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Interactions of the CCAAT-binding Trimer NF-Y with Nucleosomes*

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NF-Y is a sequence-specific evolutionary conserved activator binding to CCAAT boxes with high affinity and specificity. It is a trimer formed by NF-YA and two putative histone-like subunits, NF-YB and NF-YC, showing similarity to histones H2B and H2A, respectively. We investigated the relationships between NF-Y and chromatin using an *Artemia franciscana* chromatin assembly system with plasmids containing the Major Histo-Compatibility complex class II Ea promoter. The NF-Y trimer, but not single subunits, protects the Y box in the presence of reconstituted chromatin, and it can bind the target sequence during and after assembly. Using reconstitution assays with purified chicken histones, we show that NF-Y associates with preformed nucleosomes. Translational analysis of various Ea fragments of identical length in which the CCAAT box is at different positions indicated that the lateral fragment was slightly more prone to NF-Y binding. In competition experiments, NF-Y is able to prevent formation of nucleosomes significantly. These data support the idea that NF-Y is a gene-specific activator with a built-in capacity to interface with chromatin structures.

Regulation of gene expression is a complex set of events controlled by gene-specific *trans*-acting factors and general transcription proteins recognizing *cis*-acting elements in promoters and enhancers and operating in the context of higher order chromatin structures (1). The fundamental chromatin unit is the nucleosome, a DNA-protein complex formed by core histones (H2A, H2B, H3, H4) wrapped around 146 base pairs of DNA. Histones are among the most conserved proteins in evolution; analysis of their quasi-invariant COOH-terminal sequences revealed a 65-amino acid histone fold motif shared by all histone proteins, with low sequence identity, 14/18%, and high structural resemblance (2). It is composed of three α-helices: a long central one of 28 amino acids flanked by two short ones separated by short loops/strand regions; this structure enables histones to dimerize with the companion subunit and to make non-sequence-specific contacts with the DNA (3). The latter function results from tetramerization of H3-H4, which first nucleates the wrapping of DNA and subsequently promotes the association of two H2A-H2B dimers. Recent computational analysis of protein data banks identified additional polypeptides involved in the process of transcriptional regulation which contain putative histone fold domains (4): (i) $dTAF_{II}60$-$hTAF_{II}80$, $dTAF_{II}40$-$hTAF_{II}31$, and $hTAF_{II}20$-$dTAF_{II}30$, part of the general TFIID complex; crystallization of $dTAF_{II}60$-$dTAF_{II}40$ dimers detailed their histone-like structures (for review, see Ref. 5); (ii) the two subunits of NC2 (also called Dr1/DRAP1) which bind TBP and repress transcription (6, 7); (iii) two subunits of NF-Y, a ubiquitous CCAAT-binding heteromeric complex formed by three proteins, all necessary for DNA binding.

The CCAAT box is a widely distributed regulatory sequence, present in 25% of promoters and enhancers, very often at position −60/−80 (8). Functional experiments indicate that the CCAAT box plays an important role in essentially all such promoters. NF-Y, originally identified as the protein binding to the major histocompatibility complex class II Ea promoter Y box, has an almost absolute requirement for these five nucleotides (9) and has been implicated in the activation of more than 100 promoters (10). NF-Y genes have been cloned in different species, including yeast, plants, and parasites. Protein alignments evidenced highly conserved domains across evolution (11–14 and refs. therein). NF-YA has a 56-amino acid region that can be split into two short separable parts, responsible for contacting NF-YB-NF-YC and DNA (12, 15). NF-YB and NF-YC have conserved domains of 90 and 84 amino acids containing putative histone fold motifs (4). NF-YB and NF-YC are bound tightly, and their dimerization is a prerequisite for NF-YA association and CCAAT box binding (13). Mutagenesis of both proteins showed that the integrity of their histone fold motifs is strictly required for subunit interactions and DNA binding (16, 17). They also share a particularly high resemblance to NC2 subunits, extending beyond the histone fold motifs, within the larger yeast/human conserved domains. NF-YB and NC2β belong to the H2B-like subtype, whereas NF-YC and NC2α are closer to H2A (4, 6, 7).

Because of this peculiar histone-like structure, we felt it important to investigate the relationships between NF-Y and chromatin. In this study, we use an *in vitro* chromatin reconstitution system from the brine shrimp *Artemia franciscana* (18) and nucleosome assembly assays with purified chicken histones to understand the NF-Y-CCAAT interactions in chromatin contexts.

MATERIALS AND METHODS

Extract Preparation and Chromatin Assembly—*A. franciscana* dry cysts (U. Ghent, Belgium) were rehydrated in synthetic sea water and developed at 24 °C for 20 h. Embryos (30 g) were rinsed in water, collected, resuspended in 50 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 250 mM sucrose, 5 mM β-mercaptoethanol, 1% dimethyl sulfoxide, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml pepstatin) and homogenized. Nuclei were pelletted by centrifugation for 5 min at 8,000 rpm and resuspended in 12 ml of extraction buffer. Nuclei were disrupted by increasing the NaCl concentration to 2 M. The resulting lysate (20 ml) was clarified by centrifugation for 2 h at 60,000 rpm. Aliquots of the supernatant were stored at −80 °C. The protein concentration was usually 10–20 mg/ml.

In the assembly reaction (25 μl), 1 μg of plasmid DNA was incubated

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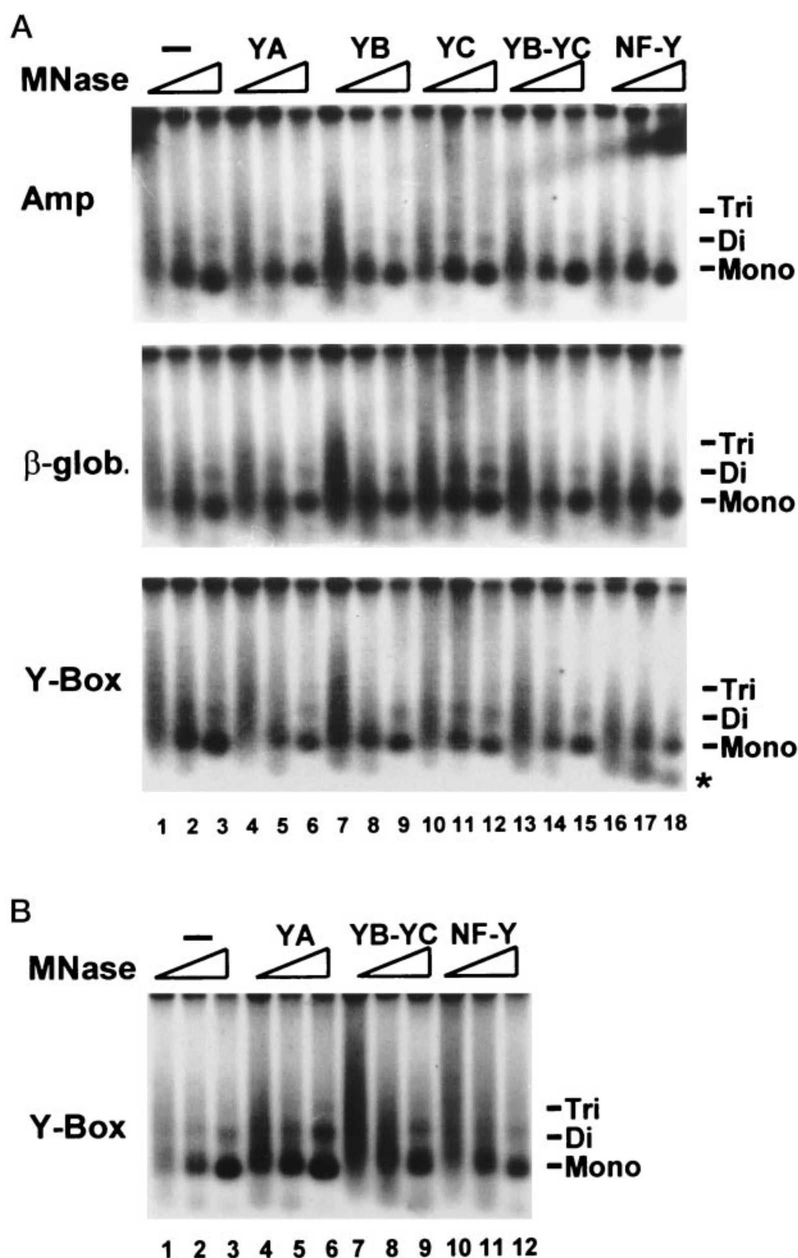

FIG. 1. **Chromatin reconstitution of the major histocompatibility complex class II Ea promoter plasmid.** *A*, micrococcal nuclease (MNase) pattern of chromatin reconstitutions, hybridized with oligonucleotides in the ampicillin region (*upper panel*), in the β-globin (*middle panel*), and Y box (*lower panel*) of pE3. NF-Y subunits (300 ng) were incubated for 30 min with pE3 as indicated, before the addition of the *Artemia* extract. Mono-, di-, and trinucleosomes are indicated; the presence of subnucleosomal bands is marked by an *asterisk*. *B*, the NF-YA, NF-YB-NF-YC dimer or the NF-Y trimer was incubated with the *Artemia* extract as in *A*, with the plasmid pE3m16, which contains a NF-Y crippling mutation in the Y box (17), and the blot was hybridized with the corresponding Y box oligonucleotide.

at 30 °C for 30 min with 10/15 μg of extract in 160 mM NaCl, 25 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM ATP, 50 ng/ml poly-L-glutamic acid (Fluka), 20 mM disodium creatine phosphate (Sigma), 1 mg/ml creatine phosphokinase (Sigma). Recombinant NF-Y subunits (300 ng) were added to each reaction as indicated in the figure legends. Micrococcal nuclease digestions were performed by adding to the reconstitution reactions 5 $CaCl_2$ and micrococcal nuclease (1 unit/mg assembled DNA) (Sigma). The reactions were incubated at 30 °C for 1, 2, and 4 min, stopped by adding SDS and EDTA to a final concentration of 0.4% and 20 mM, respectively, and the mixture incubated for 1 h at 37 °C with proteinase K (2 mg/ml final concentration). The DNA was phenol-chloroform extracted, ethanol precipitated, and run on a 1.6% agarose gel. The hybridization analyses were carried out by standard procedures with the ^{32}P-labeled oligonucleotides specified in the figures.

DNA Templates—The pE3 plasmid harbors the Major Histocompatibility Complex class II Ea proximal promoter sequences fused to the rabbit β-globin reporter gene, and pE3m16 contains a 10-bp[1] mutation in the Y box (19). Plasmids were prepared by double banding on CsCl gradients. The labeled fragments used for nucleosome assembly were generated by polymerase chain reaction from pE3, using oligonucleotides mapping to the positions indicated in Figs. 3 and 4. Fragment 2m, generated from pE3m16, contains a 10-bp mutation in the Y box (19). Oligonucleotides were labeled with T4 kinase, and the amplified fragments were purified on 7% polyacrylamide gels.

Protein Production and Purification—NF-Y subunits were produced and purified on nickel nitrilotriacetic acid-agarose (Quiagen) according to standard protocols and dialyzed against buffer BC100 (20). Histones H2A-H2B, H3-H4 were prepared from chicken erythrocyte nuclei using hydroxylapatite columns by the procedure of Simon and Felsenfeld (21).

Nucleosome Reconstitution and Electrophoretic Mobility Shift Analysis—Nucleosomes were reconstituted by adapting the method described in Ref. 22. Unlabeled DNA (500 ng of sonicated salmon sperm DNA) and approximately 2 ng of end-labeled probe (10^5 cpm) were mixed with 1 μg of purified histones in 1 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM β-mercaptoethanol (20-μl reaction volume). The reaction was incubated at room temperature, and the salt concentration was lowered to 0.1 M by stepwise addition of TE (10 mM Tris-HCl, pH 7.8, 1 mM EDTA) as described (22); a final volume of 300 μl was obtained. Reconstituted nucleosomes (15 μl of reconstitution mixture) were incubated for 5 min at room temperature in NF-Y binding buffer (20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 30 mM KCl, 1 mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride) with recombinant NF-Y. After the addition of 3% glycerol, samples were electrophoresed in a 4% polyacrylamide gel (acrylamide/bisacrylamide,

[1] The abbreviations used are: bp, base pair(s); wt, wild type.

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30:1) containing 3.5% glycerol, 0.5 mM dithiothreitol, 0.5 × Tris borate EDTA for 2 h at 250 V. Gels were dried and exposed.

DNase I Footprinting—Fragment 3 was end labeled by polymerase chain reaction, purified, and reconstituted to 90% with chicken histones; when indicated, 10 ng of NF-Y was added and incubated in NF-Y binding buffer for 20 min at 20 °C. DNase I was added, and the reactions were placed at 30 °C for 1 min, stopped by adding a final concentration of 5 mM EDTA, 0.4% SDS, phenol-chloroform, extracted, ethanol precipitated, and analyzed on a 6% sequencing gel.

RESULTS

The NF-Y Trimer Binds the CCAAT Box in Chromatin Templates—We have recently developed an *in vitro* chromatin reconstitution system using whole cell extracts from the brine shrimp *A. franciscana*; physiological spacing was obtained by the addition of ATP (18). We decided to use it to study the binding capacity of NF-Y on a plasmid containing major histocompatibility complex class II Ea promoter with the high affinity Y box (plasmid pE3, see Ref. 19). After reconstitution, we treated samples with micrococcal nuclease and separated DNA fragments on agarose gels; hybridization of the resulting blots with oligonucleotide probes mapping to different parts of the plasmid gives clues about the nucleosome patterns on a given plasmid sequence. For all of the following experiments we employed pure recombinant NF-Y, consisting of the yeast/human homology domains of the three subunits; the resulting trimer contains all of the information necessary for efficient and sequence-specific CCAAT box binding (20).

We first verified the specificity of the interactions between NF-Y and the DNA in our system, using as a probe an oligonucleotide mapping to the Amp resistance gene, some 2 kilobase pairs away from the Ea promoter sequences. As expected from experiments with other templates, incubation of the extract with pE3 leads to the formation of a regular array of physiologically spaced nucleosomes (Fig. 1*A*, *top panel*, *lanes 1–3*). No differences are observed upon preincubation of DNA with single NF-Y subunits (*lanes 4–12*); the NF-YB-NF-YC dimer (*lanes 13–15*) and the NF-Y trimer (*lanes 16–18*) gave minor destructurations of the nucleosomal pattern. Essentially the same results were obtained with a probe in the β-globin reporter gene, 140 bp downstream from the Y box (Fig. 1*A*, *middle panel*). Using as a probe an oligonucleotide corresponding to the Ea Y box (9), we observed no change in the pattern with NF-YA (Fig. 1*A*, *bottom panel*, *lanes 1–6*), NF-YB (*lanes 7–9*), or NF-YC (*lanes 10–12*), and few general modifications with the NF-YB-NF-YC dimer (*lanes 13–15*). However, preincubation of the NF-Y trimer provoked an almost complete disappearance of dinucleosomes, a decrease in the intensity of the mononucleosomes, while an intense subnucleosomal signal was apparent (Fig. 1*A*, compare *lanes 16–18* with *lanes 1–3* and *lanes 16–18* in the three *panels*). Hypersensitivity to micrococcal nuclease (MNase) generates such subnucleosomal bands that are usually caused by alterations of the nucleosomal structure induced by binding of DNA-binding proteins, a clear indication that NF-Y binds to the Ea CCAAT box in this context. The lack of such bands with the Amp and β-globin probes strongly suggests that NF-Y binding is specific. To verify this point further, we reconstituted chromatin using a template pEm16, which contains a 10-bp mutation in the CCAAT box (19). As shown in Fig. 1*B*, the addition of NF-YA, NF-YB-NF-YC, or the NF-Y trimer does not modify the nucleosomal pattern significantly; in particular, the subnucleosomal bands observed previously with the trimeric NF-Y were completely absent on this CCAAT-less plasmid (Fig. 1*B*, compare *lanes 1–3* with *10–12*). From these experiments we conclude that subnucleosomal bands indeed result from interactions between the trimeric NF-Y and an intact CCAAT box.

We then modified the order of the NF-Y addition to the reconstitution system (see scheme in Fig. 2) by incubating it before, during, or after chromatin assembly. With the control Amp probe the nucleosomal pattern was again similar (not shown); however, when probed with the Y box, we observed that although inhibition of mono- and dinucleosomes was more profound when NF-Y was incubated before or together with template DNA (Fig. 2, compare *lanes 4–6* with *7–9*), the subnucleosomal bands were evident in all conditions (Fig. 2, compare *lanes 1–6* and *10–12* with *lanes 7–9*). We conclude that CCAAT box binding by NF-Y is effective even after chromatin reconstitution. Altogether, these results indicate that in our dynamic chromatin assay (i) the single subunits of NF-Y or the NF-YB-NF-YC histone fold-containing dimer have no wide unspecific effects; (ii) NF-Y binds to DNA in the presence of preformed nucleosomes; (iii) it does not seem to have a completely obstructive role, possibly because of a non-mutually exclusive association with nucleosomes.

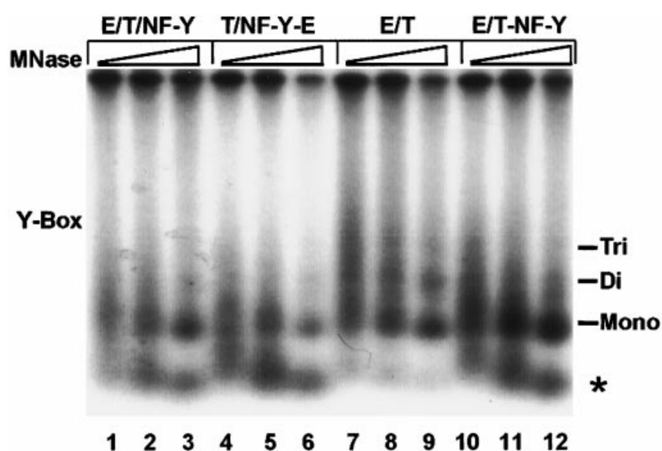

FIG. 2. **Binding of NF-Y to reconstituted chromatin templates.** The *Artemia* extract was added to template DNA either alone (*lanes 7–9*), after (*lanes 4–6*), before (*lanes 10–12*), or together with (*lanes 1–3*) the NF-Y trimer. Preincubations were for 30 min at 30 °C. *T*, template wt pE3 plasmid; *E*, *Artemia* extract. The probe used was the Y box oligonucleotide.

NF-Y Binds to Nucleosomal DNA—One possible explanation for these results is that by using *Artemia* extracts, most likely containing at least some of the chromatin-rearranging machineries recently described in man, *Drosophila,* and yeast (23), the observed effects might be caused by indirect facilitation of chromatin rearrangement by such remodeling activities. To verify whether NF-Y associates with nucleosomal DNA, we switched to a different *in vitro* system. Purified core histones prepared from chicken erythrocytes in separated couples (Fig. 3*A*) were assembled with four end-labeled Ea promoter fragments in which the CCAAT box is translationally moved or mutated (see scheme in Fig. 3*A*). Labeled DNA and a vast (250-fold) excess of cold sonicated salmon sperm DNA were added to histones in high salts; samples were then diluted progressively to decrease salt concentration, and aliquots were finally loaded on 4% polyacrylamide gels. An efficient shift in DNA mobility was seen only when stoichiometric amounts of H2A-H2B and H3-H4 were added (not shown). This complex was stable for >1 week at 4 °C and represents nucleosomized DNA, as shown by DNase I footprinting experiments (see below). We first controlled whether any of the NF-Y subunits, or the NF-Y complex, was able to bind to nucleosomal DNA in the absence of a target CCAAT box. For this, we used fragment 2m (Fig. 3*A*) containing the same mutated CCAAT box described in Fig. 1*B*. As evidenced in Fig. 3*B*, neither the single NF-Y subunits nor the trimeric complex was able to associate with DNA, either nucleosome-bound (*lanes 1–8*) or free (*lanes 9–16*).

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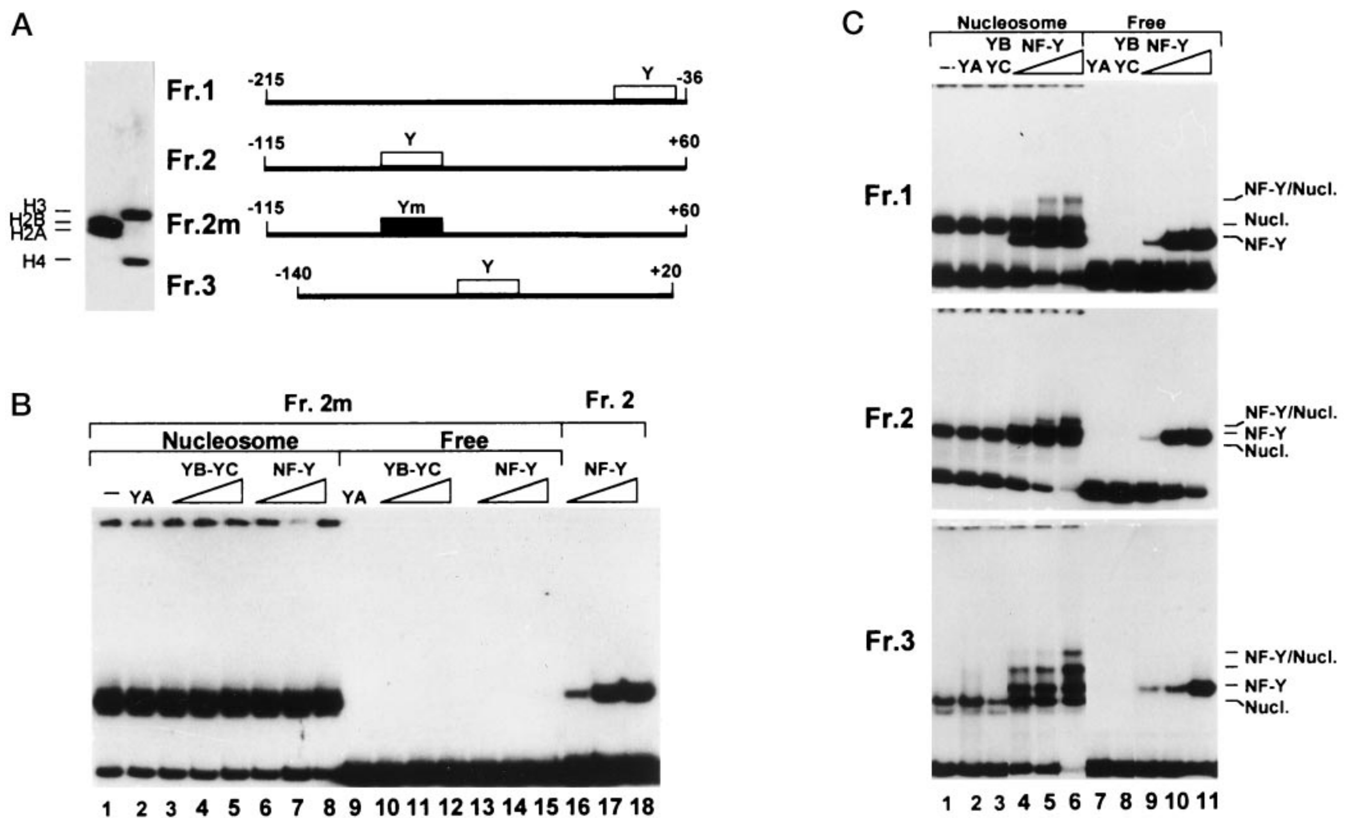

FIG. 3. **Electrophoretic mobility shift analysis of nucleosome reconstitution with purified core histones on Ea promoter sequences.** *A*, pure H2A-H2B and H3-H4 histones from chicken erythrocytes were run on a 17% SDS gel, stained with Coomassie Blue. The scheme depicts the Ea fragments used, showing the position of the Y box; *numbers* refer to the Ea +1 transcriptional initiation signal. *B*, fragment 2m contains the same mutation in the Y box as pE3m16 described in Fig. 1*B* (17). Fragment 2m, either nucleosomized (*lanes 1–8*) or naked (*lanes 9–15*), was incubated with 5 ng of NF-YA9 (*lanes 2* and *9*), increasing amounts of NF-YB4-NF-YC5 alone (0.2, 1, 5 ng, *lanes 3–5*, *10–12*), or with NF-YA9 (*lanes 6–8*, *13–15*). In *lanes 16–18*, the NF-Y trimer was incubated with naked wt fragment 2. *C*, nucleosome-bound (*lanes 1–8*) and naked (*lanes 9–16*) fragments 1 (*upper panel*), 2 (*middle panel*), and 3 (*lower panel*) were challenged with NF-YA, NF-YB-NF-YC, and increasing amounts of the trimeric NF-Y. DNA-protein complexes are indicated.

As an internal control for such an experiment, we used the corresponding naked wt fragment 2; as expected, this DNA was shifted by increasing amounts of the NF-Y trimer (*lanes 17–19*). We then turned to wt Ea fragments, employing templates that were only 30% nucleosome-bound, reasoning that free DNA in the reaction would help us compare NF-Y binding affinity for naked *versus* nucleosomal DNA. The dose responses of single subunits and of the trimer on fragments 1, 2, and 3 are shown in Fig. 3*C*. Upper bands of slower mobilities were apparent in all fragments only when the NF-Y trimer was added to nucleosomal DNA (Fig. 3*C*, *lanes 4–6*) but absent in the nucleosome complex (*lane 1*), when NF-YA or NF-YB-NF-YC was added (*lanes 2* and *3*), and, most notably, in samples with naked DNA (*lanes 7–11*).

Three aspects of these upper complexes should be considered: (i) their intensities increased as a function of trimer concentration; (ii) they appear already under conditions where free DNA is still available for NF-Y to bind (see *lanes 5*, for example); and (iii) as expected, the mobilities of the NF-Y complexes are different among the naked fragments because of NF-Y-induced DNA bending (24, 25). In fragment 3, where the CCAAT box is central, the NF-Y complex is slower than nucleosomal DNA, but it is faster in fragment 1 (Fig. 3*C*, compare *lanes 4–6* and *9–11*). Note that the upper complexes also show slightly dissimilar electrophoretic behaviors.

To define better the influence of translational positioning on the affinity of NF-Y, we decided to move progressively by 20 bp the position of the CCAAT box on fragments of identical length; in addition to fragment 1, we labeled and reconstituted the three additional fragments depicted in Fig. 4*A*. A parallel dose response of NF-Y indicates that nucleosomized fragments 4–6 are slightly less prone to bind NF-Y compared with the lateral fragment 1 (compare *lanes 1–4*, *28–32*). Moreover, the latter and the central fragment 6 showed a reduction in the intensities of the nucleosomal bands. These data suggest that, with the exception of fragment 1, in which the CCAAT box is eccentric and NF-Y binding can partially overlap with nucleosomes, the three more central positions are recognized with similar affinity.

Because of the putative histone-like structure of NF-YB-NF-YC, we wanted to investigate whether the upper complexes contain all NF-Y subunits. It is possible, in fact, that they might result from association of NF-YB-NF-YC with H3-H4, H2A-H2B, or both. Since it is known that only the trimer has CCAAT binding specificity (11), we performed competition experiments on the upper complexes; if the CCAAT-binding trimer is present, we expect it to dissociate from the nucleosomal DNA. In these experiments, we used fragment 2, 30% (Fig. 5, *lanes 1–6*) and 60% reconstituted (*lanes 7–12*). Note that in the latter case the nucleosomal band is shifted completely at the lowest dose of NF-Y, and the upper complexes are readily seen (Fig. 5, compare *lanes 2–4* with *7–9*). Incubation of cold short oligonucleotides containing the wt but not the mutant Y box after the addition of NF-Y to nucleosomes resulted in the disappearance of the upper complexes as well as of the NF-Y band (Fig. 5, *lanes 4–6* and *10–12*), while the nucleosomal bands increased in intensity (Fig. 5, compare *lanes 4* and *5*, *10* and *11*). Parallel competition on NF-Y alone on naked DNA abol-

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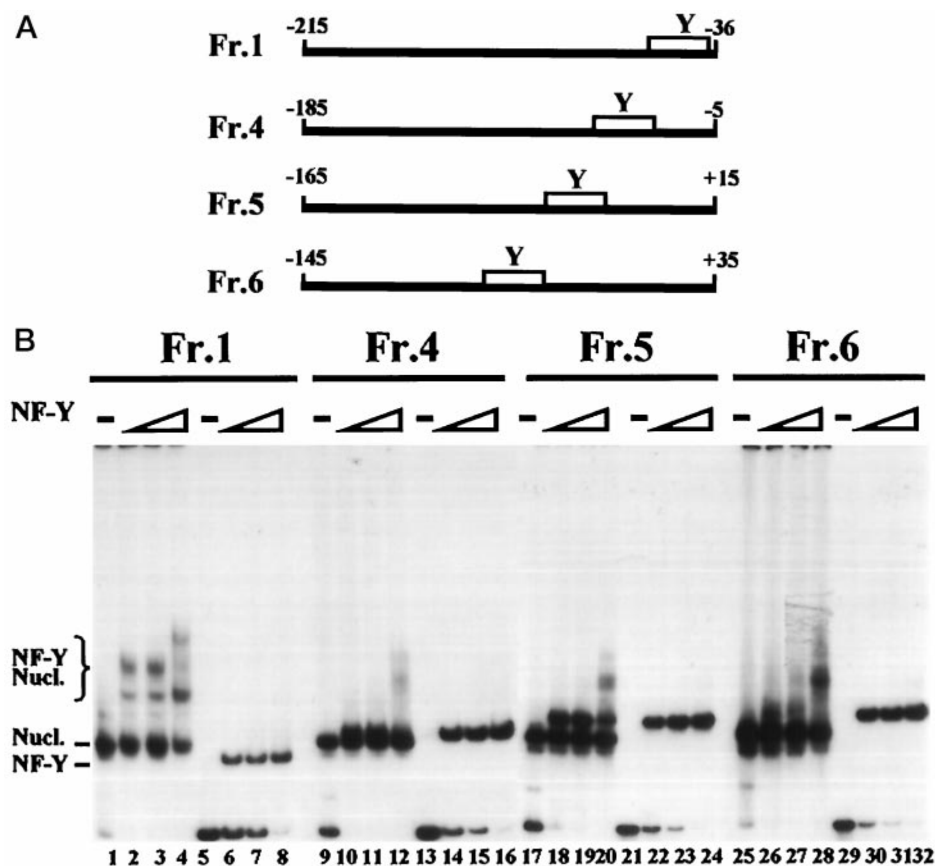

FIG. 4. **Binding of NF-Y to translationally moved Y boxes.** *A*, scheme of the DNA fragments of identical length used for the translational analysis. Fragment 1 is identical to the one described in Fig. 3. Fragments 4–6 are derived by polymerase chain reaction, designing oligonucleotides so that the CCAAT box is moved progressively toward the center of the fragment. *B*, nucleosomized fragments are in *lanes 1*, *9*, *17*, and *25*. Free DNAs are in *lanes 5*, *13*, *21*, and *29*. Dose response, 0.2, 0.5, and 1 ng, of NF-Y on nucleosomized DNAs (fragment 1, *lanes 2–4*; fragment 4, *lanes 10–12*; fragment 5, *lanes 18–20*; fragment 6, *lanes 26–28*) are compared with naked DNA (*lanes 6–8*, *14–16*, *22–24*, *30–32* for fragments 1 and 4–6, respectively). The nucleosome, NF-Y, and nucleosome/NF-Y bands are indicated.

ished, as expected, the NF-Y band (Fig. 5, *lanes 10–12*).

We further characterized the upper nucleosome-NF-Y complexes on fragment 3 by DNase I footprinting (Fig. 6). A clear footprint over the CCAAT box was seen when the NF-Y trimer was incubated with DNA (Fig. 6, compare *lanes 1* and *2*); when DNase was added after nucleosome reconstitution, protection of some sites and emergence of hypersensitive sites with a typical 10-bp period were observed (*lane 3*). The addition of NF-Y after reconstitution provoked no changes in the DNase cutting pattern outside the CCAAT box region but caused a clear decrease of the major hypersensitive site in the CCAAT box and disappearance of the two neighboring sites (Fig. 6, compare *lanes 3* and *4*). Overall, the pattern is consistent with the simultaneous presence on DNA of nucleosomes and NF-Y and suggests that NF-Y locally modifies histone-DNA interactions.

Altogether, these data prove that the NF-Y trimer can bind to nucleosomal DNA with some (positive) influence of the translational position of the CCAAT box in case the latter is lateral and overlapping the nucleosome(s) border.

NF-Y Interferes with Nucleosome Reconstitution—We felt that our *in vitro* nucleosome reconstitution system could help us answer an important question: what is the assembly efficiency of nucleosomes when reconstitution is performed in the presence of NF-Y? To address this point, we reconstituted nucleosomes by adding together core histones (Fig. 7, *lane 1*), increasing concentrations of the NF-Y trimer (*lanes 2–5*) or NF-YA, with core histones (*lanes 6* and *7*). The NF-Y-DNA complex is formed in the presence of H2A-H2B-H3-H4. Comparison with parallel mock reconstitutions of NF-Y and NF-YA in the absence of histones indicates that the efficiency of NF-Y-CCAAT binding is not reduced and indeed is increased slightly (Fig. 7, compare *lanes 2–5* with *8–11*). Note that at relatively high NF-Y concentrations, still insufficient to shift completely free DNA, the nucleosomal band was essentially not formed. We take these results as an indication that NF-Y can compete successfully with histones for binding to DNA.

DISCUSSION

Histones associate with DNA in a highly stable and compact way. The resulting structure, the nucleosome, is originated and stabilized by a number of strong protein-protein interactions between histones and by multiple ionic interactions with DNA (2, 3). This structure is generally inhibitory for the binding of transcriptional proteins to promoter sequences. Therefore, one key question in the physiological activation of gene expression is how gene-specific activators and the general transcription factors can reach their target sites when they are embedded in chromatin structures. Consequently, the relationships between different transcription factors and nucleosomes have been investigated in several *in vitro* studies.

The ability of sequence-specific transcriptional activators to associate with nucleosomal DNA is dependent upon several circumstances. The number of binding site(s), their translational and rotational positions, the presence of intact histone tails or their hyperacetylation, and the activity of remodeling proteins are all important factors. The two systems used in this study are to a large extent complementary: the *Artemia* extract

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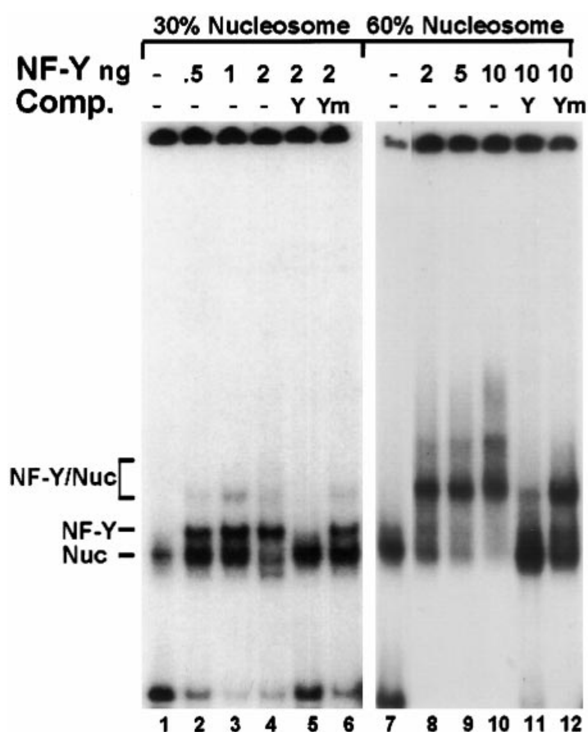

FIG. 5. **Competition of nucleosome-NF-Y complex with Y box oligonucleotides.** Increasing concentrations of NF-Y indicated above each *lane* were incubated with fragment 2 either 30% (*lanes 1–6*) or 60% nucleosomized (*lanes 7–12*). After formation of the nucleosome-NF-Y complexes, 50 ng of wt (*lanes 5* and *11*) or mutant (*lanes 6* and *12*) Y box oligonucleotides was added, and incubation proceeded for further 20 min.

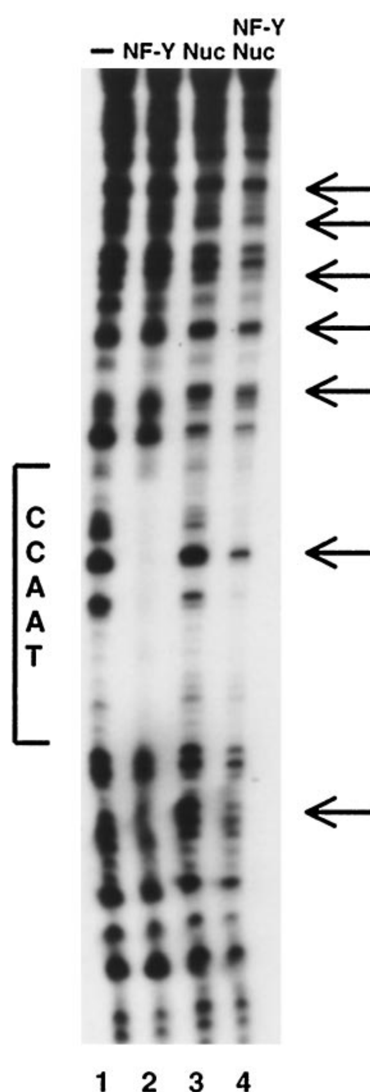

FIG. 6. **Footprinting of nucleosomes and NF-Y on the central fragment 3.** The figure shows DNase I footprinting of end-labeled fragment 3 alone (*lane 1*) after the addition of 5 ng of the NF-Y trimer (*lane 2*), after nucleosome reconstitution (*lane 3*), and after nucleosome reconstitution and NF-Y addition (*lane 4*).

is a dynamic situation requiring energy-consuming machines for proper spacing of nucleosomes (18), whereas reconstitution with purified histones on short DNA fragments rules out possible intervention of additional polypeptides and focuses on the fundamental chromatin unit. In chromatin reconstitutions, NF-Y clearly binds the Ea Y box; protection appears to be relatively limited, as a probe 100 bp downstream shows no sign of nucleosome displacement. Moreover, binding leads to incomplete inhibition of nucleosome formation, possibly because of non-mutually exclusive DNA binding. That this is the case is also suggested by the dose response, competition, and footprinting experiments with purified histones, indicating that NF-Y is able to form complexes with preformed nucleosomes. With the *Artemia* extract this could be influenced by chromatin-rearranging machineries, but this is certainly not the case with purified histones. In both systems NF-Y requires only a single CCAAT box to bind to nucleosomal DNA. The translational position appears to be important only in the case of the most lateral fragment, which shows a slightly higher affinity, probably because the nucleosome position only partially overlaps with the NF-Y binding site.

How does this capacity compare with other transcription factors? The affinity of a given specific DNA-binding activator for a nucleosome falls essentially into three classes. (i) The majority (TFIIIA, c-EBP, NF-1, MYC, HSF, CREM, NF-κB, and TBP) have little capacity on their own to associate a preformed nucleosome (26–33). (ii) Some activators interact efficiently under certain conditions but poorly in others; Gal4-AH and derivatives dramatically increase their affinity when multiple binding sites are present, when the histone amino termini are removed, when another transcription factor binds nearby, or when chromatin-remodeling proteins, such as the SWI-SNF complex, nucleoplasmin, and NAP1, are present (32, 34–37). Sp1 requires multiple sites for binding (38). Max alone can bind to intact nucleosomal DNA but with much lower affinity (2 orders of magnitude) with respect to naked DNA, whereas Myc, another member of the same family, has negligible affinity for nucleosomal DNA (29). (iii) The hormone receptors, glucocorticoid receptor and progesterone receptor, are able to associate nucleosomes efficiently (39–41). Glucocortoid receptor binding has been analyzed extensively with rotational and translational studies, and in general, it is only 2–3-fold reduced compared with naked DNA. Moreover, GR binding enhances nucleosome disruption by the SWI-SNF complex (42). A factor with a high propensity for nucleosome binding is *Drosophila* GAGA (43), whereas AP1 members are efficient in displacing preformed nucleosomes (44).

We conclude therefore that NF-Y belongs to the restricted class of transcription factors with a good capacity to bind their target sequences, both when free of chromatin structures and when covered by nucleosomes, without the help of nearby transcription factors or additional remodeling proteins. For this, the NF-Y trimer is required, and NF-YB-NF-YC dimers are unable to associate with preformed nucleosomes despite their histone-like domains. They do, however, have a small but re-

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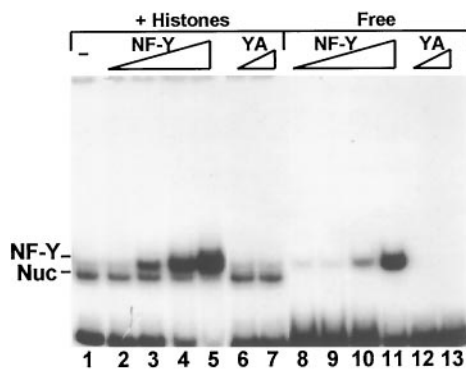

FIG. 7. **Competition between NF-Y and histones.** Fragment 2 was reconstituted together with equimolar amounts of core histones and increasing amounts of NF-Y (0.5, 2.5, 10, 50 ng, *lanes 2–5*) or NF-YA (5, 50 ng, *lanes 6* and *7*). *Lanes 8–13*, equivalent concentrations of NF-Y or NF-YA were reconstituted without core histones. About 10% of each reconstitution was loaded in each lane.

producible negative effect on chromatin reconstitutions which is seen in all areas of the template DNA and thus is largely independent of CCAAT boxes. What are the intrinsic structural features enabling NF-Y to bind nucleosomes? We propose that the built-in histone-like structures of NF-Y are essential for this function. Bending and phasing studies on the γ-globin double CCAAT boxes prove that the NF-Y-mediated distortions are remarkably similar to the ones observed with crystallographic studies on nucleosomes. DNA is bent by an angle of about 80° and twisted rotationally by about 100° (24, 25).

These results provide new and different data leading to the hypothesis that NF-Y, itself not possessing very strong transcriptional activation domains, plays a dominant role in determining promoter architecture. (i) Analysis on 503 promoters revealed a strong position bias at −60/−100 for NF-Y binding CCAAT sites (10); the distance between CCAAT and the neighboring transcription factor binding sites is conserved across species and among gene families. (ii) NF-Y has been shown to increase the affinity of structurally unrelated activators (RFX, SP1, SREBP) to their, sometimes poor, sites (45–48). (iii) Occupancy of NF-Y sites *in vivo* is an obligatory requirement for gene activation in all CCAAT-containing promoters tested so far. Interestingly, Landsberger and Wolffe (49) used a *Xenopus* oocyte chromatin assembly system to show that intact Y boxes of the HSP70 promoter serve multiple functions in transcriptional regulation: they facilitate basal, as well as heat shock factor-activated transcription, and they are required for disruption of repressive chromatin structures on the promoter, allowing protein interactions on the TATA box and facilitation of HSF binding (49). Although the DNA-binding protein responsible for this behavior has not been identified unambiguously, NF-Y seems by far the likeliest candidate based on the sequence of the CCAAT sites (10, 49). The data presented here on the Ea promoter are in full agreement with this scenario and represent the starting point for the mechanistic dissection of these phenomena.

Binding of H3-H4 tetramers to DNA represents the initial step in the formation of a nucleosome, followed by the ordered association of H2A-H2B (50, 51); H3-H4 interactions with DNA are less stable and more restricted than those of an intact nucleosome (52, 53). The reconstitution experiments performed with histones and NF-Y together indicate that NF-Y is able to compete effectively with nucleosome formation. This is particularly impressive considering that a large amount, 250-fold molar excess, of random sonicated salmon sperm carrier DNA is added in our reconstitution system. Based upon the expected frequency of CCAAT boxes (10), we calculated that in our experiments one cold NF-Y target site is present for every labeled molecule of Ea probe. Thus the relatively low amounts of NF-Y, as judged from incomplete binding of the free DNA, required to inhibit nucleosome formation are particularly remarkable. The mechanism by which this is accomplished is not clear. One straightforward possibility is that NF-Y binding precedes and prevent H3-H4 tetramer association with DNA and hence impedes subsequent nucleation of the octamer. Alternatively, it could be a stepwise phenomenon. NF-YB-NF-YC, having no DNA binding capacity on their own, could compete with H2A-H2B for binding to H3-H4 and prevent the assembly of a complete nucleosome; it should be kept in mind that the NF-YB-NF-YC dimer is stable at the high salt concentrations used in the initial steps of our reconstitution, whereas NF-YA might not be associated because it was shown to dissociate from the dimer at high salts. However, as salt concentrations are lowered, NF-YA would start to associate with NF-YB-NF-YC and the resulting trimer bind to the CCAAT box. For the second hypothesis to mimic a physiological situation *in vivo*, one has to assume that NF-YB-NF-YC dimers exist in the absence of NF-YA. Indeed evidence for the presence of such dimers has been recently obtained by biochemical means (54).

By the same line of reasoning, it is possible to hypothesize that the histone-like subunits of NF-Y might play structural roles other than associating NF-YA and binding to the CCAAT box. By stabilizing the H3-H4 tetramer and/or competing with H2A-H2B, they might locally prevent correct nucleosome formation on promoter regions and represent docking spots to which NF-YA subsequently associates, finally leading to sequence-specific binding to nearby CCAAT sequences. Interestingly, a recent report showed that NF-Y is associated in complexes with histone acetylase activity and that the NF-YB-NF-YC dimer binds P/CAF and hGCN5 (55). Thus the reductionistic approach taken here might underscore the importance of NF-Y in histone modifications. Although the present study proves the remarkable NF-Y facility in penetrating chromatin structures, higher order NF-Y-containing complexes with histone acetylase activity might modify the histone tails of neighboring nucleosomes, thus further facilitating access of additional transcriptional activators.

In summary, the peculiar NF-Y structure makes it an ideal candidate for a pivotal role in associating promoters embedded in chromatin, promoting the binding of other transcription factors to neighboring activating sequences, and the formation of stable multiprotein transcriptionally competent promoter complexes. Understanding the fine details of the NF-Y-nucleosome interactions will have to await structural studies on the intimate relationship among these conserved proteins.

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Interactions of the CCAAT-binding Trimer NF-Y with Nucleosomes

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