Differential Role of the Proline-rich Domain of Nuclear Factor 1-C Splice Variants in DNA Binding and Transactivation*

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Félix Prado‡, Guillermo Vicent, Carina Cardalda, and Miguel Beato§

From the Institut für Molekularbiologie und Tumorforschung (IMT), Philippus-Universität, E.-Mannkopff-Str. 2, D-35033 Marburg, Germany

We have addressed the functional significance of the existence of several natural splice variants of NF1-C* differing in their COOH-terminal proline-rich transactivation domain (PRD) by studying their specific DNA binding and transactivation in the yeast Saccharomyces cerevisiae. These parameters yielded the intrinsic transactivation potential (ITP), defined as the activation observed with equal amounts of DNA bound protein. Exchange of 83 amino acids at the COOH-terminal end of the PRD by 16 unrelated amino acids, as found in NF1-C2 and splicing out the central region of the PRD, as found in NF1-C7, enhanced DNA binding in vivo and in vitro. However, the ITP of the splice variants NF1-C2 and NF1-C7 was found to be similar to that of the intact NF1-C1. Additional mutations showed that the ITP of NF1-C requires the synergistic action of the PRD and a novel domain encoded in exons 5 and 6. Intriguingly the carboxyl-terminal domain-like motif encoded in exons 9/10 is not essential for transactivation of a reporter with a single NF1 site but is required for activation of a reporter with six NF1 sites in tandem. Our results imply that differential splicing is used to regulate transcription by generating variants with different DNA binding affinities but similar ITPs.

Regulation of transcription operates by the combinatorial action of sequence-specific trans-acting factors bound to upstream regulatory regions of promoters and enhancer/silencer regions. The particular linear disposition of these cis-regulatory sequences and their topological organization in chromatin, along with the cellular repertoire of transcription factors results in the formation of gene- and cell-specific constellations of chromatin bound factors. The particular array of proteins on the chromatin target modulates the transcriptional rates by multiple interactions with chromatin remodeling complexes, co-regulators, and components of the basal transcriptional machinery (1). Transcriptional regulators often exhibit a modular structure with independent DNA-binding domains (DBDs) and regulatory regions. The transactivation domains are classified according to their amino acid composition as either rich in acidic side chains, glutamine, or proline residues (2). Although the precise fashion by which transactivators regulate transcription of a specific gene depends on many different promoter features and cell-specific factors, the mechanisms of action for acidic and proline-rich domains appear to be conserved in eukaryotic organisms from yeast to man (3, 4).

Most transcription factors are expressed in several variants that can be grouped in large families, whose members show only subtle differences. The expression of these variants is tightly regulated in a cell-type or developmental-stage specific manner. Alternative splicing is the mechanism most widely used to generate this precisely regulated, diversity of transcription factors. However, with few exceptions, the functional significance of transcription factor variants generated by alternative splicing is poorly understood (5, 6).

The prototype of the proline-rich class of activators is nuclear factor 1 (NF1), originally identified through its role in stimulating adenovirus DNA replication (7, 8). NF1 is expressed in vertebrates from at least four different genes (NF1-A, NF1-B, NF1-C/CTF, and NF1-X), each of them giving rise to different variants by alternative splicing of the COOH terminus (9–13). The variants bind as homo- and heterodimers to the consensus binding site TTGGC(N5)GCCAA (14). This wide variety of forms is differentially expressed during mouse development and regulates the activity of many genes expressed in multiple organs (Ref. 15 and references therein). Disruption of the NF1-A gene in mice causes multiple developmental defects and perinatal lethality (16), suggesting that at least some functions of the various NF1 proteins are not redundant. However, the functional implications of the existence of a large variety of NF1 proteins remain largely unknown.

Comparison of the primary structures of different NF1-C variants reveals their modular organization (Fig. 1A). All variants share a very well conserved NH2 terminus containing the DBD and the dimerization domain. This NH2-terminal half activates Adenovirus 2 DNA replication by interacting with the viral DNA polymerase (17, 18). The COOH-terminal half can be divided in a central region, which is specific for the products of each of the four genes, and a variable carboxy region, which is specific for each splice variant (13, 18, 19). A functional comparison of NF1-C variants showed different efficiencies of transcriptional activation, indicating that the very COOH-terminal region determines the transactivation potential of NF1 (20, 21).

This region contains the proline-rich transactivation domain (PRD), whose importance in the transactivation functions of NF1 has been largely confirmed (3, 18). A detailed analysis of this region identified a sequence homologous to the COOH-terminal domain (CTD) of the largest subunit of RNA polymerase II as an essential element of the PRD. In addition, a stretch of hydrophobic amino acids contributes strongly to the
activity of the CTD-like motif (22–24). The very last 20 amino acids at the COOH terminus of NF1-C1 have been reported to interact with the globular domain of histone H3 and may mediate regulation of NF1-C1 activity by transforming growth factor-β (25). However, some variants lacking most of the PRD and the CTD-like motif enhance transcription to a greater extent than the full-length NF1-C1 (20, 21). These observations suggested the existence of additional regulatory sequences in NF1-C1, and placed a question mark on the role of the variable PRD in transactivation by NF1-C.

Here we report studies in Saccharomyces cerevisiae showing that although the absolute transactivation obtained with three natural splice variants of NF1-C (NF1-C1, NF1-C2, and NF1-C7, differing in their PRD) are different, this is largely due to differences in DNA binding. Once corrected for DNA binding activity, the intrinsic transactivation potential (ITP) of the three isoforms is similar and requires the synergistic action of the PRD and an internal region encoded by exons 5 and 6. The CTD-like motif exhibits a reporter-specific behavior. We show that the splice variants NF1-C2 and NF1-C7 bind to DNA with higher affinity than the full-length NF1-C1 and conclude that splicing out part of the PRD regulates transactivation by increasing the DNA affinity of the resulting protein without affecting its ITP once bound to DNA.

MATERIALS AND METHODS

Yeast and Growth Conditions—The yeast strain used in this study was YPH499 (a ade2-101 his3 Δ500 leu2-3,112 trpl-1Δ ura3-52 lys2-801). Standard media, such as rich medium YEPD and synthetic complete medium (SC) with bases and amino acids omitted as specified, were prepared as described previously (26). Yeast strains were transformed using the lithium acetate method (27) modified according to Schiestl and Gietz (28).

Plasmids—All plasmid constructions were performed using Escherichia coli strain DH5α, pLR-NF1(x6) (21) and pSch105 (29) are YEplasmids based on the URA3 gene containing the NF1(x6)-GAL1(UAS3)-lacz and the MMTVα-lacz fusion constructs, respectively. pAA-CTF1, pAA-CTF7 (21), and pAA-CTF2 are YEpl expression vectors for pig NF1-C1, NF1-C7, and NF1-C7, respectively, derived from pAAH5 (30). pAA-CTF1ΔCT7 is a YEpl expression vector for pig NF1-C1 ΔCT7 constructed by cloning the C7-2) (23) into the blunted XbaI site of pAAH5. p415MCTF1, p415MCTF7, constructed by cloning the C229, based on the pBR-CTF1mbd fragment from pBR-CTF1mbd (21) into the blunted HindIII site of pAAH5. pBR-CTF1 consists of a yeast C-1 (at position 687 of the NF1 coding sequence) ORF and made blunt ended)-HindIII site of pAAH5. pAA-CTF-(1–406) was cloned into the HindIII site of pAAH5. pAA-CTF-(1–406), pAA-CTF-(1–362), or 1 to 406 were amplified by PCR and inserted at the HinIII site of pBR322. pAA-CTF-(1–406). It was constructed using the lithium acetate method (27) modified according to Schiestl and Gietz (28). The yeast strain used in this study was YPH499 (a ade2-101 his3 Δ500 leu2-3,112 trpl-1Δ ura3-52 lys2-801). Standard media, such as rich medium YEPD and synthetic complete medium (SC) with bases and amino acids omitted as specified, were prepared as described previously (26). Yeast strains were transformed using the lithium acetate method (27) modified according to Schiestl and Gietz (28).

RESULTS

NF1-C1, NF1-C2, and NF1-C7 Activate Reporter Genes to a Different Extent but Have a Similar ITP—To gain insight into the mechanisms of transcriptional activation by members of the NF1 family we have studied the ITP of three natural NF1-C splice variants (Fig. 1A). We have chosen the yeast S. cerevisiae for these studies because NF1-C is known to transactivate in this organism and endogenous NF1 homologues are not known. NF1-C1 encompasses all 11 exons of the gene (Fig. 1A). In NF1-C2, the splicing of exon 9 deletes 83 amino acids of the PRD and creates a new reading frame leading to the COOH-terminal addition of 16 amino acids with a high proline content. NF1-C7 lacks exons 7, 8, and 9 including most of the PRD (Fig. 1A). We transformed yeast with plasmids expressing each of these variants together with the reporter plasmid pLR-NF1(x6) (21). In this plasmid, which we will name pLR-NF1(x6), the lacZ gene is driven by the GAL1 promoter with its regulatory region replaced by six NF1-binding sites. As previously reported, NF1-C7 transactivated this promoter to a much larger extent than NF1-C1 (Fig. 1A), despite lacking most of the PRD (21). NF1-C2 transactivated with similar efficiency (48%) as NF1-C1 (Fig. 1A), confirming previous findings in Drosophila Schneider cells (9, 18). We also tested the behavior of the three NF1-C variants in the context of a natural promoter with only one NF1 site, namely a mutant MMTV promoter (MMTVa), truncated just upstream of the NF1-binding site to remove the hormone responsive region (29). Although the activity on this promoter was lower, the results with the NF1-C variants were qualitatively similar to those obtained with NF1-GAL1 (Fig. 1A). NF1-C7 was by far the best transactivator, while NF1-C2 was very similar to NF1-C1. Thus, independent of the reporter used, NF1-C7 was a better transactivator than NF1-C1 and NF1-C2.

Most studies on the transactivation properties of NF1 in yeast have not taken into account possible differences in levels of expression or in DNA binding affinity of the different variants. To incorporate these parameters in our studies, we determined the extent of specific DNA binding to calculate the ITP.
Different DNA Affinity of Natural NF1-C Splice Variants

Transactivation by NF1-C Requires the Synergistic Action of the PRD and a Central Domain—Previous work on NF1 has defined the COOH-terminal PRD as the main transactivation determinant of the protein (3, 18). However, the results obtained with NF1-C7 show that most of the PRD of NF1-C1 could be spliced out without affecting the ITP. Along the same line, the natural variant NF1-C406, which lacks exons 9 and 10, acts as a strong transactivator in yeast (20). These observations suggest the presence of additional transactivation functions in NF1-C. To address this issue, we have analyzed different deletion mutants of the full-length NF1-C1 for their ability to transactivate the two reporter genes in yeast (Fig. 3A). As for the natural NF1-C variants, the activities of the mutant proteins have been corrected for their corresponding DNA binding activities (Fig. 3B) to calculate their ITP once bound to DNA (Fig. 3C).

In previous studies the core PRD has been defined as the region encoded in exons 9–11 (amino acids 407–506). Deletion of this region, as in NF1-C406, led to a dramatic reduction in transactivation of both reporters (Fig. 3A). This was accompanied by a 5-fold reduction in DNA binding activity (Fig. 3B). After correction for DNA binding, the ITP of NF1-C406 on the

of each variant by comparing amounts of protein yielding equivalent DNA binding activity. To establish the reliability of our measurements, we first expressed NF1-C1 under control of the regulated MET25 promoter, whose activity depends on the methionine concentration in the medium. We prepared extracts from cells grown at different methionine concentrations and measured β-galactosidase activity and specific binding to an oligonucleotide with a NF1-binding site by GEMSMA. As expected, DNA binding increased with progressive activation of the MET25 promoter (Fig. 2A). Within the range tested, the NF1-C1 dependent activation of the NFGAL1 reporter was a linear function of the amount of DNA binding activity (Fig. 2B, circles). The same is true for the MMTVΔ promoter (data not shown), confirming the validity of our quantitation of DNA binding.

Next we compared the DNA binding activity of extracts from cells expressing the three natural variants of NF1-C. While extracts containing NF1-C1 and NF1-C2 showed very similar binding to the NF1 oligonucleotide, extracts containing NF1-C7 showed a 7-fold higher specific DNA binding (Fig. 1B). The activation values obtained with the three NF1-C variants in these experiments fitted very well in the standard curve constructed with NF1-C1 under the control of the inducible MET25 promoter (Fig. 2B, squares). Correction of the transactivation activities (Fig. 1A) by the corresponding DNA binding values of the NF1-C variants yielded their respective ITP, which were very similar on the MMTVΔ reporter (Fig. 1C, light columns). On the NFGAL1 reporter, NF1-C2 exhibited a 2-fold lower intrinsic transcriptional potential when compared with NF1-C1 and NF1-C7 (Fig. 1C, dark columns) (see below). These results demonstrate that the splicing of different regions of the PRD of NF1-C1 generates two proteins, NF1-C2 and NF1-C7, with different DNA binding activity, but with similar ITP.

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NF1βGAL1 and the MMTVΔ reporter was 5 and 15% that of NF1-C1, respectively (Fig. 3C), confirming that the core PRD is important for transactivation. As exon 8 is also rich in proline (19.5%), we tested the behavior of a deletion of all the proline-rich regions (exons 8–11). This deletion, NF1-C362, showed slightly higher transactivation than NF1-C406 (Fig. 3A), but a 13-fold higher DNA binding activity compared with NF1-C406 (Fig. 3B). Thus, the ITP was further reduced 3-fold, to 1.3% of NF1-C1 with the NF1βGAL1 reporter and 5% with the MMTVΔ reporter (Fig. 3C). These results confirm the importance of the proline-rich region, and show that it encompasses sequences encoded in exons 8–11 of NF1-C1. Constructions lacking this region exhibited 3-fold higher residual activity on the MMTVβ reporter than on the reporter with six NF1-binding sites, suggesting that the PRD is also involved in the synergism between DNA-bound NF1 molecules. Although part of the proline-rich region can be deleted and replaced by different sequences without influencing the ITP, as in NF1-C2 and NF1-C7 (Fig. 1), other regions are obviously essential. It remains to be elucidated what additional features of these regions, besides the high proline content, determine their transactivation properties.

If the PRD were the only transactivation function within the COOH-terminal half of NF1-C, a protein with this domain fused to the DBD, as generated by deleting exons 5–7, should display similar activity as the intact protein, provided the DBD has no transactivation function. We confirmed that the NF1-CDBD, although able to transactivate to some extent when expressed from a strong promoter (Fig. 3A), had negligible ITP (Fig. 3C, C229) after correction for the high DNA binding activity (Fig. 3B, C229). The internal deletion of exons 5–7 in construct NF1-C Δ234–406 led to a 2–2.5-fold decrease in transactivation (Fig. 3A) and to a 4-fold increase in DNA binding activity (Fig. 3B). After appropriate correction, this construction showed a 10-fold decrease of ITP compared with intact NF1-C1 (Fig. 3C). Nevertheless, these values were still 7–10-fold higher than those obtained with the DBD of NF1-C (Fig. 3C, compare CΔ234–406 and C229). These findings identify a transactivation function in sequences encoded by exons 5–7. Since NF1-C7 shows maximal activity and lacks amino acids 320–472 (Fig. 1), and deletion of exon 7 encoded sequences does not reduce the ITP (compare C319 and C362), the novel transactivation function must be located between amino acids 234–319 in the region encoded by exons 5 and 6. A construction including just the DBD and exons 5–6, NF1-C319, exhibited low but reproducible ITP, in particular with the MMTVΔ reporter (Fig. 3C) (see below). Thus, our results with natural variants and mutations support the existence of a weak internal transactivation domain in the region encompassing exons 5 and 6. As the sum of the ITPs of the PRD and this internal domain accounts only for 15–20% of the ITP of NF1-C1, the two domains seem to act synergistically in the intact protein.

The CTD-like Motif of NF1-C1 Is Not Essential for Transactivation of a Promoter with a Single NF1 Site—The PRD of NF1 contains a sequence with a striking similarity to the heptapeptide repeats of the CTD of the largest subunit of RNA polymerase II. This CTD-like motif has been claimed to be essential for the transactivation function of the PRD (22–24). However, this claim is in conflict with the strong ITP of the natural variants NF1-C2 and NF1-C5, which lack the CTD-like motif (Fig. 1 and Ref. 20). Therefore, we tested a selective deletion of this motif. The resulting protein, NF1-CΔCT7, exhibited a lower transactivation than NF1-C1 (Fig. 3A) and an almost normal DNA binding activity (Fig. 3B). These changes result in a 5-fold decrease in its ITP on the NF1βGAL1 promoter (Fig. 3C), in agreement with previous results with similar constructs (23, 24). However, the ITP of NF1-CΔCT7 was hardly affected when tested on the MMTVΔ promoter, which contains a single NF1-binding site (Fig. 3C). Except C229, all constructions lacking the CTD-like motif (C2, C319, C362, and C406) exhibited 2–3-fold lower ITP on NF1βGAL1 than on MMTVΔ, whereas this difference was not found with constructions including the motif (C1, C7, and CΔ234–406). Although we cannot exclude other promoter-specific features, these results suggest that the CTD-like motif may be more important for cooperation between multiple NF1-C proteins bound to adjacent DNA sites.

Splicing Out Part of the PRD Increases the DNA Affinity of NF1-C—We have observed significant differences in DNA binding activity among extracts from cells expressing different NF1 variants and mutants (Figs. 1B and 3B). To determine whether these differences were due to different levels of expression of the various proteins or to different DNA binding affinities, we measured the amount of protein by Western blotting (Fig. 4A). As the only region shared by all constructions is the DBD and the available antibodies against this region are of low affinity, we cloned an epitope at the NH2 terminus and expressed the epitope-tagged NF1-C variants and mutants under control of the ADH1 promoter. In agreement with previous studies (17), epitope tagging did not alter the DNA binding properties of the different proteins, determined by GEMSA (data not shown). Western blots with different amounts of the various cell extracts showed that most of the tagged proteins (C1, C7, CΔCT7, C362, and C229) were present at comparable levels. The exceptions were NF1-C2, which was present at 4–5-fold lower levels, and NF1-C Δ234–406, which was present at 15-fold higher levels (Fig. 4A). Using these expression values and the binding activities determined by GEMSA, we calculated the specific DNA affinity of each protein in the extracts (Fig. 4B). The results showed that splicing out part of the PRD in variants NF1-C2 and NF1-C7 increased the affinity for DNA 4–5-fold, suggesting the existence of DNA binding inhibitory domains in NF1-C1. As the region lacking in both variants is exon 9, this
In agreement with this idea, all the other proteins lacking exon 9 exhibited higher DNA affinity (C229 and C362), whereas the internal deletion of exons 5 and 6, which brings exon 9 closer to the DNA-binding domain (NF1\(\text{C}^234-406\)), led to a reduced affinity for DNA. We can exclude a role for the CTD-like motif, as mutant NF1\(\text{C}^234-406\) displayed similar DNA binding affinity as the intact NF1-C1 (Fig. 4B).

To ascertain the relevance of these in vitro results for the situation in intact cells, where the NF1-binding sites are organized in chromatin, we performed genomic footprinting experiments with NF1-C1, NF1-C7, and NF1\(\text{C}^234-406\) on the NF1\(\text{GAL1}\) promoter. NF1-C1 and NF1\(\text{C}^234-406\) bound to the promoter with similar affinity, protecting the six NF1 sites and generating a series of DNase I-hypersensitive sites at the upstream border of the footprint (Fig. 5, asterisks). Hence, the transactivation defect of NF1\(\text{C}^234-406\) in the reporter with six NF1-binding sites is not due to lower affinity for its target DNA. Most important, NF1-C7 displayed a stronger protection of the DNA-binding sites than NF1-C1 (Fig. 5). This increase in DNA binding affinity within the cell validates the results obtained with cell extracts and provides an explanation for the high activity displayed by NF1-C7 compared with NF1-C1 despite their similar ITP (Fig. 1, A and C). Therefore, splicing out part of the PRT domain enhances the actual transactivation by NF1-C7 by increasing the specific DNA affinity in chromatin without changing the ITP once bound to DNA.

**DISCUSSION**

The existence in most animal cells of multiple NF1 variants generated by alternative splicing of the transcripts from four different genes raised the question of their functional differences. The presence of ubiquitous combinations of splice variants makes virtually impossible the study of this problem in metazoan cells. As all the natural variants share the conserved NH\(_2\)-terminal DBD and exhibit similar DNA binding specificity, potential differences in function have been assigned to the variable COOH-terminal half of the proteins, which encompasses the PRD and a central subtype-specific domain. We have compared NF1-C1 and two natural variants, NF1-C2 and
NF1-C7, with different PRDs, in *S. cerevisiae*, in which the PRD of NF1 has been shown to be active (3, 35). Using two reporters with either one or six NF1-binding sites we find that transactivation is highest with NF1-C7 and comparable for NF1-C1 and NF1-C2. Similar results have been previously reported, and have been interpreted as reflecting a bipartite structure of the PRD (21). In NF1-C7, deletion of amino acids encoded in exons 7–9 would bring closer together the two subdomains and thus result in higher transactivation efficiency. However, alternative interpretations are possible.

Three Natural Variants of NF1-C Show Similar ITP—Differences in transcriptional strength of NF1-C variants could not only be due to differences in the strength of their transactivation domains, but also to differences in their levels of expression, nuclear localization, and DNA binding properties. Binding to an NF1-specific oligonucleotide probe was 7-fold higher in cell extracts containing NF1-C7 compared with those containing NF1-C1 or NF1-C2. Correction for these differences in DNA binding activity yielded the transactivation achieved by equivalent amounts of DNA-bound proteins. This parameter, that we have called ITP, did not change significantly among the three variants, when determined with the reporter gene containing a single NF1-binding site. With the reporter containing six NF1-binding sites no difference in ITP was observed between NF1-C1 and NF1-C7, while a slight but significant reduction was seen with NF1-C2 (18). The fact that in a previous study NF1-C2 was found to transactivate much less efficiently than NF1-C1, was probably due to the use of NF1-C2 constructs lacking a few amino acids at the NH2 terminus, which are important for DNA binding (21). Our findings suggest that the well conserved 20 amino acids at the COOH-terminal end of NF1-C1, which are missing in NF1-C2, do not play an essential role in transactivation in yeast. We do not know whether the lack of function of this region, which has been shown to interact with histone H3 (25), is due to a pecu-
liarity of yeast chromatin or to the lack of appropriate intermediary factors. However, the most important conclusion from this group of results is that splicing out the central part of the PRD encoded in exons 7–9, as in NF1-C7, while leading to higher DNA binding activity, does not change the ITP of the resulting protein.

**Identification of a Central Transactivation Domain That Synergizes with the PRD**—As the natural NF1-C variants did not exhibit major changes in their ITP they were not helpful for the delimitation of the activation domains. The results obtained with additional mutations showed that deletion of the core PRD (exons 9–11) leads to a significant reduction of the ITP. However, even the construct containing only the sequences encoded in exons 1–7 exhibited a significant ITP on the reporter with a single NF1 site, pointing to the existence of additional transcriptional activation functions in the NH2-terminal half of NF1-C1. This notion is supported by the results obtained with an internal deletion of sequences encoded by exons 5–8, which fuses the core PRD to the DBD. This construct exhibited only 11% of the ITP of NF1-C1, showing that the central region contributes significantly to the full activity of the intact protein. As neither the DBD (exons 1–4) nor the sequences encoded in exon 7 exhibit significant transcriptional activation, the novel central transactivation domain must be encoded by exons 5 or and 6. This region is relatively rich in negatively charged amino acids and could represent an acidic type of transactivation domain different from the COOH-terminal PRD. The sum of the contributions of the acidic domain and the PRD accounts only for about 20% of the ITP of the intact NF1-C1. Therefore, both transactivation domains must synergize in the intact protein. How this synergism is brought about is not known but there are indications suggesting that acidic and proline-rich domains use different classes of co-activators (36, 37). A similar synergism between the PRD and a central subtype-specific domain has been described for the *Xenopus* NF1-X subtype based on the study of natural variants and deletion mutants (19). Thus, this kind of modular organization may be conserved in the NF1 family.

**Reporter-specific Requirement of the CTD-like Motif**—The COOH-terminal end of NF1-C1 contains a conserved amino acid sequence, SPTSPSYSP, with homology to the CTD repeat found in the largest subunit of RNA polymerase II, that has been claimed to participate in transcriptional activation (22–24). However, this claim is based on experiments with fusions to the GAL4 DNA-binding domain and is in conflict with the observation that variants lacking this CTD motif, such as NF1-C2, show almost normal ITP. A selective deletion of CTD-like motif in NF1-ΔCT7 reduced the binding to a NF1 oligonucleotide by only 40%. A similar reduction (35%) was observed in the ITP of this mutant on a reporter with a single NF1-binding site. Moreover, the specific affinity of NF1-ΔCT7 for the NF1 oligonucleotide, calculated after correction for expression levels, was the same as that of the full-length NF1-C1. Thus, the CTD-like motive is not required for activation of a reporter containing a single NF1-binding site. However, NF1-ΔCT7 exhibited a 5-fold reduction in the ITP measured with the reporter containing six NF1-binding sites. Thus, the effect of deleting the CTD motif depends largely on the nature of the reporter promoter. This could reflect a major effect of the mutation on the synergistic binding of NF1-C dimers to multiple adjacent NF1 sites, but this seems unlikely in view of the genomic footprinting results, which showed similar protection of the six NF1 sites in cells expressing NF1-C1 or NF1-ΔCT7. Therefore the CTD-like motif seems to be involved in activation of reporters with multiple NF1 sites at steps subsequent to DNA binding. The requirement for the CTD-like motif is strong in the context of the full-length NF1-C1, whereas NF1-C2, which also lacks the rest of the COOH-terminal 83 amino acids, showed a much less dramatic decrease in ITP with the reporter containing six NF1 sites.

*A Region of the PRD Reduces DNA Binding*—The results obtained with NF1-C7 suggest the existence within the PRD of NF1-C1 of a region that reduces DNA binding activity of the protein. However, the increased DNA binding activity of this variant could simply reflect a higher level of expression or a better nuclear localization. The latter possibility seems unlikely as the nuclear localization signal of NF1-C has been assigned to the DBD (5, 18), which is conserved in the various isoforms of NF1-C. However, we cannot formally exclude differences in the nuclear localization of the different NF1 isoforms. The expression levels of the various proteins were assessed by quantitative Western blotting with epitope-tagged constructs of the different variants and mutants. With the exception of the internal deletion NF1-CΔ234–406, which accumulated to high levels, and NF1-C2, which was poorly expressed, all the other proteins were present at similar levels in whole cell extracts. After correction for levels of expression, proteins with deletions in the COOH-terminal half, including NF1-C7, bound with higher affinity to DNA. The exception was NF1-CA7CT7, which bound with similar affinity as NF1-C1, and the internal deletion mutant NF1-CΔ234–406, which showed only 40% of the DNA affinity of NF1-C1. This latter result is questionable due to the very high expression levels of NF1-CΔ234–406. Genomic footprinting experiments showed that NF1-CA7CT7 binds with similar affinity as NF1-C1, while NF1-C7 binds with higher affinity to its target sequences even when these are organized in chromatin. Thus, our *in vitro* binding data reflect the behavior or the corresponding proteins within the cell.

One possible interpretation of our findings is that removal by alternative splicing of the central part of the PRD generates proteins with higher specific DNA affinity. This region of the PRD, different from the CTD-like motif, reduces the specific DNA affinity of NF1-C1 in intact cells and in cell extracts. A DNA binding inhibitory domain has been localized to the COOH-terminal end of the central subtype-specific domain of *Xenopus* NF1-X (19). However, in this case the inhibitory effect on DNA binding is only observed in *in vitro* experiments with the recombinant proteins, whereas it is not apparent in the intact cell. Previous experiments with similar variants of NF1-C expressed in *E. coli* and purified by DNA affinity chromatography did not detect significant differences in DNA binding behavior (9, 18). Although we have not tested the binding properties of our recombinant NF1 variants, the results with bacterially expressed suggest that the differences we observed *in vivo* and in cell extracts could reflect either cell-specific post-translational modifications or interactions with other components of the extract. Examples of these kind of intramolecular modulation of DNA binding have been described for other transcription factors, including p53 (38) and SWI4 (39). Post-translational modifications of the protein remain an interesting and attractive possibility, which could contribute to the physiological regulation of NF1 function (25). Although mutation of all the possible phosphorylation sites at the very COOH-terminal end of NF1-C1 does not influence its regulation by growth factors (25), other sites of phosphorylation or other modifications, such as acetylation, could play a role.

Our findings suggest that the control of transcription factors affinity for DNA via alternative splicing is a widespread mechanism for regulation of gene expression. Modulation of DNA binding may be achieved by structural modifications of either the DBD or regions adjacent to the DBD that regulate its
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activity (5). Examples of the latter possibility have been reported for some members of the basic helix loop helix family of transcription factors. Thus, in the human E12/E47 immunoglobulin enhancer binding factors, the E12 isoform includes an NH2-terminal region of 11 amino acids that functions as an inhibitor of DNA binding (40). Examples of alternatively spliced inserts which enhance DNA binding affinity by basic helix loop helix factors are found in two isoforms of either Mi (41) or Max proteins (42). This type of regulation of DNA binding affinity by alternative splicing may be particularly relevant for NF1-C, since several of its reported functions, such as the synergism with progesterone receptor on the MMTV promoter (43) and the activation of adenovirus DNA replication (44), are exclusively attributed to its DNA-binding domain.

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