NF-Y Organizes the γ-Globin CCAAT Boxes Region*

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The CCAAT-binding activator NF-Y is formed by three evolutionary conserved subunits, two of which contain putative histone-like domains. We investigated NF-Y binding to all CCAAT boxes of globin promoters in direct binding, competition, and supershift electrophoretic mobility shift assay; we found that the α, ζ, and proximal γ CCAAT boxes of human and the prosimian Galago bind avidly, and distal γ CCAAT boxes have intermediate affinity, whereas the ε and β sequences bind NF-Y very poorly. We developed an efficient in vitro transcription system from erythroid K562 cells and established that both the distal and the proximal CCAAT boxes are important for optimal γ-globin promoter activity. Surprisingly, NF-Y binding to a mutated distal CCAAT box (a C to T at position −114) is remarkably increased upon occupancy of the high affinity proximal element, located 27 base pairs away. Shortening the distance between the two CCAAT boxes progressively prevents simultaneous CCAAT binding, indicating that NF-Y interacts in a mutually exclusive way with CCAAT boxes closer than 24 base pairs apart. A combination of circular permutation and phasing analysis proved that (i) NF-Y-induced angles of the two γ-globin CCAAT boxes have similar amplitudes; (ii) occupancy of the two CCAAT boxes leads to compensatory distortions; (iii) the two NF-Y bends are spatially oriented with combined twisting angles of about 100°. Interestingly, such distortions are reminiscent of core histone-DNA interactions. We conclude that NF-Y binding imposes a high level of functionally important coordinate organization to the γ-globin promoter.

The CCAAT box is a widespread regulatory sequence found in promoters and enhancers of several genes (1), whose functional importance has been well established in different systems (2–12). NF-Y (also termed CBF) has an almost absolute requirement for these five nucleotides and a strong preference for additional flanking sequences (13, 14). Based on supershift experiments with anti-NF-Y antibodies, on competition analysis with the original Ea Y box oligo,1 or on the heteromeric nature of the DNA-binding complex, NF-Y has been identified as the CCAAT box activator in over 100 promoters (7–11, 14, 15). The CCAAT consensus derived statistically by Bucher (1) (RRCCAT(C/G)(A/G)) fits well with the optimal NF-Y-binding site.

NF-Y is a ubiquitous heteromeric protein composed of three subunits, NF-YA, NF-YB, and NF-YC, all necessary for DNA binding (16, 17). The cloning of NF-Y genes from several species including yeast, maize, lamprey, and sea urchin, evidenced highly conserved domains (16–22). The NF-YA homology domain can be divided into subunit association and DNA-contacting subdomains (20). The N-terminal contains a hydrophobic and Gln-rich activation surface (18). The NF-YC gene has been recently cloned and is specular with respect to NF-YA, since the homology domain is at the N terminus, whereas the C-terminal 180 amino acids are rich in glutamines and hydrophobic residues. NF-YB and NF-YC tightly interact with each other, and their association is a prerequisite for NF-YA binding and sequence-specific DNA interactions (16, 22). Both the NF-YB- and NF-YC-conserved domains contain putative histone fold motifs. This motif, common to all core histones, is responsible for the formation of the histone octamer (24) and is composed of three α-helices, separated by short loops/strand regions, enabling histones to dimerize with companion subunits (24, 25). Recent experiments on the yeast HAP3 (26), NF-YB/CBF-A (27), and NF-Y/CBF-B (28) indicate that this 65 amino acid long motif is necessary for subunit interactions and DNA binding. NF-Y has additional interesting features as follows: (i) CCAAT boxes are not able to activate alone even if multimerized, but they increase the activity of neighboring enhancer motifs. (ii) NF-Y appears to increase the affinity of transcription factors for their target sequence (29). However, the exact mechanisms of transcriptional activation by NF-Y are still elusive.

Globin genes are transcribed in a tissue-specific and developmentally regulated manner by means of various regulatory elements in their clusters (30). In addition to the locus control regions which have been characterized in transgenic mice, the promoters of each globin gene contain sequences that usually impart tissue-specific control in transfection experiments. Several lines of reasoning point to CCAAT sequences as important elements in globin gene expression as follows: (i) they are present in all globin promoters; (ii) they have been remarkably conserved in different species at a fixed distance from the cap site; (iii) genomic footprinting of all globin promoters in erythroid cells showed invariable protection of CCAAT sequences in vivo, indicating binding of activators (31); (iv) the functional importance has been documented in the β, α, ε, ζ, and δ-globin promoters (2–5, 15); (v) the γ-globin duplicated CCAAT boxes are the target of mutations affecting developmental silencing of γ-globin expression in adult life. In particular, strong genetic evidence suggests that point mutations in the CCAAT box region are causative of the HPFH syndromes, characterized by increased fetal globin levels in adults (32–37).

Several studies investigated factors binding to CCAAT; CDP, NFE3, and e/EBP appear to recognize different globin promoters (3, 32–36). One CCAAT-binding protein, α-CP1, was purified to homogeneity using an α-globin CCAAT box affinity...
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RESULTS

NF-Y Binds to CCAAT Boxes of All Globin Promoters—To determine which of the globin CCAAT boxes is recognized by NF-Y, we labeled oligonucleotides (see Table I) for EMSA experiments with nuclear extracts, challenging the resulting complexes with anti-NF-Y- and anti-NF-YA-purified antibodies (12). Bands of different mobilities and intensities are generated with all oligos (Fig. 1), with the a (lanes 4–6), b (lanes 8–10), hY (human γ proximal, lanes 16–18), gY/P (prosimian Galago crassicaudatus γ proximal, see Ref. 36, lanes 28–30), hD (human γ distal, lanes 20–22) and gY/D (G. crassicaudatus γ distal, lanes 32–34) bands being the most prominent. To verify that these complexes are supershifted by specific anti-YB and anti-YA antibodies (12). In each case we ran parallel migrations of the Y box oligo incubated with recombinant NF-YA and purified NF-YB/NF-YC; as shown in Fig. 1, the bands have electrophoretic behaviors identical to the endogenous K562 bands shifted by the anti-NF-Y antibodies. On the other hand, with the β and ε CCAAT multiple bands are visualized (lanes 12–14 and 24–26); for the ε a weak band comigrating with NF-Y and supershifted by the antibodies is observed, and for the β the four major bands detected have different mobilities compared with the Y/NF-Y band, and no supershift is evident. These data show that NF-Y binding is readily visualized on all globin CCAAT boxes and is the most prominent binding protein, with the exception of ε and β, which show a much higher affinity for proteins unrelated to NF-Y.

To determine the relative affinity of NF-Y for each of the globin CCAAT boxes, we then incubated purified NF-YB/
NF-YC and recombinant NF-YA with the different labeled oligos in the absence (Fig. 2, see lane 1) or in the presence of 20- and 100-fold excess of cold competing oligos containing the different CCAAT boxes examined above. Results are shown in lanes 2–19. Y box binding is efficiently competed only by z, whereas α and the γP, both from human and Galago, have a somewhat lower affinity (see quantification of the data in Fig. 2B). All these CCAAT boxes clearly have a higher affinity in cross-competitions than ε and the very low affinity β. Note that the complex generated with the ε and β-globin CCAAT boxes is visible only after prolonged exposures (3 and 7 days, respectively, in the experiment shown). In general, all data are consistent with the fact that the better binders in direct EMSA are also the most avid competitors. Cross-competition experiments establish that the relative affinity of NF-Y for the different CCAAT boxes varies profoundly by more than 2 orders of magnitude, ranging from very high (ζ and Y) to high (α, hγP, and γγP) to medium (γγD) to low (ε and β).

Both γ-Globin CCAAT Boxes Contribute to Promoter Activity in Vitro—We next focused our attention on the developmentally regulated γ-globin promoter. We fused the minimal tissue-specific γ-globin promoter (−299 to +35) to a rabbit β-glo-
bin reporter gene (plasmid pAG1) and generated mutants in the proximal or in the distal CCAAT boxes (see scheme in Fig. 3A). The wt and mutated constructs were tested in a functional in vitro assay with transcriptionally competent erythroid K562 extracts. RNA was purified and hybridized to a single-stranded end-labeled DNA probe; subsequent S1 mapping allowed the determination of qualitative and quantitative changes in the transcription rate. As an internal control we added a plasmid containing the adenovirus major late promoter TATA box devoid of any activating sequences and fused to the same reporter gene. Fig. 3B shows that our system efficiently transcribes the \( \gamma \)-globin promoter and faithfully reproduces the correct start site used in vivo. We tested the different mutants: alteration of the proximal CCAAT box or destruction of the distal by a 13-base pair deletion (pAG3 and pAG4) decreases transcription 3–4-fold (Fig. 3C, lanes 3 and 4). Swapping the weak e \( \beta \)-globin CCAAT box into the distal CCAAT box position partially restores transcription when the proximal CCAAT is mutated, while having minor effects when the proximal CCAAT is intact (pAG2 and pAG5, respectively; Fig. 3C, lanes 1, 2 and 5). A mutant promoter containing mutations in both CCAAT boxes (pAG6) was also compared with wt pAG1 and resulted in the lowest transcriptional rate (6-fold down, compare Fig. 3C, lanes 6 and 7). Note that the signals in Fig. 3C result from a 4-h exposure. These data indicate that both CCAAT elements contribute to the optimal promoter activity. However, other important activators are probably operating, since mutations in the CCAAT boxes do not abolish transcription completely.

**Binding of NF-Y to the \( \gamma \)-Globin Double CCAAT Boxes Region**—By having shown that both \( \gamma \)-globin CCAAT boxes bind NF-Y and are important for in vitro promoter activity, we investigated their interplay; we labeled a long oligo encompassing the two CCAAT boxes, incubating increasing concentrations of recombinant NF-Y, containing wt NF-YA and NF-YB and the homology domain of NF-YC. Fig. 4 shows that two bands of different electrophoretic mobility are generated (lanes 1–4); to ascertain whether the slow migrating complex corresponds to DNA fragments bound by two NF-Y molecules, we used fragments mutated in the distal CCAAT (C\(_{2}T_{114}\)), in the proximal CCAAT (the corresponding C to T mutation at 287), or in both. We have deliberately chosen this mutation because genetic evidence strongly associates it with HPFH syndromes in humans (34). Mutations in such position are known to essentially abolish NF-Y binding to all CCAAT boxes tested so far, including the \( \gamma \)-globin (13–15, 40).

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\(^{2}\) A. Ronchi, unpublished observations.
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Only modestly affected by mutations in the distal CCAAT; the slower complex is greatly diminished in the −87 CCAAT mutant and in the double mutant (compare lanes 4, 12 and 16). Surprisingly, the −114 mutant exhibited a considerable level of the upper complex (compare lanes 4 and 8 and see the calculated ratios in Fig. 4B). With the double mutant, both the slower and the faster complexes were also crippled (lanes 13–16). These data suggest that the faster band corresponds to NF-Y binding to either the proximal or the distal site, whereas the upper one results from double occupancy of the two CCAAT boxes. Interestingly, consistent with the cross-competition experiments, binding of NF-Y to the proximal CCAAT is predominant and compensates for a crippling mutation in the distal CCAAT, whereas the reverse is not true.

To better understand the mechanisms leading to double occupancy and rule out the alternative explanation of the data in Fig. 4, namely that the upper band is due to formation of an NF-Y dimer on single CCAAT boxes, we rotationally moved the position of the two CCAAT by introducing short deletions of 3, 5, and 8 base pairs between them. Dose-response experiments with labeled oligos; deletion of three nucleotides has relatively little effect on double binding, whereas a 5 and even more with the wt oligos, the double occupancy band was more resistant to competition than the single CCAAT-NF-Y (Fig. 6, C and D, lanes 1–5; see 6E). The latter result tends to suggest some form of cooperative behavior among the two NF-Y molecules on the Δ5 CCAAT boxes and some interplay in the wt. However, upon turning the reciprocal rotational positions of the two CCAAT sequences by 80/100°, as in the Δ3 and Δ8, this effect is not observed.

NF-Y Induces Bending of γ-Globin CCAAT Boxes—We have recently shown that NF-Y is able to induce distortions in the double helix, with angles that vary depending on the surrounding sequences (14). By using the circular permutation assay we checked the degree of distortion of the proximal and distal CCAAT boxes separately. We cloned the two oligos of identical length in the pBend2 vector (14); we then cut with different enzymes so that the CCAAT boxes were at different distances from the extremities of the fragments, and we performed EMSA with NF-Y. To calculate precisely the angles, we maximized the differences in mobilities of the protein-DNA complexes, using the small NF-YA9 mutant and purified endogenous NF-YB/NF-YC. We have shown that such combination does not alter significantly the distortion angles of four NF-Y sites (14). Clear indication that NF-Y induces distortions on both γ-globin CCAAT boxes was evident from the different electrophoretic mobilities of the fragments (Fig. 7). Calculations of the angles gave similar results for both sites, and the values, 66° and 72° for distal and proximal, respectively, are similar to those observed for the murine sarcoma virus and ε-globin and slightly lower than for the Ea Y box and HSP70 NF-Y-binding sites. As expected, the flexures are indeed centered on the CCAAT sequences (Ref. 14 and data not shown).

To verify the effect of two bound NF-Y molecules on the overall distortion, we then repeated the circular permutation experiments with the long γ-globin oligo (Fig. 7). As expected, two bands are visible in EMSA. (i) The lower corresponds to occupancy of either the distal or the proximal CCAAT and is therefore a mixture of proximal and distal NF-Y binding. The arch formed by these bands is very similar to the one observed in circular permutation assays with the single CCAAT. Note, however, that in some of the lateral fragments the shifted bands are rather wide; since the distances from the extremities of the proximal versus the distal CCAAT are different, we do expect to see slightly altered electrophoretic behaviors. (ii) The upper complex mobilities are much more similar, with a calculated overall angle of 45°, suggesting compensation; much bigger differences between the fragments would have been scored had NF-Y bending angles been additive. Nonetheless, despite the presence of the two sites on opposite sides of the double helix (they are separated by 27 bp, hence 2.5 helical turns), a perfect straightening of the helix is not observed, as judged by this circular permutation EMSA, as one might have expected considering that the amplitudes of the two angles are nearly identical.

Phasing Analysis of CCAAT Boxes—To verify whether NF-Y induces directed bends, as suggested by the previous experiment, and to seek further information about the relative distortions of the CCAAT region, we performed phasing analysis. This assay is usually tackled with appropriate vectors contain-
ing a fixed angle of known curvature, obtained exploiting the distortions caused by short AT-rich sequences spaced by one turn of the helix (Ref. 40 and references therein). Having fixed one angle, the binding site is rotationally moved on different sides of the double helix, by increasing the distance from the fixed angle, taking into account that one turn corresponds to

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**Fig. 6.** EMSA competition analysis on oligos with CCAAT deletions. A, a 5-, 20-, 100-, and 500-fold molar excess of competing wt (lanes 2–5 and 12–15) or mutated Y box oligos (lanes 7–10 and 17–20) were incubated with NF-YA/NF-YB/NF-YC5 (5 ng) before addition of the wt and Δ3 labeled oligos (see scheme). B, same preincubation as in A, except that labeled Δ5 and Δ8 oligos were used. C, same as A except that the labeled wt and Δ3 oligos were first incubated with NF-YA/NF-YB/NF-YC5 (22) and then challenged with the competing oligos (see scheme). D, same as B, except that the same order of addition as in C was used. For the Δ5 and Δ8 experiments a 10-fold higher amount of NF-Y was necessary to visualize the upper NF-Y bands. E, quantification of the data in A–D.
10.5 base pairs. The relative mobilities of the protein-bound complexes give clues about the orientation of the angles. Rather than adopting this strategy, which would give information about one isolated CCAAT, we took advantage of the two $g$-globin-binding sites with their previously calculated distortions. We cloned in pBend2 the $D_3$, $D_5$, $D_8$ oligos, and a $A_3$ oligo, in which we added 3 bp between the two CCAAT. Cutting the resulting plasmids with any of the enzymes generates nearly identical fragments with NF-Y-binding sites rotationally displaced with respect to the wt situation. EMSA analysis of the central $Xho$I fragments is shown in Fig. 8, and comparable amounts of protein generated the single occupancy band with identical electrophoretic mobility for all fragments. The double occupancy band is visible with the $A_3$, wt, and $D_3$ fragments, with the $D_5$ only after prolonged exposures, and is absent in the $D_8$. The mobilities of the latter bands are different, a clear indication that indeed the DNA is oriented upon NF-Y binding (40). The slowest fragment should be the one in which the two bends are in phase with respect to the helical turn of DNA, whereas the fastest is the one in which the two bends are facing opposite sides and counteract each other; the $A_3$, in which the two CCAAT boxes are only 1.5 bp away from perfect alignment on the same side of the helix, is the slowest, and the $A_3$ and $D_5$ are the fastest. This is consistent with overall distortion angles of about 100° among the two CCAAT in the wt configuration (see Fig. 9). These results are indeed confirmed by experiments with a “mini” NF-Y protein (22) that is able to generate double bands in the $A_8$ as well, which give a phasing “period” of about 8 bp, corresponding to a rotation of about 100°.3

DISCUSSION

In this study we undertook a systematic analysis of NF-Y binding to the CCAAT boxes of globin gene promoters and established that most, but not all, contain bona fide high affinity sites. We focused then on the human $g$-globin, and we developed an efficient in vitro functional assay with K562 extracts and provided for the first time evidence that both CCAAT boxes add to the overall level of expression. NF-Y binds better to the proximal than to the distal site, and DNA binding is neither cooperative nor mutually exclusive. The bending angles of the isolated CCAAT boxes are similar, and double binding induces compensatory alterations; the combined twisting angles are about 100°.

NF-Y and the Globin CCAAT Boxes—Previous experiments with nuclear extracts identified several proteins binding to the CCAAT boxes of various globin promoters. CDP binds to the duplicated $g$-globin CCAAT box region (32, 33), NF-E3 to the $g$-distal CCAAT box and to the $e$ box (32, 36), and c/EBP to the $b$ CCAAT (3). The $e$ CCAAT box region is bound by a number of factors (36). Binding of NF-Y has been more than suspected;

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of 45°. This value should be reconciled with the fact that the overall angle, as shown in Fig. 9, with a combined angle that binding of two NF-Y molecules are compensating in term mutation experiments on the long double CCAAT oligo show attitudes have been shown to be due to nucleotides flanking the proximal. This is not surprising since differences in amplification experiments presented by Jackson et al. (42) suggest that NF-Y binding dramatically increases the affinity of SREBP-1a for its neighboring target site, without even forming a stable ternary complex on the DNA. With this line of reasoning, mutations of the distal CCAAT might alter the reciprocal interplay between the two NF-Ys, and possibly the association with additional neighboring factors, rather than the affinity of NF-Y for the CCAAT sequence per se.

Role of CCAAT Boxes in γ-Globin Transcription—The efficient in vitro transcription system for the γ-globin promoter allowed us to determine the role of the CCAAT boxes. Our data are consistent with the idea that both NF-Y-binding sites contribute to the optimal activity, and mutations in both CCAAT resulted in transcription levels that were lower (6-fold) than alterations in either CCAAT alone. A point mutation in the proximal and removal of the distal CCAAT box led to a comparable 4-fold decrease in transcription; and swapping an ε-like CCAAT box, which is a poor NF-Y-binding site, in the place of the distal γ CCAAT weakens the promoter both when the proximal CCAAT is intact (2-fold) and even more if it is mutated, as in pAG5. Thus, from the functional point of view, the twin CCAAT boxes seem to have an additive rather than a multiplicative effect, a result that is in line with the lack of cooperativity between the two proteins observed in the binding assays. Either CCAAT is nevertheless sufficient to impart rather efficient levels of transcription. This might be entirely due to strong TATA box and basal elements or to the activity of additional upstream factors, as suggested by challenging tran-

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scription with anti-GATA antibodies. Recent findings by Ronchi et al. (37) with mutated γ-globin CCAAT boxes in transgenics suggested that they are important for γ-globin expression in the adult (−117 HPFH) mutation but redundant in the embryonic/fetal period; mutations in the distal or in the proximal sites do not prevent efficient embryonic expression of the normal γ-globin gene, a clear indication that additional strong activators are indeed operating. These findings are in sharp contrast with the data obtained in another transgenic system; removal of the Y box from the MHC class II Ea promoter dramatically decreases transcription, altering start site selection (43). In MHC class II promoters, NF-Y was shown to help the binding of the neighboring X-binding proteins, and cooperation between the two complexes is strictly necessary for efficient activation (29). The less profound effect of CCAAT mutations observed in the γ-globin system is reminiscent of the albumin promoter, in which NF-Y contributes to 

in vitro transcription rates by a factor of 3 (12). One possible explanation for these observations is that complex, highly efficient promoters, such as globins and albumin, rely on multiple activators and might be less critically dependent on a single factor, whereas in the simpler MHC class II system NF-Y plays a more fundamental role. We favor a scenario in which NF-Y might have distinct roles depending on the promoter context; in Ea it would directly participate in the formation of a preinitiation complex and ultimately help choosing the start site, in conjunction with an Initiator element (12, 44); in globin promoters it might help build the overall architecture and the proper interactions of additional DNA binding activators. NF-Y might also have a direct influence on the developmental regulation of globin promoters; the fact that the α-like promoters have among the most avid NF-Y-binding sites and somewhat simpler promoters suggest a less refined level of regulation. Recent transgenic experiments on the human δ showed that 67 base pairs, including only the NF-Y-binding CCAAT box and TATA box, are sufficient to direct LacZ expression in erythroid cells in the correct developmental period (5). In the β-like locus, whose genes are switched on and off three times, the very low affinity CCAAT boxes of embryonic ε and adult β genes are most likely not the target of NF-Y. The hemoglobin switching model based on promoter competition proposed by Grosveld and colleagues (30) predicts that transcription complexes formed on the differentially expressed globin promoters play a fundamental role, being alternatively connected with locus control regions. As a result of the combined CCAAT bending and twisting, activators binding to the more distal CCAAC, GATA, and −200 regions might be brought closer to the TATA box and to the basal transcriptional machinery. Indeed evidence that NF-Y subunits can interact with TATA-binding protein has been recently obtained (45).

Consistent with this hypothesis are two findings, the "stage selector" element found between the CCAAT and TATA boxes (46) and the occurrence of several mutations (mostly point mutations) in alleles associated with persistence of fetal hemoglobin in adults (HPFH). Three such alterations, HPFH −117, −114, and Δ13, involve the distal CCAAT box region. Our results on NF-Y binding to the −114 mutation in the context of the double CCAAT boxes help explain the apparent discrepancy of a crippling mutation in a functionally important element, retaining functional activity; based on the positive effect of the proximal CCAAT, we anticipate only minor consequences of the −114 for the total effectiveness of the mutant promoter. Nonetheless, HPFH subtle alterations might alter the fine interplay of factors involved in the developmental regulation of the γ gene, by modifying the bending and twisting of reciprocal angles.

**NF-Y Binding to Duplicated CCAAT Boxes—** CCAAT boxes show a strong position bias within promoters being usually positioned between −60 and −100 (1). Functional experiments indicated that a single CCAAT box is not able to increase transcription over basal levels, and multimerized CCAAT boxes also fail to do so. NF-Y can greatly improve the activity of diverse upstream transcription factors, and in some cases, it has been proved that it does so by dramatically improving the affinity of neighboring factors for their target DNA sequence (29, 42). Since no data were available on the binding of NF-Y in promoters harboring more than one CCAAT box, the γ-globin represents an excellent model to study their relationship. Several findings in our study bear implications for other systems as follows: (i) bringing two CCAAT sequences closer than 24 base pairs essentially abrogates double binding, and (ii) NF-Y complexes are more stable on the DNA only with the Δ5 mutant, a deletion bringing the two CCAAT boxes on the same side of the helix. This suggests that cooperativity is possible, provided that the correct rotational position is respected. From the long list of CCAAT-containing promoters activated by NF-Y, a growing number contains two or more sites (see Table II); we note that in all, bar the gp91

**TABLE II**

A list of multiple CCAAT-containing promoters

| Gene | Organism | Distance | Ref. |
|------|----------|----------|------|
| TK   | Human    | 30       | 47   |
| FPP  | Rat      | 45       | 42   |
| HMG-CoA Red | Mouse | 124     | 54   |
| Hexokinase II | Rat  | 56       | 8    |
| Interleukin-4 | Human | 68       | 7    |
| Cdc2 | Human    | 32       | 48   |
| Cdc25C | Human | 64       | 49   |
| Topoisomerase IIα | Human | 40      | 50   |
| gp91phox | Human | 14       | 10   |
| TAT  | Rat      | 212      | 51   |
| LRSV | RSV      | 65       | 9    |
| E2F1 | Mouse    | 43       | 52   |
| H2b1 | Sea urchin | 32    | 53   |
| HSP70| Xenopus  | 84       | 2    |

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Page 16885, Fig. 6E: This part of Fig. 6 was printed without the legends. The complete version of Fig. 6E is shown below.

Fig. 6

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