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Germ Cell Nuclear Factor Is a Response Element-Specific Repressor of Transcription[1]

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We have shown that the orphan receptor Germ Cell Nuclear Factor (GCNF) binds to a direct repeat of the sequence AGGTCA with zero base pair spacing (DR0). Here, we further characterize the binding characteristics of GCNF. We demonstrate that GCNF binds specifically to DR0s as a homodimer, and does not bind with high affinity to DR1-DR6 sequences. GCNF is the first nuclear receptor shown to bind specifically to DR0s. The wild type GCNF is unable to transactivate the reporter plasmid DR0(2)tkCAT. Lacking a ligand to activate GCNF, we fused the activation domain from the viral protein VP16 to GCNF, and observed activation of DR0(2)tkCAT. This activation is specifc to DR0s, and is not observed when that sequence is replaced by DR1-DR6 sequences. In addition GCNF does not transactivate through an SF-1 response element. At increasing concentrations, wild type GCNF is able to repress basal transcription. Repression is again specific to DR0s. The preference of GCNF for the DR0 sequence both *in vitro* and in transfections suggests that GCNF defines a novel nuclear receptor signaling pathway.

Germ cell nuclear factor (GCNF), an orphan receptor is expressed in the adult mouse predominantly in germ cells in the adult (1–3), and during development in the placenta and the central nervous system (4). GCNF is a putative transcription factor, based on homology to the nuclear receptor superfamily of ligand-activated transcription factors (5). These receptors are genetically and structurally related, even though they differ in their ligand-binding properties and are involved in different biological functions. They generally contain a variable N-terminus, that contributes to the transactivation function; a highly conserved DNA-binding domain (DBD), which is responsible for DNA recognition and dimerization; and the conserved C-terminus, which is often involved in ligand binding, nuclear localization, receptor dimerization, silencing, and transactivation (5). Although there is a high degree of conservation among the DBD of nuclear receptors, each nuclear receptor still has specific DNA binding properties that determines the set of genes it regulates. In this way, each receptor defines a unique signaling pathway.

One aspect of orphan receptor function that has been highly characterized is their DNA binding properties (5). The non-steroid binding members of the nuclear receptor superfamily, including the orphan receptors, have been shown to bind to DNA elements that consist of an AGGTCA halfsite (5). While some orphan receptors have been shown to bind to single halfsites (6), the predominant arrangement is as repeats, either direct repeats (DRs) or symmetrical repeats. The spacing between the repeats allows different receptors to specifically recognize response elements (7,8). It has been shown that various nuclear receptors bind to DR sequences with between 1 and 8 bp spacings (5). Thus, it is believed that different nuclear receptors discriminate between genes based on their ability to bind to a DR element with a particular spacing between the repeats. In this way, the endpoint of an orphan receptor's signaling pathway may be defined. Binding to specific DR elements by nuclear receptors can also be determined by the dimerization state of the receptors. Orphan receptors typically bind as either homodimers or as heterodimers with RXR, and many, such as VDR, RAR and TR, do both (9).

We and others have previously shown that GCNF can bind to a DR0 (1,10). In this report we further characterize the DNA binding properties of GCNF, demonstrating that it preferentially binds as a homodimer with high affinity to DR0s and with much lower affinity to the DR1 and HS sequences. We also demon-

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strate its ability to regulate transcriptional activation in a DR0-dependent manner. In the absence of ligand, we demonstrate that GCNF is a transcriptional repressor. We thus postulate that GCNF is the mediator of a novel nuclear receptor signaling pathway involved in regulation of DR0-containing genes.

MATERIALS AND METHODS

Expression vector and reporter plasmid construction. The Hind III/Nco I fragment of pT7β containing the 5′ untranslated region, Kozak sequence and start codon in the β globin gene (11) was subcloned into the plasmid pBSSK$^-$ digested with Hind III/Xho I. To generate ΔN-GCNF the DBD (amino acids 75-140) of GCNF was generated by PCR as previously described (12), so that it had a start codon and Nco I site at its N-terminus inframe with the start codon. The 5′ primer was AGGCCATGGCCTGTCTCATCTGTGGGGAC and the 3′ primer was AGGCCATGGTTGCTCCAGTGATTGGCTTC. The rest of the GCNF cDNA was liberated from bluescript by a Nco I/Xho I digestion (nucleotides 761-1883) (1). The Hind III/Nco I pT7β, Nco I GCNF DBD PCR product and the Nco I/Xho I GCNF cDNA were all ligated into Hind III/Xho I digested pBSSK$^-$ (Stratagene, La Jolla, CA) (13) to form pΔN-GCNF. The GCNF (nucleotides 1-1883) cDNA was subcloned into the Bgl II/Acc65 I sites of the mammalian expression vector pCMV4 to generate pCMV4GCNF (14).

The VP16 activation domain was subcloned in-frame and C-terminal to GCNF. To achieve this, the 3′ end of the GCNF cDNA was modified using PCR (12), the GCNF LBD was amplified by PCR from the Nco I site to the last codon encoding an amino acid (nucleotides 761-1688) using the PCR primers: 5′ primer GGGCCATGGTGACAGCGACCACAGTTC; 3′ primer CCCGGTACCCTCCTTCACCGTACTTGTCTTG. The 5′ primer contained an endogenous Nco I site at 761 to permit subcloning of the rest of the GCNF cDNA. The 3′ primer contains a Kpn I site to allow subcloning of VP16 in-frame with GCNF and removes the endogenous GCNF stop codon. The VP16 activation domain (amino acids 1-84) was generated by PCR from the vector pGLVP (15) using the PCR primers: 5′ primer GCAGGTACCTCCTGCTCCTTC; 3′ primer GGAAAGCTTTCAACTCGTCAATTCCAAGG. The 5′ primer contains a Kpn I site to permit in frame subcloning of the VP16 with GCNF. The 3′ primer contains a stop codon and a Hind III site to subclone into pCMV4GCNF. The expression vector pCMV4GCNF was digested with NcoI and Hind III and the Nco I/Kpn I GCNF and Kpn I/Hind III VP16 PCR products were ligated into the vector to generate pCMV4GCNFVP16. The N-terminal GCNF: VP16 vector, pCF-VP-GCNF, was obtained by fusing the 78 amino acids of the VP16 activation domain (18) preceded by the Flag recognition sequence and the Kozak consensus sequence (17) to the N-terminal end of GCNF and cloning the resulting fragment into pBK-CMV (Stratagene).

The series of direct repeat tkCAT reporters (DR0-DR5(2)tkCAT) was generated by subcloning two copies of the following double stranded oligonucleotides into the Bam HI/Bgl II sites of tkCAT ΔH/N (18). DR0(2)tkLuc and DR6(2)tkLuc contain 2 copies of the DR0 or DR6 repeats, respectively, upstream of the tk promoter and the luciferase gene. p-65-Luc contains five copies of the -65-element from the steroid 21-hydroxylase promoter upstream of the prolactin promoter (19).

DR0: gatccCTTCTCAAGGTCAAGGTCATCTTa
gatctAAGATGACCTTGACCTTGAGAAGg
DR1: gatccAAGGGTTCACCGAAAGTTCACTCGCATa
gatctATGCGAGTGAACTTTCGGTGAACCCTTg
DR2: gatccCTAGAGTTAGGTCATGAGGTCAGTa
gatctACTGACCTCATGACCTAACTCTACg
DR3: gatccGATTCAGGTCAAGGTAGGTCAGCACa
gatctGTGCTGACCTCCTTGACCTGAATCg
DR4: gatccTAGGGGTCAAATAAGGTCAAATGa
gatctCATTTGCCCTTATTTGACCCCTAg
DR5: gatccGATTCAGGTCACCAGGAGGTCAGCa
gatctGCTGACCTCCTGGTGACCTGAATCg

Gel-mobility shift assays. The gel mobility shift assays were essentially performed as previously described (20). GCNF and ΔN GCNF were *in vitro*-translated using a TNT T3-coupled reticulocyte lysate system (Promega, Madison, WI). The oligonucleotides for the EMSA and competition are described above. The oligonucleotides were radiolabeled by a fill-in reaction with either Klenow enzyme or Sequenase enzyme (Amersham, Arlington Heights, IL) in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP (ICN, Costa Mesa, CA). An aliquot of *in vitro* translated protein (2 μl) was incubated with probe (6×10^4 cpm) in binding buffer (1) for 15 min at room temperature in the presence or absence of unlabeled competitor oligonucleotides.

Cell transfections. CV-1 cells, plated to a density of 1.5×10^5 cells/well/6 well plate in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) with 10% stripped fetal bovine serum (FBS), were transfected using the Lipofectin according to the manufacturers instructions (Amersham). The cells were incubated at 37°C, 5% CO_2 for 36 hrs and then harvested, cell lysates prepared and protein determinations made, as previously described (21,25). CAT activity was then assayed using 15 μg total protein from each sample in the presence of butyryl CoA (0.25 mg/ml) and 2×10^5 cpm/sample of (^{3}H)chloramphenicol (specific activity = 37.10 Ci/mmol). Samples were incubated overnight, and the amount of radioactivity in the resulting products was counted.

RESULTS

Analysis of DNA binding properties of GCNF. To investigate the specificity of GCNF DNA binding, oligonucleotides containing direct repeats with 0-5 base pairs separating the halfsites (DR0-DR5) were individually labeled and incubated with *in vitro*-translated GCNF. Whereas a strong GCNF complex was formed on the DR0 probe (Fig. 1A, lane 1) and weaker complexes were formed on the DR1 and HS probes (lanes 2 and 7), no GCNF complexes were observed on the probes containing the sequences for DR2-DR5 (lanes 3-6). These results were confirmed by competition analysis of labeled DR0 oligonucleotide with 50-fold molar excess of DR0-DR5 and HS oligonucleotides (Fig. 1B). Binding to GCNF was competed by the DR0 (lane 3) and HS oligonucleotides (lane 9), but not by the DR1-DR5 oligonucleotides (lanes 4-8). GCNF therefore binds specifically to the DR0 and HS element with high affinity, and to the DR1 element with very low affinity. Thus, GCNF displays specificity of binding for DR0 elements.

To determine whether GCNF binds DNA as a dimer or a monomer we generated an N-terminal deletion mutant (ΔN-GCNF) that is predicted to have a faster mobility in EMSA than the wild type (wt) GCNF. N-terminal truncations generally have little effect on the overall DNA binding capabilities of nuclear receptors (21), to confirm this we performed EMSA analysis (Fig. 2A). In the presence of GCNF, we observed a retarded complex (lane 1) that was specifically competed with increasing amounts of unlabeled DR0 oligonucleotides (lanes 2–5). In the presence of ΔN-GCNF (lanes 6–10) two faster migrating complexes were observed, both

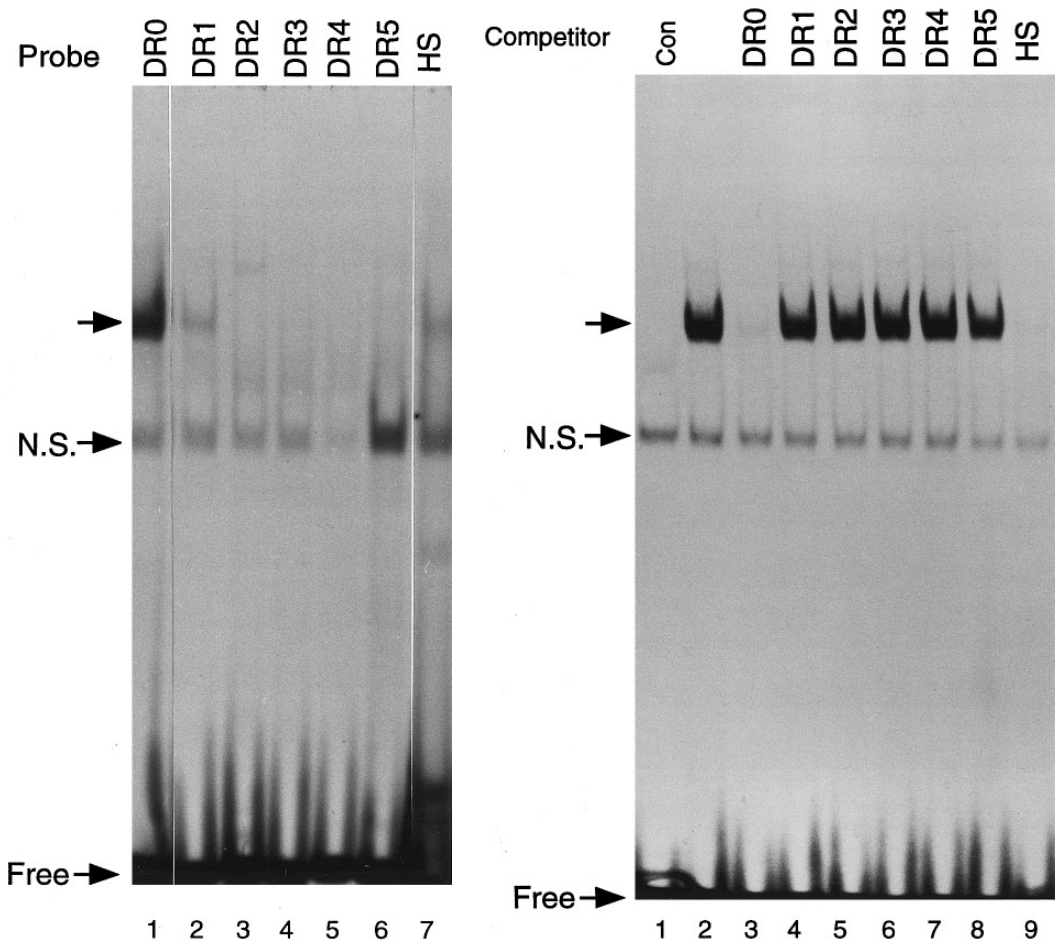

FIG. 1. EMSA analysis of the specificity of binding of GCNF to the DR0 sequence. *In vitro*-translated GCNF was incubated with ^{32}P-labeled oligonucleotide probes containing DR0-DR5 sequences alone (A), or with labeled DR0 probe in the presence or absence of 50-fold molar excess of unlabeled DR0-DR5 and HS oligonucleotides (B). DR = direct repeat; HS = SF-1 halfsite; con = control rabbit reticulocyte lysate; N.S. = non-specific band; arrow = GCNF/DNA complex; free = free probe.

of which were specifically competed for by increasing amounts of the unlabeled oligonucleotide (lanes 7–10). Thus, the affinity of ΔN-GCNF for the DR0 was comparable to that of wtGCNF.

It was expected that a complex consisting of a dimer of wtGCNF and ΔN-GCNF would have an intermediate mobility compared to that of either the wt or ΔN-GCNF homodimers. One retarded complex was observed when DR0 probe was incubated in the presence of wtGCNF (Figure 2B, lanes 2 and 3), while the two ΔN-GCNF complexes were observed (lane 5). When the DR0 probe was incubated in the presence of both wtGCNF and ΔN-GCNF, a complex with intermediate mobility was observed (lane 4). The presence of an intermediate complex clearly indicates that wtGCNF dimerized with ΔN-GCNF on a DR0. The faster migrating complex (arrow labeled monomer) is a monomer of ΔN-GCNF (lanes 4, 5, 10, and 11).

We also examined whether GCNF binds to probes containing either a DR1 or an SF-1 element as a dimer using EMSA analysis (Fig 2B, lanes 6-8 nad 9-11). A complex co-migrating with the intermediate complex observed for the DR0 probe is clearly observed for both the DR1 and HS probes (lanes 7 and 10). GCNF is therefore capable of forming a homodimer on the DR0, the DR1, and the HS sequences. Interestingly, no ΔN-GCNF monomer was observed binding to the DR1 probe (lanes 7 and 8).

GCNF response element specificity and transactivation properties in cells. To analyze the ability of GCNF to transactivate through DR0s in cells, we tested the function of GCNF in transient transfections in CV-1 cells using a CAT reporter construct driven by two copies of the DR0 sequence upstream of the tk promoter (DR0(2)tkCAT). Increasing amounts of pCMV4GCNF plasmid failed to activate transcription of the reporter gene (Fig. 3A left hand panel). These transfections showed that wtGCNF, presumably unliganded, is unable to activate transcription, and therefore is not a constitutively active transcriptional activator.

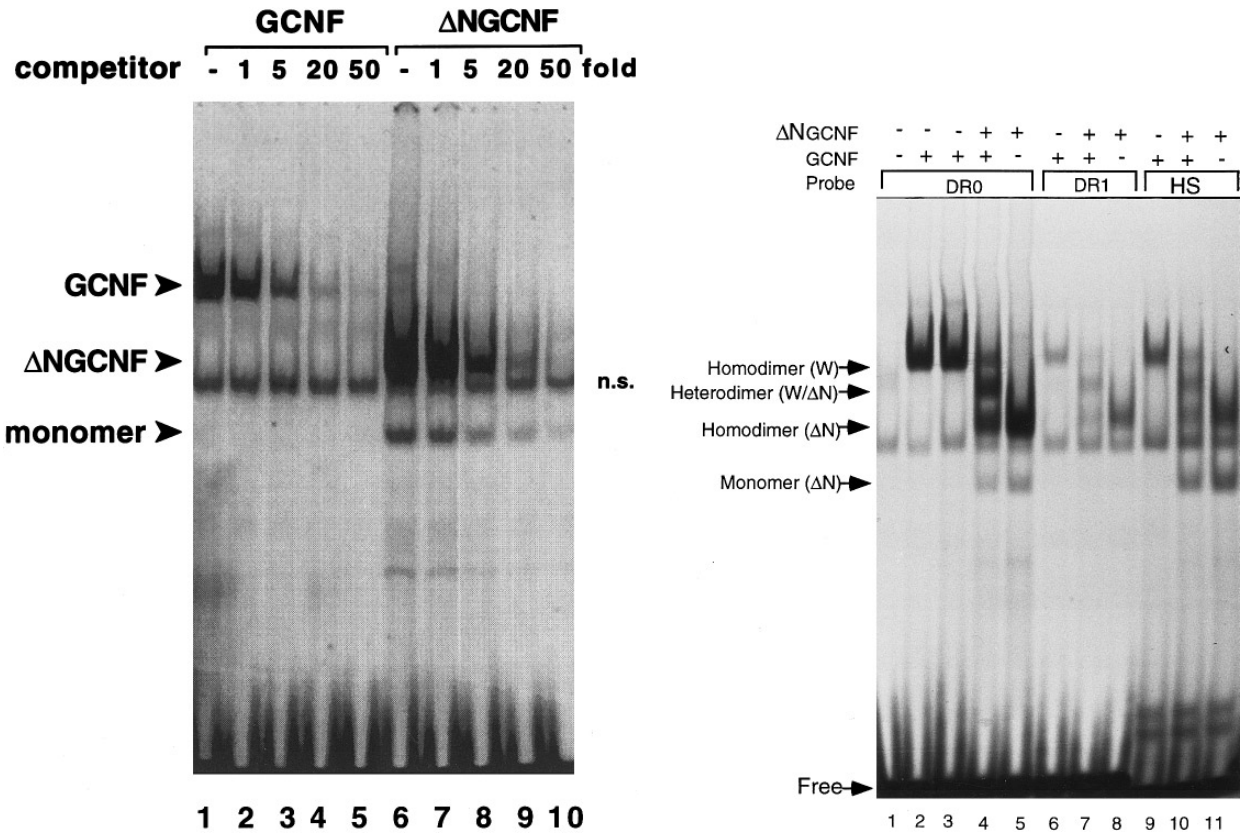

FIG. 2. Analysis of GCNF dimerization. A, Characterization of the ability of ΔN-GCNF to bind to the DR0 sequence. *In vitro*-translated GCNF or ΔN-GCNF were incubated with ^{32}P-labeled DR0 oligonucleotide probe. Unlabeled DR0 was added at 1- to 50-fold molar excess of labeled DR0 probe. B, Analysis of the ability of wtGCNF to dimerize with ΔN-GCNF. *In vitro*-translated GCNF and ΔN-GCNF were incubated singly and in combination in the presence of labeled DR0, DR1, and HS probes. W = wt GCNF; ΔN = ΔN-GCNF.

To circumvent the lack of activation by wtGCNF we fused the transactivation domain from the viral transactivator VP16 N- and C-terminal to GCNF to generate a chimeric factors (pCF-VP-GCNF and pCMV4VP16G-CNF), which we predicted would be constitutive transactivators. The right hand side of figure 3A shows a dose-dependent transactivation of the DR0tkCAT reporter gene by increasing amounts of pCMV4GCN-FVP16 (lanes 2-6). The DBD of GCNF is therefore able to functionally interact with the DR0 sequence in cells.

To determine the specificity of response element-dependent transactivation by GCNF, we co-transfected pCMV4GCNFVP16 with a series of reporters containing DR0-DR6 response elements. Figure 3B shows approximately 12-fold activation of the DR0(2)tkCAT reporter, by pCMV4GCNF-VP16. No activation was observed on any of the plasmids containing DR1-DR5 (Fig. 3B). In addition no transactivation was observed on DR6(2)tkLuc or the SF-1 half site reporter, -65-21OHase(5)pI-Luc with pCMV4VP16GCNF, while a robust response was observed on DR0(2)tkLuc (Fig. 3C). These results indicate that GCNF functionally interacts specifically with the DR0 sequence in cells to transactivate reporter gene expression, confirming the *in vitro* DNA-binding interaction seen in the EMSA analysis.

While wtGCNF does not display a transactivation function, the results in figure 3A show the protential for GCNF-mediated repression. This potential repression function of GCNF was further investigated. The plasmid pCMV4GCNF was co-transfected to higher levels with plasmids for either DR0(2)tkCAT, DR5(2)tkCAT, or the pBLCAT2 (Fig. 4). No reproducible effects were observed on either pBLCAT2 or DR5(2)tkCAT in the presence of increasing amounts of pCMV4GCNF. However, increasing amounts of pCMV4GCNF showed a significant reproducible, dose-dependent decrease in CAT activity from DR0(2)tkCAT. GCNF therefore shows a constitutive repression function on a DR0 element.

DISCUSSION

In order to extend our understanding of the function of the orphan nuclear receptor GCNF and its signaling pathway, we have further characterized its DNA binding and transcriptional properties. The preference of GCNF for binding and transactivation through a DR0 elements and the lack of activity on the DR1 or SF-1

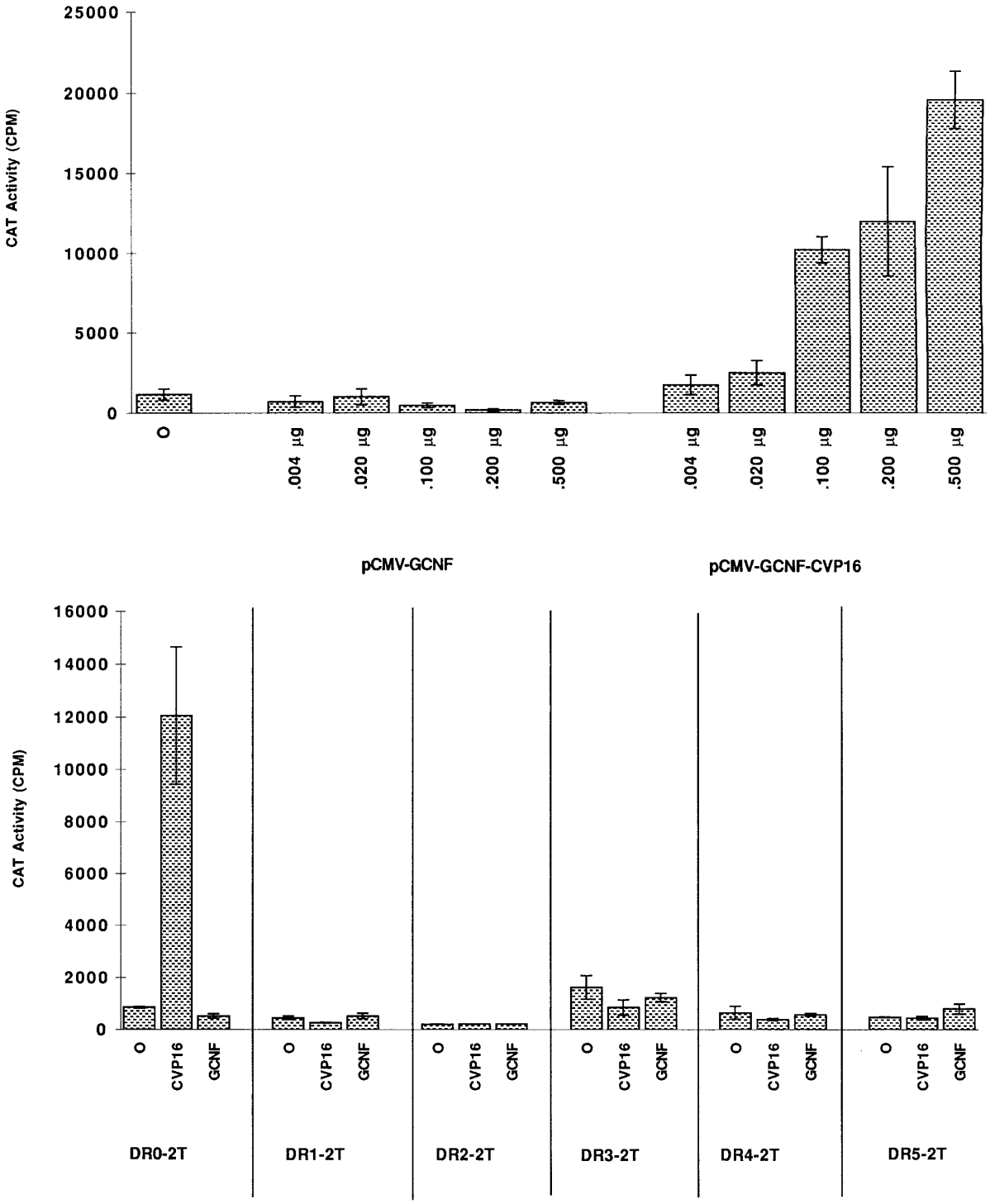

FIG. 3. Analysis of the transactivation properties of GCNF. The expression plasmids pCMV4GCNF and pCMV4GCNFCVP16 were co-transfected into CV-1 cells with each of the reporter plasmids pDR0(2)tkCAT through pDR5(2)tkCAT. A, 1 μg of pDR0tkCAT was transfected into CV-1 cells with increasing amounts of either pCMV4GCNF (left side) or pCMV4GCNFCVP16 (right side). Column marked O represents CAT activity of cells transfected with 1 μg empty pCMV4 expression plasmid plus the reporter plasmid. B, 1 μg of each of the reporter plasmids pDR0(2)tkCAT to pDR5(2)tkCAT was transfected into CV-1 cells with 0.25 μg of either pCMV4 (column O), pCMV4GCNFCVP16 (CVP16), or pCMV4GCNF (GCNF). CAT activity is represented as total CPM for 15 μg of total protein. C, CV-1 cells were co-transfected with either pCMV4GCNF or pCF-VP-GCNF together with the luciferase reporter constucts DR0(2)tkLuc, DR6(2)tkLuc, or p-65-Luc as well as a β-galactosidase control plasmid. Luciferase activity shown is normalized to β-galactosidase activity.

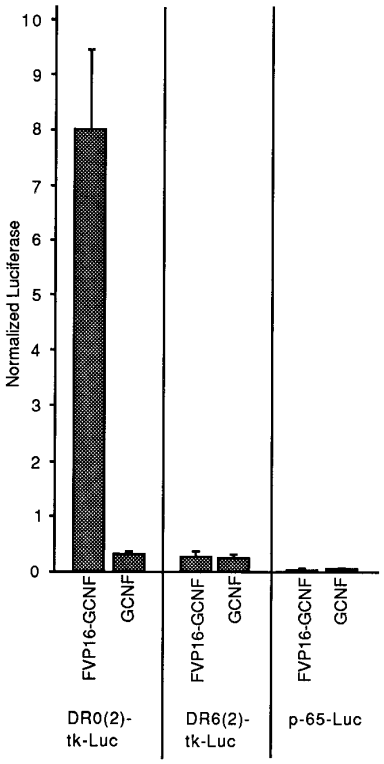

FIG. 3—*Continued*

reporters suggests that high affinity binding of GCNF to its target element is necessary for transactivation (Fig. 1 & 3). This specificity is unique among nuclear receptors, although COUP-TF has been shown to bind to a DR0 (24), however it is more promiscuous than GCNF in its DNA binding properties, and binds to DR1-DR5 elements (25, 26). There may be some interaction between the GCNF and COUP-TF signaling pathways on DR0 elements. The absence of ΔN-GCNF monomer binding to the DR1 (Fig. 2B) indicates that the DBD of GCNF needs more than the AGGTCA sequence to bind, the DR0 has the TCA sequence immediately 5′ to the half-site. This sequence is necessary for proper recognition by the GCNF DBD. Therefore, binding of GCNF to the DR0 sequence will most likely prove to be the most physiologically relevant interaction of GCNF with DNA, establishing the potential for a novel signaling pathway involving GCNF, an unknown ligand, and responsive genes containing DR0 response elements.

The ability of GCNF to repress transcription in the absence of ligand (Fig. 4) suggests that GCNF may be constitutively bound to DNA *in vivo*, waiting for a signal to activate transcription of its target genes. Thus, the mechanism of the novel signaling pathway mediated by GCNF may be similar to that of TR, in that it represses the expression of genes in the absence of a signal and then activates them upon binding ligand (26). The repression function is likely mediated by co-repressors such as NCoR or SMRT (27, 28).

GCNF mediates its activities as a homodimer binding to DR0s (Fig. 2B). Four classes of nuclear receptors have been defined based on DNA binding and dimerization properties (5). GCNF is a class III receptor that binds to direct repeats as a homodimer (Fig.2). Yan et al. (1997) reported that GCNF binds to HS sequences as a monomer (10), in contrast we observe GCNF binding as a homodimer (Fig. 2B). This is not surprising since the HS sequence is really an extended halfsite of the sequence TCAAGGTCA. The faster migrating complex observed in figure 2B that occurs only in the presence of ΔN-GCNF is a monomer, and can be seen binding the DR0 and HS probes.

The binding of ΔN-GCNF but not the wt monomer to DNA indicates that the N-terminus either plays a role in the stabilization of the homodimer, or disrupts monomer binding of the wtGCNF. In either case, its absence permits ΔN-GCNF to bind DNA as a monomer. wtGCNF is prevented from binding DNA as a monomer by the presence of the N-terminus, and therefore only binds to the DR0 as a homodimer. The modulation of GCNF DNA binding may be important when considering the various human isoforms that have been cloned, which vary in their N-termini (22). Indeed, subtle modulation of DNA binding by the N-termini of orphan receptors was observed.

In this report, we present evidence to show that the orphan receptor GCNF may mediate a novel signaling pathway. Several lines of evidence lead to this conclusion: 1) GCNF preferentially binds to DR0 response elements, marking it as the only nuclear receptor discovered to date that does so specifically; 2) GCNF homodimerizes and does not dimerize with the RXR; and 3) GCNF represses target gene expression in the absence of ligand. In support of this hypothesis we have shown

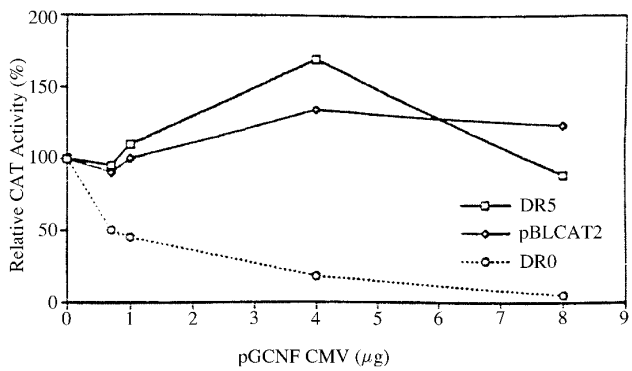

FIG. 4. Analysis of the repression properties of GCNF. CV-1 cells were co-transfected with increasing amounts of pCMV4GCNF (0-8 μg) and either pDR0tkCAT (circle), pDR5tkCAT (square), or pBLCAT2 (diamond). The empty expression vector pCMV4 was transfected to equalize total DNA transfected. Basal level CAT activity with 8 μg pCMV4 and 0 μg pCMV4GCNF designated as 100%.

that, the protamine genes, which are co-expressed with GCNF and contain DR0 elements in their promoters are candidate responsive genes for such a novel GCNF based signalling pathway (29).

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