected with extracts from non-heat-shocked cells (Fig. 3A). Consistent with known requirements for NGFI-B binding, the closely related 21-OHase −140 element did not interact with NGFI-B.

To determine whether the sequences bound by NGFI-B corresponded to those previously defined as the 21-OHase −65 element, we performed DNase I footprinting experiments. As defined in previous studies (15), Y1 nuclear extracts produced footprints centered at −65, −120, and −140 (Fig. 3B) and at −210 (data not shown). The extract from heat-shocked hsB-CHO cells protected a region at −65 that is similar to the footprint obtained with Y1 extracts but did not protect the region at −120 or −140 (Fig. 3B) or at −210 (data not shown). The control hsB-CHO extract did not protect any region of the 21-OHase promoter fragment from DNase I digestion. These results establish that NGFI-B interacts with the 21-OHase −65 element in a highly specific manner.

The interaction of the −65 element with Y1 nuclear extracts is quite complex, with three or four distinct complexes detected in different gel mobility shift assays (15). To determine whether NGFI-B participates in any of these complexes, gel mobility shift assays were performed by incubating the −65 probe with Y1 extracts in the absence or presence of a monoclonal antibody that specifically recognizes NGFI-B (5). The addition of these antibodies specifically diminished the intensity of a discrete complex of intermediate mobility while apparently increasing the intensity of a less mobile complex (Fig. 4). Similar experiments with an antibody against steroidogenic factor 1 (SF-1), a second nuclear receptor that interacts with the −65 element, confirmed the specificity of this effect. The anti-SF-1 antibody did not affect the intermediate complex but specifically inhibited the formation of the complex of greatest mobility (12a).

The complex whose abundance appeared to increase upon addition of the anti-NGFI-B antibody coincided with a complex seen in the control Y1 reaction, precluding definitive demonstration of a supershift. Direct comparisons of the anti-NGFI-B-sensitive Y1 complex and the complex formed by heat shock-induced CHO extracts were complicated by the multiple complexes formed by Y1 nuclear extracts and by potential differences in the posttranslational modification and/or primary structure of NGFI-B from the two sources. Nevertheless, the selective disappearance of the intermediate complex upon incubation with the anti-NGFI-B antibodies strongly implicates NGFI-B in its formation.

Coexpression of NGFI-B augments the activity of the 21-OHase promoter region in transfected Y1 cells. The studies described above identified the 21-OHase −65 element as a likely target for NGFI-B induction. To explore further the role of NGFI-B in 21-OHase gene regulation, we analyzed its ability to affect the activity of the 21-OHase −65 element in Y1 adrenocortical cells. Y1 cells were cotransfected with the NGFI-B expression plasmid (NGFI-Bwt) in various doses and different derivatives of p-40GH, a plasmid which contains the core promoter of the aldosterone synthase gene directing the expression of the hGH reporter gene. As detailed in Materials and Methods, the derivatives included p-40GH(−65), which contains two copies of the −65 element, and p-40GH(−65M), which contains two copies of the −65 element in which the half-site has been mutated, thus destroying the ability to bind NGFI-B. Coexpression of NGFI-B markedly augmented the expression of p-40GH (−65) in a dose-dependent manner, with up to a 12-fold increase at the highest dose (Fig. 5). In contrast, no significant effects of NGFI-B were seen with the parental plasmid or with p-40GH(−65M), which contains the mutated NBRE.

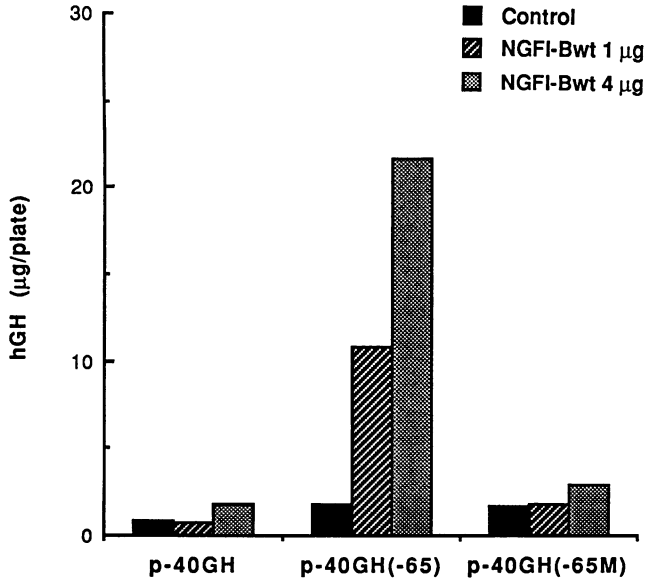

FIG. 5. Activation by NGFI-B of transcription from the 21-OHase −65 element in transfected Y1 adrenocortical cells. Y1 cells were transfected with the minimal promoter construct (p-40GH), with p-40GH containing two copies of the −65 element [p-40GH(−65)], or with p-40GH containing two copies of a mutated −65 element [p-40GH(−65M)] as described in Materials and Methods. To maintain a constant amount of DNA in each transfection, cells were cotransfected with various ratios of pCMV and the NGFI-B expression plasmid (NGFI-Bwt). Levels of hGH expression were determined by radioimmunoassay 48 h after transfection and are reported as the means of four independent transfections.

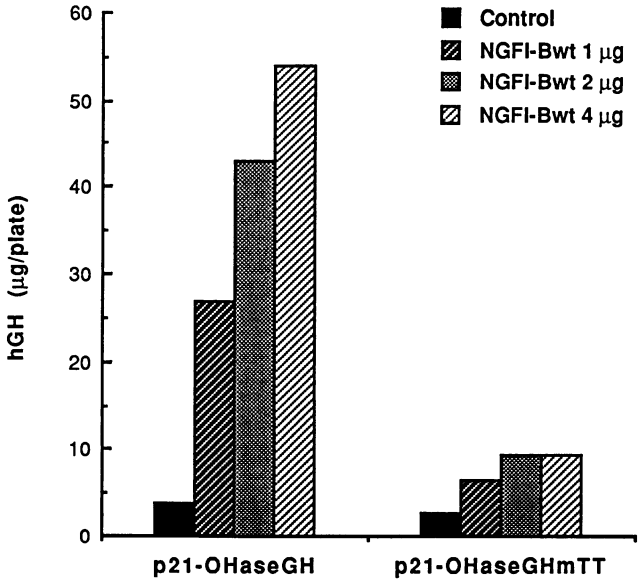

FIG. 6. Evidence that transcription from the 21-OHase promoter is increased by NGFI-B. Y1 adrenocortical cells were cotransfected with NGFI-Bwt and p21-OHaseGH, which contains the intact 21-OHase promoter driving hGH reporter gene expression, or with a derivative (p21-OHaseGHmTT) in which the NBRE at −65 was mutated. In all cases, the total amount of DNA was kept constant by the addition of plasmid pCMV. Levels of hGH expression were determined by radioimmunoassay 48 h after transfection and are reported as the means of four independent transfections.

Having established that NGFI-B activates transcription from the −65 element, we next investigated its effect on the intact 21-OHase promoter. Cotransfection experiments were performed in Y1 adrenocortical cells by using p21-OHaseGH, which contains 6.4 kb of 5′-flanking sequences from the mouse 21-OHase gene placed upstream of the hGH reporter gene, and NGFI-Bwt. NGFI-B expression markedly augmented the activity of the 21-OHase promoter region, with a 14-fold increase at the highest dose of NGFI-B (Fig. 6). To confirm the importance of the NGFI-B/−65 element interaction in this effect, we took advantage of a distinctive feature of NGFI-B. High-affinity binding of NGFI-B requires nucleotides 5′ of the half-site (21, 22). We therefore used a reporter construct (p21-OHaseGHmTT) that is identical to p21-OHaseGH except that two adenine residues immediately 5′ of the AGGTCA half-site in the −65 element were mutated to thymidines. NGFI-B does not bind to this mutated NBRE in vitro (data not shown), and cotransfection experiments with this plasmid demonstrated that both basal expression and NGFI-B-stimulated expression from this promoter are significantly reduced (Fig. 6).

The identification of a cellular target of NGFI-B induction allowed us to confirm in a more physiological setting previous structure-function studies of NGFI-B that used an idealized NBRE (13, 22). We therefore tested several variants of NGFI-B that lack previously characterized DNA-binding and/or transcriptional activities. One mutation (Cys-283 to Ala) alters a residue in the zinc finger region that is critical for DNA binding. As expected, if NGFI-B is acting directly, this plasmid (BmC283A) does not enhance transcription from the 21-OHase −65 element (Fig. 7). Interestingly, the activity of the mutated NGFI-B plasmid BmR345S,L348A, which contains a mutation in the A box that impairs DNA binding in vitro (21; data not shown), was also reduced, but not to the same degree observed with BmC283A. Finally, a mutation (BΔ25-195) that is unable to activate transcription from the NBRE linked to a heterologous promoter (13) was also totally inactive when tested against the 21-OHase −65 element.

NBREs render a heterologous promoter responsive to ACTH. To examine more directly the effect of ACTH on NBREs, we analyzed the ability of these sequences to confer ACTH responsiveness to a heterologous promoter in transfected Y1 cells. We first analyzed the effect of ACTH on NBRE-luc, a plasmid containing eight copies of the NBRE-containing B1a oligonucleotide (13) upstream of the prolactin core promoter and a luciferase reporter gene (21). The parental plasmid (Pro36-luc) directed very low levels of luciferase expression in transfected Y1 cells and did not show significant response to ACTH (data not shown). NBRE-luc directed very low levels of basal expression of luciferase in transfected Y1 cells, consistent with the low basal levels of NGFI-B expression. Luciferase expression was markedly increased upon treatment with either ACTH (22-fold) or 8-Br-cAMP (18-fold). Thus, ACTH treatment significantly increases the activity of an NBRE, supporting the role of NGFI-B in ACTH action.

We next examined the effect of ACTH on the 21-OHase −65 element. Y1 adrenocortical cells were transfected with p-65-luc, which contains five copies of the 21-OHase −65 element upstream of the prolactin promoter and luciferase reporter gene. The −65 element significantly increased the basal expression of the luciferase reporter gene (data not shown). This effect on constitutive expression presumably reflects its ability to interact with SF-1, another important

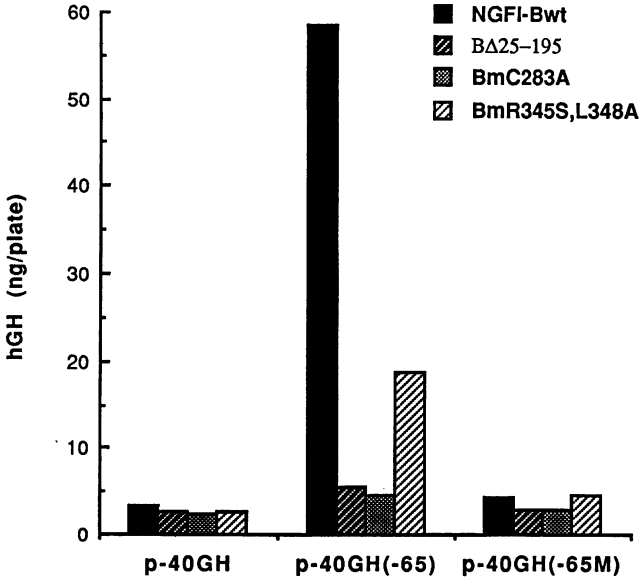

FIG. 7. Evidence that mutated NGFI-B plasmids are markedly impaired in their ability to activate the 21-OHase promoter. Y1 adrenocortical cells were transfected with p-40GH, p-40GH(−65), or p-40GH(−65M) as described in Materials and Methods. Cells were cotransfected with 4 μg of either NGFI-Bwt or mutated NGFI-B expression plasmids. These mutants included NGFI-BΔ25-195, which lacks a transcriptional activation domain; BmC283A, which contains a mutation in a critical Cys in the zinc finger region; and BmR345S,L348A, in which two residues within the A box have been mutated. Levels of hGH expression 48 h after transfection were determined by radioimmunoassay and are reported as the means of three independent transfections.

regulator of steroid hydroxylase gene expression (10). Even with this higher basal expression, ACTH and 8-Br-cAMP treatments both significantly increased expression. Treatment with either ACTH or 8-Br-cAMP led to sevenfold increases in luciferase expression directed by p-65-luc. These results thus demonstrate direct effects of ACTH on activities of both an idealized NBRE and the 21-OHase −65 element, strongly supporting the importance of this nuclear receptor in ACTH action.

DISCUSSION

Studies of the transcriptional regulation of the mouse 21-OHase gene identified several elements in the 5′-flanking region which bind factors present in Y1 adrenocortical cell extracts and which are necessary for expression of the 21-OHase gene in transfection experiments (15). One element, located at nt −65, includes a sequence known as a half-site that is present in the recognition sites of nuclear receptors. The disruption of this half-site markedly impaired promoter activity (15), suggesting that a nuclear receptor was involved in 21-OHase regulation. The subsequent identification of the recognition site for the nuclear receptor NGFI-B as AAAGGTCA (21, 22), the precise sequence present in the −65 element, led us to investigate its role in 21-OHase expression. We observed that high levels of NGFI-B are present in the adrenal cortex of the adult rat. In vitro DNA-binding assays demonstrated that recombinantly expressed NGFI-B forms a complex with the −65 element with the same specificity as a naturally occurring, NGFI-B-containing complex seen with Y1 adrenocortical nuclear extracts. In addition, cotransfection experiments in Y1 cells demonstrated that NGFI-B stimulates 21-OHase promoter activity in a fashion that requires the −65 element. Collectively, these results implicate NGFI-B in the regulation of 21-OHase gene expression, thus identifying a target gene for this immediate-early member of the nuclear receptor family.

The response of adrenocortical cells to ACTH, the predominant regulator of glucocorticoid biosynthesis, can be divided temporally into two phases (19). The acute phase occurs within seconds to minutes and largely reflects increased delivery of cholesterol substrate to the side chain cleavage enzyme. In contrast, the chronic response requires hours to days and largely involves increased transcription of the genes encoding the steroidogenic cytochrome P-450 enzymes. Although treatment of adrenocortical cells with cAMP analogs mimics the chronic response, the increase in transcription of genes encoding steroidogenic enzymes is delayed relative to that of most other cAMP-responsive genes and is blocked by inhibitors of protein synthesis (9). In addition, the 5′-flanking regions of these genes generally lack sequences similar to known cAMP-responsive elements (17). It therefore seems likely that an intermediate step requiring de novo protein synthesis, such as the induction of an early-response gene product, is involved in the regulation of these genes by ACTH.

The results presented here show that ACTH rapidly activates the expression of the nuclear receptor NGFI-B in Y1 adrenocortical cells. They further demonstrate direct effects of NGFI-B on a promoter element that regulates the expression of 21-OHase, one of these ACTH-induced steroidogenic cytochrome P-450 enzymes. Finally, they show direct effects of ACTH on reporter genes containing either an idealized NBRE or the 21-OHase −65 element. Although definitive proof that the NGFI-B/−65 element interaction regulates ACTH induction in the intact animal will require documentation that a 21-OHase transgene lacking nucleotides essential for NGFI-B binding is impaired in ACTH induction, these results provide strong evidence that NGFI-B is an important early-response component of ACTH induction of this essential steroidogenic enzyme.

We previously used an idealized NBRE that lacks known biological function to study structure-function relationships of the NGFI-B protein, including requirements for DNA binding (22) and transcriptional activation (13). In this report, we verify that these same structure-function relationships apply to NGFI-B-dependent transcription of a bona fide cellular promoter element. First, it is clear that the amino terminus of NGFI-B is absolutely required for transcriptional activation (mutation BΔ25-195 in Fig. 7). Second, these results with a cellular promoter element support a role of the apparent monomer/DNA-binding modality identified for NGFI-B, which is atypical for nuclear receptors, in transcriptional activation. Mutations either of nucleotides outside of the NBRE half-site (p21-OHasemTT) or of the A-box residues downstream of the zinc fingers (BmR345S,L348A) significantly affected activity from the 21-OHase promoter. In neither case, interestingly, was the activity decreased to the extent seen with a mutation of a zinc finger cysteine (BmC283A); the lesser degrees of impairment may reflect a degree of flexibility in the mechanism of DNA binding by NGFI-B that is unavailable in the Cys mutation.

Previous studies implicated another nuclear receptor, COUP-TF, as a component of certain gel mobility shift complexes that Y1 adrenocortical extracts form with the steroidogenic regulatory elements (16). The demonstration

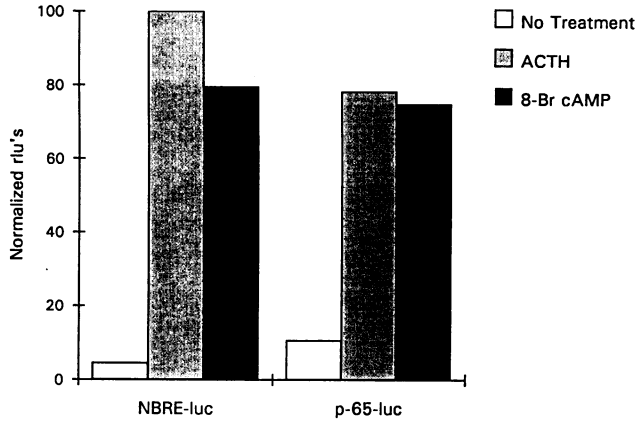

FIG. 8. Evidence that ACTH increases the activity of the 21-OHase −65 element. Y1 adrenocortical cells were transfected either with NBRE-luc, which contains eight copies of an idealized NBRE upstream of the core promoter of the prolactin gene driving the firefly luciferase gene, or with p-65-luc, which contains five copies of the 21-OHase −65 element cloned upstream of the prolactin promoter/luciferase sequences. Where indicated, ACTH (25 mU/ml) or 8-Br-cAMP (1 mM) was added to the culture medium at 48 h after transfection, and luciferase activity was determined following an additional 7 h. For a given transfection experiment, relative luciferase units (rlu's) were normalized to the value obtained for NBRE-luc with ACTH treatment to allow comparisons between experiments. Results are presented as the means of three independent transfections.

that NGFI-B regulates the expression of 21-OHase thus increases the evidence linking the nuclear receptor family and steroidogenic enzyme expression. This link is of great interest for several reasons. First, it raises the possibility that NGFI-B and other nuclear receptors have a more generalized role in transcriptional regulation of the overall class of ACTH-inducible genes. With regard to NGFI-B, this putative role in regulating the body's response to stress provides a possible rationale for its identity as an early-response protein (i.e., one that is synthesized in response to environmental change). Second, the great potential for competition (at the same or overlapping half-sites) and cooperativity (via homo- or heterodimerization) within the nuclear receptor family provides a possible mechanism for implementing the complex regulatory circuits that control steroidogenic enzyme expression. In this regard, it is intriguing that a number of steroidogenic regulatory elements, including the 21-OHase −65 element, interact with a protein designated SF-1, a nuclear receptor whose expression in the adult mouse is limited to steroidogenic cell types (10). Although our transfection studies (Fig. 8) suggest that both NGFI-B and SF-1 bind the 21-OHase −65 element and increase transcription, further studies will be needed to provide a better understanding of cooperative and/or antagonistic interactions of these two transcriptional regulators on the endogenous 21-OHase promoter in adrenocortical cells. Finally, the link between orphan receptor action and control of steroidogenic enzyme gene expression raises the intriguing possibility that metabolic intermediates of steroid production serve as ligands for these orphan receptors.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant NS01018 from the National Institute of Neurological and Communicative Disorders and Stroke, grant P01 CA49712 from the National Cancer Institute, and a grant from Ronald McDonald Children's Charities (to J.M.) and by a grant from the Howard Hughes Medical Institute (to K.L.P.).

We thank Bernard Schimmer for helpful discussions, Sue Frese and Steven Carroll for help with the in situ hybridization analysis, and Jeana Meade for superb technical assistance.

REFERENCES

1. **Adler, S., M. L. Waterman, X. He, and M. G. Rosenfeld.** 1988. Steroid receptor mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. Cell **52**:685–695.
2. **Bogerd, A., D. A. Rice, A. Franklin, B. P. Schimmer, and K. L. Parker.** 1990. Identification and characterization of two upstream elements that regulate adrenocortical expression of steroid 11β-hydroxylase. Mol. Endocrinol. **4**:845–850.
3. **Domalik, L. J., D. D. Chaplin, M. S. Kirkman, R. C. Wu, W. Liu, T. A. Howard, M. F. Seldin, and K. L. Parker.** 1991. Different isozymes of mouse 11β-hydroxylase produce mineralocorticoids and glucocorticoids. Mol. Endocrinol. **5**:1853–1861.
4. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240**:889–895.
5. **Fahrner, T. J., S. L. Carroll, and J. Milbrandt.** 1990. The NGFI-B protein, an inducible member of the thyroid/steroid receptor family, is rapidly modified posttranslationally. Mol. Cell. Biol. **10**:6454–6459.
6. **Handler, J. D., B. P. Schimmer, T. R. Flynn, M. Szyf, J. G. Seidman, and K. L. Parker.** 1988. An enhancer element and a functional cAMP-dependent protein kinase are required for expression of adrenocortical 21-hydroxylase. J. Biol. Chem. **263**:13068–13073.
7. **Hazel, T. G., D. Nathans, and L. F. Lau.** 1988. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc. Natl. Acad. Sci. USA **85**:8444–8448.
8. **Herschman, H. R.** 1991. Primary response genes induced by growth factors and tumor promoters. Annu. Rev. Biochem. **60**:281–319.
9. **John, M. E., M. C. John, V. Boggaram, E. R. Simpson, and M. R. Waterman.** 1986. Transcriptional regulation of steroid hydroxylase genes by ACTH. Proc. Natl. Acad. Sci. USA **83**:4715–4719.
10. **Lala, D. S., D. A. Rice, and K. L. Parker.** 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. Mol. Endocrinol. **6**:1249–1258.
11. **Milbrandt, J.** 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron **1**:183–188.

11a. **Milbrandt, J.** Unpublished data.

12. **Miller, W. L.** 1988. Molecular biology of steroid hormone biosynthesis. Endocrine Rev. **9**:295–318.

12a. **Parker, K.** Unpublished data.

13. **Paulsen, R. E., C. A. Weaver, T. J. Fahrner, and J. Milbrandt.** 1992. Domains regulating transcriptional activity of the inducible orphan receptor NGFI-B. J. Biol. Chem. **267**:16491–16496.
14. **Rice, D. A., L. D. Aitkens, G. R. Vandenbark, A. R. Mouw, A. Franklin, B. P. Schimmer, and K. L. Parker.** 1989. A cAMP-responsive element regulates expression of the mouse steroid 11β-hydroxylase gene. J. Biol. Chem. **264**:14011–14015.
15. **Rice, D. A., M. S. Kronenberg, A. R. Mouw, L. D. Aitken, A. Franklin, B. P. Schimmer, and K. L. Parker.** 1990. Multiple regulatory elements determine adrenocortical expression of steroid 21-hydroxylase. J. Biol. Chem. **265**:8052–8059.
16. **Rice, D. A., A. R. Mouw, A. Bogerd, and K. L. Parker.** 1991. A shared promoter element regulates the expression of three steroidogenic enzymes. Mol. Endocrinol. **5**:1552–1561.
17. **Roesler, W. J., G. R. Vandenbark, and R. W. Hansen.** 1988. Cyclic AMP and the induction of eukaryotic gene expression. J. Biol. Chem. **263**:9063–9066.
18. **Shapiro, D. J., P. A. Sharp, W. Wahli, and M. J. Keller.** 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA **7**:47–55.

19. **Simpson, E. R., and M. R. Waterman.** 1988. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu. Rev. Physiol. **50:**427–440.
20. **Wanaka, A., E. M. Johnson, and J. Milbrandt.** 1990. Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization. Neuron **5:**267–281.
21. **Wilson, T. E., T. J. Fahrner, M. Johnston, and J. Milbrandt.** 1991. Identification of the binding site for NGFI-B by genetic selection in yeast. Science **252:**1296–1299.
22. **Wilson, T. E., R. E. Paulsen, K. A. Padgett, and J. Milbrandt.** 1992. Participation of non-zinc finger residues in DNA binding by two orphan receptors. Science **256:**107–110.