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Identification of the Transcriptional Repression Domain of Nuclear Factor 1-A

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We previously showed that nuclear factor 1-A (NF1-A) binds to the silencer elements in the glutathione transferase P (GST-P) gene, and the carboxy terminal region of NF1-A represses the transcription activity of human metallothionein IIA (hMTIIA) promoter. In this study, we identified a repression region which is divided into two 100 amino acid domains (RD1 and RD2). RD1 increased the repression activity of RD2 to the hMTIIA promoter activity. The NF1-A repression domain inhibited the promoter activities of not only the hMTIIA gene but also those of the GST-P and CCAAT/enhancer binding protein δ genes. RD1 and RD2 had abundant serine and glycine residues, and proline and serine residues, respectively. Whereas some repression domains identified previously are enriched with alanine, proline, or serine, and are associated with the general transcription factors, the NF1-A repression domains did not interact with transcription factor IIB, TATA-binding protein (TBP), or TBP-associated factors *in vitro*. © 1997 Academic Press

Transcriptional repression and activation are two of the most important mechanisms underlying gene regulation. However, studies of the regulation of transcription have usually focused on the mechanism of transcriptional activation; little is known about the repression mechanism. Many eukaryotic transcriptional activators which stimulate transcription by the binding of the target genes to *cis*-elements have been identified. In general, the transcriptional activators are divisible into two domains, the DNA-binding domain and the activation domain. The latter domain has been well characterized and interacts with one or more components of the general transcription machinery (1-3).

A variety of transcriptional repression models have recently been proposed, including competition, quenching, direct inhibition, squelching, and recruitment of inhibitory chromatin components to the promoter (4-7). However, the number of transcriptional repressors that have been identified and characterized is still small.

We previously demonstrated that nuclear factor 1-A (NF1-A) binds to the silencer element in the glutathione transferase P (GST-P) gene which is repressed completely in rat liver, and we observed that the carboxy terminal region of NF1-A showed the repressing activity (8). NF1-A is a member of the NF1 family of proteins. It was reported that other members of the NF1 family, NF1-C and NF1-X, act as activators and that the activation regions of these *trans*-acting factors were narrowed down (9-12). The repression domain for NF1-A has not been yet characterized.

In the present study, we identified the repression domains of NF1-A that repress the promoter activities of several genes and do not bind to TATA binding protein (TBP), TFIIB, or TBP-associated factors (TAFs). We will also and discuss the mechanism of the repression by NF1-A.

EXPERIMENTAL PROCEDURES

Plasmid constructions. 5xGAL4-MTIIA-luciferase has been described previously (8). For the construction of 5xGAL4-91GST-luciferase and 5xGAL4-167C/EBPδ-luciferase, a GAL4 binding site was synthesized and multimerized to 5 mer, and cloned into the *Xho*I site of pBluescript KS+. The sequence of the GAL4 binding site of the upper strand was as follows: 5′-TCGACGGAAGACTCTCCTCCGT-3′. The *Sma*I-*Kpn*I and *Kpn*I-*Xba*I fragments including 5 mer of the GAL4 binding site were cloned into the *Sma*I-*Kpn*I and *Kpn*I-*Nhe*I sites of the −91GST-luciferase (13) and −167C/EBPδ-luciferase, respectively. To generate −167C/EBPδ-luciferase, the fragment from base pairs −167 to +42 in the promoter region of CCAAT/enhancer binding protein δ (C/EBPδ) gene (14) was made by polymerase chain reaction (PCR) techniques and subcloned into *Xho*I-*Hin*dIII sites in the luciferase vector, PGV-B (Toyo Ink Mfg. Co., Tokyo, Japan).

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Abbreviations: NF1, nuclear factor 1; GST-P, glutathione transferase P; TBP, TATA-binding protein; TFIIB, transcription factor IIB; TAF, TBP associated factor; GTF, general transcription factor; C/EBP, CCAAT/enhancer binding protein; MT, metallothionein; PCR, polymerase chain reaction.

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GAL4-A1 has been described previously (8). GAL4-A1 (318-509), GAL4-A1 (318-427), GAL4-A1 (427-509), GAL4-A1 (318-365), GAL4-A1 (365-427), and GAL4-A1 (145-315) were made by digesting NF1-A1 cDNA with *Sph*I and *Hinc*II, *Sph*I and *Alw*44I, *Alw*44I and *Hinc*II, *Sph*I and *Hinf*I, *Hinf*I and *Alw*44I, and *Sca*I and *Sph*I, respectively, blunt ending with T4 polymerase, and subcloning into pSG424 (15). GAL4-A2 (145-315) and GAL4-A3 (145-315) were generated by digesting NF1-A2 and NF1-A3 cDNAs with *Sca*I and *Sph*I, respectively, and ligating to pSG424. GAL4-A1 (209-315) and GAL4-A1 (209-427) were constructed by PCR.

For the construction of GST-RD1 and GST-RD1·RD2, the fragments corresponding to the amino acid positions 209-315 and 209-427 were cloned into pGEX-3X (Pharmacia, Uppsala, Sweden). GST-RD2 was generated by subcloning a *Sph*I-*Alw*44I fragment into pGEX-3X. All fragments generated by PCR were checked by sequencing by the dideoxy method (16).

Cell culture, transfection and luciferase assay. HeLa cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, and passaged by trypsinization at confluence. Transfection was carried out by the calcium phosphate co-precipitation technique (17), using 3.5 μg of the luciferase reporter plasmid, 1.0 μg of the effector plasmid, and 0.5 μg of pRSVGAL (a eukaryotic expression vector which contains the *Escherichia coli* β-galactosidase structural gene controlled by the Rous sarcoma virus long terminal repeat) as an internal control. The luciferase activities were measured by Pikka Gene (Toyo Ink). All of the transfection experiments were performed four to twelve times with two different preparations of DNA, and the relative luciferase activity was derived from the mean values of the results. The activity of β-galactosidase was assayed as described (18).

Purification of bacterially expressed proteins and GST pull-down assay. GST fusion proteins and histidine-tagged TFIIB, TFIIFβ, TBP, TAF28α, and TAF28β were expressed in *E.coli* and purified as described previously (8). The histidine-tagged general transcription factor (GTF) expression vectors, the proteins-expressing baculovirus including TAF230, TAF110, TAF85 and TAF42, and their antisera were kindly provided Dr. Y. Nakatani (NIH). The GST pull-down analysis was performed according to a previously described protocol (19).

RESULTS AND DISCUSSION

NF1-A recognized the specific DNA sequence through the DNA-binding domain (amino acids 1 to 236) (20). We previously isolated four NF1-A cDNA isoforms generated by alternative splicing. All of the NF1-A carboxy terminals repress the metallothionein IIA (MTIIA) promoter activity when fused to a heterologous DNA-binding domain of the yeast transcription factor GAL4 (8).

To further define the domain important for repression, we constructed a series of deletion mutants of the carboxy terminal regions (Fig. 1). When subdivided into two regions, the region conserved among the isoforms (318-509), and the non-conserved region (145-316), these segments repressed luciferase activity, but their repressing abilities were less than that of GAL4-A1. When the region 318-509 was separated into two regions, 318-427 and 427-509, the former showed the repression activity and the latter did not. To minimize the repression region, the region 318-427 was divided into two regions, 318-367 and 365-427. Since the repression activities of these fragments were slightly decreased in comparison with that of the region 318-427, we determined that the minimum repression domain is the region 318-427, which we named RD2. Next we examined the repression activity of the region 145-316. The experiments using deletion mutants showed that the region 209-315, RD1, does not act as a repression domain by itself, while RD1 with the segments 145-209 or 145-187 repressed the transcription. When RD1 and RD2 were fused, this region (209-427) showed the same repression activity as that seen in the intact carboxy terminal region, GAL4-A1. We concluded that RD2 is a minimum repression domain, but for the maximum repression activity, RD1 is also required for the enhancement of the repression activity.

To define the mechanism of repression by NF1-A, we tested its ability to repress the GST-P and C/EBPδ gene promoters. MTIIA promoter exhibits a high level of transcriptional activity, because it contains basal level enhancers that do not exist in the GST-P and C/EBPδ genes (14, 21, 22). Cells were cotransfected with effector plasmid, GAL4DBD, GAL4-A1, or GAL4-A1 (318-427), also termed GAL4-RD2, together with reporter plasmid containing five GAL4 binding sites in front of the GST-P or C/EBPδ promoter (Fig. 2). GAL4-A1 and GAL4-RD2 repressed the luciferase activities derived from the various promoters, but the ability of GAL4-RD2 was less than that of GAL4-A1. These results might indicate that NF1-A represses different types of promoter activity through interactions with the general transcription machinery.

Some repressors have recently been isolated, and the amino acid sequences specific for the repression were reported. These sequences have abundant alanine, alanine and proline, proline, serine and threonine, and charged amino acids (6, 7, 23). The NF1-A repression domains RD1 and RD2 are rich in serine (22%) and glycine (11%), and in proline (15%) and serine (14%), respectively (Fig. 3A). The transcriptional repressors Even-skipped (which is rich in alanine and proline) and Krüppel (which is abundant for alanine), interact directly with TBP and the TFIIEβ subunit, respectively (24, 25). Since NF1-A represses the promoter activities of various genes, a direct repression mechanism in which repressors interact with the general transcription machinery is possible (4-7). To determine whether NF1-A repression domains associate directly with GTFs, we performed *in vitro* binding assays using GST fusion proteins and histidine-tagged GTFs including TBP, TFIIB, TFIIFβ, TAF230, TAF110, TAF85, TAF42, TAF28α and TAF28β. We constructed three expression plasmids for GST fusion proteins, GST-RD1, GST-RD1·RD2, and GST-RD2 (Fig. 3B). None of the three GST and repression domain fusion proteins interacted directly with the GTFs tested (Fig. 3C; data not shown), although GST-yTAF130, known as the associated factor of TBP, interacted with TBP in our system (Fig. 3C, lane 6). Thus, NF1-A repression domains

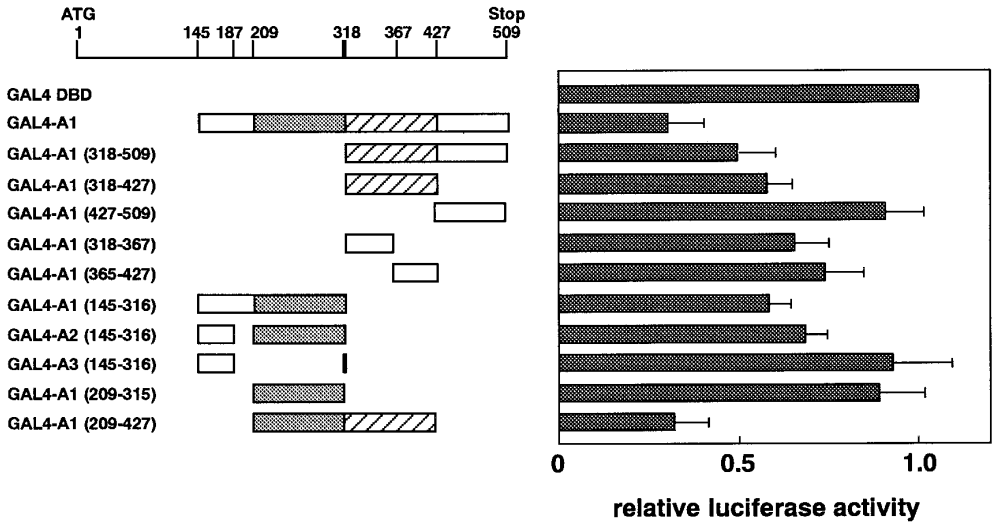

FIG. 1. Identification of NF1-A repression domain. The left panel shows the effector plasmids which contain the GAL4 DNA binding domain and the carboxy terminal region of various NF1-As. Each dish received the reporter plasmid containing MTIIA promoter and GAL4 binding sites and β-galactosidase expression vector as well as the effector plasmid. Luciferase activity was measured in the cell extracts and normalized by the β-galactosidase activity. The hatched and striped boxes indicate RD1 and RD2, respectively. The right panel shows the luciferase activity ± standard deviation (n = 6-12) relative to the activity of cotransfection of the GAL4 DNA binding domain alone.

do not associate with several GTFs *in vitro*. Further analysis will contribute to the definition of the mechanism of repression by NF1-A.

NF1 proteins contain highly conserved amino terminal regions that are required for DNA-binding and dimerization, and their binding sites are indistinguishable (9, 26, 27). Since NF1-C and NF1-X, the activation domains of which have been identified, function as the transcriptional activators (10-12), NF1-A acts as a repressor by competing for DNA-binding sites. In this study, we identified the NF1-A repression domain, indicating that the mechanism of repression by NF1-A might be not only the competing for DNA-binding sites but also the direct repression, which means that the repressor interacts with the general transcription machinery. Though we did not detect the interaction with GTFs, the repression domains might interact with other GTFs or molecules with properties suggestive of corepressor function. E4BP4, a transcriptional repressor rich in charged amino acids, interacts with TBP-binding protein Dr1 (28). KAP-1 has been cloned as a universal corepressor for a large family of Krüppel-associated box repression domain-containing transcription factors (29). We have identified the repression domains of NF1-A; it was also reported that NF1-A activates the transcription from the myelin basic protein promoter which is required for the brain-specific transcription in the neural/glial hybrid cell line NG108-15 (30). When RD2 was fused to the LFB1 DNA-binding domain, it activated the transcription in yeast (31). Therefore, it is possible that NF1-A functions as both a repressor and an activator. Whereas RD2 repressed the transcription of hMTIIA and the GST-P and C/EBPδ promoters, RD2 might activate the brain-specific transcription. One possibility regarding functional differences might be derived from cofactors. To define the mechanism utilized by the NF1-A repression domain, we are attempting to identify the cofactors as well as GTF interacting with the NF1-A repression domain.

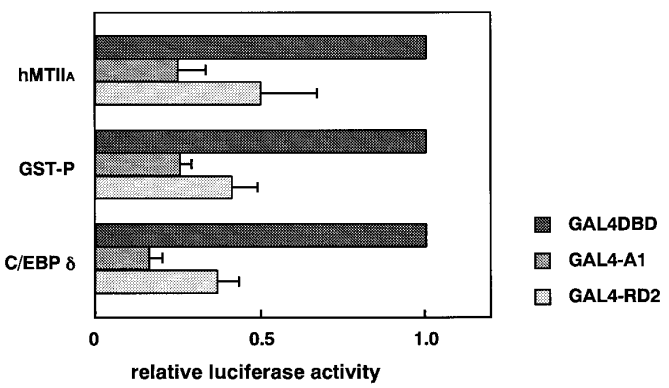

FIG. 2. Effect of NF1-A repression domain on the transcription activity of various promoters. Cells were cotransfected with the effector plasmids together with the reporter plasmid containing MTIIA, GST-P or C/EBPδ promoter, and GAL4 binding sites. Relative luciferase activities and the standard deviations are shown as in Fig. 1.

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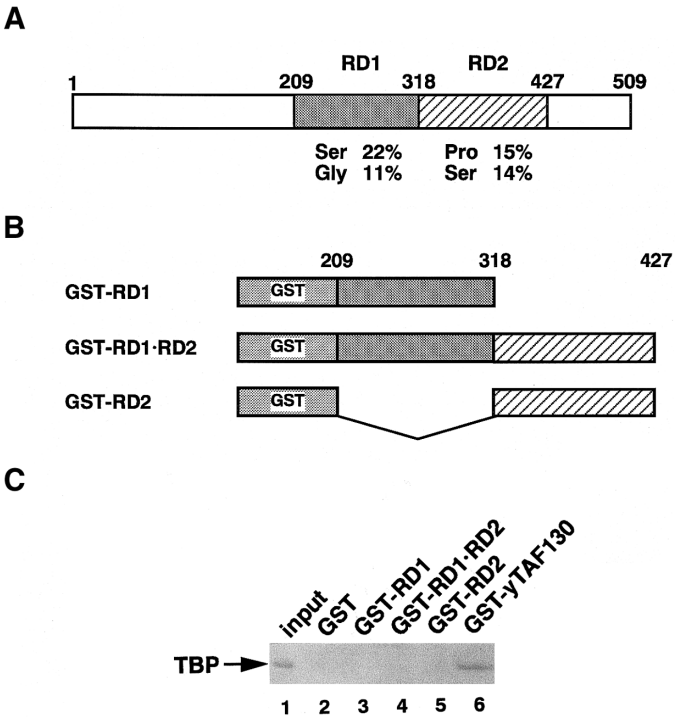

FIG. 3. Characteristics of NF1-A repression domain. (A) amino acid components of the NF1-A repression domains, RD1 and RD2. (B) diagram of the GST and NF1-A repression domain fusion proteins containing RD1 and/or RD2. (C) GST pull-down assays were performed by incubating histidine-tagged TBP with GST fusion proteins indicated or GST-yTAF130 (200 pmol each) (*lanes* 2-6). The proteins eluated by the 20 mM glutathione were resolved by SDS polyacrylamide gel electrophoresis and visualized by Coomassie Brilliant Blue staining. The amount of TBP in lane 1 is equivalent to 20% of the total histidine-tagged TBP used in the assay. The arrow designates the mobility of the histidine-tagged TBP.

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