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Transcriptional Activity of the Zinc Finger Protein NGFI-A Is Influenced by Its Interaction with a Cellular Factor

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***NGFI-A* is an immediate-early gene that encodes a transcription factor whose DNA-binding domain is composed of three zinc fingers. To define the domains responsible for its transcriptional activity, a mutational analysis was conducted with an NGFI-A molecule in which the zinc fingers were replaced by the GAL4 DNA-binding domain. In a cotransfection assay, four activation domains were found within NGFI-A. Three of the activation domains are similar to those characterized previously: one contains a large number of acidic residues, another is enriched in proline and glutamine residues, and another has some sequence homology to a domain found in Krox-20. The fourth bears no resemblance to previously described activation domains. NGFI-A also contains an inhibitory domain whose removal resulted in a 15-fold increase in NGFI-A activity. This increase in activity occurred in all mammalian cell types tested but not in *Drosophila* S2 cells. Competition experiments in which increasing amounts of the inhibitory domain were cotransfected along with NGFI-A demonstrated a dose-dependent increase in NGFI-A activity. A point mutation within the inhibitory domain of the competitor (I293F) abolished this property. When the analogous mutation was introduced into native NGFI-A, a 17-fold increase in activity was observed. The inhibitory effect therefore appears to be the result of an interaction between this domain and a titratable cellular factor which is weakened by this mutation. Downmodulation of transcription factor activity through interaction with a cellular factor has been observed in several other systems, including the regulation of transcription factor E2F by retinoblastoma protein, and in studies of c-Jun.**

NGFI-A (also called *Egr-1* [35], *Zif268* [11], and *Krox-24* [20]) is an immediate-early gene originally identified by virtue of its rapid activation by nerve growth factor (NGF) in PC12 cells (26) and by serum in fibroblasts (11, 35). This gene encodes a transcription factor whose DNA-binding domain is composed of three zinc fingers of the C_2H_2 subtype. It is a member of a gene family that includes the immediate-early genes *Krox-20* (7), *NGFI-C* (13), *Egr-3* (28), and the Wilms' tumor gene (30), all of which encode nearly identical zinc fingers and a number of additional regions of homology outside the DNA-binding domain (14). NGFI-A is a phosphorylated nuclear protein with a short half-life that is synthesized rapidly in response to various stimuli, including growth factors, membrane depolarization, seizure, brain ischemia, synaptic activity, and nerve injury (5, 15).

NGFI-A binds to the nucleotide sequence GCG(G/T)GGGCG (10, 21), a sequence motif present in the promoter regions of *NGFI-A* itself and other immediate-early genes (10) as well as a number of other genes (platelet-derived growth factor A chain [36], insulin-like growth factor II [IGF-II] [16], adenosine deaminase [1], and α-myosin heavy chain [17]). In transient-transfection assays, NGFI-A has been reported to activate transcription from its own promoter (24) and the platelet-derived growth factor A chain and α-myosin heavy-chain promoters, which it regulates in a cell type-dependent manner (17, 38). Both the amino-terminal (24, 37) and carboxy-terminal (37) portions of this factor have been implicated in its transcriptional activity. Interestingly, it appears that NGFI-A may also produce alterations in gene expression by competing with other transcription factors that recognize the same or overlapping sequences. For example, the promoter of the murine adenosine deaminase gene contains overlapping binding sites for Sp1 and NGFI-A. Transcription of this gene is activated by Sp1 and repressed by NGFI-A, suggesting that the binding of one may exclude binding of the other (1). Interestingly, the Wilms' tumor gene product WT1 represses transcription of the IGF-II and platelet-derived growth factor A chain promoters, and the activation of the *NGFI-A* promoter mediated by NGFI-A is diminished by the coexpression of WT1 (24). However, the physiologic relevance of NGFI-A regulation of these genes is unclear. As several encode growth factors, it is reasonable to propose that NGFI-A may play a central role in normal growth, development, and senescence and that perturbations of this regulation may contribute to various pathologic conditions, including Wilms' tumor.

NGFI-A is a transcription factor whose synthesis is coincident with alterations in the cellular environment. It is therefore presumed that it promotes cellular responses by regulating the transcription of a number of target genes. The present study focused on defining domains within NGFI-A that are important in regulating its transcriptional capabilities. By examining a series of NGFI-A deletion mutants, four domains which contribute to the transcriptional activation ability of NGFI-A were identified. Interestingly, we also identified a potent inhibitory domain whose deletion resulted in a 15-fold increase in activity. This increased activity was observed in all mammalian cell types tested but not in *Drosophila* S2 cells. Furthermore, this domain was active when linked to a heterologous DNA-binding domain. The

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inhibitory domain appears to function by interacting with a cellular factor, since its coexpression in *trans* can titrate out a factor which inhibits the activity of NGFI-A. Mutation analysis reveals that a single point mutation can abolish this interaction, as shown by the loss of the enhancement properties of competitors and by the increased activity of an NGFI-A protein with this mutation. The NGFI-A inhibitory domain bears no sequence similarity to regions in Jun or E2F that have been shown previously to interact with cellular factors that modulate their transcriptional activity (4).

MATERIALS AND METHODS

Recombinant plasmids. The mammalian expression vector pCMV and the luciferase reporter construct containing two GCGGGGGCG motifs with a minimal prolactin promoter (A2ProLuc) have been described before (13). The reporter G5BCAT, which contains five GAL4 binding sites upstream of a minimal E1b promoter and the chloramphenicol acetyltransferase gene (22), and pSG424 (32) were the generous gift of M. Ptashne. The reporter G5B-luc was created by a *Hin*dIII-*Asp*718 transfer of the promoter region of G5BCAT immediately 5′ of the firefly luciferase gene. Internal deletion mutants of *NGFI-A* were constructed by cleavage of the cDNA (26) with appropriate restriction enzymes and insertion of *Bam*HI linkers of appropriate length to maintain the correct reading frame. Deletion mutants are designated with a Δ followed by the numbers of the residues which were deleted. Carboxy-terminal truncation mutants were constructed by cleavage with a unique restriction enzyme and insertion of an oligomer with termination codons in all three reading frames.

The chimeric NGFI-A/GAL4 transcription factor construct AG was made by standard cloning procedures, including polymerase chain reaction with pSG424 as a template to derive a fragment encoding the GAL4 DNA-binding domain (amino acids [aa] 1 to 147). This fragment was cloned into the *NGFI-A* cDNA so that it replaced the native *NGFI-A* DNA-binding domain and encoded a molecule containing the first 338 residues of NGFI-A followed by the GAL4 DNA-binding domain (aa 1–147) and then by NGFI-A residues 420 to 536. NGFI-A/GAL4 chimeras with deletions extending to residue 1 of GAL4 were constructed by cloning fragments from *NGFI-A* deletion mutants into a *Bam*HI site introduced 5′ to the sequence encoding the GAL4 DNA-binding domain. By virtue of this intervening *Bam*HI site, the residues Asp-Pro-Gly-Met-Arg were added immediately amino-terminal to the GAL4 residues. *NGFI-A/GAL4* chimeras encoding deletions at residue 147 of GAL4 were constructed by truncating the molecule at the unique *Afl*II site directly flanking the *GAL4* portion of the molecule. Competitors were constructed with synthetic oligonucleotides inserted into a *Bam*HI site at the internal deletion of AΔ29–322/420–536. They contain the first 29 residues of NGFI-A, the specified insert, and then the native NGFI-A DNA-binding domain. Point mutations at codons 292 and 293 were introduced with synthetic oligonucleotides into a full-length *NGFI-A* cDNA containing multiple silent mutations which introduced cloning sites. In the construction of these point mutations, it was necessary to first add five codons (Leu-267–Gly–Thr–Ala–Asp–Pro–Gly-268) to the full-length construct, creating the parent construct Ains268. The identity of all constructs described above was confirmed by nucleotide sequencing (33) before they were cloned into the expression vector pCMV.

Cell culture, transfection, and luciferase assay. CV1, NIH 3T3, JS1, *Drosophila* Schneider S2 (a gift of P. Cherbas), PC12, and COS7 cells were cultured as described before (29). The cells were transfected by the calcium phosphate precipitation method with 100 ng of expressor, 5 μg of reporter, and 4.9 μg of pBSKS carrier DNA per plate unless otherwise indicated. HeLa cells were grown in Earle's minimal essential medium with 10% fetal bovine serum, seeded at 10^6 cells per 10-cm plate, and transfected by the calcium phosphate technique 24 h later with 5 μg of expressor and 5 μg of reporter DNA. Cells were harvested as described before (29) 48 h after transfection. Saos-2 cells (a gift of Stan Korsmeyer) were treated similarly except that Iscove's medium with 10% fetal bovine serum was used. Luciferase assays were performed as described before (13). The results shown are the means of the indicated number of independent experiments (*N*), each performed as the transfection of duplicate plates. At least two independent preparations of each plasmid were tested and found to give indistinguishable results. The full-length NGFI-A/GAL4 chimera transcriptional activity was taken as 100% activity, while the transcriptional activity of the DNA-binding domain of GAL4 alone was assigned a value of 0%.

Gel retardation and protein blot analysis. Gel retardation assays with COS7 whole-cell extracts (29) were performed as described before (41). Protein blot analyses of whole-cell lysates with rabbit anti-NGFI-A (A310) or anti-GAL4 (gift from M. Ptashne) antiserum were performed as described before (15).

Immunohistochemistry. Immunohistochemistry was performed as described (25a). Briefly, 48 h after transfection, CV1 cells grown on multichamber slides were washed, fixed, permeabilized, and blocked prior to the addition of the A310 antiserum. After further rinsing, fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G was used to visualize the location of the chimeric proteins.

RESULTS

NGFI-A has several activation domains. To determine the domains responsible for the transcriptional activity of NGFI-A, a luciferase reporter construct containing two cognate NGFI-A binding sites (GCGGGGGCG) was used (13). This reporter contains no Sp1 sites yet had a high basal activity in a variety of cell lines, including *Drosophila* S2 cells. Despite this high background, a sevenfold increase in luciferase activity was observed when this reporter was cotransfected with *NGFI-A* expression vectors (13, 21, 24). CV1 cells transfected with 5 mg of A2ProLuc and 5 mg of the pCMV control, a plasmid expressing wild-type NGFI-A, or AΔ226–322 showed 82×10^3, 545×10^3, and $2{,}310 \times 10^3$ relative luciferase units of transcriptional activity, respectively. This level of activity was not sufficient to allow definitive characterization of mutants with decreased activity; however, during these studies, we observed that deletion mutant AΔ226–322 had much greater activity than wild-type NGFI-A.

Because of the relatively high basal activity from the *NGFI-A* reporter, further analysis of NGFI-A transcriptional domains required the use of another reporter system. For this purpose, a chimeric *NGFI-A* transcriptional activator in which the sequences encoding the *NGFI-A* zinc fingers (aa 337–420) were replaced with sequences encoding the DNA-binding and nuclear localization domain from *GAL4* (aa 1–147) (6) was constructed. The reporter contained the luciferase gene with five *GAL4* sites upstream of the E1b TATA box (G5B-luc) (see Materials and Methods). The

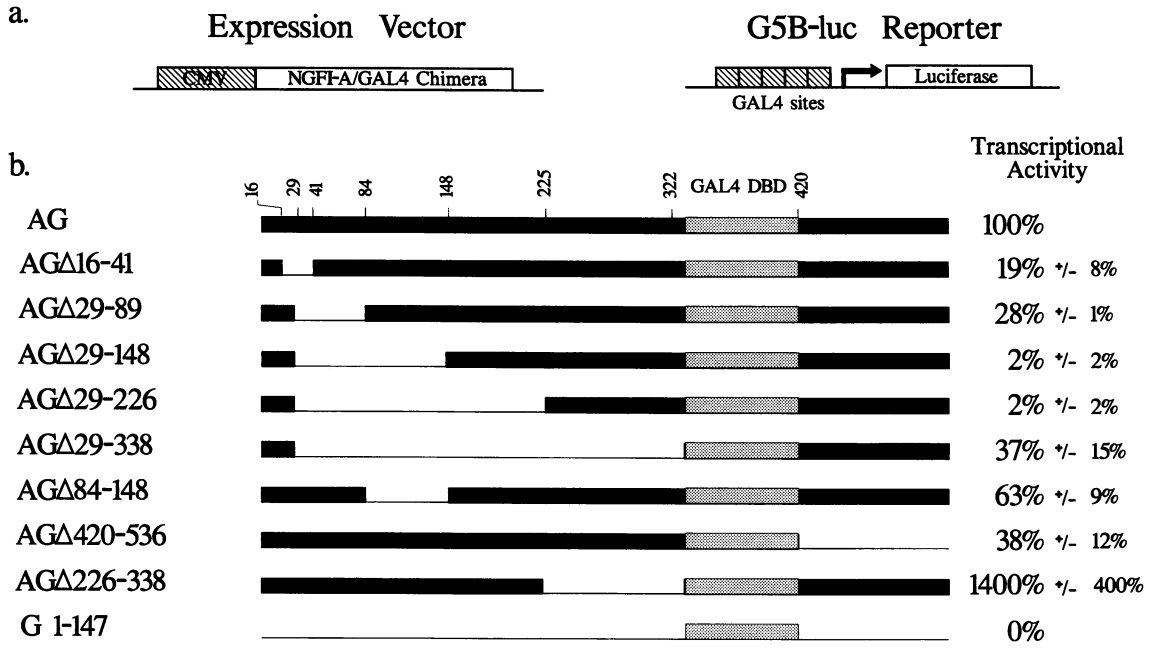

FIG. 1. Transcriptional activity of selected NGFI-A/GAL4 chimeras. (a) Schematic representation of expression vectors and reporter G5B-luc. (b) Schematic representation of full-length and deleted NGFI-A/GAL4 (AG) chimeras. CV1 cells were transfected with the indicated NGFI-A/GAL4 mutant and G5B-luc, cultured for 2 days, and assayed for luciferase activity ($N = 5$). Residues are numbered relative to translation initiation occurring at nucleotide 268 (26). Values are expressed as a percentage of the value obtained with construct AG. CMV, cytomegalovirus; DBD, DNA-binding domain.

relative transcriptional activity of this full-length chimera and selected deletion mutants was examined by cotransfecting CV1 cells with the G5B-luc reporter plasmid and various activating plasmids and then monitoring luciferase activity. These studies revealed the presence of at least three domains that contribute to the transcriptional activity of NGFI-A (Fig. 1). One domain (A1) lies between residues 16 through 41, as detected by the reduction in activity to 15% of the wild-type level. Further deletion up to residue 89 had no further effect, but a second activation domain (A2) was detected when residues up to 148 were removed, as the transcriptional activity of this construct (AGΔ29–148) was almost negligible. A third activation domain (A3) was found in the region carboxy terminal to the DNA-binding domain (residues 420 to 536) that reduced the activity to 40% of the wild-type level when deleted.

Inspection of the sequences of these domains revealed that A2 is enriched in acidic residues. Region A1 has weak homology to a portion of an activation region found by deletion analysis of Krox-20 (37), while the other is not homologous to previously described activation domains. The relative levels of the chimeric proteins were found to be similar in an immunoprecipitation assay with anti-GAL4 antibodies, indicating that differences in transfection efficiency or protein half-life were not responsible for the observed differences in transcriptional activity (data not shown). Each construct has an identical nuclear localization and DNA-binding domain (i.e., GAL4 residues 1 to 147), so that differences in nuclear localization are unlikely to explain these observations. Further, immunolocalization experiments in CV1 cells revealed the nuclear localization of these proteins.

NGFI-A has a potent inhibitory domain. In our initial experiments with native NGFI-A and again with the NGFI-A/GAL4 chimeras, we observed a striking increase in transcriptional activity when residues 226 to 338 were deleted (Fig. 1). We reasoned that this increase in activity was due to the disruption of an inhibitory domain, as has been postulated for the increased activity of v-Jun over c-Jun and attributed to the absence in v-Jun of a 27-amino-acid inhibitory domain referred to as δ (3, 4). To further delimit this apparent inhibitory domain of NGFI-A, additional chimeric constructs were tested (Fig. 2). The deletion of residues 226 to 267 (AGΔ226–267) results in a decrease in activity to 20% of the wild-type level, indicating the presence of a previously undetected strong activating domain (A4) in this region. The removal of residues 269 to 338 (AGΔ269–338) resulted in a very active chimeric NGFI-A protein, indicating that this construct lacked the inhibitory domain.

To further delimit the residues important for this phenomenon, additional mutants were constructed in which residues were added back to AGΔ269–338 in an attempt to reduce its activity to wild-type levels. When residues 269 to 297 were included, as in construct AGΔ298–338, the transcriptional activity was greatly reduced. A further reduction, back to nearly wild-type levels, was observed after the addition of six residues to produce AGΔ304–338, indicating that the inhibitory domain, termed R1, is located between residues 269 and 303.

To determine whether the R1 inhibitory domain of NGFI-A was active in a cell type-specific manner, the activities of the deletion construct AGΔ226–338, lacking the inhibitory domain, and its full-length AG counterpart were compared in a variety of cell types. The transcriptional activity of constructs lacking the R1 inhibitory domain was greater than that of the full-length chimera in all mammalian cell types tested (CV1, COS, PC12, JS1, HeLa, Saos-2, and 3T3), but they were not more active when tested in *Drosophila* S2 cells (data not shown).

Interaction with a cellular factor is responsible for inhibition. Two general mechanisms for the inhibitory phenome-

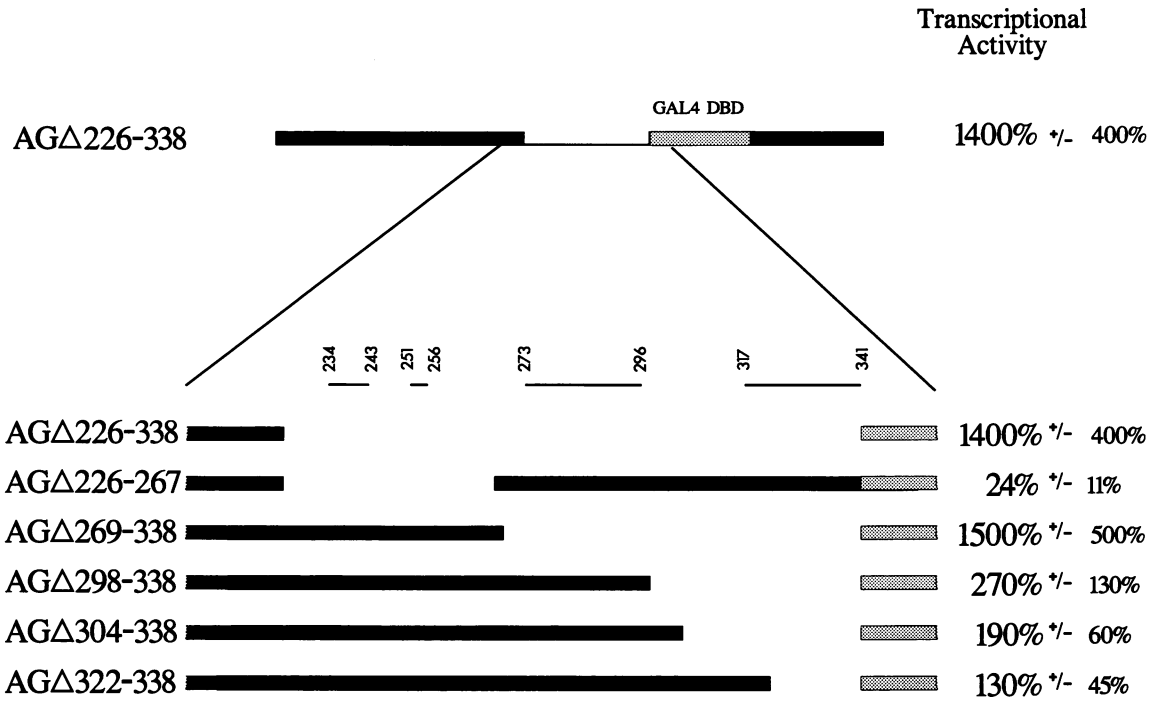

FIG. 2. Localization of the NGFI-A transcriptional inhibitory domain. The initial chimera with deletion of an inhibitory domain (AGΔ226–338) is shown to scale at the top. An expanded view of deletion chimeras within this region is shown below. Regions of identified sequence homology with Krox-20 and Egr-3 (14) are numbered and denoted by lines above the respective positions. CV1 cells were transfected with the indicated mutants and G5B-luc, cultured for 2 days, and assayed for luciferase activity ($N = 4$). Other details are as in Fig. 1.

non have been considered. First, as has been proposed for an inhibitory domain within c-Jun (3), the NGFI-A inhibitory domain located between residues 269 and 304 may interact in *cis* with one or more of the activation domains or, possibly, with the DNA-binding domain. This could occur via intra- or intermolecular interactions to mask or attenuate the activity of a particular domain. Second, the inhibitory domain might interact with another cellular component in *trans* and thereby alter either the frequency or nature of the interaction of NGFI-A with the transcriptional machinery. A combination of these mechanisms can also be envisioned.

To investigate possible *cis* interactions, constructs with deletions of the various activation domains, with and without the inhibitory domain, were cotransfected into CV1 cells. If the inhibitory domain functions by attenuating the activity of a particular activation domain, removal of that activation domain should render the construct insensitive to the absence of the inhibitory domain. We found that the inhibitory domain remained functional in the absence of each of the activation domains (Fig. 3), suggesting that it does not act solely through a specific activation domain. In addition, we investigated whether the presence of the inhibitory domain altered NGFI-A DNA-binding affinity, providing a possible mechanism for the observed effects on transcriptional activity. To obtain lysates with adequate amounts of protein to perform these assays and protein blot analysis, we transfected COS cells with 5 μg of either AΔ226–322 or native NGFI-A (Fig. 4). Equal portions of these lysates were found to contain equivalent amounts of the respective chimeric proteins. Analysis of the retarded complex in the gel shift assay by a phosphoimager revealed a less than twofold difference in the amount of oligomer bound by native NGFI-A and the AΔ226–322 mutant. These results show that the DNA-binding properties of these two constructs are similar and that the increased activity of the deletion construct AΔ226–322 cannot be explained on the basis of either increased protein levels or increased affinity for its cognate site on the reporter construct.

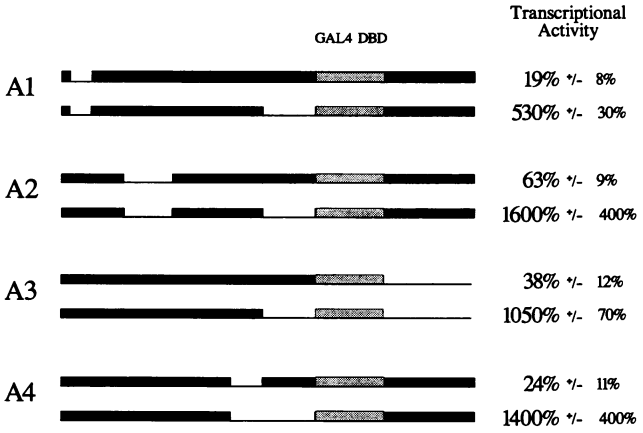

FIG. 3. Inhibitory domain does not act through a specific activation domain. The activity of mutants lacking the indicated activation domain was compared in the presence and absence of each inhibitory domain. Transcriptional activity is given as a percentage of the activity of the full-length AG chimera ± standard deviation ($N = 2$). Plasmids used for the pairwise comparison were: A1, AGΔ16–41 and AGΔ16–41/269–338; A2, AGΔ84–148 and AGΔ84–148/269–338; A3, AGΔ420–536 and AGΔ269–338/420–536; and A4, AGΔ226–267 and AGΔ226–338. DBD, DNA-binding domain.

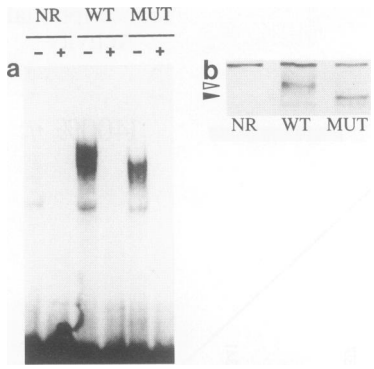

FIG. 4. Inhibitory domain does not affect the protein level or apparent DNA-binding properties of NGFI-A. COS7 cells were transfected with 10 μg of the indicated expression vector and cultured for 48 h, and whole-cell lysates for use in gel shift assays were prepared. (a) Gel shift assays with lysates from 10^6 cells transfected with nonrecombinant pCMV (NR), NGFI-A (WT), or AΔ226–322 (MUT) and an oligonucleotide containing two copies of the GCGGGGGCG NGFI-A binding site were performed in the presence (+) and absence (−) of a 200-fold excess of unlabeled specific competitor. Analysis of the gel shift with a phosphoimager revealed a less than twofold difference in the quantity of oligomer retained by the wild-type and mutant NGFI-A proteins. (b) Protein blot analysis of lysates representing 5×10^6 cells from the same plates as in panel a was performed with anti-NGFI-A (A310) antiserum (15). Open and solid arrowheads denote the wild-type and mutant NGFI-A proteins, respectively.

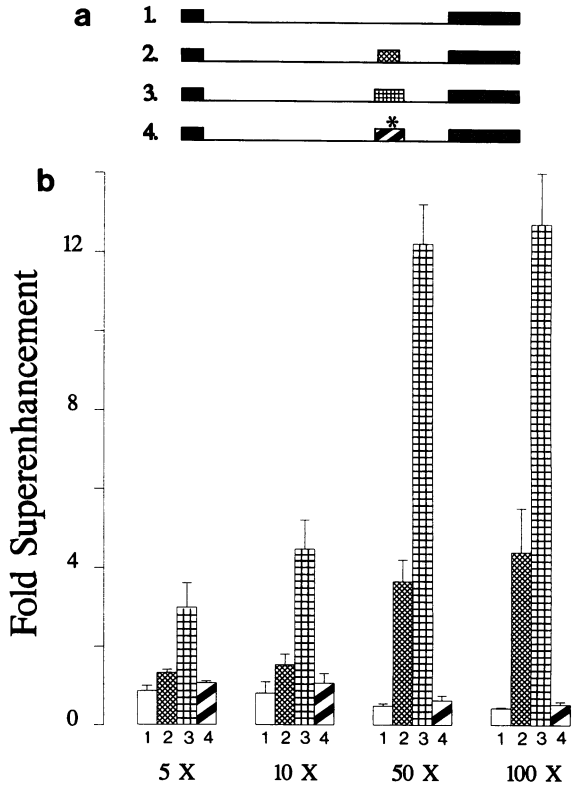

FIG. 5. Competition experiments demonstrate the presence of a titratable repressor. (a) Four competitors with native NGFI-A DNA-binding domains, identical except for their inserts, are depicted (not to scale). See text for inserts. (b) CV1 cells were transfected with 0.01 μg of full-length AG, 5 μg of G5B-luc, and the indicated excess of competitor. Total DNA transfected per plate was brought to 10 μg by adding the appropriate amount of pBSKS plasmid DNA (Stratagene), and the plates were harvested after 48 h of incubation. Two independent preparations of each competitor plasmid were used in two independent experiments. The results shown are the means and standard deviations of the fold enhancement, with a value of 1 assigned to luciferase activity in the absence of any competitor.

To address the second possibility, that another cellular component may interact with this domain of NGFI-A to cause this inhibition, we developed a competition assay. If the inhibitory domain of NGFI-A interacts with a cellular component, mutant NGFI-A molecules that contain the inhibitory domain but are unable to bind to the GAL4 luciferase reporter should act as competitors for this factor. If effective competition occurs, a decreased amount of this cellular component will be available to interact with the NGFI-A/GAL4 chimera, preventing its inhibitory effects and resulting in an increase in transcriptional activity similar to that observed with NGFI-A deletion mutants lacking the inhibitory domain.

Four competitors were used to assess the effect of overexpression of this inhibitory domain on the activity of a constant amount of NGFI-A/GAL4 chimera on its reporter. Each contains NGFI-A residues 1 to 29 and 322 to 420 (DNA-binding domain) and either no insert (competitor 1), NGFI-A residues 269 to 298 (competitor 2), residues 269 to 304 (competitor 3), or residues 269 to 304 with an Ile-to-Phe mutation at 293 (I293F) (competitor 4). Competitor 4 is identical to competitor 3 except for the mutation of Ile-293 to Phe. These competitors were cotransfected into CV1 cells in amounts ranging from 5-fold to 100-fold greater than that of the full-length NGFI-A/GAL4 chimera expressor, and the activity of the chimera was determined (Fig. 5). Overexpression of competitors 2 and 3 resulted in significant increases in the transcriptional activity of the chimera. The additional six residues of chimera 3 increased this effect, while the mutation of residue 293 in competitor 4 abolished its ability to increase NGFI-A activity and gave results similar to those of the control competitor 1. These experiments suggest that residues 269 to 304 are sufficient for an interaction with a titratable cellular component necessary for transcriptional inhibition of NGFI-A and that the substitution of Phe for Ile at residue 293 disrupts this interaction.

To further support our hypothesis that the interaction of NGFI-A with this titratable cellular factor is important for the inhibitory phenomenon, we also compared the effects of competitor constructs 1 and 2 on various NGFI-A/GAL4 deletion mutants (Fig. 6). The results of these experiments showed that the activity of mutants which contain the R1 inhibitory domain (residues 269 to 304) was increased only in the presence of competitor 2, while those lacking these residues were unaffected by either competitor. Together, these data suggest that a cellular factor binds to NGFI-A residues 269 to 304, decreasing its transcriptional activity.

To determine whether residue 293 is significant only in the competition assay used here or might have a critical role in the native molecule, NGFI-A was mutated at residue 293 (I293F), and its transcriptional activity was assessed (Fig. 7). A construct with a mutation at an adjacent residue (T292I) was also studied in these assays. As these mutant constructs

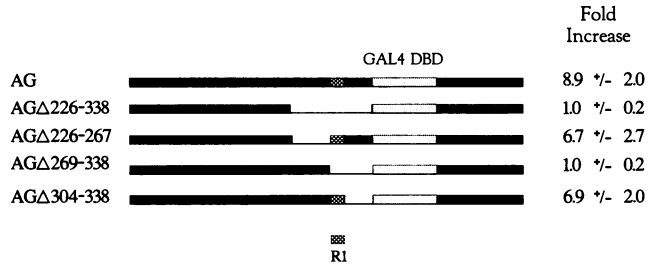

FIG. 6. Enhancement of NGFI-A/GAL4 chimeras is dependent on the presence of the inhibitory domain. CV1 cells were transfected with 0.1 μg of the indicated AG chimeras, 4.9 μg of G5B-luc reporter, and 5 μg of competitors 1 and 2 (depicted in Fig. 5), cultured for 48 h, and assayed for luciferase activity. The fold increase for each AG chimera is calculated by taking the ratio of its transcriptional activity with competitor 2 to that with competitor 1. Each experiment (N = 2) was conducted within the linear range of the luminometer and of luciferase production by CV1 cells (data not shown). DBD, DNA-binding domain.

are based on a parent NGFI-A construct (Ains268) with an additional five residues adjacent to the inhibitory domain introduced by cloning manipulations, the transcriptional activity of this parent molecule was also tested. As Fig. 7 demonstrates, the five extra residues had little if any effect on transcriptional activity, while the I293F mutant construct had much greater activity than the wild-type construct. Its activity appears to be greater than that of the deletion mutant, which has lost activation domain A4 as well as R1. Furthermore, unlike the I293F mutant, the T292I mutant is indistinguishable from the wild type in transcriptional activity. This confirms the results obtained in the competition experiments and demonstrates that residue 293 is necessary for effective interaction of NGFI-A with the cellular inhibitor.

DISCUSSION

NGFI-A is a transcription factor that is rapidly induced in response to a variety of environmental stimuli, positioning it in the nucleus at a time optimally suited for orchestrating the changes in gene transcription required to promote appropriate cellular responses. NGFI-A recognizes a GC-rich element that is also recognized by a number of other transcription factors, such as NGFI-C (13), Krox-20 (7), the Wilms' tumor gene product WT1 (30), and Egr-3 (28), suggesting that target genes for these transcription factors will be under complex control, a supposition substantiated by investigations of the effects of WT1 and NGFI-A on the activity of the *NGFI-A* promoter (24). As part of our investigations of the molecular events accompanying cellular responses to environmental alterations, we have pursued a structure-function analysis of NGFI-A (15, 41). Four activation domains, A1 (aa 16 to 41), A2 (aa 84 to 148), A3 (aa 420 to 536), and A4 (aa 226 to 267), were found to contribute to the transcriptional activity of NGFI-A. Each of them is capable of supporting transcriptional activation by itself when linked to the GAL4 DNA-binding domain (data not shown). The relative contribution of each activation domain to the overall activity of NGFI-A in CV1 cells is A4 = A1 > A3 > A2.

The most common characteristic of transcriptional activation domains is the presence of acidic residues, which give these regions a net negative charge (12). Within the A2 domain is a region (residues 84 to 126) that has a net negative charge of 6 and multiple sites for potential phosphorylation, including sites for protein kinase C, casein kinase II, and tyrosine kinases. As phosphorylation events modulate the activity of a number of transcription factors, this could impart another level of regulation on NGFI-A activity (reviewed in reference 19). Another type of activation domain is characterized by a high content of prolines, as seen in CCAAT-binding transcription factor (CTF1) and AP2 (40), or glutamines, found in the activation domain of Sp1 (27). The A4 domain is similar to these types of domains, as it is enriched in proline (19%) and glutamine (12%) residues. The A1 domain has limited homology to portion of a domain of Krox-20 found to be necessary for its transcriptional activity, with 23% identity over 26 residues, with one break (37). The A3 domain has no homology to previously identified activation domains. Notably, however, each of the activation domains contains sequences conserved among other members of this family (e.g., NGFI-C, Krox-20, and Egr-3 [14]). Intriguingly, the A3 domain contains eight repeats (26) which are similar to the carboxy-terminal domain repeats of RNA polymerase II that are critical to its function via their interactions with elements of the basal transcriptional machinery (34). It is also notable that domains A1 and A2 are separated by a repeating trinucleotide motif that is similar to those whose variation in copy number has been implicated in the pathogenesis of several human genetic diseases (reviewed in reference 31).

In addition to the four activation domains, we have identified a potent inhibitory domain (R1), located between residues 269 and 304, whose removal yields an NGFI-A molecule with 15-fold greater transcriptional activity than native NGFI-A. This increased activity is not dependent

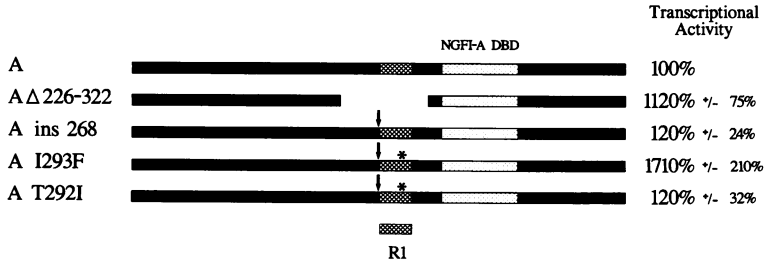

FIG. 7. Point mutations in R1 domain affect NGFI-A/GAL4 chimera activity. CV1 cells were transfected with 1 μg of the indicated expressor plasmids, 5 μg of A2ProLuc reporter plasmid, and 4 μg of pBSKS, harvested 48 h later, and assayed for luciferase activity (N = 2). The activity with native NGFI-A is taken as 100% activity and that of the nonrecombinant expression plasmid as 0%. The arrow indicates the area of insertion of five additional residues (see text). The asterisks represent the locations of the point mutation. DBD, DNA-binding domain.

upon any one activation domain, works on native and heterologous DNA-binding domains, and does not alter the abundance or DNA-binding properties of the protein. Rather, competition assays suggest that R1 interacts with a titratable cellular factor whose interaction with NGFI-A decreases its transcriptional activity. We have shown that NGFI-A activity is increased when *NGFI-A* is cotransfected with a competitor construct that encodes the wild-type R1 domain, while a competitor construct encoding an R1 with an I293F mutation has no effect. Furthermore, the competitor encoding the R1 domain increases the activity only of NGFI-A constructs that contain the inhibitory domain, further substantiating the idea that the inhibitory effect is realized by direct interaction of the cellular factor with this domain. When the I293F mutation, which converts the otherwise potent competitor to a nonfunctional competitor, is introduced into the native NGFI-A molecule, the result is a mutant transcription factor with activity 17-fold greater than that of the wild type. This provides further functional evidence in support of a significant interaction between the R1 domain of NGFI-A and a cellular factor which results in downmodulation of NGFI-A's transcriptional activity. Together with data shown above, we also conclude that residues 269 to 304 are sufficient, and that residue Ile-293 is necessary, for this interaction to occur.

The functional evidence indicating an interaction of a cellular factor with NGFI-A suggests a possible physiologic mechanism by which NGFI-A transcriptional activity might be rapidly modulated. For example, phosphorylation of NGFI-A and/or the cellular factor could result in altered interactions between these proteins, as has been demonstrated to modulate the interactions between Rb and E2F (8) and of the Jun repressor with c-Jun (2). A region with homology to protein kinase C phosphorylation sites is present in the inhibitory domain at Thr-292. Peptides based on the sequence of the physiologic protein kinase C substrate neurogranin have been used in kinetic studies (9) and demonstrate that a peptide with a Phe at the position corresponding to NGFI-A residue 293 has a 30-fold-lower K_m than the Ile-containing peptide, suggesting that NGFI-A constructs with the I293F mutation may be better substrates for protein kinase C. As the constructs with the I293F mutation appear to have lost the ability to interact with the cellular inhibitory factor, phosphorylation at Thr-292 may be the mechanism responsible for the disruption of this interaction. Consistent with this model of regulation, the activity of a mutant lacking this putative phosphoacceptor residue (A-T292I) is equivalent to that of wild-type NGFI-A (Fig. 7).

Enhancement of a transcription factor's activity by coexpression of a defined region of that transcription factor to titrate out interacting factors has been observed previously. The yeast transcription factor GAL4 is inhibited by GAL80 through an interaction with a 30-amino-acid domain in the carboxy terminus of GAL4. When those 30 residues are coexpressed with native GAL4, the inhibition by GAL80 is ameliorated and an increase in GAL4 activity is observed (23). The cell type-specific inhibitor of Jun interacts with the δ and ε regions of Jun, and overexpression of these domains relieves this inhibition (3, 4). The sequence of the R1 domain bears no resemblance to the Jun domains or to the 30 carboxy-terminal residues of GAL4. The cellular factor that interacts with NGFI-A, unlike the factor interacting with Jun, is present in a wide variety of mammalian cell types, but it is absent in *Drosophila* S2 cells, suggesting that it may be a general modulator of transcription factors. The p53 oncogene product physically interacts with the WT1 zinc finger protein, and its presence is required for WT1 to act as a transcriptional repressor, whereas in its absence, WT1 acts as a transcriptional activator (25). Similarly, the retinoblastoma gene product (Rb) has been shown to repress the transcriptional activity of E2F (18, 39), and these proteins were therefore considered possible candidates for the unidentified cellular factor acting on NGFI-A. However, when tested in the osteosarcoma cell line Saos-2, which lacks both functional Rb and p53, chimeric NGFI-A/GAL4 proteins without the R1 domain had activity much greater than that of the full-length chimera, suggesting that the cellular inhibitory factor was indeed present in these cells and is unique from p53 or Rb (data not shown).

The R1 domain is not homologous to any previously identified domain responsible for transcriptional modulation, but it is conserved in Krox-20 and Egr-3 (but not NGFI-C [14]), in which it might be expected to contribute similar properties. A deletional analysis of Krox-20 (37) did not identify any domains with inhibitory activity. However, these studies were done in *Drosophila* Schneider S2 cells, which lack the cellular factor responsible for the inhibition of NGFI-A activity. If the cellular factor interacting with the R1 domain also modulates the activity of some but not all members of this family, it would perhaps provide a mechanism by which differential regulation of potential target genes could be achieved. This is increasingly important, as abnormal target gene expression mediated by this family of transcription factors has been postulated to play a role in the pathogenesis of Wilms' tumor (16) and may be involved in other neoplasias. Further understanding of the function of NGFI-A and related proteins will be aided by the isolation and identification of this cellular factor.

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