Crystal structure of the NF-YB/NF-YC dimer

The NF-YB/NF-YC structure gives insight into DNA binding
and transcription regulation by CCAAT factor NF-Y

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Summary

The heterotrimeric transcription factor NF-Y recognizes with high specificity and affinity the CCAAT regulatory element which is widely represented in promoters and enhancer regions. The CCAAT box acts in concert with neighbouring elements and its bending by NF-Y is thought to be a major mechanism required for transcription activation. We have solved the structure of the NF-YC/NF-YB sub-complex of NF-Y, which shows that the core domains of both proteins interact through histone fold motifs. This histone-like pair is closely related to the H2A/H2B and NC2α/NC2β families, with features that are both common to this class of proteins and unique to NF-Y. The structure, together with the modelling of the non-specific interaction of NF-YC/NF-YB with DNA and the full NF-Y/CCAAT box complex, highlight important structural features which account for different and possibly similar biological functions of the transcriptional regulators NF-Y and NC2. Especially, it emphasizes the role of the newly described αC helix of NF-YC, which is both important for NF-Y trimerization and a target for regulatory proteins, such as MYC and p53.
Introduction

Transcription initiation by RNA polymerase II at class II gene promoters is a finely regulated process requiring the interplay of many different transcription factors\(^1\). General transcription factors (GTFs), namely TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIH, recognize specifically the core promoters, recruit the RNA polymerase and help melting the DNA, thus enabling the initiation of transcription at the correct start site\(^2\). Assembly of this preinitiation complex is controlled by a large set of transcriptional activators and repressors which recognize, in a sequence specific way, DNA sequences located on proximal or distal enhancer regions of the promoters, and function by contacting either directly or indirectly, through co-activators and co-repressors, the GTFs\(^1\).

The eukaryotic transcription factor NF-Y (also termed CBF) specifically recognizes the regulatory CCAAT element found in either orientation in the proximal and distal enhancer regions of many genes\(^3\text{--}^4\). In higher eukaryotes, this element is found in about 30\% of the promoters, preferentially in the -60/-100 region, and analysis of various CCAAT boxes showed that specific flanking sequences are required for efficient binding\(^5\text{--}^7\). NF-Y is a heterotrimeric complex composed of NF-YA, NF-YB and NF-YC which are all required for CCAAT binding\(^8\). Each subunit contains a core region which has been highly conserved throughout evolution and which is sufficient for subunit interactions and CCAAT-binding, while the flanking regions, which include the activation domains, are much less conserved\(^8\text{--}^{13}\). In yeast, the activation function is encoded by a fourth subunit with no apparent homologues in other species\(^14\).

NF-YC and NF-YB core regions are homologous in sequence to histones H2A and...
H2B, respectively, and are required for heterodimerization, a prerequisite for NF-YA association and CCAAT binding. NF-YC and NF-YB show an even higher sequence similarity with subunits $\alpha$ and $\beta$ of NC2, a protein that represses TATA-box dependent transcription while increasing the activity of the Distal Promoter Element, DPE. The recent structure of a NC2/TBP/TATA element complex confirmed that NC2 $\alpha$ and $\beta$ subunits interact through histone fold motifs and that NC2 recognizes the preformed TBP/TATA complex.

The NF-YA core domain is less than 60 amino acids long and is sufficient for DNA binding when complexed with NF-YC/NF-YB. Contrary to NF-YC and NF-YB, careful examination of available databases failed to reveal homologues of NF-YA. Several studies have divided the NF-YA core domain into two segments: a N-terminal domain responsible for NF-YC/NF-YB binding, and a C-terminal domain implicated in specific recognition of the CCAAT element.

Once the trimeric complex is formed, it binds DNA with very high specificity and affinity. Specific recognition of the bases seems to involve both minor and major groove interactions, and circular permutation assays indicated that, upon binding, the DNA is bent by about 60 to 80 degrees. Footprinting and photo-crosslinking experiments have shown that the DNA is contacted by a subset of the three subunits at three different locations, spanning about 24-26 nucleotides on each strand. In agreement with these results, it was shown that two CCAAT boxes cannot be occupied simultaneously, unless they are separated by at least 22-24 base pairs.

A major role of NF-Y is to act synergistically with other transcription factors for
activation. The CCAAT box is generally found in the close vicinity of other promoter elements, and in many cases a precise distance is required for proper transcription. Evidence that this process requires CCAAT box bending and/or direct protein/protein interactions has been repeatedly reported. Several lines of evidence also indicate that NF-Y interacts directly with GTFs, especially TFIID. Additionally, NF-Y has been shown to be the target of regulatory proteins such as c-Myc and p53 (Imbriano et al., manuscript in preparation).

We have started the structural characterization of transcription factor NF-Y and have solved the structure of the complex between the conserved regions of human NF-YB and NF-YC by X-ray crystallography. The structure was refined at 1.6 Å resolution and shows that both proteins interact through histone fold motifs in a head-to-tail fashion. The structure is very close to that of H2A/H2B and especially of NC2α/NC2β, but changes at the sequence and secondary structure level provide explanations for various functional roles played by these different complexes. Based on this overall structural homology, which extends up to the electrostatic properties, the interaction between the NF-YC/NF-YB dimer and DNA was modelled, and further extended to the full NF-Y/CCAAT element complex, in agreement with several biochemical studies performed on NF-Y, including footprinting experiments and mutational analyses. EMSA experiments were also carried out which emphasized the importance of the NF-YC/NF-YB histone-like pair in DNA binding and bending. Finally, the structure reveals an important element of secondary structure, the αC helix of NF-YC, which is not only involved in NF-YA binding, but plays also a role in the regulatory pathway of important growth regulators such as MYC and p53.

**Experimental procedures**
Cloning, expression and purification

The various constructs used for the co-expression study were amplified by standard PCR procedures. All NF-YB constructs were inserted in the pACYC184-11b vector, whereas all NF-YC constructs were inserted in the pET15b (Novagen) and pGEX4T-2 (Amersham Pharmacia) vectors, using NdeI and BamHI restriction sites. Co-expression tests were carried out using a standard procedure described previously. For large scale expression, 6 x 1 liter cultures were grown, either in 2xLB medium for native complexes, or in M9 medium supplemented with Seleno-methione (Sigma) for seleno-methionylated complexes. Cells were grown at 37°C to an absorbance of 0.3 at 600 nm and the temperature was then switched to 25°C. Growth was then carried on until cells reached an absorbance of 0.8-1.0 at 600 nm. At this point, co-expression of the complex was induced by adding a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (Euromedex) and cells were further grown overnight at 25°C. Cells were then collected by low speed centrifugation, resuspended in buffer A (10 mM Tris pH 8.0; 400 mM NaCl) and lysed by sonication. The soluble fraction recovered by high-speed centrifugation was mixed with 1 ml Talon resin (Clontech) in the case of a his-tagged complex or 1 ml Gluthatione sepharose 4B resin (Amersham Pharmacia) in the case of a GST-tagged complex. After 1 hour incubation, the supernatant was removed and the resin washed extensively with buffer A. The resin was then resuspended in 2 ml of buffer A and 5 U of bovine thrombin (Sigma) were added overnight at 4°C, for cleaving off the tag. The supernatant containing the soluble dimer was recovered and applied onto a gel filtration column Hiload 16/60 Superdex 75 (Amersham Pharmacia) equilibrated with buffer B (Buffer A + 2 mM 1.4-dithiothreitol, Boehringer Mannheim). The purified complexes were concentrated on Microsep 10K Omega (Pall Filtron) to a final concentration of around 10-14 mg/ml as assayed with Bio-Rad protein assay (BioRad).
Crystal structure of the NF-YB/NF-YC dimer

**Crystallisation**

For crystallisation of the NF-YC3/NF-YB3 complex, 2 µl of protein complex solution were mixed with an equal volume of the reservoir solution containing 0.1 M NaHepes (Sigma) pH 7.5, 0.2 M Mg(OAc)₂ (Merck) and 10 to 14% PEG 4000 (Fluka). Crystals appeared after a few hours and continued to grow for a few days or weeks to reach a size of approximatively 0.5x0.1x0.1 mm³. For the NF-YC2/NF-YB3 complex, the percentage of PEG 4000 had to be raised to 20-24% to obtain crystals that were smaller and more clustered than for the NF-YC3/NF-YB3 complex. Only the latter dimer was used for solving the phase problem with seleno-methionylated proteins. For data collection, crystals were briefly transferred in a cryoprotectant solution of 0.05 M NaHepes, 0.1 M Mg(OAc)₂, 0.2 M NaCl, 13 or 22% PEG 4000, 20% glycerol, and then quickly frozen in liquid ethane.

**Structure determination**

Data collection on native and seleno-methionylated crystals was carried out on beamline BM30A at the European Synchrotron Radiation Facility. A three wavelengths MAD experiment with collection of data up to 1.8 Å resolution was first carried out, and native data sets for the NF-YC3/NF-YB3 and NF-YC2/NF-YB3 complexes were then collected at 1.57 and 1.67 Å resolution, respectively. All data were processed and scaled using Denzo/Scalepack⁴⁵. Location of 5 of the 6 selenium atoms was done using Shake and Bake⁴⁶. Their positions were refined within the phasing program SHARP³⁷ and the phases further improved with the solvent flattening program SOLOMON³⁸.

**Model building, refinement and modelling**

Model building was carried out using program TURBO-FRODO⁴⁹. The model built
in the initial 1.9 Å resolution MAD electron density map was further refined independently against both native data sets by several cycles of manual building and refinement using standard protocols within CNS. B-factor restraints for bonded main chain and side chain atoms were 1.5 and 2.0, respectively. B-factor restraints for angle main chain and side chain were 2.0 and 2.5, respectively. The coordinates of the NF-YC2/NF-YB3 complex have been deposited in the PDB databank under PDB # 1N1J.

Superimposition of NF-YC/NF-YB, H2A/H2B, H3/H4 and NC2α/NC2β complexes was carried out using poly-glycine models with our in-house program Superpose (B. Rees, unpublished data), and the transformations applied onto the full models of the nucleosome core particle (PDB # 1AOI) and of the NC2/TBP/TATA element complex (PDB # 1JFI). The r.m.s. differences were obtained from the superimposition of the poly-glycine model, removing additional residues but also helix α1 loop L1 in the case of superimpositions with H2A and H3, since these elements have clearly a different trajectory with respect to those of NF-YC or NC2α.

Modelling of the NF-YC/NF-YB/DNA complex was made by extracting a DNA fragment from the structure of the nucleosome core particle once superimposed as described above. Replacement of the bases and the modelling of the interaction between NF-YA α-helices and the NF-YC/NF-YB/DNA complex was carried out manually in TURBO-FRODO. The coordinates of the model are available upon request.

**Production of NF-YC mutants and EMSA experiments.**

NF-YC mutants were produced by PCR mutagenesis with the appropriate oligonucleotides in the backbone of the YC5 mutant. The recombinant His-tagged YC5 mutants were obtained in inclusion bodies from BL21 bacteria, renatured with equimolar
amounts of NF-YB and purified over NTA-columns\textsuperscript{29,42}. The resulting dimers were assayed in immunoprecipitations and EMSA experiments with recombinant NF-YA and the Mab7 monoclonal antibody\textsuperscript{42}. Production and purifications of NF-Y and off rates EMSA experiments were done under conditions described previously\textsuperscript{29,43}.

**Results**

**Structure determination of the NF-YC/NF-YB complex**

All three subunits of NF-Y contain a core region which has been highly conserved throughout evolution and, in the case of NF-YC and NF-YB, which displays sequence homology to the histone fold motifs of H2A/NC2\(\alpha\) and H2B/NC2\(\beta\), respectively (Figure 1). The core domains of NF-YB and NF-YC have been shown to be necessary and sufficient for DNA binding in the context of the trimeric complex\textsuperscript{15-16,20,41}. However, less conserved stretches at their N- and C-termini seem to influence this process\textsuperscript{29}. The majority of histone fold proteins are produced in bacteria as insoluble material in inclusion bodies. We have studied the formation of the NF-YC/NF-YB pair with protein constructs of different lengths, by testing protein solubilisation using the technique of co-expression in *Escherichia coli*\textsuperscript{34}. The results summarized in Table 1 indicate that only the evolutionary conserved domains of NF-YC and NF-YB, but not the less conserved regions, are necessary for complex formation.

For the subsequent crystallisation trials, four of the six soluble complexes obtained were used: NF-YC2/NF-YB3, NF-YC2/NF-YB4, NF-YC3/NF-YB3 and NF-YC3/NF-YB4 (see Table 1). Small crystals were initially obtained with the NF-YC3/NF-YB3 pair. Further refinement of the crystallisation conditions showed that crystals of NF-YC2/NF-YB3...
could also be obtained. Both crystals belong to the same space group with the same cell parameters (Table 2). Crystals of the seleno-methionylated NF-\textsubscript{YC}3/NF-\textsubscript{YB}3 complex were also grown and used for solving the phase problem by Multiwavelength Anomalous Diffraction (MAD)\textsuperscript{44}. An initial model was built manually into the experimental electron density map at 1.9 Å resolution and was further refined independently against native NF-\textsubscript{YC}3/NF-\textsubscript{YB}3 and NF-\textsubscript{YC}2/NF-\textsubscript{YB}3 data sets at 1.57 and 1.67 Å resolution, respectively. The final models include 87 residues of NF-\textsubscript{YB}, 78 residues of NF-\textsubscript{YC}, about 300 water molecules and have R-factors around 18\% and R-free factors around 20\%, with very good deviations from ideal geometry (Table 2).

In NF-\textsubscript{YB}3, no density was observed for the first seven residues. Mass spectrometry revealed that all these residues except for the initial methionine were present in the protein used for crystallisation and also in the crystals (data not shown). Thus, the N-terminal residues of NF-\textsubscript{YB}3, which point towards a solvent channel, are probably disordered. In NF-\textsubscript{YC}3, only the residual thrombin site residues Gly-Ser at the N-terminus were not unambiguously found in density. In the case of the NF-\textsubscript{YC}2 construct, which is 16 residues longer than NF-\textsubscript{YC}3, no additional residues could be built at the N-terminus either. Once again, mass spectrometry revealed that all the unobserved residues are present in the crystals (data not shown). Since the initial experimental phases were obtained for the NF-\textsubscript{YC}3/NF-\textsubscript{YB}3 complex, it could be assumed that the initial model was not good enough to provide phases for these residues. However, several loops in other parts of the structure, which could not be seen in the initial electron density map, appeared during refinement, whereas density at the N-terminus of NF-\textsubscript{YC}2 never improved. A large solvent channel being found where the residues should be located, it seems reasonable to assume that these residues are disordered.

\textbf{NF-\textsubscript{YC}/NF-\textsubscript{YB} forms a histone-like pair}
Crystal structure of the NF-YB/NF-YC dimer

As expected, the core domains of NF-YB and NF-YC adopt a histone-like fold and interact in a head-to-tail fashion, forming a histone-like pair (Figure 2A). Interestingly, comparison of the NF-YC/NF-YB, NC2α/NC2β, H2A/H2B, but also H3/H4 histone pairs reveals relatively little differences between their core histone motifs (helix α1-loop L1-helix α2-loop L2-helix α3; see Figure 2A) both in terms of sequence identity (ranging from 10 to 20%) or pairwise main chain r.m.s. differences (ranging from 1.5 to 1.1). Actually, that NF-YC/NF-YB belongs to the H2A/H2B family is confirmed by the presence of additional elements of secondary structure, at the C-termini of both proteins, characteristic of H2A and H2B, although H3/H4 features are also observed (see below). The interactions between the various elements of secondary structure of NC2α/NC2β and the comparison of this pair with the H2A/H2B dimer have already been described at length19. The conclusions mostly apply to NF-YC/NF-YB and will not be discussed further. Rather, we will focus on the differences and the specificities we observe.

One feature concerns the presence in both NF-YB and NF-YC of an intra-chain arginine-aspartate bidentate pair which is found in histones H3 and H4, but not in H2A and H2B45 (Figure 2A). In NF-YC this pair is formed by residues Arg93 (loop L2) and Asp100 (helix α3) and both are absolutely conserved in the NF-YC, but neither in H2A nor in NC2α families (Figure 1C). In NF-YB, residues Arg108 (loop L2) and Asp115 (helix α3) form an identical pair and are also absolutely conserved throughout evolution. Once again, this pair is not conserved in NC2 and is replaced by an arginine/lysine-glutamate pair in H2B (Figure 1B). In this latter case, however, the pair is not formed and the arginine contacts the DNA: interestingly, this is not seen in H3 and H4, where the pairs are formed even if the arginines are in the vicinity of the DNA backbone45.
Another specific feature is the presence in NF-YC of an absolutely conserved tryptophan at position 85, at the end of helix \( \alpha_2 \), sandwiched between loop L2 of NF-YC and loop L1 of NF-YB (Figure 2). Such a bulky residue at this position clearly influences the overall structure of this region. Interestingly, this amino acid is not conserved in NC2\( \alpha \) and the conformation of L1 of NC2\( \beta \) is different from that of NF-YB. Since L2 of NC2\( \alpha \) was not seen, it is impossible to compare it to that of NF-YC. The difference in L1 loop conformation is certainly dependent on their lengths: that of NC2\( \beta \) is in general one residue shorter, although in yeast it has the same length. The hydrophobic cores organising these regions are rather different, with little differences observed in the rest of the structures. These cores may be the reason why NF-YB/NC2\( \alpha \) and NF-YC/NC2\( \beta \) pairs cannot be formed\(^{42}\). Notably, when both structures are superimposed, it is clear that steric clashes would occur between residues of NC2\( \beta \) L1 and Trp85 which are not likely to be accommodated by conformational changes.

Characteristic of the H2B family, a long \( \alpha_C \) helix is found in NF-YB (Figure 2). This helix is shorter than those of H2B and NC2\( \beta \), but since additional residues at its C-terminus seem to prevent crystallization, it is possible that it extends further. In NF-YC, a loop-short helix-loop motif is found C-terminal to the core histone fold (Figures 2 and 3). A short \( \alpha_C \) helix is also found in H2A, but is positioned rather differently: it packs against the C-terminus of helix \( \alpha_3 \) of H2A on one side, and loop LC/start of helix \( \alpha_C \) of H2B on the other side, making little other interactions with the rest of the dimer (Figure 2C). The packing is totally different in the case of NF-YC, where the \( \alpha_C \) folds back onto \( \alpha_3 \) and participates in a large hydrophobic core formed by residues of \( \alpha_2 \) and \( \alpha_3 \) of NF-YC and \( \alpha_2 \) of NF-YB. The interactions between loop LC/helix \( \alpha_C \) of NF-YC and loop LC/helix \( \alpha_C \) of NF-YB are fewer than in H2A/H2B, where loop LC/start of helix \( \alpha_C \) of H2B are closer and interacts
strongly with helix $\alpha C$ of H2A (Figure 2C), especially with an absolutely conserved glutamate of H2A being fixed by the dipole effect of helix $\alpha C$ of H2B. Interestingly, the differences between these short $\alpha C$ helices extend further, as in the case of NF-YC this region does not fold as an $\alpha$-helix, but a 310-helix (however, for clarity, the term $\alpha C$ has been kept). For technical reasons, the sequence spanning this region in NC2$\alpha$ was replaced by unrelated residues and this chimera was subsequently used during crystallization studies of the NC2/TBP/TATA element complex. Based upon the strong sequence homology between NF-YC and NC2$\alpha$ in the $\alpha C$ region (residues 109 to 114) and in the rest of the secondary structure elements participating in the hydrophobic core stabilizing it, we anticipate that a helix is also present in NC2$\alpha$ at the corresponding position. Whether additional residues at the C-terminus of this helix adopt the same loop conformation seen in NF-YC is not clear, as NF-YC and NC2$\alpha$ sequences tend to diverge from this point.

**Minimal DNA fragment required for proper binding by NF-Y**

Previous footprinting experiments have shown that three regions of the CCAAT boxes of the pro-$\alpha2(1)$ and pro-$\alpha1(1)$ collagen promoters are protected upon NF-Y binding. We have performed EMSA experiments on the Ea promoter to assess the minimal DNA fragment required for proper NF-Y binding. The results of dose-response and off-rate experiments performed with full length as well as with a mutant containing the conserved domains of the three subunits are summarized in Table 3. Essentially, all the protected DNA stretches are important for proper recognition by NF-Y. Partial removal of one of these regions generally leads to a decrease in binding (oligonucleotides Ea-6, 12-Ea and 8-Ea-2). The effect observed is rather weak when considering the region the farthest from the CCAAT pentanucleotide (see oligonucleotides Ea-4, Ea-6 and 8-Ea-2), but deletion of this site...
results in an almost complete loss of binding (oligonucleotide Ea-10). Besides, the comparison between oligonucleotides of identical length (Ea-10 and 8-Ea-2) clearly shows the asymmetry in position of the CCAAT pentanucleotide on the minimal DNA fragment required for NF-Y binding.

Mutational analysis on NF-YC αC residues

NF-YC helix αC has been shown to be important for NF-YA binding. We have mutated certain residues of this helix (Figure 3 and Table 4), either solvent exposed or buried in hydrophobic cores, to further characterize its role in dimer and trimer formation and DNA binding, using EMSA experiments. None of the mutations affected dimerization, showing that the helix does not play an important role in the interaction between NF-YB and NF-YC. The mutation of the solvent exposed aspartate 112 into asparagine leads to a decrease in trimerization but not in DNA binding (Table 4), showing that this residue most probably plays a role in NF-YA binding, but that in the presence of DNA the trimeric interaction is stabilized. Two mutations, F111S and L114T, were supposed to destabilize the hydrophobic core in which the αC helix participates. From their positions, the F111S mutation should destabilize the overall hydrophobic core, whereas the L114T mutation should weaken the anchoring of helix αC to the rest of the dimer. Both F111S and L114T are indeed highly reduced in association with NF-YA. However, as for the D112N mutant, DNA binding was not affected, confirming that in presence of DNA the interaction with NF-YA is stabilized. Two more radical mutations were performed on solvent exposed isoleucine residues, I115P and I117P, which are outside helix αC. Interestingly, both mutations prevent trimerization but also DNA binding (Table 4). On the other hand, we mutated isoleucine 115 into a lysine, the only residue of human NC2α that is deviant within this conserved stretch. Contrary to the I115P mutant, the I115K behaves like wild-type NF-YC in dimerization, trimerization and
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DNA-binding assays (Table 4), showing that the conformation of the loop following helix $\alpha_C$ is also important for NF-YA binding.

Discussion

DNA binding by NF-YC/NF-YB

The structure described here confirms that the NF-YC/NF-YB histone pair is structurally closely related to the H2A/H2B and NC2$\alpha$/NC2$\beta$ dimers, and suggests that DNA binding by NF-YC/NF-YB might also be similar. Both H2A/H2B and NC2$\alpha$/NC2$\beta$ interact directly and non-specifically with DNA in a multiprotein context, within the histone octamer and with TBP, respectively. In these complexes, few direct protein/DNA contacts are made by the core histones motifs (Figure 1). A stable interaction between these histone pairs and the DNA seems to require other protein stretches, e.g. the histone tails and helix $\alpha_5$ of NC2$\beta$. Such additional regions do not seem to exist in NF-YB and NF-YC, and it is clear that to obtain the remarkable specificity and affinity for the CCAAT sequence, NF-YA must stabilize the complex, although photo-crosslinking experiments have confirmed that all three subunits of NF-Y interact with DNA directly$^{28}$.

The DNA fragments recognised by H2A/H2B (within the nucleosome core particle) and NC2$\alpha$/NC2$\beta$ (in complex with TBP and the TATA element) have rather different conformations (in the latter case the DNA is strongly distorted upon TBP binding). However, both complexes display similar DNA binding properties$^{19}$, and the trajectory of these fragments on the surface of these complexes is extremely similar (Figure 4A). The electrostatic properties of NF-YC/NF-YB are almost identical to those of H2A/H2B and NC2$\alpha$/NC2$\beta$; thus, it is tempting to postulate that the CCAAT box would also follow an identical trajectory onto NF-YC/NF-YB (Figure 4B).

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Such an interaction was modelled by superimposing the NF-YC/NF-YB structure onto a H2A/H2B dimer from the nucleosome core particle (see Experimental procedures). Upon modelling, no steric clashes between the NF-YC/NF-YB dimer and the DNA are observed (Figure 4C). As in the nucleosome structure, both the DNA interactions sites L1L2 (formed by loops L1 and L2 at both extremities of the dimer) and α1α1 (formed by both α1-helices) are able to make contacts with the DNA, and the dipoles of helices α1 and α2 also point toward phosphate groups. On the other hand, the two arginines of H2A and H2B penetrating into the minor groove have not been conserved. The same observation is true for Lys/Arg29 of NC2α, the only residue contacting directly a base of the TATA element, which is replaced by an absolutely conserved methionine in NF-YC. In fact, careful inspection of the model could not identify any residue from the core domains of NF-YB and NF-YC which would be able to make specific contacts with a base of the CCAAT box. Besides, in this model the histone dimer spans about 24 to 26 base pairs, which is in excellent agreement with biochemical data.

The interaction between NF-Y and the CCAAT box was further modelled by replacing the DNA bases found in the nucleosome structure by those of the pro-α2(1) collagen promoter. Two locations for the CCAAT box are possible (depending on the strand chosen as the plus strand) which are related by the pseudo two-fold axis of the histone dimer (Figure 4C). One of them agrees better with NF-YA binding (see below). This model is in good agreement with the footprinting and methylation interference experiments made on several promoters. Especially, the L1L2 sites would be responsible for interacting with the protected sites at both extremities of the footprinted region and are actually sufficient to explain the protection by hydroxyl radical cleavage at these sites. Since these protein regions
are not supposed to make specific contacts with the DNA, it would also explain why no interference by methylation has ever been observed at these locations. As for the α1α1 site, it only partially accounts for the central region footprint: specifically, the CCAAT element itself, the only region of the DNA where methylation interference occurs\textsuperscript{6,26}, is not protected by any region of the dimer (Figure 4C). This clearly suggests that this protection is brought by the third subunit, NF-YA.

The model further provides a good explanation for the results of our EMSA experiments. Indeed, both strands are contacted by the dimer at all the protected sites. Partial removal, on one strand only, of one of the external sites, would lead to smaller effects in terms of binding, as is seen experimentally (Table 3). On the other hand, complete removal of one of these sites on both strands should have a much drastic effect, which is the case when considering oligonucleotide Ea-10. It is interesting to note that in the case of NC2 where the histone pair recognizes a preformed TBP/TATA element complex, the requirement for three interaction sites seems to be less stringent\textsuperscript{19}.

**Recognition of NF-YA and CCAAT box binding by NF-Y**

Many biochemical studies have attempted to decipher the set of interactions between yeast and mammalian NF-YA, NF-YC/NF-YB and the CCAAT box\textsuperscript{15-16,20-24,29,42}. Most of these studies were mutational analyses, performed either by point or deletion mutations. Recollection of all these data in the light of our structure and of the proposed model reveals that most of the mutants described can affect dimer and/or dimer/CCAAT interaction by (i) interfering with the packing of the NF-YC/NF-YB dimer, (ii) destroying the dipole effect of α-helices α1 and α2 supposed to fix phosphate groups, and (iii) abolishing interactions, or causing steric or electrostatic hindrance, between the histone dimer...
and the DNA. Essentially, the vast majority of the mutations, particularly those falling in the last two classes, favour the NF-YC/NF-YB/CCAAT element model. The remaining mutations that do not fall into these three classes have been further considered to model the interaction of NF-YA with both the histone dimer and the DNA.

Two regions of the core domain of NF-YA have been identified: a N-terminal region (NF-YA1; residues 234 - 257) recognizing the NF-YC/NF-YB dimer, and a C-terminal region (NF-YA2; residues 269 - 289) responsible for specific recognition of the DNA20-24 (Figure 1A). The N-terminus of NF-YA1 forms an \( \alpha \)-helix in solution and only residues on one side of this helix are functionally important\(^{23}\). In NF-YB, mutations on helix \( \alpha 2 \) (E90R and S97R) were shown to influence NF-YA binding\(^{16,22}\). In NF-YC, both helices \( \alpha 1 \) and \( \alpha C \) have been shown to be important for NF-YA binding\(^{15,42}\) (our mutational analyses). These three elements of secondary structure are on one side of the NF-YC/NF-YB dimer and form a groove where NF-YA1 N-terminal \( \alpha \)-helix could bind (Figure 4D). Such an interaction was modelled, showing that functionally important residues of NF-YA1, such as Arg245 and Arg249, could contact residues at the surface of the dimer, including Glu90 and Ser97. Interestingly, in this model the conserved NF-YA Ile246 would pack against Ile117, which is solvent exposed in the loop following NF-YC helix \( \alpha C \). We have shown that a mutation of the latter isoleucine into proline completely abolishes NF-YA binding (Table 4), a result that further highlight the importance of NF-YC C-terminal region in trimer formation. Intriguingly, the model does not provide an explanation to the fact that the D112N mutant is impaired in NF-YA recognition (Table 4), showing that NF-YA1 binding possibly requires other interactions than the ones mentioned above.

From secondary structure prediction analysis, the NF-YA2 domain can be divided

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into an α-helical N-terminal region (residues 269–281) and a small coiled C-terminal region (residues 282–289), as for NF-YA1 (data not shown). Several mutational experiments indicate that helix α1 of NF-YB influences DNA recognition by NF-YA2 and suggest that these two regions interact directly, with NF-YB helix α1 possibly positioning NF-YA2 α-helix in a correct orientation. We have already mentioned that modelling of the CCAAT box left two possible locations for this DNA sequence. Interestingly, NF-YB helix α1 is positioned exactly underneath one of these two locations, on its major groove side.

Modelling of the interaction between the helical part of NF-YA2 with both NF-YB helix α1 and the CCAAT pentanucleotide was rendered difficult by the fact that essential residues of NF-YA2 might be involved either in specific base recognition, in phosphate backbone recognition, or might interact with NF-YB. Besides, one cannot exclude that NF-YA binding distorts DNA into a conformation that would be preferred for proper recognition by NF-YC/NF-YB, a fact which could not be accounted for by our model. However, as it stands, the model could fully explain the footprinting pattern observed for NF-Y on the collagen promoter and our EMSA experiments.

Still, several questions remain. First, the orientation of the NF-YA2 helix is not known since the linker connecting NF-YA1 to NF-YA2 could possibly either go through the space left between the NF-YC/NF-YB dimer and the DNA, or cross over the DNA (Figure 4D). Second, NF-Y was shown to bind into the minor groove, a fact that could possibly be explained by having the NF-YA linker region crossing over the DNA, although it cannot be excluded that the supposed coiled C-terminal region of NF-YA2 could also play such a role. Third, the flanking regions of the CCAAT pentanucleotide are crucial for efficient binding of NF-Y. It is not known whether these bases can be recognized specifically by NF-Y, or are necessary for proper distorsion of the DNA, or both. Finally, another unresolved
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question concerns NF-YA and NF-YC having been shown by photo-crosslinking studies to interact more extensively with CCAAT elements than the model would account for\(^28\). We suspect that this might be due to the interaction between the activation domains of these two proteins and the DNA. Such open questions, and possibly others, will only be answered with the determination of the structure of the quaternary complex.

**Structural and functional differences with H2A/H2B and NC2\(\alpha\)/NC2\(\beta\)**

A lot of controversy arises from the fact that the NF-YC/NF-YB, H2A/H2B and NC2\(\alpha\)/NC2\(\beta\) histone pairs share sequence and structural similarity, but have different functional roles inside the nucleus. This is clearly reinforced by the fact that their DNA binding characteristics are strongly conserved, and that the NF-YC/NF-YB pair has been shown to interact with protein partners of the two other dimers. Indeed, NF-YC/NF-YB can associate with H3/H4, but not with H2A/H2B, to form higher order structures\(^43\). After superposition of our dimer onto a H2A/H2B dimer of the core nucleosome particle, we looked at the possibility of forming \((H3/H4)_2(NF-YC/NF-YB)_2\) octamers reminiscent of the histone octamer. Clearly, such an hypothesis is not valid, as many steric clashes occur at the different interfaces between the pairs (data not shown). This result is in agreement with previous data and with the fact that NF-YC/NF-YB can also associate with formed nucleosomes, suggesting indeed that the interactions between these dimers is rather different\(^43\).

Second, NF-YC/NF-YB has also been shown to interact *in vitro* with TBP, but not with a preformed TBP/TATA element\(^30,42\). In the NC2/TBP/TATA structure, NC2 makes relatively few protein-DNA contacts, that could also be formed by NF-YC/NF-YB, and it recognizes TBP on both sides at two locations, thus encircling the DNA with TBP\(^19\). The strongest interaction corresponds to numerous contacts between the \(\alpha5\) helix of NC2\(\beta\) and the
C-terminal domain of TBP, whereas at the other location an arginine side chain from TBP contacts a main chain carbonyl and is stacked against other side chains of NC2α. From sequence alignments, it is not clear whether NF-YB contains a fifth helix which might contact TBP as NC2β does. If there is any interaction between NF-YB and TBP, it would most probably be different. In the case of NF-YC, the replacement of the absolutely conserved Gly28 in NC2α by Lys/Arg59 would cause a strong steric hindrance, preventing interaction with TBP. In conclusion, and in agreement with experimental data, although the determinants for DNA binding in the core histone regions of NC2α/NC2β are conserved in NF-YC/NF-YB, recognition of TBP by this latter complex must be rather different and cannot be achieved in the context of a preformed TBP/TATA element, as observed with NC2.

The previous examples suggest the existence of specific determinants implicated in the functionality of each pair. Another aspect in which the NF-YC/NF-YB dimer might play a role independently from NF-YA association is related to the positive transcription function of NC2, recently unmasked on DPEs, for which the histone folds are sufficient. The mechanistic details are poorly understood at present, but clearly independent from TBP-binding, and possibly related to facilitation of correct connections between the DNA and the H3/H4-like TAFIIIs within TFIID. Since NF-YC/NF-YB is known to interact with histone-like TAFs, it would be interesting to investigate whether NF-YC/NF-YB might, in this case, play an essentially identical positive role than NC2 at the DPE, or whether other determinants make this process once again NC2 specific.

**The NF-YC αC region is a target for regulatory proteins**

The distorsion of the DNA by NF-Y, as modelled here, possibly coupled to the recruitment operated by NF-YA and NF-YC activation domains, would make it possible for...
other gene specific regulatory factors (e.g. RFX, SREBP, Sp1, C/EBP) to come in close vicinity of the GTFs, thus facilitating transcription activation. More globally, the emergence in highly regulated promoters of several CCAAT elements located 30-40 bases apart clearly raises the question of the precise three-dimensional arrangement mediated by NF-Y, and of the requirement of such large DNA distortions for the recruitment of incoming positive, as well as negative co-factors. Recent studies reveal that this process might be influenced by different regulatory proteins in a promoter dependent way, and that the nearly invariant NF-YC αC region, besides its role in NF-YA binding, is a target element for these proteins. First, it has been shown that the C-terminus of the NF-YC core region is the docking site for c-MYC, and that this interaction is absolutely necessary for transcriptional repression by c-MYC on the PDGF β-receptor. Second, p53 transcriptional repression on promoters having multiple CCAAT boxes has been shown to be dependent on NF-Y and, once again, the αC region of NF-YC is required for this process (Imbriano et al., manuscript in preparation). The different location of NF-YC helix αC, compared to that of H2A, reveals a unique specificity of the NF-Y, and also most probably of the NC2, sub-family. The overall strong evolutionary sequence conservation between these both histone pairs raises the question whether NF-Y and NC2, contrary to their different functional roles, could share common regulatory pathways. In this respect, it would be interesting to study whether c-MYC and p53 would have any effects on NC2 known functions.

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from MIUR-COFIN (Chromatin dynamics in transcription) and AIRC.
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Figure legends

**Figure 1**: NF-Y subunits sequence alignments.

Sequence alignments of the core regions of (A) NF-YA, (B) NF-YB, and (C) NF-YC from human, *X. laevis*, *D. melanogaster*, *C. elegans*, *A. thaliana*, *S. pombe*, and *S. cerevisiae*. In the case of *A. thaliana*, one sequence was included for each subunit, but actually several different genes coding for each subunit are found in its genome. For NF-YB and NF-YC, the alignments also include the sequences from human H2B/NC2 and H2A/NC2α, respectively, and are based on the superposition of the three structures. Human NF-Y subunits numbering is used. (A) Fully and almost totally conserved residues of NF-YA core domain are coloured red and blue, respectively. Domains implicated in NF-YC/NF-YB (NF-YA1) and DNA (NF-YA2) binding are indicated, with important residues for each function boxed. (B)(C) Fully conserved residues in all, in the NF-Y/NC2, and only in the NF-Y sequences are coloured red, green and blue, respectively. Secondary structure elements (bars for α-helices and solid lines for coils), as observed in the structure, are coloured orange above the alignments. Black solid lines indicate regions present in the crystals that are not seen in density. Intra-chain arginine-aspartate pairs have been schematically represented in red. Boxed residues indicate amino acids of H2A/H2B and NC2α/NC2β pairs that hydrogen bond directly the DNA backbone with at least main chain atoms (red boxes), or only their side chains (blue boxes).

**Figure 2**: Comparison of NF-YC/NF-YB, H2A/H2B, NC2α/NC2β and H3/H4 histone pairs.

(A) Ribbon representation of the NF-YC/NF-YB dimer. NF-YC and NF-YB have been coloured orange and green, respectively, with elements of secondary structure indicated. Arginine-aspartate pairs are displayed, together with Trp85 of NF-YC which may play an...
important role in the specific interaction between NF-YB and NF-YC, in comparison to NC2. (B) Stereo Cα traces of the superimposition of the NF-YC/NF-YB (orange), H2A/H2B (grey), NC2α/NC2β (blue) and H3/H4 (green) histone pairs. The tails of the histone proteins and H3 αN helix have been removed for clarity. (C) Superimposition of NF-YC/NF-YB (orange) and H2A/H2B (grey) histone pairs. The tails of H2A and H2B have been removed for clarity. The elements of secondary structure showing major differences have been labelled. (D) Superimposition of NF-YC/NF-YB (orange) and NC2α/NC2β (blue) histone pairs. Helix α5 of NC2β has been removed for clarity. NF-YC Trp85 is displayed.

Figure 3. View of helix αC of NF-YC.

(A) Ribbon representation of the NF-YC/NF-YB dimer with a close-up view of the NF-YC αC region. Residues which have been mutated in our study are shown and labelled (see also Table 4). (B) Stereo figure showing the 2Fo-Fc electron density map contoured at 1.2 σ around helices α3 and αC of NF-YC. For clarity, the orientation has been slightly changed compared to (A).

Figure 4. Comparison of DNA binding by H2A/H2B and NC2, and models of NF-YC/NF-YB/DNA and NF-Y/CCAAT complexes.

(A) GRASP representation of the electrostatic potential at the surface of the NC2 dimer missing the α5 helix of NC2β. The electrostatic potentials 8 and +8 kBT (kBT, Boltzmann constant; T, temperature) are coloured red and blue, respectively. The DNA fragment of the NC2/TBP/TATA structure is shown as ribbons coloured yellow/green. The DNA fragment spanning a H2A/H2B dimer of the nucleosome is shown as ribbons coloured red/blue. The figure was made after superimposition of both histone pairs (see text). (B) Electrostatic potential at the surface of the NF-YC/NF-YB dimer. The modelled DNA is shown as ribbons...
coloured red/blue. (C) Model of the complex of NF-YC/NF-YB with the CCAAT element from the pro-α2(1) collagen promoter. The DNA backbone is shown as ribbons (purple) with the bases displayed. The two possible locations of the CCAAT box, according to the modelling, have been coloured cyan. (D) Model of the NF-Y/CCAAT complex. NF-YC, NF-YB and DNA are coloured as in (C), while NF-YA is coloured blue. The two alternative positions for the linker connecting NF-YA1 and NF-YA2 sub-domains are shown as dotted blue lines. Secondary structure elements of the histone pair which are implicated in NF-YA1 and NF-YA2 recognition (see text) are labelled and coloured in red and grey, respectively. For clarity, only the bases for the CCAAT pentanucleotide are shown and labelled.
Table 1. Summary of co-expression experiments

|                | NF-YC1 (21-120)a | NF-YC2 (27-120) | NF-YC3 (44-120)b |
|----------------|------------------|-----------------|------------------|
| NF-YB1 (49-122) | -                | -               | -                |
| NF-YB2 (49-131) | -                | -               | -                |
| NF-YB3 (49-141)b | +                | +               | +                |
| NF-YB4 (49-149) | +                | +               | +                |

a Human numbering
b Evolutionary conserved domains
Table 2. Data collection and refinement statistics

| Pairs          | NF-Y-C2/NF-YB3 | NF-YC3/NF-YB3 |
|----------------|---------------|---------------|
| Data sets      | Native        | Native        | Se-Met λ1    | Se-Met λ2    | Se-Met λ3    |
| Wavelength (Å) | 0.920023      | 0.920023      | 0.979650     | 0.979407     | 0.977775     |
| Space group    | P2₁2₁2₁       | P2₁2₁2₁       | P2₁2₁2₁      | P2₁2₁2₁      |
| Cell constants |               |               |              |              |              |
| a (Å)          | 51.6          | 51.6          | 51.6         | 51.6         | 51.6         |
| b (Å)          | 60.3          | 60.6          | 60.6         | 60.6         | 60.6         |
| c (Å)          | 61.6          | 61.8          | 62.0         | 62.0         | 62.0         |
| Resolution (Å) | 24. – 1.67    | 24 – 1.57     | 24. – 1.78   | 24. –        |
| Reflections measured/unique | 110854/22726 | 151344/27411  | 131407/35755 | 130502/35753 | 132289/36007 |
| Redundancy overall/last shell | 4.9/2.5       | 5.5/2.9       |              |              |
| Completeness (%) overall/last shell | 97.9/99.0      | 97.9/93.5     | 98.7/99.5    | 98.8/99.3    | 98.9/99.0    |
| Rsym (%) overall/last shell | 2.6/5.3       | 4.5/8.3       | 4.8/6.5      | 5.0/6.2      |
| I/σ(I) overall/last shell | 43.8/17.3     | 28.9/13.6     | 20.2/10.8    | 19.8/11.2    |

Refinement statistics...
Resolution (Å) 
N° of protein atoms 
N° of water molecules 
N° of reflections (working/test sets) 
R-factor (%) 
R-free (%) 
Deviations from ideal geometry 
Mean temperature factors (Å²) 

Table 3. EMSA experiments

| Oligonucleotide | Sequencea | Interaction |
|-----------------|-----------|-------------|
| Ea (39 mer)     | agcactcaacttttaaCCAATcagaaaaaatgtttcagac tctttttttacaaagtc | ++++ |
| Ea-2            | agcactcaacttttaaCCAATcagaaaaaatgtttcag tctttttttacaaagtc | ++++ |
| Ea-4            | agcactcaacttttaaCCAATcagaaaaaatgtttc tctttttttacaaagtc | ++++ |
| Ea-6            | agcactcaacttttaaCCAATcagaaaaaatgtttc tctttttttacaaagtc | +++ |
| Ea-10           | agcactcaacttttaaCCAATcagaaaaaatgtttc tcttttttttttttttttt | + |
| 4-Ea            | ctcacttttaaCCAATcagaaaaaatgtttcagac gagggagttttaaGAATtccttttttacaaagtc | ++++ |
### Table 4. Mutational analysis on NF-YC αC helix

| NF-YC | Dimerization | Trimerization | DNA-binding |
|-------|--------------|---------------|-------------|
| wt    | +            | +             | +           |
| F111S | +            | +/-           | +           |
| D112N | +            | +/-           | +           |
| L114T | +            | +/-           | +           |
| I115K | +            | +             | +           |
| I115P | +            | -             | -           |
| I117P | +            | -             | -           |

*a* Ea equivalent of the footprints on the pro-α1(1) collagen promoter are underlined.
Figure 2
The NF-YB/NF-YC structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y
Christophe Romier, Fabienne Cocchiarella, Roberto Mantovani and Dino Moras

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