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Identification of YY1 Sequences Necessary for Association With the Nuclear Matrix and for Transcriptional Repression Functions

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Abstract YY1 is a zinc finger-containing transcription factor that can both repress and activate transcription. YY1 appears to use multiple mechanisms to carry out its diverse functions. Recently, it was observed that YY1 can exist in multiple nuclear compartments. In addition to being present in the nuclear extract fraction, YY1 is also a component of the nuclear matrix. We show that YY1 can be sequestered in vivo into a high-molecular-weight complex and can be dislodged from this complex either by treatment with formamide or by incubation with an oligonucleotide containing the YY1 DNA binding site sequence. By transfecting plasmids expressing various YY1 deletion constructs and subsequent nuclear fractionation, we have identified sequences necessary for association with the nuclear matrix. These sequences (residues 256–340) co-localized with those necessary for in vivo sequestration of YY1 into the high-molecular-weight complex. We have also characterized YY1 sequences necessary for repression of activated transcription (residues 333–371) and those necessary for masking of the YY1 transactivation domain (residues 371–397). Sequences that repress activated transcription partially overlap YY1 sequences necessary for association with the nuclear matrix. However, these sequences are distinct from those that appear to mask the YY1 transactivation domain. The potential role of nuclear matrix association in controlling YY1 function is discussed. J. Cell. Biochem. 68:484–499, 1998.

Key words: YY1; zinc finger; high-molecular-weight complex; plasmid transfection; nuclear matrix association

Yin-Yang-1 (YY1) is a 414-amino acid, ubiquitously expressed nuclear protein that can either activate or repress transcription under a variety of conditions. Some of the genes repressed by YY1 include the Moloney murine leukemia virus long terminal repeat, the adeno-associated virus P5 promoter, the skeletal α-actin promoter, the β-casein promoter, ϵ- and γ-globin genes, the serum amyloid A1 promoter, the human immunodeficiency virus promoter, and the human papilloma virus (HPV) type 18 promoter [reviewed in Shi et al., 1997; Shrivastava and Calame, 1994]. In addition, YY1 can activate many genes including the c-*myc* gene, the ribosomal protein L30 and L32 genes, and the intracisternal A-particle gene [reviewed in Shi et al., 1997; Shrivastava and Calame, 1994].

The ability of YY1 to both activate and repress transcription suggests the existence of distinct domains responsible for each function. YY1 contains four zinc fingers of the C_2H_2-type at its carboxy terminus (amino acids 298–414) and a region rich in alanine and glycine between amino acids 154 and 201 [Shi et al., 1991; Hariharan et al., 1991; Park and Atchison, 1991]. Sequences 43–53 contain 11 consecutive acidic residues, while amino acids 70–80 consist of 11 consecutive histidine residues. In addition, sequences 16–29 have the potential to form an amphipathic negatively charged helix and sequences 80–100 are rich in proline and glutamine. Several groups have identified YY1 sequences responsible for transcriptional repression [Shi et al., 1991; Lee et al., 1994, 1995; Lewis et al., 1995; Bushmeyer et al., 1995; Yang et al., 1996]. These studies indicate that two YY1 segments, one rich in alanine and glycine and the other within the zinc finger region, can

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contribute to transcriptional repression. YY1 sequences important for transcriptional activation reside near the amino terminus and include a region with the potential to form an amphipathic negatively charged α-helix and sequences rich in proline and glutamine [Lee et al., 1994, 1995; Bushmeyer et al., 1995; Austen et al., 1997].

YY1 may activate or repress transcription by a variety of mechanisms. In some cases, YY1 can repress transcription through competition for DNA binding sites with other transcription factors [Lu et al., 1994; Ye et al., 1996; Gualberto et al., 1992; Meier and Groner, 1994; Peters et al., 1993; Raught et al., 1994]. DNA bending may also influence the activation and repression properties of YY1 [Natesan and Gilman, 1993]. In some experimental systems, YY1 shows either no affect on promoter activity, or very weak activation. However, deletion of C-terminal YY1 sequences leads to high-level constitutive transactivation [Bushmeyer et al., 1995]. Therefore, the YY1 activating sequences may be masked by YY1 carboxy terminal sequences and exposure of the activation domain may require interactions with other proteins. Indeed, YY1 function can be greatly influenced by certain protein interactions. For example, Shi et al. [1991] demonstrated that YY1 can bind to and repress activity of the adeno-associated virus P5 promoter. However, in the context of adenoviral E1A protein, YY1 strongly activated the same promoter. This E1A-mediated relief of YY1 repression requires the CREB binding protein-related factor, p300, which physically interacts with YY1 [Lee et al., 1995]. YY1 may also repress transcription by recruitment of a co-repressor protein such as the mammalian homologue of the yeast global regulator RPD3 [Yang et al., 1996]. Protein interactions with YY1 can also lead to loss of activated transcription. For instance, interaction of YY1 with transcriptional activators such as ATF/CREB and AP1 can lead to loss of activated transcription [Zhou et al., 1995; O'Connor et al., 1996]. In addition, YY1 can repress human papilloma virus type 16 transcription by quenching AP1 activity [O'Connor et al., 1996].

An enigmatic aspect of the diverse functions of YY1 is its ubiquitous expression pattern. Therefore, YY1 function must be modulated by either interaction with other proteins (as described above), post-translational modifications, or intracellular localization. YY1 is known to be a phosphoprotein and phosphorylation can influence its ability to bind to certain DNA sequences [Becker et al., 1994; Austen et al., 1997]. However, no other properties of YY1 are currently known to be controlled by post-translational modifications. Recently, however, it was observed that YY1 can exist in two distinct nuclear compartments. YY1 is present in both the soluble nuclear extract, as well as the insoluble nuclear matrix compartments. This adds a potential new dimension to the control of YY1 function. The nuclear matrix is operationally defined as the nuclear structure that remains after extraction of cells or nuclei with detergents and salt, and after removal of chromatin with nucleases (RNase A and DNase I). The structure appears to consist of residual nucleoli, nuclear pore-lamina complexes, and the internal nuclear matrix [Fey et al., 1984; Berezney, 1991]. The nuclear matrix has been implicated in DNA replication, transcription, RNA splicing and organization of DNA within the nucleus [Hozak et al., 1993; Carter et al., 1993; Jackson et al., 1993; Blencowe et al., 1994; Durfee et al., 1994; Holzman et al., 1997; Stein et al., 1996; Chabot et al., 1995]. A number of DNA binding proteins and other proteins involved in transcription have been found to associate with the nuclear matrix [Guo et al., 1995; Mancini et al., 1994; Buhrmester et al., 1995; Chang et al., 1995; Carvalho et al., 1995; Bidwell et al., 1993; van Wijen et al., 1993; Sun et al., 1996a; Vincent et al., 1996; Morillaro et al., 1996; Herrscher et al., 1995; Merriman et al., 1995].

Little is known concerning the mechanisms by which proteins are compartmentalized within the nucleus. Stein and coworkers showed that a 31-amino acid segment of the AML transcription factor is necessary and sufficient for nuclear matrix targeting [Zeng et al., 1997]. However, other mapping studies are less precise. A somewhat large region of the human androgen receptor and the DNA binding domain of the human glucocorticoid receptor have been implicated in association with the nuclear matrix [van Steensel et al., 1995]. In addition, the amino terminus of Rb binds to a nuclear matrix protein that co-localizes to centers for RNA processing [Durfee et al., 1994]. Identification of the sequences within YY1 that associate with the nuclear matrix would have an immediate impact on our understanding of compartmentalization of proteins within the nucleus. It would also aid in our understanding of YY1

function because the identified sequences could be directly compared to those identified as important for other YY1 functions, such as transcriptional repression.

We report here the identification of YY1 sequences necessary for association with the nuclear matrix. These sequences co-localize with YY1 sequences necessary for sequestration of YY1 into a large protein complex. The sequences necessary for association with the nuclear matrix are distinct from those involved in "masking" of the YY1 activation domain, but may overlap with those necessary for transcriptional repression.

MATERIALS AND METHODS

Plasmid Constructions

Plasmids GAL-DBD, GAL-1–414, GAL-1–102, GAL-1–143, GAL-1-414Δ16–99; GAL-1-200Δ16–99, GAL-1-414Δ371–380, GAL-1-414C360S, GAL-1–340, GAL-1–397, and GAL-1–256 were previously described [Bushmeyer et al., 1995]. Plasmid CMV-PU.1 was described in Pongubala et al. [1993]. To prepare GAL-1-414Δ381–397 and GAL-1-414Δ371–397, mutagenesis was performed using the BioRad Muta-Gene kit with the appropriate oligonucleotides (Table I) on a plasmid subclone containing a *Hin*dIII-*Xba*I fragment encoding the 3′ half of the YY1 cDNA. Mutations were confirmed by dideoxynucleotide sequence analysis and inserted into the parent YY1 full-length clone at the *Hin*dIII site. To prepare Core($YY1_4$)TKCAT a plasmid containing the immunoglobulin kappa (Igκ) 3′ enhancer sequences 391–523 linked to TKCAT (clone E) [Pongubala and Atchison, 1991] was digested with SalI and blunt end ligated to four copies of the wild-type YY1 binding site derived from the Igκ 3′ enhancer (Table I). For YY1-1–333, an *Eco*RI-*Hin*dIII DNA fragment encoding YY1 sequences 1–333 was cloned into the *Eco*RI-*Hin*dIII sites of CMV expression vector pCB6+ [Patwardhan et al., 1991]. CMV-Pip was a gift from H. Singh (University of Chicago). CMV-*fos* and CMV-*jun* were provided by F. Rauscher (Wistar Institute, Philadelphia, PA).

TABLE I. Oligonucleotides

YY1 wt	TACCCCACCTCCATCTTGTTTGATA
Δ381–397	GATGTGAGATTTCAGGTTAGTGTCTCCGGTATGGATTCG
Δ371–397	GATGTGAGATTTCAGGTTAGTCAAATTGAGGTCCAGTCA
GAL4	CGGAGTACTGTCCTCCGAACTGAAAACAGAAG

Cell Culture and Transfection

NIH 3T3 cells were grown in Dulbecco's modified Eagle's high glucose medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and Pen-Strep. Transfections were performed by the calcium phosphate method of Graham and van der Eb [1973]. Transfections typically contained 5 µg reporter plasmid, 5 µg effector plasmid and 1 µg of a pCB6+ based β-galactosidase expression plasmid to normalize transfection efficiency. For preparation of nuclear extracts and nuclear matrices, transfections contained 15 µg expression plasmid. Cells were harvested 44 h after transfection. Cell extracts and CAT assays were performed according to Gorman et al. [1982].

Nuclear Extract and Nuclear Matrix Preparations

Mininuclear extracts were prepared by the method of Schrieber et al [1989] with minor modifications [Bushmeyer et al., 1995]. Nuclear matrix preparations were prepared from transfected cells by the method of Fey et al. [1984]. Cell monolayers were washed twice with cold PBS and placed on ice with 5 ml cold cytoskeleton buffer (CSK: 100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, and 1.2 mM PMSF) for 10 min with rocking. The supernatant was aspirated and replaced with 5 ml of 250 mM NH_4SO_4, 300 mM sucrose, 10 mM PIPES pH6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, and 1.2 mM PMSF for 10 min. After aspiration, the remaining cell structures were scraped from the plates and recovered by micocentrifugation for 3 min. The pellet was resuspended in CSKbuffer containing 100 µg/ml pancreatic RNaseA and 100 µg/ml pancreatic DNase and incubated on ice for 20 min. NH_4SO_4 was added to give a final concentration of 0.25M and samples were incubated for 5 min at 25°C. Insoluble material containing the nuclear matrix was recovered by microcentrifugation for 5 min, washed twice with cold PBS and dissolved in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS).

Western Blots

Protein samples were electrophoresed on 10% or 12% SDS-polyacrylamide gels and trans-

ferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH). Following blocking overnight in TBST (0.1 M Tris–HCl, 0.15 M NaCl, 0.05% Tween) containing 5% milk at 4°C, blots were incubated with GAL4 (Santa Cruz Biotechnology) or YY1 [Bushmeyer et al., 1995] antibodies at 1:500 dilution for 2 h at room temperature. After treatment with donkey antirabbit secondary antibody (Amersham) for 2 h at room temperature, blots were developed using the ECL detection system (Amersham).

Electrophoretic Mobility Shift Assays

Each reaction was done in a final volume of 20 µl containing 4 µg poly dI-dC, 10 mM Tris–HCl pH7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 5,000–6,000 cpm ^{32}P end-labeled GAL4 DNA binding site probe (Table I) and either 10 µg mininuclear extract protein, or 2 µl protein prepared by in vitro translation (Promega, Madison, WI). For antibody pretreatment, reactions were incubated with 2 µl of either anti-GAL4 or anti-YY1 antisera for 15 minutes at room temperature prior to addition of the DNA probe. For formamide treatment, formamide was added to nuclear extracts at concentrations of 15%, 20%, or 25% (v/v). Upon addition of the remaining binding assay components, the final concentration of formamide was 3.75%, 5%, or 6.25%, respectively. Other additions to the binding reactions are noted in the figure legends.

RESULTS

Dominance of the YY1 Weak Transcriptional Activation Phenotype

Previously, we identified YY1 sequences involved in transcriptional repression and activation [Bushmeyer et al., 1995]. For these studies, we prepared YY1 deletion constructs fused to the GAL4 DNA binding domain. Our results showed that the YY1 activation domain lies between residues 16–29 and 80–100, whereas the repression domain lies in the C-terminal portion of the protein (residues 333–414). Full-length YY1 protein yields very weak activation potential in NIH 3T3 cells when assayed with a GAL4-responsive promoter. However, deletion of YY1 C-terminal sequences (370–414) results in constitutive transcriptional activation [Bushmeyer et al., 1995]. Therefore, YY1 carboxy terminal sequences appear to mask the activation domain. Alternatively, the C-terminal repression domain may function in competition with the N-terminal activation domain. Repression domain deletion could then expose activation domain function. Each of these possibilities need not be mutually exclusive. For simplicity, we will refer to this phenomenon as activation domain masking.

The GAL-YY1 fusion proteins that we previously prepared [Bushmeyer et al., 1995] can dimerize through the GAL4 DNA binding domain sequences. Therefore, we sought to exploit this property of GAL4 to determine whether the full-length YY1 phenotype (weak transcriptional activation) is dominant over the high-level constitutive transcriptional activity of the deletion constructs. In other words, in a heterodimer of full-length plus truncated YY1, would full-length YY1 repress the transcriptional activation potential of the constitutively active YY1 deletion constructs, or would the constitutive activation potential of the deletion constructs be dominant over full-length YY1?

Plasmids expressing the GAL4 DNA binding domain (GAL-DBD) alone, or fused to various portions of YY1 were transfected into NIH 3T3 cells with a reporter plasmid containing 5 GAL4 binding sites adjacent to the thymidine kinase promoter driving the chloramphenicol acetyltransferase gene (GALTKCAT). Plasmids GAL:1–102 and GAL:1–143 (GAL4 sequences 1–147 fused to YY1 amino acids 1–102 and 1–143, respectively) lack the YY1 repression domain and confer constitutive activation potential, whereas GAL:1-414 contains full-length YY1 and yields very low transactivation potential. As expected, GAL:1–102 and GAL:1–143 strongly activated GALTKCAT, while activation by GAL:1-414 was weak (Fig. 1A, lanes 1–4). Co-transfection of GAL:1-102 and GAL:1–143 resulted in additive transcriptional activation (lane 9). However, inclusion of GAL:1-414 resulted in 50% and 75% drops in activation by GAL:1-102 and GAL:1-143, respectively (cf. lanes 2–3 with 6–7). Therefore, when the full-length YY1 protein is tethered to a highly active YY1 deletion protein via GAL4 dimerization, it exerts a dominant phenotype over the YY1 deletion constructs leading to reduced transcriptional activity.

The presence of the GAL4 DNA binding domain on these constructs enabled us to monitor the expression of transfected chimeric proteins in electrophoretic mobility shift assays (EMSA) using the GAL4 DNA binding site as probe. We previously showed that chimeric proteins that

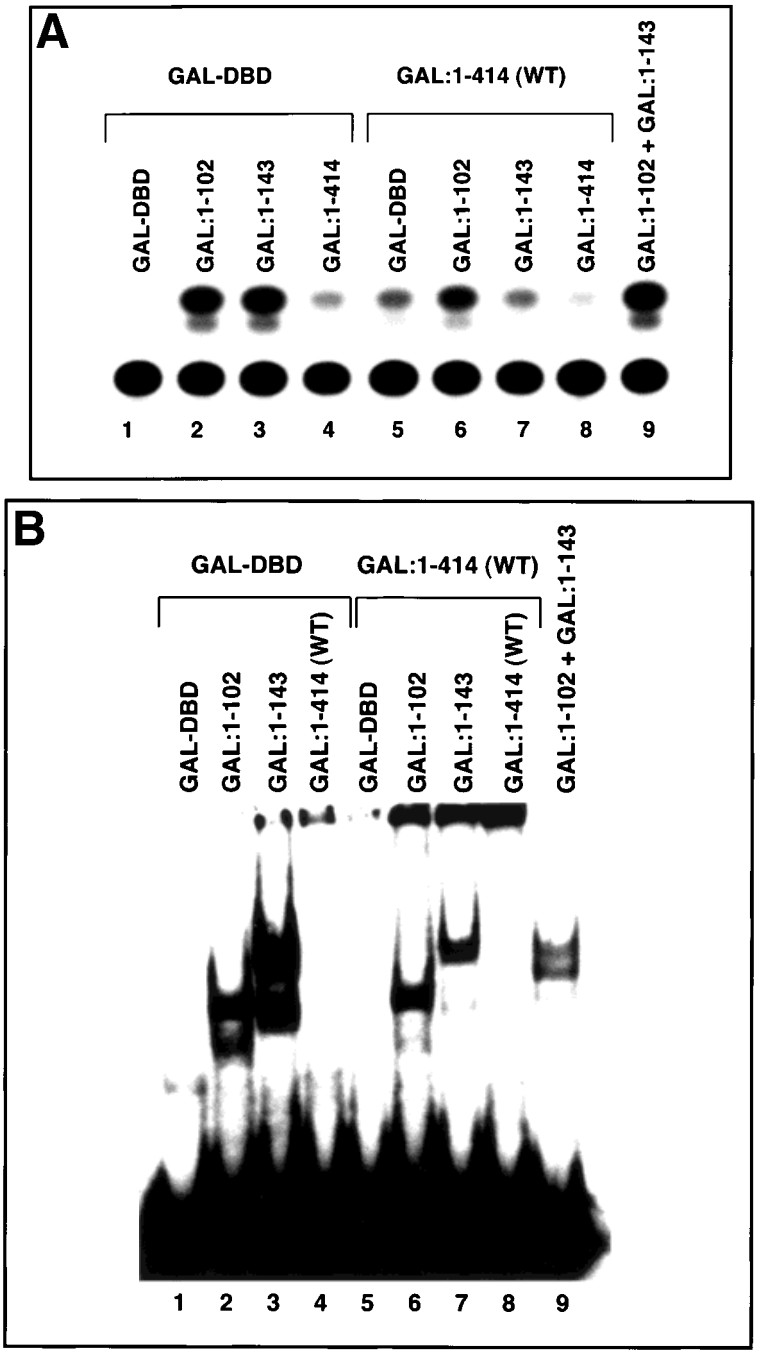

Fig. 1. The function of full-length YY1 is dominant over YY1 deletion constructs. Plasmids expressing the GAL4 DNA binding domain or various YY1 fusion constructs were co-transfected with the GALTKCAT reporter plasmid. Cells were harvested 2 days after transfection. **A:** CAT assay of transfected cells; 2.5 μg of the expression plasmids indicated above each lane was co-transfected with 2.5 μg of either GAL-DBD (lanes 1–4) or GAL:1-414 (lanes 5–8). Full-length YY1 inhibited the high-level activity of the YY1 truncation mutants. **B:** EMSA with the GAL4 DNA binding site probe and nuclear extracts prepared from transfected cells. The identity of each transfected plasmid is indicated above each lane.

include YY1 sequences between residues 256 and 341 are expressed efficiently within the cell but do not yield typical EMSA complexes [Bushmeyer et al., 1995]. Instead, these proteins either migrate aberrantly or yield no complex at all. We therefore sought to determine whether full-length YY1 also exerted a dominant phenotype on the EMSA pattern of the GAL:1-102

and GAL:1-143 proteins. As expected, the GAL-DBD, GAL:1-102, and GAL:1-143 proteins isolated from transfected cells generated EMSA complexes of the appropriate size while GAL:1-414 yielded essentially no complex (Fig. 1B, lanes 1–4). Co-transfection of GAL:1-102 and GAL:1–143 resulted in mixture of each complex and the appearance of an intermediate complex indicative of heterodimerization through the GAL4 sequences (lane 9). Interestingly, co-transfection with GAL:1–414 greatly reduced the EMSA complexes generated by the GAL:1–102 and GAL:1–143 proteins (Fig. 1B, lanes 6 and 7). This reduced DNA binding by the GAL:1–102 and GAL:1–143 proteins parallels their lost ability to activate transcription (Fig. 1A).

YY1 can be sequestered within the cell

One possible explanation for the above results is that YY1 carboxy terminal sequences make contacts with other proteins within the cell resulting in sequestration into a protein complex. If true, we reasoned that we may be able to disrupt YY1 from this complex. NIH 3T3 cells were transfected with plasmids expressing GAL-DBD, GAL:1–143, or GAL:1–414. Mini-nuclear extracts were prepared from the transfected cells and assayed by EMSA with the GAL4 DNA binding site as probe. GAL-DBD and GAL:1–143 proteins yielded protein DNA complexes of expected size, whereas GAL:1–414 yielded the aberrant, slowly migrating complex near the top of the gel (Fig. 2, lanes 1–3). Treatment of extracts with RNase had no effect on the EMSA pattern (lanes 4–6). However, inclusion of an oligonucleotide containing the YY1 binding site induced the appearance of a complex with a mobility expected for the GAL:1–414 protein (lane 9). The same treatment had no effect on the GAL-DBD and GAL:1-143 proteins (lanes 7 and 8). Heat treatment abolished all protein–DNA complexes (lanes 10–12) and inclusion of a nonspecific oligonucleotide had no effect (lanes 13–15). We also tested the abil-

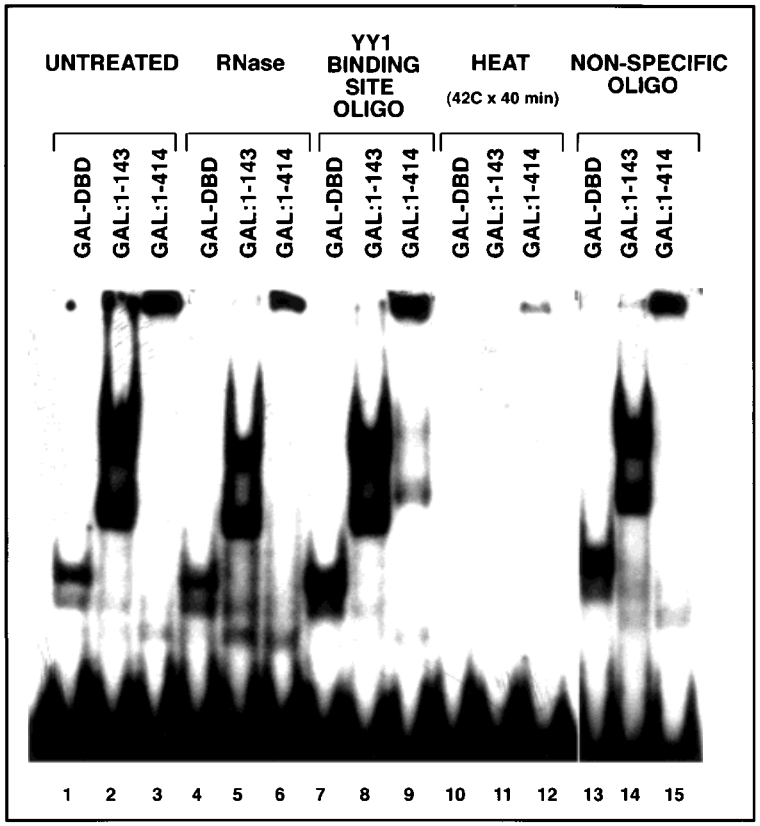

Fig. 2. YY1 can be sequestered into a large complex in vivo and can be dislodged with YY1 binding site sequences. NIH 3T3 cells were transfected with plasmids GAL-DBD, GAL:1–143, or GAL:1–414 and nuclear extracts were prepared. Each extract was subjected EMSA with the GAL4 DNA binding site probe. Samples were either left untreated or were treated with RNase, YY1 binding site oligonucleotide (8 μg), heat (42°C), or a nonspecific oligonucleotide (8 μg). The identities of the transfected plasmids and the specific treatments are listed above the lanes.

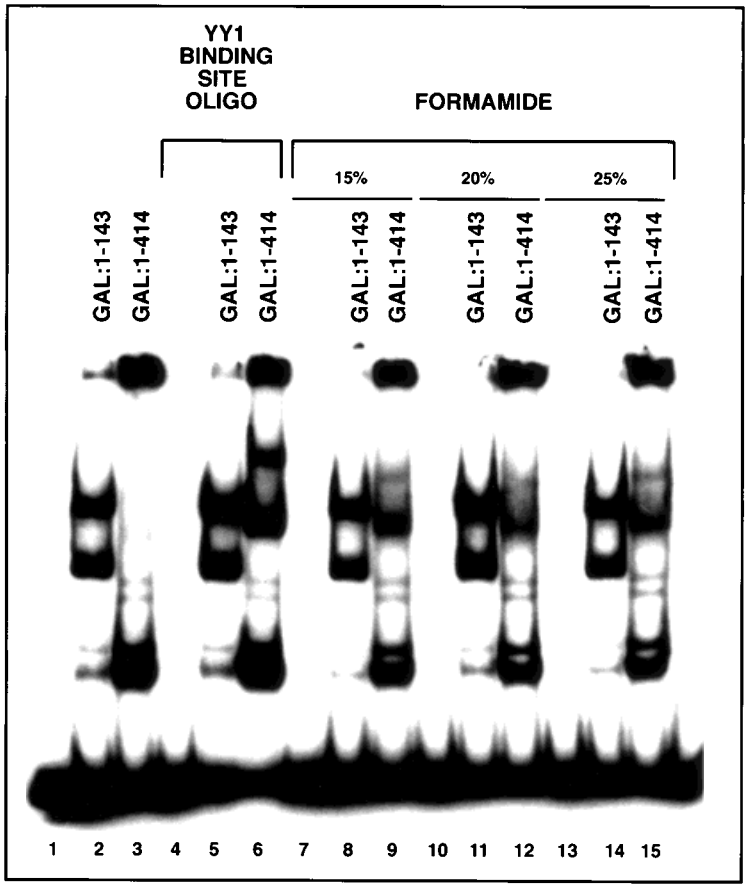

Fig. 3. Formamide can dissociate YY1 from the large complex. NIH 3T3 cells were transfected with plasmids expressing either GAL:1–143 or GAL:1–414 and nuclear extracts were prepared. Each extract was subjected to EMSA with the GAL4 DNA binding site probe. Samples either received no treatment (lanes 1–3), YY1 binding site oligonucleotide (8 μg) (lanes 4–6), or various concentrations of formamide (lanes 7–15). Initial formamide concentrations of 15%, 20%, and 25% with the nuclear extract were reduced to final concentrations of 3.75%, 5%, and 6.25%, respectively, upon addition of the remainder of the binding assay components. The transfected plasmids and the various treatments are indicated above the lanes. Lanes 1, 4, 7, 10, 13, contain probe with no nuclear extract.

ity of formamide to disrupt YY1 from the large complex (Fig. 3). Treatment of nuclear extracts with formamide had no effect on the GAL:1-143 EMSA pattern, but induced the appearance of the proper complex with the GAL:1-414 fusion protein (Fig. 3, cf. lane 3 with lanes 9, 12, and 15). Inclusion of the YY1 binding site oligonucleotide served as a positive control for induction of the full-length YY1 complex (cf. lane 3 with lane 6). The above results suggest that full-length YY1 is present in the cell within a complex. YY1 can be dissociated from this complex by formamide treatment or by binding to its specific DNA binding sequence.

To determine whether recombinant YY1 could assemble into a higher order complex in vitro, we incubated GAL:1–414 or GAL-DBD produced by in vitro translation with soluble proteins isolated from NIH 3T3 nuclear extracts. Both proteins yielded the expected EMSA complexes with the GAL4 DNA binding site probe (Fig. 4, lanes 1 and 2) and reacted properly with anti-GAL4 and anti-YY1 antisera (lanes 3–8). Incubation with NIH 3T3 cell nuclear extract, however, did not sequester GAL:1-414 into a higher-order complex indicating that soluble nuclear extract proteins cannot regenerate the higher order complex in vitro.

YY1 Sequences Responsible for Association With the Nuclear Matrix

Guo et al. [1995] showed that YY1 can associate with the nuclear matrix. Because of the unusual EMSA pattern of some GAL-YY1 mutants, we sought to determine whether YY1 sequences necessary for nuclear matrix associa-

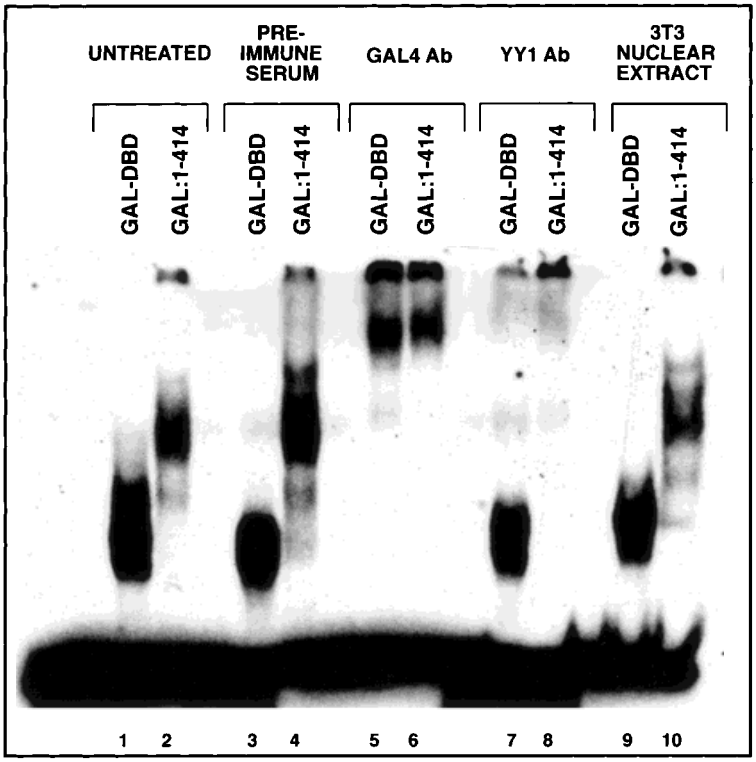

Fig. 4. The high-molecular-weight complex cannot be reconstituted in vitro. GAL-DBD and GAL:1–414 proteins were prepared by in vitro transcription and subjected to EMSA with the GAL4 DNA binding site probe. Reactions were either left untreated (**lanes 1, 2**) or treated with preimmune *sera* (**lanes 3, 4**), GAL4 antibody (**lanes 5, 6**), YY1 antibody (**lanes 7, 8**), or NIH 3T3 cell nuclear extract (**lanes 9, 10**). Untreated samples migrated at the positions expected for each protein, and antibodies yielded the expected supershifts. However, addition of NIH 3T3 cell nuclear extract did not lead to the appearance of the unusual high-molecular-weight complex.

tion correlate with those that cause the unusual EMSA pattern. Cells were transfected with GAL:1–397 and GAL:1–370 (which yield the unusual EMSA pattern) [Bushmeyer et al., 1995], and GAL:1–256 (which yields the normal EMSA pattern [Bushmeyer et al., 1995]. One portion of each transfection was used for isolation of nuclear extract proteins, whereas the other portion was used for isolation of nuclear matrices. Samples were then analyzed by Western blot with GAL4 antisera. All proteins were present in equivalent amounts in the nuclear extract fraction (Fig. 5A, lanes 1–3). However, GAL:1–370 and GAL:1–397 proteins were greatly enriched in the nuclear matrix fraction as compared to GAL:1–256 (lanes 4–6). This finding suggests that sequences important for association with the nuclear matrix lie between residues 256 and 370. Consistent with these results, proteins containing YY1 sequences 1–102 and 1-200Δ16–99 were found in the nuclear extract fraction but not within the nuclear matrix (Fig. 5B, lanes 1–2 and 6–7). On the other hand, proteins that included residues 256–370 were found to be enriched in the nuclear matrix. For instance, a protein with a transactivation domain deletion (GAL:1-414Δ16–99) was found in both fractions, as was full-length YY1 (Fig. 5B, lanes 4, 5 and 8, 9). Similarly, GAL:1-414Δ371-397 was found in both the extract and matrix fractions (Fig. 5C). Finally, GAL:1-340, but not GAL:1–256, was present in the nuclear matrix (Fig. 5D). This last pair of constructs narrowed the YY1 sequences necessary for association with the nuclear matrix to between residues 256 and 340. Interestingly, these are the same sequences that confer unusual EMSA patterns on GAL-YY1 fusion proteins with the GAL4 DNA binding site probe [Bushmeyer et al., 1995].

The nuclear matrix associating sequences identified above overlap with the YY1 zinc fingers. Sequences important for several other YY1 functions map to the zinc finger region. For instance, residues important for repression of basal level transcription map to sequences 333–

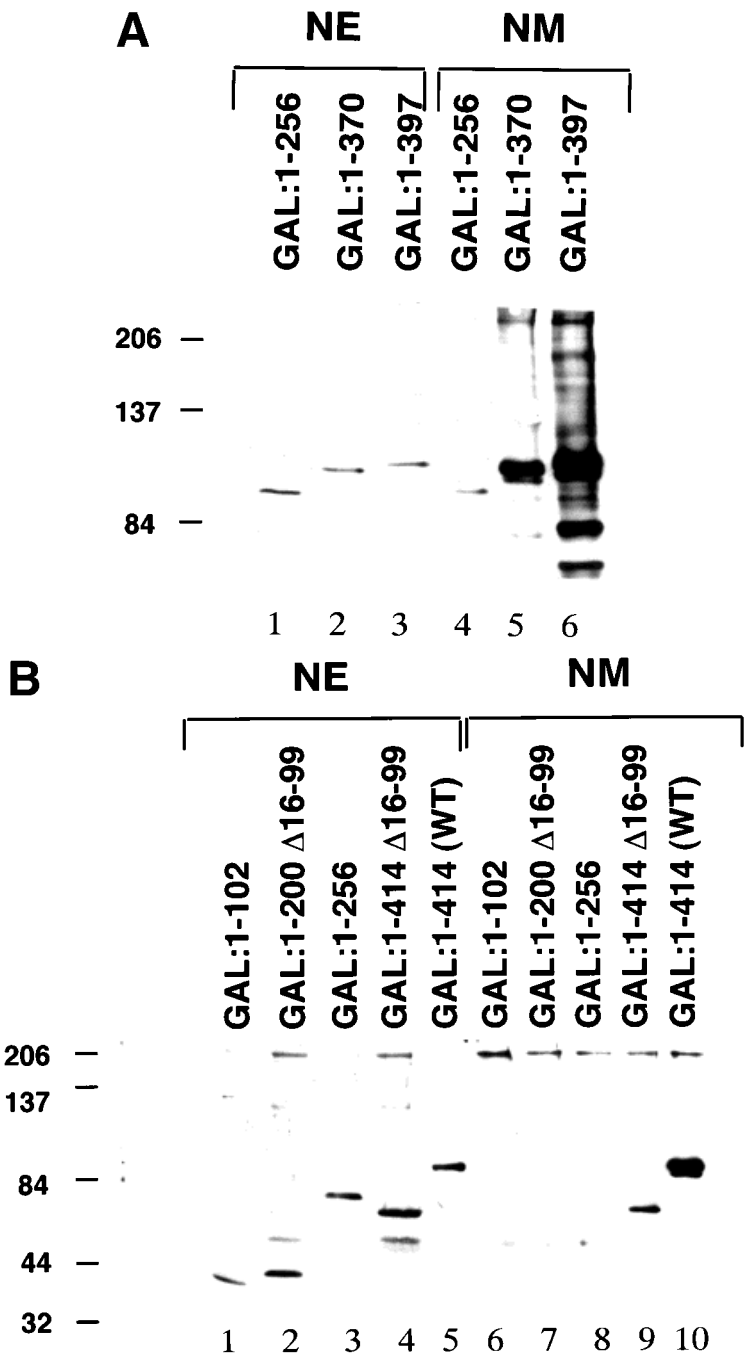

Fig. 5. Identification of YY1 sequences necessary for association with the nuclear matrix (residues 256–340). **A-D:** NIH 3T3 cells were transfected with various GAL4-YY1 fusion constructs and cells were harvested 2 days later; 25% and 75% of each transfection was used for preparation of nuclear extract (NE) and nuclear matrix (NM) fractions, respectively. Samples were subjected to the Western blot procedure with either GAL4 antibodies (A–C) or YY1 antibodies (D). The identity of each transfected plasmid is indicated above each lane and the source of each protein is indicated either NE or NM.

414 [Bushmeyer et al., 1995]. This segment partially overlaps the sequences necessary for nuclear matrix association (residues 256–340). In addition, YY1 sequences that potentially mask the YY1 activation domain, and those that repress activated transcription (see below) reside within the YY1 zinc fingers. Therefore, we set out to determine whether sequences

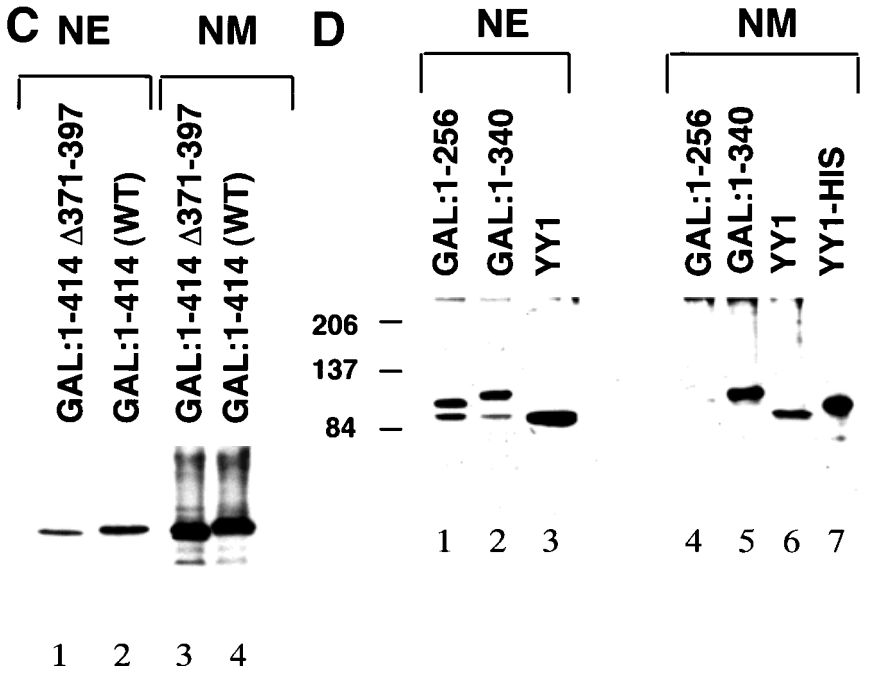

Fig. 5. (Continued.)

important for YY1 transcriptional functions co-localized with the YY1 sequences necessary for nuclear matrix association.

Identification of YY1 Sequences Involved in Activation Domain Masking

We previously showed that progressive C-terminal deletions of YY1 can convert the protein from a weak to a strong transcriptional activator in NIH 3T3 cells [Bushmeyer et al., 1995]. To better map the sequences important for this apparent activation domain masking, various YY1 mutants were transfected into NIH 3T3 cells with the GALTKCAT reporter and assayed for their transactivation potential. As expected, full-length YY1 (GAL:1–414) yielded weak activation potential (Fig. 6A, lane 2). Deletion GAL:1–397 behaved similar to wild-type YY1, while GAL:1–370 and GAL:1–341 showed strong activation (Fig. 6A, lanes 3–5). These results suggest a possible role for zinc finger 3, which lies between residues 355–384 in masking the activation domain function. We therefore, prepared mutations that disrupt zinc finger 3. Mutation of cysteine 360 to serine, or deletion of sequences 371-380 and 381-397 had no effect on YY1 activation potential (Fig. 6A, lanes 6-8) indicating that zinc finger structure is unnecessary for activation domain masking function. However, a larger deletion encompassing sequences 370–397 resulted in constitutive transcriptional activation (Fig. 6A, lane 9). Thus, sequences 370–397 are necessary for masking YY1 transactivation function but smaller mutations within this region do not disrupt masking function. The sequences identified above do not co-localize with those necessary for nuclear matrix association (residues 256–340). Therefore, there is not a direct correlation between the sequences important for activation domain masking and localization to the nuclear matrix.

Low transactivation potential could be due to poor expression of certain YY1 mutants within the transfected cells. To be certain that this was not the case, mininuclear extracts were isolated from the transfected cells and assayed by western blot with anti-GAL4 antisera. Results from these studies indicated that each protein was efficiently expressed in vivo (Fig. 6B).

YY1 Sequences Necessary for Repression of Activated Transcription

We then characterized YY1 sequences important for repression of activated transcription. We originally cloned YY1 based on its ability to bind to a negative-acting DNA sequence within the immunoglobulin kappa 3′ enhancer [Park

and Atchison, 1991]. This enhancer binds to the transcription factors PU.1, Pip, c-*fos*, and c-*jun* within a 132-bp region termed the enhancer core [Pongubala and Atchison, 1991, 1995]. Cotransfection of PU.1, Pip, c-*jun*, and c-*fos* greatly stimulates a 3′ enhancer core-dependent reporter plasmid [Pongubala and Atchison, 1997]. To determine whether YY1 can repress this activated transcription, we prepared a reporter plasmid containing 4 copies of the YY1 binding site adjacent to the enhancer core (Core ($YY1_4$) TKCAT). This reporter plasmid was transfected with plasmids expressing PU.1, Pip, c-*jun*, or c-*fos* in the absence, or presence, of YY1. In the absence of YY1 (with cotransfected plasmid GAL-DBD as a negative control), PU.1, Pip, c-*jun* and c-*fos* greatly stimulated transcription (Fig. 7, lane 2). However, inclusion of YY1 abolished this activated transcription (lanes 5, 6, and 10). Surprisingly, YY1 also repressed transcription of a reporter plasmid containing mutated YY1 binding sites (data not shown). These results suggest that YY1 can repress activated transcription in this system by a DNA binding site-independent method (squelching). To ensure that YY1 was not repressing transcription by binding to a cryptic YY1 binding site within the reporter plasmid, we performed experiments with YY1 mutants incapable of binding to DNA (the cysteine 360 to serine mutant and Δ371-397). Both mutants repressed activated transcription as efficiently as wild-type YY1 (Fig. 7, lanes 3 and 4). Therefore, DNA binding by YY1 is unnecessary for repression of activated transcription. However, further deletion of YY1 sequences to residue 333 (construct 1–333) abolished its ability to repress activated transcription (lane 9). Together, our results indicate that YY1 residues 333–371 are necessary for repression of Igκ 3′ enhancer activity. These sequences are encompassed within those necessary for repression of basal transcription (333–414)[Bushmeyer et al., 1995] and share a short region of overlap with sequences necessary for association with the nuclear matrix (256–340) (Fig. 5). However, these sequences

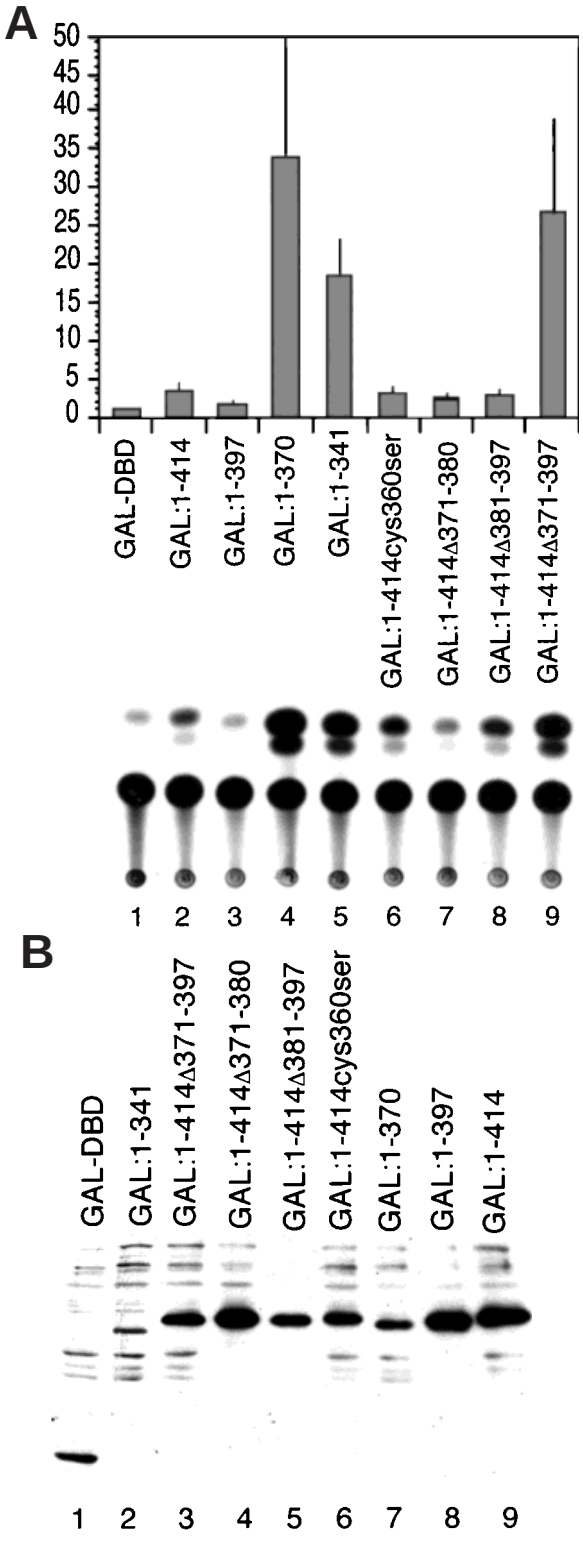

Fig. 6. The YY1 transactivation masking domain requires sequences 371–397. NIH 3T3 cells were transfected with plasmids expressing various GAL-YY1 deletion proteins and the GALTKCAT reporter. **A:** CAT activities were determined from cell lysates prepared from the transfected cells. Bottom, representative CAT assay. Top, average of 3–5 independent experiments normalized to the level of GAL-DBD defined as 1. Error bars = standard deviation of the data. The identities of the transfected constructs are indicated. **B:** To demonstrate the expression of each transfected protein, mininuclear extracts were prepared from each transfection and proteins were subjected to the western blot procedure with anti-GAL4 antibodies. The identities of the transfected constructs are indicated above each lane.

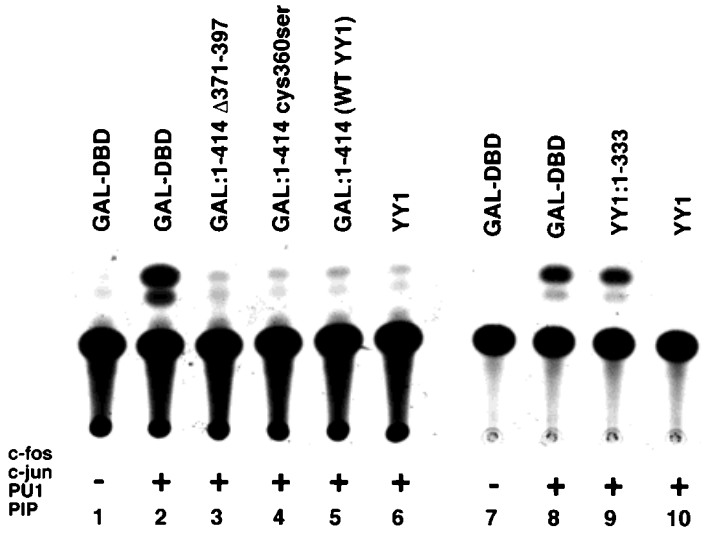

Fig. 7. YY1 sequences necessary for repression of activated transcription lie between residues 333 and 371. NIH 3T3 cells were transfected with the Core($YY1_4$)TKCAT reporter and plasmids expressing the transcription factors PU.1, Pip, c-*fos*, and c-*jun* (+ or - sign below each lane). Included in each transfection was either the GAL-DBD vector **(lanes 1, 2, 7, 8)**, various GAL-YY1 fusion constructs **(lanes 3–6, 9)**, or wild-type YY1 **(lane 10)**.

are distinct from those necessary for masking the YY1 activation domain (371–397).

DISCUSSION

Previously, we showed that full-length GAL-YY1 fusion protein, but not certain truncated derivatives yield unusual EMSA complexes with the GAL4 DNA binding site probe. These unusual EMSA complexes are not an intrinsic feature of the full-length protein because GAL-YY1 produced by in vitro translation yields the expected complex (Fig. 4). The unusual EMSA complexes are only observed with GAL-YY1 produced in transfected cells. This observation suggested to us that full-length GAL-YY1 may be sequestered in vivo into a large complex. If true, we reasoned that we may be able to dislodge YY1 from this complex. Indeed, we found that GAL-YY1 could be dislodged from this complex by treatment of nuclear extracts with formamide, or by inclusion of the YY1 DNA binding site oligonucleotide (Figs. 2 and 3). The ability of the YY1 binding site sequence to dislodge GAL-YY1 from the complex is consistent with the fact that the YY1 sequences responsible for in vivo sequestration overlap the YY1 DNA binding domain [Bushmeyer et al., 1995]. However, DNA binding is not necessary for YY1 sequestration because many YY1 mutants incapable of binding to DNA are still sequestered into the complex. The absence of a DNA binding requirement coupled with the inability of RNase to remove YY1 from the complex (Fig. 2) suggest that protein–protein interactions are necessary for YY1 sequestration. The ability of formamide to dislodge YY1 from the complex supports this conclusion.

The proteins that interact with YY1 in vivo are currently unknown. However, the ability of YY1 to associate with the nuclear matrix [Guo et al., 1995] afforded a possible site for YY1 sequestration in vivo. Interestingly, we found that YY1 sequences responsible for sequestration in vivo co-localize with YY1 sequences necessary for association with the nuclear matrix (sequences 256–340)[Bushmeyer et al., 1995] (Fig. 5). Therefore, there is a direct correlation between the YY1 sequences that yield unusual EMSA patterns and those which associate with the nuclear matrix. Apparently, the full-length GAL-YY1 isolated from mininuclear extracts of transfected cells remains associated with some of these proteins resulting in altered electrophoretic mobility in nondenaturing gels. The normal EMSA pattern induced by inclusion of the YY1 DNA binding site oligonucleotide suggests that when YY1 is bound to DNA, it is incapable of associating with the proteins that cause the unusual electrophoretic mobility, and perhaps with the nuclear matrix. This could provide a potential mechanism for regulating YY1 function.

We also identified YY1 sequences necessary for masking of the YY1 activation domain, and

sequences necessary for repression of activated transcription. These studies showed that sequences 371–397 are necessary for masking of the YY1 activation domain (Fig. 6). Rather than abolishing a masking domain, deletion of these sequences could also destroy a YY1 repression domain whose function competes with the YY1 activation domain leading to very low activation potential of the full-length protein. The ability of sequences 333–414 to repress basal transcription is consistent with this possibility [Bushmeyer et al., 1995]. However, it is curious that two half deletions of this region (i.e., Δ371–380 and Δ381–397) had no effect on activation domain masking. If sequences 371–397 constitute part of a repressor domain, then each half of this sequence must carry out a redundant function. If these sequences physically mask the activation domain, it suggests that removal of the entire region is necessary to expose the YY1 activation domain. In either case, the activation domain masking sequences (i.e., residues 371–397) are distinct from those necessary for association with the nuclear matrix (residues 256–340). Therefore, activation domain masking and association with the nuclear matrix are not directly related.

On the other hand, sequences necessary for repression of activated transcription (333–371; Fig. 7) partially overlap the matrix associating sequences. Deletion of YY1 sequences between residues 333 and 371 abolished its ability to repress enhancer activity. Enhancer repression by YY1 did not require direct DNA binding because mutation of the YY1 binding site in the reporter plasmid did not abolish repression. Similarly, mutants of YY1 incapable of binding DNA (Cys360Ser and Δ371-397), nonetheless repressed activated transcription. Therefore, this repression function by YY1 most likely results from protein interactions. A number of proteins are known to physically interact with YY1, some within the zinc finger region. These YY1 interacting proteins include TBP, CBP, p300, TFIIB, c-myc, Sp1, ATF2, CREB, AP1, $TAFII_{55}$, and E1A [Usheva and Shenk, 1994, Chiang and Roeder, 1994, Lee et al., 1993, 1995; Seto et al., 1993; Zhou et al., 1995; O'Connor et al., 1996; Shrivastava et al., 1993; Yang et al., 1996; Austen et al., 1997; Shi et al., 1991]. YY1 sequences 333–371 could potentially inhibit enhancer activity by physically interacting with one or more of these proteins thereby disrupting transcriptional activity. Interestingly, Galvin et al. [1997] recently showed that YY1 zinc finger 2 (residues 327–347) is involved in certain YY1 transcriptional repression functions. These sequences overlap those that we show are necessary for association with the nuclear matrix. Determination of whether the sequences responsible for these functions co-localize will require further studies. The YY1 regions responsible for the functions studied here are diagrammatically represented in Figure 8.

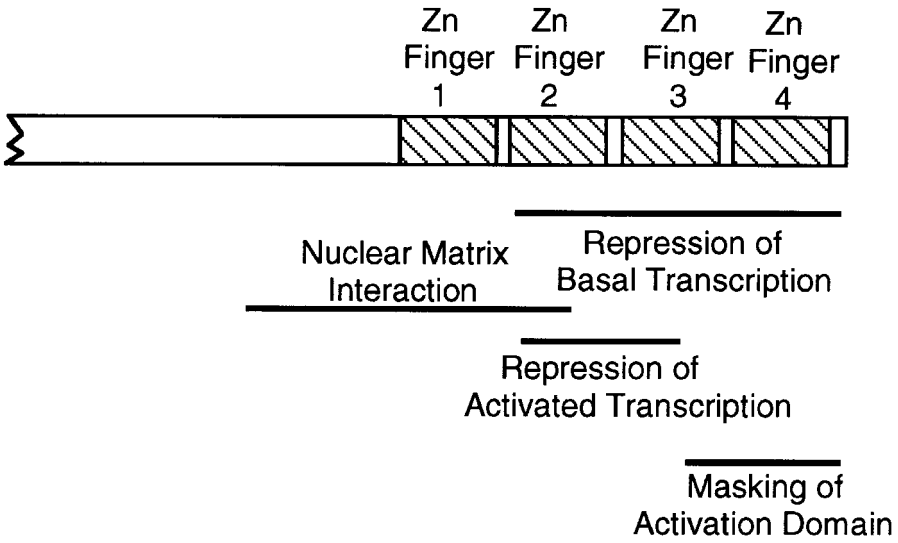

Fig. 8. Summary of the YY1 sequences involved in either nuclear matrix interaction (256–340), repression of basal transcription (333–414, from Bushmeyer et al. [1955]), repression of activated transcription (333–371), or masking of the YY1 transactivation domain (371–397). The location of each YY1 zinc finger is indicated.

It is somewhat surprising that sequences responsible for YY1 repressor activity and association with the nuclear matrix lie within the YY1 zinc finger region. Zinc fingers are generally believed to function in DNA binding and the YY1 zinc fingers do constitute its DNA binding domain. However, zinc fingers are also known to be involved in certain protein interactions [Webster and Ricciardi, 1991; Geisberg et al., 1994, Abdel-Hafiz et al., 1993; Perkins et al., 1994; Ha et al., 1993; Sanchez-Garcia and Rabbits, 1994; Crossley et al., 1995; Merika and Orkin, 1995; Sun et al., 1996b; Tsang et al., 1997]. Therefore, the YY1 zinc fingers apparently can bind to DNA and can contact proteins necessary for transcriptional activity and for association with the nuclear matrix.

The ability of the YY1 zinc fingers to participate in multiple functions suggests at least some of these functions may be mutually exclusive. Indeed, as mentioned above, DNA binding and nuclear matrix association functions may be mutually exclusive. This raises the possibility that association with the nuclear matrix could be used as a mechanism to control the function of YY1. The function of some DNA binding proteins such as PML and Rb can be regulated by nuclear matrix associations [Mancini et al., 1994; Durfee et al., 1994; Chang et al., 1995]. For instance, disruption of nuclear compartmentalization of PML due to the PML-RARα fusion protein produced by the t(15;17) translocation in acute promyelocytic leukemia appears to directly relate to the disease state [Carvalho et al., 1995; Dyck, 1994; Dong et al., 1994]. Thus, the function of a nuclear factor may be controlled by its nuclear compartmentalization. Disruption of this compartmentalization may lead to a disease state, as has been suggested for the PML-RARα fusion protein.

It will be interesting to determine whether association of YY1 with the nuclear matrix is controlled within the cell and whether this influences YY1 function. Our transfections with full-length GAL-YY1 and various GAL-YY1 truncations showed that the full-length YY1 phenotype (weak activation) is dominant over high-level activation exhibited by YY1 deletion constructs. Whether this phenomenon relates to dominance of the repression domain function of YY1, or to intranuclear compartmentalization is unclear. Additional experiments will be required to distinguish between these two possibilities.

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