Identification of a Novel gp130-responsive Site in the Vasoactive Intestinal Peptide Cytokine Response Element*

Elizabeth A. Jones, Jill Conover, and Aviva J. Symes‡

From the Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

The neurotrophic cytokine ciliary neurotrophic factor (CNTF) potently induces transcription of the vasoactive intestinal peptide (VIP) gene through a 180-base pair (bp) cytokine response element (CyRE) in the VIP promoter. We have previously shown that CNTF induction of STAT and AP-1 protein binding within the CyRE is necessary to mediate CNTF induction of VIP gene transcription. We now show that a third, previously uncharacterized site at the 3′-end of the CyRE is also critical to CNTF induction of CyRE transcription. A 4-bp mutation in this 3′-region reduced CNTF-mediated induction of transcription ~80%. Whereas mutations in both the STAT and AP-1 sites substantially reduced CNTF induction of transcription, mutations in these sites together with the novel 3′-site completely abolished the ability of CNTF to induce CyRE-mediated transcription. Gel shift analysis indicated that a complex in neuroblastoma cells bound specifically to this 3′-site. This complex was not altered by CNTF treatment. Mutations in an 8-bp sequence (TTACTGGAA) eliminated binding of this protein complex and markedly reduced transcriptional activation of the CyRE by CNTF. Thus, we have identified a protein complex binding to a novel DNA sequence that is necessary for full CNTF induction of VIP gene transcription.

Ciliary neurotrophic factor (CNTF), a gp130 cytokine with neurotrophic activity, performs many functions in the central and peripheral nervous systems. CNTF mediates cell survival in several different neuronal populations including motor and sensory neurons (1–4), induces reactive gliosis (5), and may stimulate differentiation of precursors toward the astrocytic lineage (4, 6, 7). CNTF also initiates an adrenergic and may stimulate differentiation of precursors toward the sympathetic neurons (3, 8–11). As part of this phenotypic to-cholinergic switch in the neurotransmitter phenotype of pri-

* This work was supported by National Institutes of Health Grant R29 NS-35839 (to A. J. S.) and the American Heart Association Mid-Atlantic Affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 301-295-3234; E-mail: Asymes@usuhs.mil.

The abbreviations used are: CNTF, ciliary neurotrophic factor; VIP, vasoactive intestinal peptide; LIF, leukemia inhibitory factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; bp, base pair(s); CyRE, cytokine response element; C/EBP, CAAT/enhancer-binding protein; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; RSV, Rous sarcoma virus; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; HNF-1, hepatocyte nuclear factor-1; HMG, high mobility group.
examined the proteins binding to the 3′-region and characterized the sequences to which they bind to understand the combinatorial mechanisms through which CNTF induces VIP gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents were obtained from Mediatech (Herndon, VA); fetal bovine/horse serum was from Life Technologies, Inc.; and culture plates were from Costar (Corning, NY). Recombinant human CNTF was a gift from Regeneron Pharmaceuticals (Tarrytown, NY). Oligonucleotides encoding the consensus sites for the transcription factors STAT, C/EBP, ETS, NFAT, AP-1, AP-2, AP-3, OCT-1, and nuclear factor-1 were purchased from Promega (Madison, WI). The remaining oligonucleotides were synthesized on a PE Applied Biosystems 394 synthesizer by the Uniformed Services University of the Health Sciences in-house oligonucleotide facility. This included all PCR and mutagenic primers listed in Table I and the electrophoretic mobility shift assay (EMSA) probes G9 (GGG CAG GAT ATT CTT TTA CTG GAT CAG TCT GA), G10 (GGG CAT AGC AGG ATA TTC TTT TAC ATT AGG ACA TCG), M6 (GGA CCA CAG TTG GGA TTT CCC AAC CTG ACC A), acute phase response element (GGA CCA CAG TTT TAC TGG ATC AGT CTG ACT TTG AAC G), C/EBP (AGC TTG TGG), G11 (GGG TGG ATC AGT CTG ACT TTG AAC G), p28 (GGG GAT CAG TCT GA), G10 (GGG CAT AGC AGG ATA TTC TTT TAC ATT AGG ACA TCG), acute phase response element (GGA CCA CAG TTT TCC ACC ACG CTG ACC A), M6 (GGA CCA CAG TTT TCC ACC ACG CTG ACC A), and CyR (GAA AAT ATG ATT AAG CAT AGA GCA GA).

**Cell Culture and Transfection**—NBFL cells were maintained and transfected as described previously (37). Cells were plated at 1 or 4.5 × 10^5 cells/well in 6-well plates and transfected overnight by calcium phosphate precipitation. Each well received 1 μg of luciferase reporter construct, 0.5 μg of RSV-luc, and 2.5 μg of carrier DNA. CNTF was added in serum-free medium 6 h after the DNA precipitate was removed and for 40 h before cell harvesting. Samples were assayed for luciferase activity (41) and β-galactosidase activity (Galacto-Light Plus kit, Promega Inc., Bedford, MA). Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency.

**Plasmids**—The details of Cy1luc, Cy1mG3luc, Cy1mG2luc (previously termed mG2GCy1uc), and VIP1300luc have been described (38, 42). The 3bp substitution mutant VIPm1300luc was constructed using the CLONTECH Transformer site-directed mutagenesis kit with the mutagenic primer 5′-GCG GGT ACC TTA AAA AAG TAG TGG ACT GGT ATT AAG CCA CAG GAA CTC TGG GAT CAG TCT GA and the antisense primer 5′-32P[dCTP]-labeled oligonucleotides. Binding reactions were electrophoresed on 5% nondenaturing polyacrylamide gel in 0.5× Tris borate/EDTA buffer at 200 V. The EMSA time course, NBFL cells were serum-starved overnight and then activated with CNTF for 0, 0.5, 1, 3, 6, and 24 h prior to the extraction of nuclear proteins. When used, competitor oligonucleotides (5–200 ng) were incubated with the nuclear extracts for 10 min at room temperature prior to adding probe.

**RESULTS**

We have previously characterized the STAT and AP-1 sites within the VIP CyRE and shown both sites to contribute to the CNTF-mediated induction of CyRE-dependent transcription (38, 40). However, our previous deletion studies on the VIP CyRE also revealed that a 28-bp region at the 3′-end of the CyRE, distinct from the STAT and AP-1 sites, contributes substantially to the transcriptional activity of the CyRE (38). To determine the exact sequences within the 3′-end of the VIP CyRE that mediate the actions of this 28-bp 3′-region, we compared murine and human genomic sequences (38). Although the 180-bp CyRE is 84% homologous between mouse and human, the distal 28-bp 3′-sequence is only 53% conserved. However, one 4-bp motif (G9 site) is conserved between species, suggesting functional significance of this site. To investigate whether this 4-bp motif is important in mediating CNTF induction of CyRE transcription, we transfected NBFL neuroblastoma cells with a Cy1luc luciferase reporter plasmid mutated within these 4 bp (Cy1mG9luc) (Table I). CNTF induction of transcription driven by Cy1mG9luc was reduced to 28% of that mediated by Cy1luc (Fig. 1). These data suggest that this 4-bp motif may form part of a binding site for a protein complex that contributes to CNTF-induced transcription through the VIP CyRE.

We wanted to investigate the relationship between the newly identified G9 site and the previously identified STAT and noncanonical AP-1 sites that contribute to CNTF-mediated induction of CyRE-driven transcription. To determine whether the 3′-G9 site acts independently of the STAT and AP-1 sites, we constructed a series of luciferase plasmids with individual, double, or triple substitution mutations at these sites within the wild-type Cy1luc plasmid. Cy1luc directed the highest level

**TABLE I**

| Primer | PCR primers for site-directed mutagenesis | 5′ → 3′ |
|--------|------------------------------------------|---------|
| A1     | CCGGATCCTTACAAAAAGAGATCTGTTG             |         |
| mA1    | CCGGATCCTTACAAAAAGAGTACTGTTG             |         |
| A4     | CACCTGACGCTTTAAGGATCTGTTG               |         |
| mG3    | CCG GTT ACC TAA AAA AGA AGA TGG ACT GGT ATT AAG CCA CAG GAA CTC TGG GAT CAG TCT GA |         |
| a/s mG9| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mG2| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS3| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS4| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS5| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS7| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS8| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS9| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |
| a/s mS10| CAG CTC GAG GTT ACA AAG TCA GAG TCA GAG TCA TCC AGT AA A A A A A A A A A A A A A A |         |

a/s, antisense.
of transcription in unstimulated cells. CNTF induction of CyRE-mediated transcription was reduced in all of the mutant constructs. In the CyRE-luciferase construct with mutations in both STAT and AP-1 sites (Cy1mG2mG3mG9luc), CNTF induced luciferase activity 9-fold (Fig. 2). This remaining CNTF inducibility mediated by a CyRE-luciferase plasmid without a functional AP-1 or STAT site supported the existence of additional functional sites such as the 3′-CyRE in the CyRE. CNTF induction of CyRE transcription was reduced to ~17% of Cy1luc with single mutations in either the STAT or 3′-CyRE site (Fig. 2). Mutations in both the STAT (G3) and 3′-G9 sites further reduced CNTF induction of transcription to 8% of that produced by the wild-type Cy1luc plasmid. Thus, the two sites each contribute to the CyRE-mediated CNTF response. Mutation of the AP-1 site (G2) reduced CNTF-induced transcription by 44%, and double mutants of the AP-1 site together with either the STAT or G9 site reduced CNTF induction of transcription by 90 and 84%, respectively. Introduction of mutations into all three sites (Cy1mG2mG3mG9luc) abrogated the response to CNTF. These data suggest that the STAT, AP-1, and 3′-G9 sites all independently contribute to CNTF induction of CyRE transcription.

To investigate whether CNTF treatment of NBFL cells altered nuclear protein binding to the 3′-region of the CyRE, we performed EMSAs with four overlapping probes of the 3′-region of the CyRE (Fig. 3A). Three protein complexes of different mobility bound to different probes from the 3′-CyRE in nuclear extracts prepared from untreated NBFL cells (Fig. 3B). Complex A bound strongly to the G9 probe, complex B to the G10 probe, and complex C to the G11 probe (Fig. 3B). CNTF treatment did not alter binding of any of these nuclear protein complexes, showing that these complexes are able to bind to DNA sequences constitutively. We also investigated which complexes bound to a probe containing the entire distal 28 bp of the 3′-end (p28) and detected a single band with the same mobility as the G9-binding complex A, which did not change with CNTF treatment (Fig. 3C and data not shown). The protein complex binding to p28 was competed by a 100-fold molar excess of G9, but not G10 or G11, suggesting that this complex was probably the G9-binding nuclear protein complex (Fig. 3C). Therefore, nuclear protein binding to this region of DNA is complex; several protein complexes may be responsible for mediating the CNTF induction of CyRE-driven transcription at the 3′-end.

To determine whether nuclear protein binding to the G9, G10, and G11 probes was specific and to identify whether similar proteins were bound to each of the probes, we performed EMSAs in the presence of varying concentrations of unlabeled competitor oligonucleotides. Binding of NBFL nuclear protein to each probe was specific, as its binding was competed by a 100-fold molar excess of unlabeled oligonucleotide (Fig. 4). The mG9 oligonucleotide failed to compete for binding to the G9 probe (Fig. 4A) or to the p28 probe (Fig. 3C), showing that this mutation markedly reduces the ability of complex A to bind. As this oligonucleotide is mutated in the same 4 bp as the Cy1mG9luc plasmid (Fig. 1), these data suggest that complex A binding to the G9 site may contribute to the CNTF induction of CyRE transcription. The G9 oligonucleotide competed for the protein complexes binding to the G10 and G11 probes; in contrast, G10 and G11 were unable to compete for the protein complexes binding to the G9 probe (Fig. 4, B and C). Thus, complex A binding to the G9 probe appears to require sequence additional to that in either G10 or G11, despite the considerable overlap between the oligonucleotide probes. Our results implicate complex A binding to the G9 site as critical for
the CNTF induction of CyRE-mediated transcription.

In our initial attempts to identify the components of the G9-binding complex A, we used a variety of known transcription factor-binding sites to compete for binding of complex A to the G9 probe. None of the oligonucleotides shown or oligonucleotides containing CREB, SMAD, or NF-κB consensus sites competed for complex A binding (Fig. 4D and data not shown). We also competed nuclear protein binding to the CyRE probes with an AP-1 consensus oligonucleotide, as there is an AP-1-like site within this region (ATCAGTCT). The AP-1 oligonucleotide failed to compete for binding of the nuclear protein complexes specific to the G9 probe. None of the oligonucleotides used or oligonucleotides containing CREB, SMAD, or NF-κB consensus sites competed for complex A binding (Fig. 4D and data not shown).

Supershift analysis with antibodies raised against several different members of the AP-1 protein family (c-Fos, c-Jun, JunB, JunD, and activating transcription factor-2) did not identify any known AP-1 proteins contributing to complex C (data not shown). Thus, several unrelated protein complexes are able to bind to sites within the 3′-CyRE. Complex A may represent a novel constitutive factor required for CNTF-mediated transcription.

To identify which specific bases within the G9 oligonucleotide are required for complex A binding, we synthesized oligonucleotides containing a series of sequential 2- and 3-bp mutations in the G9 probe. EMSAs performed with NBFL nuclear extracts binding to these mutant oligonucleotides showed that 2- or 3-bp mutations within a large 17-bp region reduced or eliminated complex A binding to the probe (Fig. 5A). This region includes and extends from the 4-bp mutation in mG9.

The m3, m4, m5, and m6 oligonucleotides, with mutations in the sequence TTACTGGA, were unable to bind complex A or to compete for its binding to the wild-type G9 probe (Fig. 5B). The m2 and m7 oligonucleotides bound complex A weakly and were able to compete for complex A binding to G9, but with less affinity than either the wild-type G9 or m1 oligonucleotide. Interestingly, the m2, m3, m5, and m6 oligonucleotides all bound a larger complex, whereas m4 bound none. Reducing the length of the G9 oligonucleotide by 8 bp while retaining the central core recognition sequence also reduced binding of complex A. Thus, the binding site for complex A extends over 17 bp of the G9 oligonucleotide and requires adjacent sequence for high affinity binding. However, the core 8-bp sequence TTACTGGA is critical for complex A binding.

To determine the exact sequence within the 3′-end of the CyRE necessary to mediate CNTF-induced transcription, we made a series of CyRE-luciferase constructs containing sequential 3-bp mutations along the most distal 28 bp of the 3′-CyRE, within the context of wild-type Cy1luc. A notable reduction in CNTF-mediated luciferase activity was observed in cells transfected with Cy1mS7luc, Cy1mS8luc, or Cy1mS9luc (Fig. 6A). The 3-bp mutations in Cy1mS7luc and Cy1mS9luc each overlap the 4 bp mutated in Cy1mG9luc, confirming our original observation as to the importance of this site (Fig. 1). Furthermore, the 3-bp mutations in Cy1mS7luc, Cy1mS8luc, and Cy1mS9luc are located within the sequence TTACTGGA required for complex A binding (Fig. 5A). A direct comparison of the transfection data with an EMSA using a G9 probe containing the same mutations as in Cy1mS7luc, Cy1mS8luc, and Cy1mS9luc (Fig. 6, A and B) demonstrated a correlation be-
tween loss of CNTF-induced transcriptional activity of the mutants and loss of complex A binding. This was evident irrespective of whether the mutation led to a complete loss of protein binding (mS8) or to binding of slightly larger complexes (mS7 and mS9). In contrast, the mutation in mS5, to which complex A is able to bind, did not affect the transcriptional response to CNTF. The mutated mS6 probe exhibited reduced binding to complex A, but mediated a stronger transcriptional response to

![Fig. 5. EMSA analysis of G9 protein binding.](image)

![Fig. 6. Substitution mutations in the 3’-CyRE can reduce CNTF-mediated transcription.](image)
CNTF than wild-type Cy1Luc. Interestingly, the mS6 mutation introduced an artificial STAT-binding site that may be more effective than the wild-type sequence in mediating a transcriptional response to CNTF (data not shown). These data strongly suggest that protein complex A is the complex necessary within the 3’-end of the CyRE for mediating the CNTF induction of CyRE-driven transcription.

This novel sequence TTACTGGA is therefore critical both for complex A binding to the G9 probe and for mediating CNTF-induced transcription through the 3’-end of the CyRE. The sequence bears a high degree of homology to the STAT consensus sequence TTNLGGAA. However, the G9 probe does not compete with oligonucleotides containing the STAT1/8 or STAT5 consensus sequence, and complex A fails to supershift with antibodies against STAT1, STAT3, and STAT5 (data not shown). Thus, we believe that complex A is composed of novel constitutive factors and are currently in the process of identifying them.

To determine whether the G9 site could act as a classical enhancer of CNTF-driven transcription, we constructed a multimeric form of the G9 site and its surrounding sequence upstream of the basal ΔRSV promoter driving expression of the luciferase reporter gene. The multimeric site, both in the correct and reverse orientations, failed to act as a classical enhancer since the level of luciferase activity following CNTF treatment did not significantly increase over basal levels (Fig. 7). However, a multimeric CyRE AP-1 site driving a luciferase reporter gene also cannot mediate transcription in response to CNTF (data not shown). These data suggest that the G9 site contributes to CNTF-mediated transcriptional induction in a similar manner to the AP-1 site. Thus, both sites may act in a combinatorial manner, requiring additional sites in the CyRE to function.

The CyRE is one section of the entire VIP promoter regulating VIP gene transcription. We have previously shown that 1330 bp of the VIP promoter are necessary and sufficient to mediate the induction of VIP transcription to CNTF (38). To determine whether the G9 site is important to the CNTF induction of VIP transcription mediated by the wild-type VIP promoter, we introduced a 3-bp mutation in the G9 site in VIP1330 Luc to form VIPm1330 Luc. The 3-bp mutation was identical to the mutation in Cy1mSS8Luc, which eliminated complex A binding in an EMSA and substantially reduced CNTF-mediated induction in transient transfection assays (Fig. 6). Mutating the G9 site in the context of the VIP1330 promoter resulted in a 66% reduction in the level of CNTF-activated luciferase activity compared with the wild type (Fig. 8). The luciferase activity of Cy1mSS8Luc following CNTF treatment was reduced by 87% relative to wild-type Cy1Luc. These data demonstrate that the G9 site is critical to CNTF-mediated induction of the wild-type VIP promoter and confirm the importance of the G9-binding complex A to mediating the CNTF induction of VIP transcription.

**DISCUSSION**

The VIP CyRE is a very potent CNTF-responsive element, mediating induction of transcription by CNTF between 40- and 200-fold. This strong effect is produced by a combinatorial regulation of transcription with interactions between several different transcription factors, either induced by CNTF or constitutively present, that bind to sites within the CyRE. We have previously shown that STAT and AP-1 proteins are important for the CNTF induction of CyRE transcription (38, 40). In this study, we show that an additional factor, the G9-binding complex A, is also critical to mediating full CNTF-induced CyRE transcription. Binding of complex A to a novel site (TTACTGGA) at the 3’-end of the CyRE is not altered by CNTF treatment, yet mutation of this site disrupts CNTF-induced CyRE transcription to an extent comparable to mutations in the STAT site. Although the involvement of STAT activation in CNTF signaling is well delineated, the G9-binding complex A represents a previously uncharacterized protein binding to a novel site that is critical to CNTF induction of VIP gene transcription.

The VIP CyRE responds to other members of the gp130 cytokine family, functioning as a generic gp130 response element in cells that endogenously express the VIP gene (37, 38). Thus, common gp130 signaling pathways, such as those characterized for interleukin-6, are involved in the CNTF induction of CyRE transcription. Indeed, we have previously reported that CNTF induces STAT1 and STAT3 to bind to the CyRE STAT site (G3) (36, 38). CNTF also induces the AP-1 proteins c-Fos, JunB, and JunD to bind to a non-canonical AP-1 site (G2) within the VIP CyRE (40). However, when either a single STAT or AP-1 site was placed upstream of a heterologous promoter driving luciferase expression, neither was able to mediate any induction in response to CNTF (38). Multimerization of the G3 CyRE site was able to mediate a 3-fold induction in response to CNTF, in contrast to the 40–100-fold induction of the wild-type VIP CyRE (38). Additionally, neither a multimerized G2 AP-1 site nor a canonical AP-1 site was able to mediate any induction by CNTF (data not shown). These data indicate that neither the STAT nor AP-1 site is sufficient to mediate CNTF-induced transcriptional activation of the CyRE, although both contribute. Additionally, they suggest that additional regions within the CyRE also contribute to the ability of the CyRE to mediate a strong induction in response to CNTF. We now show that a third site, G9, contributes substantially to the level of transcription induced by CNTF.

Our results demonstrate that protein complex A binds specifically to the DNA sequence in the G9 oligonucleotide. The critical sequence for this interaction is the 8-bp TTACTGGA (Fig. 9). Mutation of these 8 bp both prevents complex A binding to DNA and also reduces CNTF induction of CyRE transcription in luciferase reporter assays. These data suggest that complex A is critical for mediating full CNTF induction of CyRE transcription. This nuclear protein complex was not competed by known transcription factor-binding sites, nor did a search of the TRANSFAC Database suggest that known transcription factors bind to this site. However, complex A was...
detected in a variety of different cell types, suggesting a wide expression pattern.\(^2\) Thus, our results suggest that the G9-binding complex A may be composed of widely expressed, but previously uncharacterized transcription factors.

The role of the G9 site in mediating CNTF induction of CyRE transcription is considerable since mutation of this site reduces CNTF-induced transcription by 80%. However, CNTF treatment does not alter binding of complex A to the G9 site. Thus, the mechanism through which complex A contributes to CNTF-induced transcription is unclear. One possibility is that CNTF may modify complex A post-translationally to alter its function. The cAMP-inducible transcription factor CREB, for example, binds to DNA constitutively, but is then phosphorylated to increase its affinity for binding cooperative factors (44, 45). This post-translational modification enhances the ability of CREB to interact with the transcriptional activator CBP (46, 47). CNTF may activate signaling pathways that phosphorylate complex A, although we know that this mechanism alone does not enhance transcription. Multimeric G9 cannot act as an enhancer of transcription, and so phosphorylated complex A would be dependent on other transcription factors binding to additional sites in the CyRE.

CNTF activates many kinase cascades, including the MAPK and phosphatidylinositol 3-kinase pathways (13, 15, 23), which can culminate in the phosphorylation of nuclear proteins; yet it is unclear which transcription factors are activated by these signaling events. CNTF's signaling may target C/EBPβ and C/EBPδ transcription factors since they are involved in interleukin-6-induced transcriptional responses. However, the G9-binding complex A was not competed by two independent C/EBP consensus sites (C/EBP and M6) (Fig. 4D), suggesting that C/EBP proteins are not components of this complex. Additionally, we have not found a role for C/EBP proteins in CNTF regulation of CyRE transcription in our previous experiments (39).

Alternatively, CNTF signaling may not modify complex A directly. The G9-binding complex A could prove to be an essential component of a larger transcriptional complex that forms on the CyRE after CNTF treatment. Thus, complex A may play an architectural role, stabilizing the interaction of several transcription factors in a multicomplex structure. Mutations that prevent complex A from binding DNA would therefore adversely affect the ability of CNTF to maximally induce transcription through the CyRE. Previously, the transcription factor HNF-1 was shown to be critical for function of an interleukin-6 response element in the \(\beta\)-fibrinogen gene (48). This report suggested that HNF-1, a liver-specific constitutive binding protein, might help tether interleukin-6-activated C/EBP proteins to the transcription start site. The HNF-1 site was unable to function if its position within the DNA was altered, suggesting the spatial arrangement of transcription factors was critical for function. Thus, HNF-1 represents an example of a constitutive protein that is required for gp130-mediated transcription, possibly providing an architectural role within the \(\beta\)-fibrinogen promoter. The G9-binding complex A could act in a similar manner in CNTF regulation of the VIP gene in neuronal cells.

The VIP CyRE is a complex response element with multiple functional domains. Current models of transcriptional regulation suggest that STAT, AP-1, and G9-binding complex A proteins form part of a larger complex that encourages recruitment of the basal transcriptional machinery to the VIP promoter. Commonly, DNA-binding factors can bind directly to each other or via coactivators such as CBP (46, 47). Both STAT and AP-1 can bind to CBP (49–53), suggesting a role for CBP in regulating VIP gene transcription in response to CNTF. However, in the macrophage scavenger receptor gene promoter, STAT1 antagonizes AP-1 function by competing with AP-1 proteins for binding to CBP (52). Thus, interferon-\(\gamma\)-induced STAT1 down-regulates AP-1-mediated transcription through competition for CBP. In our model, AP-1 and STAT proteins are both necessary for CNTF induction of CyRE-mediated transcription, suggesting a cooperation between these two classes of transcription factor. Indeed, another gp130 cytokine-regulated gene, TIMP-1, requires both STAT and AP-1 protein binding for full induction (54). We have found that CBP enhances...
NF-κB, interferon regulatory factor, activating transcription factor-2, and c-Jun, which bind with an architectural protein HMG I(Y), to form an enhancer (59, 60). CBP forms a bridge between this complex and the basal transcriptional machinery. Various proteins in the enhansome possess histone acetylly activity and thus may activate transcription through acetylation of histones and subsequent alteration in chromatin structure. By analogy with the interferon-β enhansome, CNTP induction of VIP transcription through the CyRE could be mediated by an enhansome-like structure. There may also be a role for the architectural HMG I(Y) proteins, as there are many AT-rich regions that bind HMG proteins within the CyRE (61). Thus, the CNTP-induced STAT and AP-1 proteins could cooperatively bind together with constitutively expressed proteins such as the G9-binding complex A, forming a more stable surface for interaction with coactivators such as CBP.

The CyRE is an important component in mediating VIP transcription (37–40, 62, 63). The CyRE and surrounding sequence are highly conserved between human and murine species, suggesting that the sequence is functionally important (64). In addition, the CyRE, together with the more proximal VIP cAMP response element, is critical for the function of a distal tissue-specific element (63, 65). We have identified a novel site (G9) at the 3′-end of the CyRE that is critical for mediating the CNTP induction of VIP gene transcription. The G9-binding protein complex A cooperates with STAT and AP-1 proteins to mediate the CNTP inducibility of CyRE transcription. We are currently in the process of purifying the proteins that bind to G9 to identify a transcription factor that binds the motif TTACTGGA. Thus, CNTP induction of VIP gene expression is dependent on the interaction of inducible and novel non-inducible proteins that act together to produce a potent transcriptional response.

Acknowledgments—We thank Regeneron Pharmaceuticals for the gift of human rCNTF and Fern Murdoch and Robert Leflecheider for many helpful discussions and suggestions.