

Dominant Negative Analogs of NF-YA*

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NF-Y is a highly conserved heteromeric CCAAT-binding transcription factor involved in the function of several promoters. The NF-YA subunit contains a domain of high homology to yeast HAP2, which we show to be necessary and sufficient to mediate interactions with the NF-YB subunit and with DNA. Using protein affinity columns derivatized with amino acid substitution mutants, we further dissect this region into two functionally separable subdomains. The subunit association function resides in a 21-amino acid stretch, which is almost perfectly conserved among different species, while interaction with DNA resides in another short segment. We also show that DNA-binding mutants act as dominant repressors of NF-Y-DNA complex formation and of NF-Y-dependent transcription.

The CCAAT box is one of the common elements of transcriptional promoters in higher eukaryotes (Bucher, 1990). A number of proteins have been reported to bind this and related sequences (see Dorn *et al.* (1987), and references therein). Among these NF-Y (also called CP1 or CBF) was originally identified as a mouse protein recognizing the Y box in MHC^I class II promoters (see Benoist and Mathis (1990) for a review). NF-Y has an absolute requirement for the CCAAT motif (Dorn *et al.*, 1987) and has been shown to bind CCAAT boxes in a wide variety of promoters: albumin (Raymondjean *et al.*, 1988; Wuarrin *et al.*, 1990), globins (Dorn *et al.*, 1987; Barberis *et al.*, 1987; Mantovani *et al.*, 1989; Kim and Sheffery, 1990), β -actin (Quitschke *et al.*, 1989), α -collagen (Hatamochi *et al.*, 1988), and interleukin 4 (Szabo *et al.*, 1993).

NF-Y is a ubiquitous heteromeric metalloprotein composed of two subunits, NF-YA and NF-YB, both contacting DNA directly; each subunit alone cannot bind stably the target sequence (Hooft van Huijsduijnen *et al.*, 1987; Hatamochi *et al.*, 1988; Chodosh *et al.*, 1988a). Maity and de Crombrughe (1992) have provided evidence that a third subunit is also part of the complex, although this would be somewhat at odds with previous observations and this subunit remains to be formally identified. The genes coding for the two NF-Y subunits have been

cloned in different species (Hooft van Huijsduijnen *et al.*, 1990; Vuorio *et al.*, 1990; Maity *et al.*, 1990; Becker *et al.*, 1991; Li *et al.*, 1992a; Olesen *et al.*, 1991; Li *et al.*, 1993). Alignment of protein sequences revealed a striking homology to the HAP2 and HAP3 genes of *S. cerevisiae*, involved in induction of cytochrome genes by non-fermentable carbon sources (Olesen *et al.*, 1987; Pinkham *et al.*, 1987; Hahn *et al.*, 1988; Forsburg and Guarente, 1989). HAP2 and HAP3 are able to heterodimerize with the corresponding mammalian subunits (Chodosh *et al.*, 1988b).

The N-terminal half of mammalian NF-YA contains a glutamine-rich activation domain (Li *et al.*, 1992b), while the C-terminal half harbors a domain of marked homology between NF-YA, HAP2, and *Schizosaccharomyces pombe* php2 (hereafter referred to as the homology domain) (Hooft van Huijsduijnen *et al.*, 1990; Maity *et al.*, 1990; Li *et al.*, 1993; Olesen *et al.*, 1991). A detailed genetic analysis of deletion and substitution mutants of HAP2 (Olesen and Guarente, 1990; Xing *et al.*, 1993), and immunoprecipitation and DNA binding studies of *in vitro* synthesized CBF-B (the rat homolog of NF-YA) (Maity and de Crombrughe, 1992) indicated that a region containing this domain is functionally essential for interaction with CBF-A/HAP2 and for DNA binding. The NF-YA homology domain does not contain any recognizable protein-protein or DNA binding motifs. To gain insights into what are apparently new modules for transcription factors in higher eukaryotes, we felt it important to better define the functional subdomains of NF-YA, and further extend the structure-function analysis of NF-YA by biochemical methods. To this end, we used bacterially produced recombinant proteins in gel retardation, protein affinity columns, and *in vitro* transcription experiments. Our data refine the delineation of the functional stretches and allow us to identify dominant negative mutants of NF-YA.

MATERIALS AND METHODS

Production of Recombinant Proteins—Recombinant proteins were produced with the *Escherichia coli* expression vector pET3b and the BL21(DE3)LysE strain. Construction of the wild type "long" NF-YA plasmid was essentially as described previously (Mantovani *et al.*, 1992), except that cDNA P8-9 was used (Li *et al.*, 1992b). The subsegments present in plasmids YA12 (amino acids 1-160), YA2 (161-346), and YA9 (262-317) were prepared by polymerase chain reaction amplification of cDNA (nucleotide positions 1-480, 481-1038, and 784-951, respectively, of the NF-YA sequence shown by Li *et al.* (1992b)); the fragments were inserted in the proper frame in the *Bam*HI site of pET3b. Point mutants of NF-YA were constructed by polymerase chain reaction, following the strategy described by Higuchi (1988); from the amplified mutated fragment, a *Kpn*I-*Bam*HI subfragment was excised and exchanged with the corresponding wild type fragment in pET3b/NF-YA. The coding region of all mutants was checked by sequencing before use.

Proteins were produced as inclusion bodies and renatured as described (Mantovani *et al.*, 1992). At least three independent preparations of each mutant were made and tested in the various functional assays.

Protein Affinity Chromatography—Coupling of recombinant protein to CNBr-activated Sepharose was performed according to the manufac-

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¹ The abbreviations used are: MHC, major histocompatibility complex; HIV, human immunodeficiency virus.

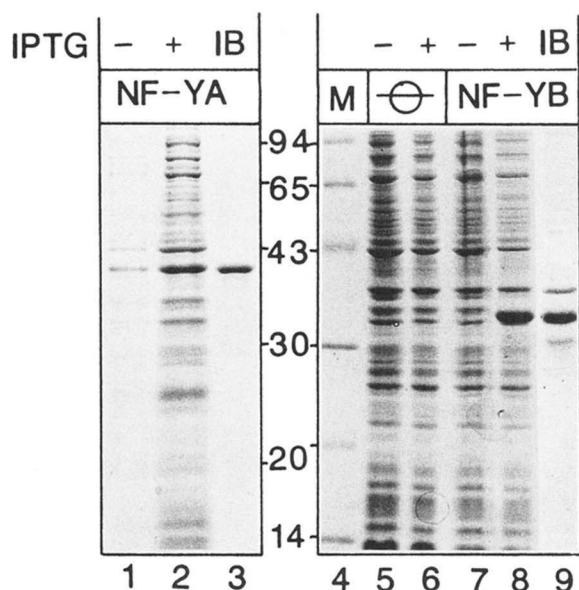


FIG. 1. Production of recombinant NF-YA and NF-YB. SDS-polyacrylamide gel electrophoresis analysis of bacterial extracts after transfection with plasmids derived from the pET3b expression vector, encoding NF-YA (lanes 1 and 2) and NF-YB (lanes 7 and 8); vector alone controls are shown in lanes 5 and 6. Production of the recombinant proteins was induced with isopropyl-1-thio- β -D-galactopyranoside in lanes 2, 6, and 8. Lanes 3 and 9 show preparations of renatured proteins from purified inclusion bodies containing NF-YA and NF-YB.

turer's instructions (Pharmacia Biotech Inc.). Protein matrix (500 μ l, 2 mg) was washed in NDB-Mg (100 mM KCl, 20% glycerol, 20 mM HEPES, pH 7.9, 2 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and rocked for 12 h at 4 °C with 2 ml of CH27 nuclear extract (8 mg/ml). The slurry was poured into a column, and the flow-through was reapplied twice. Following extensive washing with NDB-Mg (15 ml), bound proteins were eluted with 750 ml of NDB (20% glycerol, 20 mM HEPES, pH 7.9, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing increasing concentration of KCl (0.2–1.5 M). The fractions were dialyzed against NDB (250 ml for 2 h), quick-frozen, and stored at –80 °C.

For the YA21–YA29 set of mutants, three columns were prepared with each mutant protein and each was used for two independent experiments.

Gel Retardation, Western Blot Analysis, and *in Vitro* Transcription—Gel retardations with the Y box oligonucleotide were performed as described previously (Dorn *et al.*, 1987), with the indicated amount of the recombinant proteins and 1 μ l of CH27 nuclear extract (8 μ g of protein), or with 0.3–0.5 μ l of a fraction from an affinity purification of NF-YB. For the experiment with the NF- κ B protein, an oligonucleotide corresponding to the NF- κ B binding site of HIV long terminal repeat (TAGGGACTTTCCGCTGGGGACTTTCCAG) was used, together with 1 μ l (4 μ g) of nuclear extract from a phorbol 12-myristate 13-acetate-induced Jurkat T cell line; binding conditions were as reported by Osborn *et al.* (1989). Western blots were performed as described (Mantovani *et al.*, 1992). For *in vitro* transcription experiments, 100 ng of recombinant protein was incubated on ice for 15 min in transcription buffer with CH27 nuclear extract, either before addition of template DNAs or after incubation at 25 °C for 15 min. Transcription reactions were started by addition of NTPs and incubated at 30 °C for 40 min; RNA purification and S1 analysis have been detailed previously (Mantovani *et al.*, 1992).

RESULTS

Production and Functional Assays of NF-YA—We inserted the cDNAs corresponding to the A and B subunits of NF-Y into the *E. coli* expression vector pET3b (Studier *et al.*, 1990). Both proteins were efficiently produced and mostly confined to inclusion bodies in which they represent 75–90% of the proteins (Fig. 1). We considered this material pure enough to use without further fractionation in the experiments described below; it was solubilized in 8 M urea and renatured by slow dialysis

against urea-free buffer. Proper folding of bacterial rNF-YA could be evidenced by gel retardation assays together with B lymphoma nuclear extracts (data not shown) or, more readily, with purified NF-YB (see below). In contrast, we could never demonstrate DNA binding activity with bacterially produced NF-YB (soluble or renatured from inclusion bodies). To investigate the interactions between NF-YA and NF-YB, we used protein affinity chromatography, by linking renatured NF-YA to a solid matrix. We incubated CH27 nuclear extracts with the derivatized resin, with a final elution by buffers containing increasing concentrations of KCl; each fraction was analyzed in the gel retardation assays. Results of such an experiment are shown in Fig. 2 and indicate that NF-Y binding activity is no longer visible either in the flow or in the fractions eluted from the second NF-YA column (Fig. 2A, lanes 1–14); this finding suggests that the column is able to dissociate the activities necessary for DNA binding. However, addition of recombinant NF-YA to the eluted fractions restored the binding activity (lanes 16–24), indicating that they contain NF-YB.² This was also verified by Western blot analysis; NF-YB protein detected in this manner eluted with a profile identical to that observed by gel retardation (Fig. 2B). Recombinant NF-YA alone did not shift the CCAAT oligonucleotide (see below); control columns derivatized with bovine serum albumin or rNF-YB did not retain any NF-Y subunits (not shown). Thus the NF-YA column was able to bind NF-YB specifically, exchanging with natural NF-YA present in the extract. Note that the interaction was only possible when divalent cations were present in the buffer (2–4 mM MgCl₂), or when EDTA was omitted (data not shown).

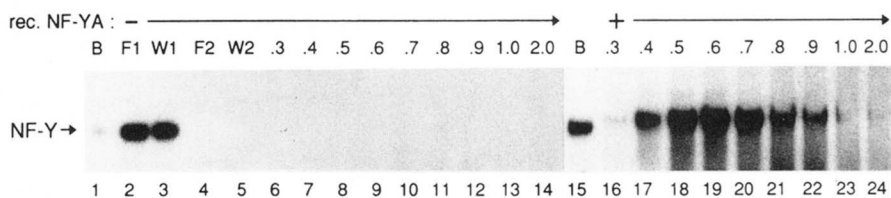
Delineation of NF-YA Segments Interacting with NF-YB and the CCAAT Box—Olesen and Guarente (1990) showed that a short segment of HAP2 is sufficient to interact with the HAP3 subunit and bind DNA. Maity and de Combrugghe (1992) also found that DNA binding activity of rat CBF-B (NF-YA) could be narrowed to the C-terminal region. We quickly verified that this assignment was also true for NF-YA, by expressing a few truncates in *E. coli* (Fig. 3); efficient DNA binding, complemented with an affinity-purified NF-YB fraction, could be found to reside solely within a 56-amino acid fragment, encompassing exactly the motif homologous to HAP2 (mutant YA9, spanning positions 262–317). Other regions appeared immaterial. (We have no clear explanation for the double band observed with YA2. It probably does not result from degradation; is it dimer/tetramer formation or an induced DNA conformation?).

These results confirm and refine the previous results of Maity *et al.* (1992) and are quite logical, since the yeast and mammalian subunits are known to cross-associate (Chodosh *et al.*, 1988). We thus focused our attention on this region, to dissect the DNA binding and subunit interaction functions of NF-YA. Analysis of amino acid composition and periodicity suggested that two stretches have potential to form α -helices: from position 264 to 290 and from position 295 to 320. We therefore wished to scan the two regions by making substitutions that would change amino acid side chains but not alter a hypothetical helical structure. We constructed five mutants in the putative C-terminal helix by polymerase chain reaction mutagenesis, replacing 3 amino acids by alanines every other 3 amino acids (Fig. 4A). Since this putative helix would have a marked charge polarity (see below), we also attempted to perturb this

² Maity and de Combrugghe (1992) have provided evidence indicating that a third subunit may be necessary for the DNA binding activity of NF-Y (CBF in their terminology). The data in the present paper have no direct bearing on this proposition, and it is certainly conceivable that the fractions purified on the NF-YA affinity column actually contain two distinct factors. We will, however, refer to the purified activity that complements NF-YA as NF-YB, for clarity.

FIG. 2. Binding of NF-YB to a recombinant NF-YA column. Nuclear extract from CH27 cells was fractionated by affinity chromatography over a Sepharose column derivatized with rNF-YA. The extract was applied twice. *F*, flow-through; *W*, wash fractions. Bound proteins were eluted with increasing concentrations of KCl, from 0.3 to 2.0 M, as indicated. *A*, the fractions were analyzed by gel retardation with a labeled Y box oligonucleotide. The DNA-binding assays were supplemented with rNF-YA (1 ng) in lanes 16–24. *B*, the same fractions were analyzed by Western blot with a polyclonal antiserum specific for the NF-YB protein (Mantovani *et al.*, 1992).

A. GEL SHIFT



B. WESTERN

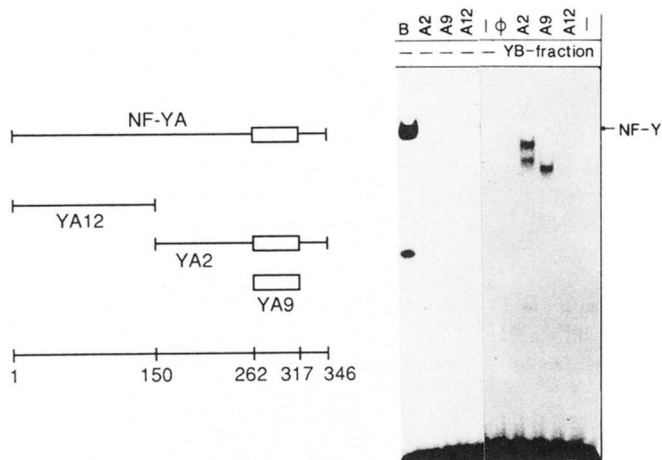
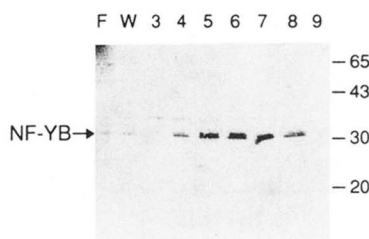


FIG. 3. DNA binding activity of the NF-YA deletion mutants. The cartoon on the left depicts the sequences contained in three deletion mutants of NF-YA. The gel on the right shows the capacity of the truncated recombinant proteins to bind the Y box oligonucleotide in a gel retardation assay, when supplemented with an affinity-purified fraction containing NF-YB. *B*, B cell lymphoma extract. Lane 0, YB fraction alone.

organization by a double Lys → Glu transition (positions 269 and 276); we also tried to disrupt the helix by a proline substitution at the non-conserved position 279. In addition, two triple alanine mutations were introduced in the putative N-terminal helix of the homology region. These mutations were placed in the context of the whole NF-YA cDNA, and the mutant proteins expressed in *E. coli*. Western blot analysis with anti-NF-YA antibody showed that all the mutant proteins were intact and had the expected size (not shown).

The recombinant proteins were tested in gel retardation assays, after addition of an affinity-purified NF-YB preparation (Fig. 4*B*). A varied spectrum of severity of the mutations could be readily visualized over the range of concentrations tested; mutant YA21 behaved as wild type, mutant YA27 was only slightly less efficient, the mutation in YA26 was more severe, and mutants YA25, 24, 23, and 22 were very strongly affected, binding DNA only at the very highest dose tested (which we estimate corresponds to a 2000-fold excess over the amounts normally present in gel shift assays with crude nuclear extract). Finally, mutants YA28 and YA29 appeared completely inactive.

A.

YA	262	EPLVYNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	317
YA21		<u>E</u> <u>A</u> <u>A</u> VNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	
YA22		EPLVYNAK <u>A</u> <u>A</u> <u>A</u> HRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	
YA23		EPLVYNAKQYHRIL <u>A</u> <u>A</u> <u>A</u> QARAKLEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	
YA24		EPLVYNAKQYHRILKRRQARAK <u>A</u> <u>A</u> <u>A</u> LEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	
YA25		EPLVYNAKQYHRIL <u>E</u> <u>R</u> <u>R</u> QARAKLEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	
YA26		EPLVYNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGGGRF	
YA27		EPLVYNAKQYHRILKRRQARAKLEA <u>A</u> <u>A</u> <u>A</u> IPKERRKYLHESRHRHAMARKRGGGRF	
YA28		EPLVYNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHR <u>A</u> <u>A</u> <u>A</u> ARKRGGGRF	
YA29		EPLVYNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARK <u>A</u> <u>A</u> <u>A</u> GGGRF	

B. GEL SHIFT

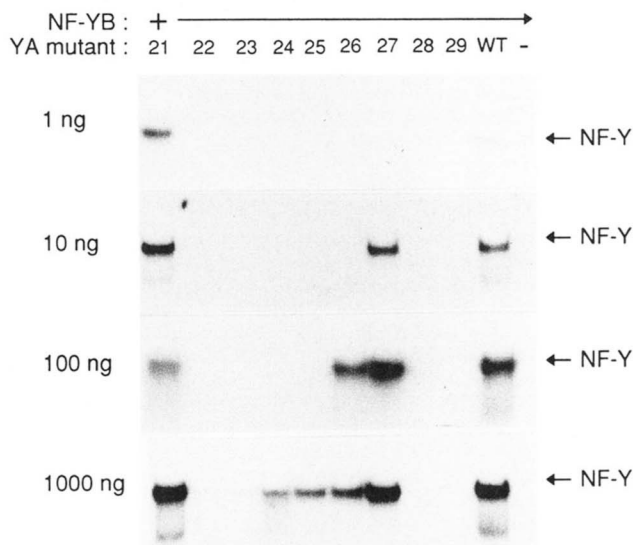


FIG. 4. DNA binding activity of the YA21–YA29 point mutants. *A*, sequences of the mutated region in mutants YA21–YA29 of rNF-YA. The sequence of each mutant is aligned under the wild type sequence, with the amino acid changes underlined. *B*, different amounts of each of the recombinant proteins (1–1000 ng) were tested in gel retardation assays with a Y box oligonucleotide, after addition of aliquots of an affinity-purified fraction containing NF-YB.

The inefficiency or absence of NF-Y-DNA complex formation with a given mutant could be due to difficulty associating with the companion subunit or to failure to interact with DNA. To discriminate between these two possibilities and compare the affinities of the NF-YA mutants for NF-YB, we used a protein-affinity column assay. Sepharose columns were prepared with

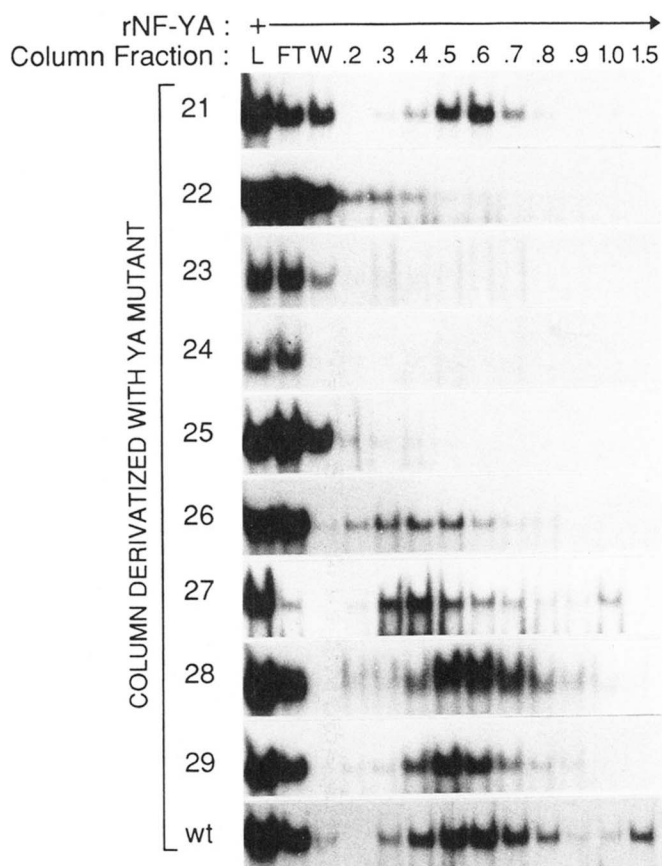


FIG. 5. Capacity of the NF-YA mutants to bind immobilized NF-YB. Affinity matrices were prepared by coupling each of the NF-YA triple mutants to Sepharose. The capacity of these columns to retain NF-YB present in CH27 nuclear extract was evaluated by testing the column fractions in gel retardation assays with a Y box oligonucleotide. rNF-YA was added in the retardation assays to reveal the presence of NF-YB. L, extract prior to loading on the column; FT, flow-through fraction; W, wash. The proteins retained were eluted with increasing concentrations of KCl, as indicated (0.2–1.5 M).

an equivalent amount of each of the mutant proteins and were incubated in parallel with CH27 nuclear extracts. Each column was step-eluted, and an aliquot of each fraction was mixed with wild type rNF-YA and analyzed in gel retardation assays.

The results of one such experiment are shown in Fig. 5. NF-YB (loaded in excess over the capacity of the columns) activity was retained efficiently and eluted at similar salt concentrations from columns derivatized with wild type NF-YA and mutants YA21, YA28, and YA29, typically with a maximum eluting around 0.5–0.7 M KCl; YA27 and YA26 did bind NF-YB, but less efficiently and with elution at slightly lower KCl concentrations. In all the other YA mutant columns, NF-YB was essentially absent from the eluted fractions, implying that the mutations prevent the protein/protein interaction between the two subunits. This pattern corresponds well to that seen in direct retardation assays with the mutants, with the clear exception of mutants YA28 and YA29.

As summarized in Table I, the data obtained in these experiments can most easily be interpreted as separating two functional regions of the DNA-binding domain of NF-YA; a stretch lying between the comparatively harmless mutations YA21 and YA27 (e.g. positions 266–286) is involved in the interactions of NF-YA with the other subunit(s) of NF-Y. The other functionally important region is defined by mutations YA28 and YA29, which do not affect protein-protein interactions, but completely abolish NF-Y·DNA complex formation. By inference, this region directly contacts the DNA bases and must be, at least in

part, responsible for the fine specificity of recognition of the CCAAT motif. This interpretation is fully consistent with the genetic data from the yeast analog (Olesen and Guarente, 1990) and extends the earlier indications obtained by Maity and de Combrughe (1992).

NF-YA Dominant Negative Mutants—Since some of these mutations appeared to affect specifically one aspect of NF-YA function, we reasoned that these defective proteins might interfere with NF-Y, if added to normal cell extracts. The set of mutant proteins was mixed with a CH27 nuclear extract before the addition of a labeled Y box oligonucleotide and was analyzed by gel retardation. While most recombinant proteins had no effect, the NF-Y band was severely decreased when the nuclear extract was preincubated with 10 ng of YA28 or YA29 (Fig. 6A). We estimate that this amount of mutant rNF-YA corresponds to a 20-fold excess over the normal NF-YA present in the nuclear extract. Control experiments were performed to establish the specificity of this inhibition; we incubated 100 ng of the wild type and mutant NF-YA proteins with nuclear extracts from phorbol 12-myristate 13-acetate-induced Jurkat cells, before addition of an HIV oligonucleotide containing an NF- κ B binding site (Fig. 6B). No inhibition of the specific NF- κ B band was observed; similar results were obtained with GATA and YY1 proteins (not shown). Since the two inhibitory mutants were precisely those with affected DNA-binding potential but a normal capacity to interact with NF-YB, we conclude that this inhibition reflects a sequestering of NF-YB into defective complexes.

These findings allowed us to assess the kinetic parameters of YA/YB and YA/YB/DNA interactions using affinity-purified NF-YB and recombinant NF-YA. An excess (20-fold) of YA28 or YA29 proteins efficiently inhibited the NF-Y band when preincubated with rNF-YA and NF-YB for 1 min before the addition of the labeled oligonucleotide (Fig. 6C, lanes 2 and 4). However, a preformed NF-Y·DNA complex was quite impervious to an excess of either YA28 or YA29 mutant proteins added 1 min after the initiation of complex formation (lanes 3 and 5). On the other hand, if the formed NF-Y·DNA complex was incubated with the inhibitor mutant for longer periods of time, some displacement occurred with time; most likely, when the NF-Y·DNA complex dissociates, the free NF-YB is trapped by the dominant negative mutant. This allowed us to measure the dissociation kinetic of the NF-Y·DNA complex. The off-rate has, under these conditions, a $t_{1/2}$ of approximately 20–40 min (Fig. 7). As expected, the complexes formed with the partially defective mutant YA27 dissociated faster. These slow dissociation kinetics contrast with the ready exchange of NF-YA and NF-YB when not bound to DNA (the off-rate in the absence of DNA must have a $t_{1/2}$ on the order of a few seconds or less, as full displacement occurs by 1 min).

These results prompted us to test our mutants in an NF-Y-dependent *in vitro* transcription system, consisting of nuclear extracts from CH27 cells eliciting the transcription of a β -globin reporter gene under the control of the mouse MHC class II Ea promoter (Viville *et al.*, 1991; Mantovani *et al.*, 1992).

Preincubation of 100 ng of YA28 and YA29 mutant proteins with the CH27 nuclear extract before the addition of template DNA severely decreased initiation from the correct Ea start site (Fig. 8A); a slight negative activity was also seen with YA26, and the other recombinant proteins had no effect. The inhibitory influence of YA28 and YA29 did not affect Ea initiation at position -25 (a spurious and artifactual initiation site observed *in vitro*, which we have found to be NF-Y-independent; Mantovani *et al.* (1992)). We noted a partial inhibition of the SV40 promoter with YA28 and YA29; while the SV40 early promoter is not thought to depend on NF-Y, we had previously observed

TABLE I
Summary of the effect of triple substitutions in NF-YA on functional interactions

Mutant	Sequence	Subunit interaction	DNA binding
YAWT	EPLYVNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	+++	+++
YA21	EAAA VNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	+++	+++
YA22	EPLYVNA ³⁰⁶ AAAHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	-	-
YA23	EPLYVNAKQYHR I ¹⁵⁹ AAARQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	-	-
YA24	EPLYVNAKQYHRILKRRQ ⁸ AAALEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	-	-
YA25	EPLYVNAEQYHRILERRQARAKLEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	-	-
YA26	EPLYVNAKQYHRILKRRPARAKLEAEGKIPKERRKYLHESRHRHAMARKRGEGRF	+	+
YA27	EPLYVNAKQYHRILKRRQARAKLE ³⁶² AAAIKERRKYLHESRHRHAMARKRGEGRF	++	++
YA28	EPLYVNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESR ²¹⁴ HRA ²¹⁴ AAARKRGEGRF	+++	-
YA29	EPLYVNAKQYHRILKRRQARAKLEAEGKIPKERRKYLHESRHRHAMARK ⁶⁴ AAAGGRF	+++	-
	-----R-----N-----N-V-----		Sea urchin
	¹⁵⁹ Q-P-----Y-----Y-----EKLRSR---KP-----K---R-P----- ²¹⁴		<i>S. cerevisiae</i>
	⁸ -G-----E-----ERLRVQT ⁶⁴ TKKP-----K---R-P--P----- ⁶⁴		<i>S. pombe</i>

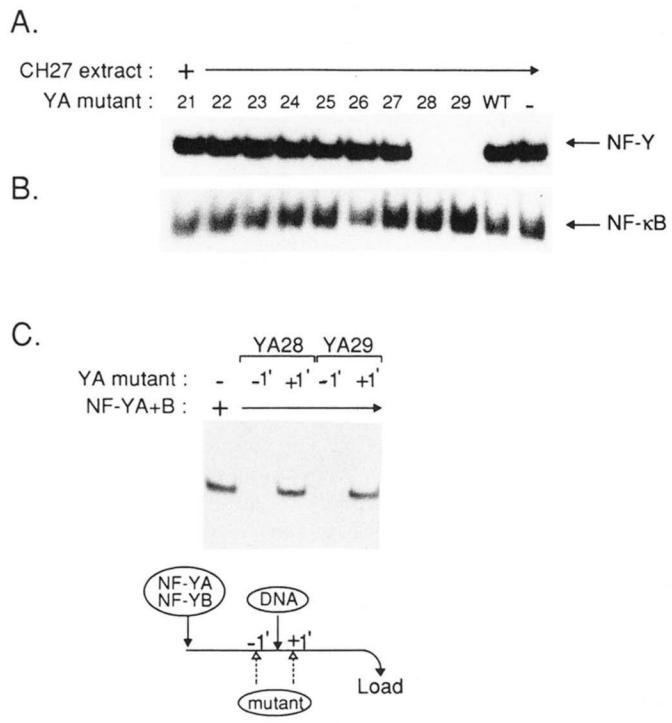


FIG. 6. Dominant inhibition of DNA binding by mutants YA28 and YA29. A and B, equal amounts (10 ng) of each of the NF-YA mutant proteins were incubated together with nuclear extract under conditions of gel retardation assays, with Y box (A) or NF-κB (B) oligonucleotides. In C, the mutant proteins were introduced either before (-1') or after (+1') the addition of labeled DNA to the incubation.

a slight inhibition of SV40-driven transcription by anti NF-Y antibodies (Mantovani *et al.*, 1992). We then changed the order of addition of components in the transcription assay, first incubating the transcription extract with DNA, thus allowing a stable preinitiation complex to form, and secondarily challenging it with the YA mutants; no inhibition of transcription was observed in this instance (Fig. 8B).

DISCUSSION

The experiments presented here confirm and extend the existing notions on the structure of the NF-YA DNA-binding region.

1) Deletion mutant analysis clearly showed that no additional sequences outside the 56 amino acids of the HAP2 homology domain are necessary for association with NF-YB and DNA binding; the sequences present in mutant YA9 are necessary and sufficient.

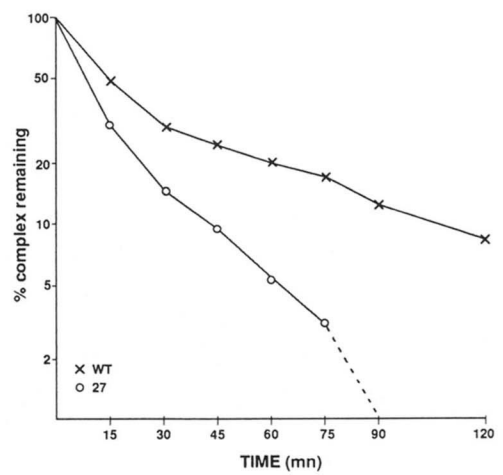


FIG. 7. Dissociation kinetics of the NF-Y-DNA complex in the presence of a dominant inhibitory mutant. NF-Y-DNA complexes were formed by incubating labeled Y box oligonucleotide together with affinity-purified NF-YB and rNF-YA (either wild type or mutant YA27). A 30-fold excess of mutant YA28 was added after 10 min, and the incubation allowed to continue at 25 °C for various times. Aliquots were loaded on the gel after the indicated times, and the proportion of NF-Y-DNA complex remaining was measured by densitometry of the autoradiograph. The two curves shown correspond to the dissociation of complexes formed with wild type or mutant NF-YA proteins.

2) Within the set of replacement mutants, all the protein-protein interaction mutants (YA22–YA26) map to the N terminus of the HAP2 homology domain, while the DNA-binding mutants (YA28, YA29) are on the C terminus. This distribution clearly delineates two subdomains within the HAP2 homology, responsible respectively for protein-protein (the N-terminal subdomain) and DNA binding (the C-terminal subdomain). The genomic organization of NF-YA supports this idea, since the homology domain is indeed split in two, being encoded by two separate exons (Li *et al.*, 1992b).

3) Binding of NF-YB to NF-YA can occur in solution and on affinity columns, DNA being apparently unnecessary but metal ions being essential. The latter probably explains the effect of metal-chelating agents on NF-Y binding to DNA (Hooft van Huijsduijnen *et al.*, 1987).

NF-YB Association Segment—Mutants YA21 and YA27, which behave in all assays essentially like wild type NF-YA molecules, delimit the subunit interaction domain to 21 amino acids: Val-266 to Ala-286. This positively charged region is almost perfectly conserved among the different species (Table I). The triple mutations within this stretch virtually abolished subunit interaction (YA22, YA23, and YA24), as did charge inversions in two conserved lysine residues (K269E, K276E). It

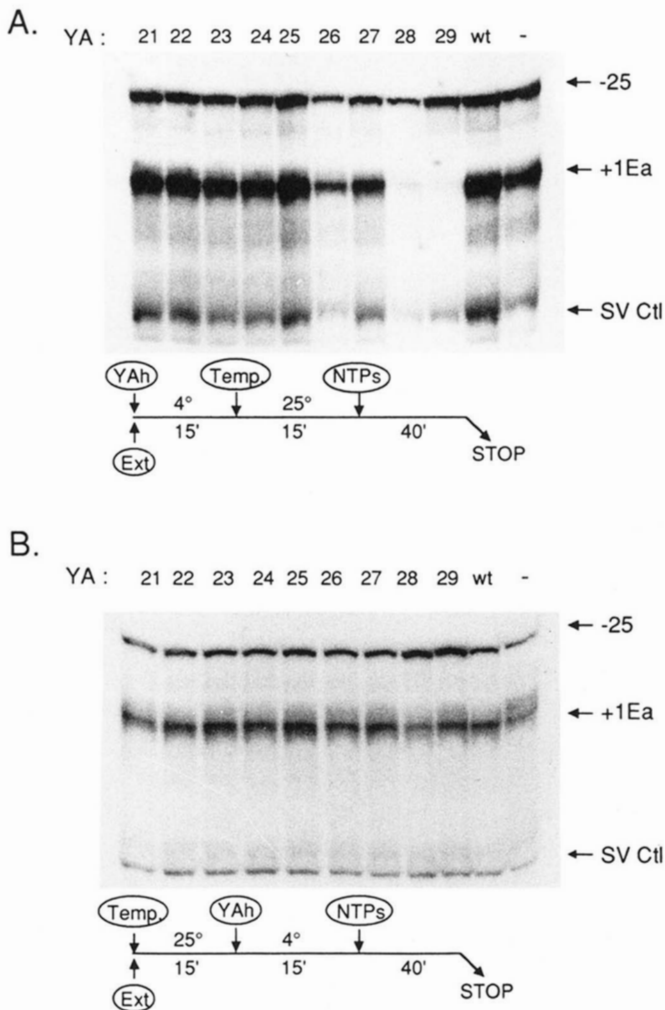


FIG. 8. Inhibition of NF-Y-dependent *in vitro* transcription by dominant negative mutants. *A*, *in vitro* transcription reactions were set up with extract from CH27 cells, supplemented with 10 ng of protein from each of the rNF-YA mutants, and template DNA consisting of the Ea promoter driving the transcription of a β -globin reporter segment. The RNAs transcribed were quantitated by S1 mapping. The three major bands produced correspond to transcripts from the SV40 promoter template used as an internal control in the reactions (SV Ctl), the main initiation site of the Ea promoter (+1 Ea), and an artificial initiation, which occurs under conditions of *in vitro* transcription and which we have shown to be independent of NF-Y (-25; see Mantovani *et al.* (1992) and references therein). *B*, as *A*, except that the order of addition of components was reversed and the mutant proteins added after preincubation of the transcription extract with the template DNA.

should be noted that Maity and de Crombrughe (1992) have found that mutating the same two lysine residues to alanines had no effect in a fairly similar test system. It thus appears that it is the introduction of negative charges at these positions that cannot be tolerated for subunit interaction. A single proline substitution at the relatively non-conserved position 279 is also quite deleterious. These results are consistent with the idea that a hypothetical α -helix (Li *et al.*, 1992b) could be responsible for association with NF-YB.

DNA-binding Segment—Although the association between subunits is required for DNA binding, a separate stretch of the HAP2 homology domain is necessary to contact the DNA, as indicated by behavior of YA28 and YA29. In the case of CBF, two double mutants mapping in this region were also shown to be crippled (Maity and de Crombrughe, 1992). For HAP2, the DNA-binding subdomain was mapped between Tyr-194 and Phe-214 (corresponding to Tyr-297 and Phe-317 in NF-YA). In our case, the DNA-binding segment is delineated on its right by

the end of the 56-amino acid homology domain, at position 317 (mutant YA9); the left boundary cannot be defined precisely with our mutants, but it extends at least to position 304, and most likely to position 297, the edge of the nearly perfect conservation from yeast to mammals.

Sequence requirements for DNA contacts appear more fastidious than those for subunit interaction; no binding whatsoever is detected even at the highest doses of mutants YA28 and YA29, while the mutations that affect the subunit interaction region almost all leave at least a trace of function, detectable with large excesses of mutant protein. One can hypothesize that the protein/protein interfaces are more flexible and better able to accommodate changes than those involved in the accurate sensing of DNA sequence.

Dominant Negative Mutants—We show that DNA-binding mutants, when preincubated with nuclear extracts, act in a dominant negative way on NF-Y activity: on DNA binding in gel retardation experiments and on transcription of the MHC class II Ea promoter *in vitro*. This is most likely due to normal association with NF-YB and formation of defective NF-Y molecules, unable to bind the Y box and to nucleate the formation of stable preinitiation complexes on the Ea promoter; in fact, when preinitiation complexes were allowed to form by preincubating template DNA with the nuclear extract, no effect of the mutants was observed (Fig. 8B). We have shown previously that multiple rounds of reinitiation take place in our *in vitro* system (Mantovani *et al.*, 1992). The data of Fig. 8B thus imply that reinitiation is not affected by the dominant negative mutants. NF-Y must remain bound to the DNA between the successive rounds of polymerase entry. This behavior of the dominant negative proteins is different from that of anti-NF-Y antibodies, which had the potential to inhibit reinitiation (Mantovani *et al.*, 1992). Steric hindrance by the antibodies was probably preventing the reentry of the polymerase or associated factors; this mechanism does not occur with the competitive inhibitors used here.

The dominant negative behavior of NF-YA DNA-binding mutants has been observed with several other heteromeric transcription factors such as Jun (Gentz *et al.*, 1989), Fos (Ransone *et al.*, 1990), Myc (Dang *et al.*, 1989), or NF- κ B (Logeat *et al.*, 1991; Toledano *et al.*, 1993; Bressler *et al.*, 1993). The negative dominant mutants of NF-Y will be important tools in establishing the role of NF-Y in several different systems in which its involvement has been so far suspected, both with *in vitro* transcription studies and transfection experiments. Similarly, our mutants can be used to test the possible functional interaction with other molecules with sequence homology to NF-YB, such as DR1 (Inostroza *et al.*, 1992). More generally, the biochemical assays described in this study can also be used to test directly the hypothetical structure of NF-Y and to investigate other important protein-protein interactions in which this transcription factor might be involved.

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