Conformation of CCAAT/enhancer binding protein alpha dimers varies with intranuclear location in living cells

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SUMMARY

The structure of a protein defines its biochemical properties, but the impact of intracellular location and environment on protein structure remains poorly defined. CCAAT/enhancer binding protein alpha (C/EBPα) is a master regulator of transcription and cellular proliferation that concentrates, and is kept inactive, at transcriptionally quiescent, peri-centromeric regions in mouse cell nuclei. C/EBPα dimer structure was measured in living cells from the amounts of fluorescence energy transferred between derivatives of the green fluorescent protein attached to different C/EBPα domains. Comparing the levels of fluorescence resonance energy transfer at peri-centromeric and non-peri-centromeric regions of the nucleus indicated that the DNA binding domains of C/EBPα dimers were further apart and interacted more poorly at peri-centromeric heterochromatin than in the more euchromatic regions of the nucleus. In contrast, the position and interactions of the transcriptional activation domains were similar throughout the nucleus. Phorbol ester treatment caused a shift in the position of the transcriptional activation domain, relative to the DNA binding domain. Thus, C/EBPα conformation varies with intranuclear location and with cellular environment. These “FRET nanoscopy” techniques will be broadly applicable for associating conformational and kinetic variations to subcompartment-specific actions of C/EBPα, or any protein, in the dynamic intracellular environment.
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INTRODUCTION

The structure of a protein specifies its interactions with itself and with other factors. X-ray crystallography and NMR characterize the structure of concentrated, isolated proteins of static composition and uniform conformation under non-physiologic conditions. Methods that investigate protein structure in living cells, where conformation may vary with localized protein interactions and with dynamic, subcellular micro-environments, would complement high-resolution X-ray crystallographic and NMR structures.

It is becoming increasingly evident that the nucleus is comprised of distinct functional subdomains at which transcription regulatory proteins dynamically associate (1-5). C/EBPα is a transcription factor that regulates gene expression and cellular proliferation through distinct mechanisms (6-9). Within mouse cell nuclei, C/EBPα accumulates at one of those subnuclear domains, the peri-centromeric heterochromatin (10,11), by binding to α-satellite DNA repeats (10)(B. Wu, F.S., unpublished) that concentrate around the centromere (12,13). In non-murine cells, the homologous repetitive element and C/EBPα are less concentrated. Although most transcription factors do not concentrate at peri-centromeric heterochromatin, some factors involved in lymphocyte and erythrocyte development transiently associate with microscopically detectable, mouse peri-centromeric heterochromatin at specific differentiation stages (14-17). This suggests that regulated compartmentalization of transcription factors may be functionally important (2).

The co-concentration of α-satellite DNA and C/EBPα at microscopically detectable peri-centromeric heterochromatin permits the study of peri-centromeric targeting of C/EBPα. Point and deletion mutations of C/EBPα that block peri-centromeric targeting still block cellular proliferation (18) indicating that peri-centromeric localization is not required for the anti-
proliferative effects of C/EBPα. In contrast, transcriptional activity is regulated by peri-centromeric location. A C/EBPα mutant that no longer binds α-satellite DNA, but that retains normal binding to some promoter binding sites, also no longer concentrates at the peri-centromeric subdomains of the nucleus. This altered-specificity mutation releases C/EBPα from sequestration at the transcriptionally quiescent (11) peri-centromeric subdomain, which results in a substantial elevation in C/EBPα activation of promoter activity (B. Wu, F.S., unpublished).

More naturally, in mouse pituitary cell cultures, C/EBPα is re-distributed to the euchromatin upon expression of the pituitary-specific transcription factor Pit-1 (19,20), whereas a Pit-1 mutation identified in human patients with combined pituitary hormone deficiency (21,22) is defective in the re-distribution of C/EBPα (19). Thus, the sequestration of C/EBPα at transcriptionally inactive heterochromatin is functionally significant and regulated.

The sequestration of C/EBPα at peri-centromeric heterochromatin may be accompanied by an alteration in C/EBPα structure. We investigated whether C/EBPα structure was different when localized at peri-centromeric heterochromatin and at euchromatin in mouse pituitary progenitor cells. C/EBPα is a member of the bZIP family of transcription factors, which are characterized by a conserved, carboxy-terminal dimerization and DNA binding domain (23-25). The bZIP domain forms a dimeric alpha-helical coiled-coil that binds DNA (26,27).

Transcription activation (TA) functions are present in more amino terminal domains of C/EBPα (28-30). However, the relative positions of the bZIP and TA domains in the C/EBPα dimer are unknown, under any in vitro or in vivo condition.

We combined fluorescence microscopy and fluorescence resonance energy transfer (FRET) techniques to define the relative positions of the TA and bZIP domains in dimers of C/EBPα at different subregions of living cell nuclei. The TA and bZIP domains of full-length
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C/EBPα were labeled with the autofluorescent green and blue fluorescent proteins (GFP, BFP) and expressed in living cells. Like endogenous C/EBPα, fluorescent protein-labeled C/EBPα was constitutively nuclear and accumulated at peri-centromeric heterochromatin (10,11,31). Since FRET only occurs if BFP and GFP are less than 80 Angstroms apart (32,33) and decreases to the sixth power of the distance separating the fluorophores (34), the amount of energy transferred from BFP to GFP indicated the relative interactions between the fluorophore-tagged TA and bZIP domains at each site within the cell (34-37).

FRET, normalized for the amounts of BFP and GFP present, was much higher when BFP and GFP were attached to the bZIP domains than to the TA domains. This indicated that the bZIP domains were closer to each other than were the TA domains in the C/EBPα dimer, or that the interactions between the bZIP domains were kinetically more favorable (36). Bimolecular interaction plots of the amount of FRET measured against the amounts of BFP and GFP-tagged C/EBPα present indicated identical kinetics for interactions between the bZIP and between the TA domains. FRET, measured at each of thousands of subregions throughout each nucleus, showed that the bZIP domains were further apart, or interacted less well, in C/EBPα dimers concentrated at the peri-centromeric subregions than in dimers at the remaining euchromatic regions of the nucleus. In contrast, the spatial relationship between the TA and bZIP domains was identical at the peri-centromeric and non-centromeric locations, but was altered by incubating the cells with a phorbol ester. Thus, we provide the first characterization of the conformational state of a gene- and cell cycle-regulatory factor at localized positions within living nuclei.
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EXPERIMENTAL PROCEDURES

Expression of C/EBPα fusions with GFP and BFP in GHFT1-5 cells—All C/EBPα fusion proteins were expressed under the control of the human cytomegalovirus after transfection by electroporation, as previously described, into pituitary progenitor GHFT1-5 cells (11). The transfected cells were plated on No. 1 borosilicate glass coverslips and grown 40-48 hours post-transfection before imaging. Transfected cells grown for 24 hours were treated with $10^{-8}$ M PMA (Sigma), or control DMSO drug vehicle, for one day prior to imaging or to extract preparation for promoter activation studies (38). The GH promoter and the C/EBPα-sensitive promoter containing the growth hormone TATA box (-33 to +8 relative to the transcription start site) and a single growth hormone C/EBPα binding site (-239 to -219) have been previously characterized (11,38,39).

Immunostaining of C/EBPα—For the quantification of C/EBPα co-localization with Hoechst 33342-stained DNA in 3T3-L1 cells, differentiated 3T3-L1 cells and transfected GHFT1-5 cells, those cells were washed with phosphate buffered saline (PBS), fixed for five minutes with methanol, treated for 5 minutes with 0.05% Triton X-100 in PBS, blocked by incubation with 5% horse serum in PBS, then incubated for one hour with a 1:500 dilution of the C-18, anti-C/EBPα primary antibody (goat) from Santa Cruz Biotechnology (sc-9314) in 0.5% horse serum in PBS. Slides were washed three times with PBS and incubated for one hour with a 1:500 dilution of donkey, anti-goat, rhodamine-conjugated secondary antibody from Santa Cruz Biotechnology (sc-2094). Following washing with PBS, slides were incubated for five minutes with 0.2 μg/ml of Hoechst 33342, then washed three times with PBS. For the 3T3-L1 cell images shown in Fig. 1B, the 14AA anti-C/EBPα primary antibody (rabbit) from Santa Cruz Biotechnology (sc-61)
was used in conjunction with the donkey, anti-rabbit rhodamine-conjugated secondary antibody from Santa Cruz Biotechnology (sc-2095).

Image Collection—All quantified FRET and co-localization data was collected on an Olympus IX-70 using Olympus 40x Plan Apochromat objective (0.95 NA). The images shown in Fig. 1A were collected using an Olympus 100x Plan Apochromat oil immersion objective (1.40 NA). Chroma Corporation (Brattelboro, Vermont) filters and multi-bandpass dichroic mirror (61000v2bs from) were used together with Sutter Instruments (Novato, California) $\lambda$-10 excitation and emission filter wheels, controlled by Universal Imaging Corporation (Downingtown, Pennsylvania) Metamorph data acquisition software. The single, immobile, multi-bandpass dichroic mirror in combination with mobile excitation and emission filters and the use of chromatically corrected objectives maximized the image registration required for pixel-by-pixel co-localization analysis and FRET nanoscopy. An Opti-Quip (Highland Hills, New York) model 1962 long-term stabilizer was used to keep light intensity constant for accurate quantitative data collection.

Antibody-stained slides were imaged by 1) exciting rhodamine with 550-560 nm light and collecting emissions from 580-630 nm, 2) exciting GFP with 480-495 nm light and collecting emissions at 500-530 nm, and 3) exciting Hoechst 33342 with 365-395 nm light and collecting emissions from 435-465 nm. Controls showed no fluorescence bleedthrough of rhodamine, GFP or Hoechst between these channels. For quantitative co-localization analysis (Figs. 1C, D), focusing was done using the Hoechst channel so as to blind data collection to the presence and amounts of expressed C/EBP$\alpha$. Once data collection parameters were established that ensured no saturation in any pixels, all integration times, camera gain and pixel binning were
kept constant to permit accurate quantitative comparisons of fluorescence amounts between different cell nuclei.

Live cell imaging was used to analyze FRET between BFP and GFP-linked C/EBPα. Images were always collected in the order: 1) acceptor (excitation filter 480-495nm / emission filter 500-530 nm, 2) donor (365-395 nm / 435-465 nm), 3) FRET (365-395 nm / 500-530 nm). The acceptor and donor channels yielded no bleedthrough, between BFP and GFP fluorescence (Fig. 2). It is particularly important to collect the FRET channel after the donor channel to eliminate any possibility of false FRET signals arising from decreased donor emissions that would occur if BFP were photobleached during an initial collection in the FRET channel. Images were collected by focusing only for the GFP-labeled C/EBPα using the acceptor filter combination. This prevented, until data collection, the excitation of BFP, which is more prone than other GFP derivatives to photobleaching (31). Practically, we had little difficulty with BFP photobleaching as was indicated by the reproducibility of our constants obtained from the control cells expressing only C/EBPα fusions with BFP (Fig. 2).

**Image Analysis: Co-localization**—All image analysis was done using Metamorph software (Universal Imaging Corporation, Downingtown, Pennsylvania). For the analysis of C/EBPα co-localization with Hoechst, a region of no fluorescence adjacent to the cell was used to determine the average background level of fluorescence in each of the rhodamine, GFP and Hoechst channels. The background amount was then subtracted from each pixel in each channel. Nuclei were identified as regions of contiguous pixels containing higher than background levels of blue fluorescence in the Hoechst channel. The selected region was transferred to the matched images collected in the rhodamine and GFP channels. For co-localization analysis of cells expressing
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C/EBPα fused to either BFP or GFP, nuclei were similarly identified as regions of contiguous pixels containing significantly higher than background levels of GFP fluorescence. Correlation coefficients were calculated by comparing the amounts of fluorescence measured in each matched pixel of two different channels using the Metamorph “correlation plot” application.

*Image Analysis: FRET*—The nuclei of cells expressing BFP or GFP-tagged C/EBPα were identified from background-subtracted BFP or GFP fluorescent images as described in the preceding section. The amounts of nuclear fluorescence collected from the acceptor (GFP) or donor (BFP) in each channel were expressed as ratios relative to the amount of fluorescence collected in the respective acceptor or donor channels (see Fig. 2). These spectral cross-talk ratios were used to calculate the contributions of BFP and GFP to each channel in cells co-expressing BFP- and GFP-labeled C/EBPα. Briefly, from the amount of acceptor fluorescence in each co-expressing cell was subtracted the minimal fluorescence contamination of C/EBPα-BFP to the acceptor channel (0.0013 x donor fluorescence amount). The minimal contribution of C/EBPα-GFP to the donor channel (0.0040 x the BFP-corrected acceptor fluorescence) was similarly subtracted. Although these contaminations were negligible in the current experiments, they can be significant with other fluorophore or filter combinations (unpublished data) and must be accounted for. For calculating FRET and FRET efficiency from the nucleus, the fluorescence contribution of the acceptor C/EBPα-GFP then was subtracted from the background-subtracted FRET channel (0.0882 x the BFP-corrected acceptor fluorescence). The amount of remaining fluorescence in the FRET channel then was divided by the amount of remaining fluorescence in the donor channel. If the resulting ratio is the same as the ratio obtained from cells containing
only donor (0.4719), then there was no FRET. If higher than 0.4719, energy was transferred from the donor to the acceptor.

It is critical that the acceptor cross-talk into the FRET channel is accounted for. We have noticed, to date, a number of attempts by other laboratories to calculate FRET without taking into account the substantial contributions of the acceptor itself into the FRET channel. Simply measuring the ratio of the amount of fluorescence in the FRET and donor channels with only donor excitation incorporates acceptor bleedthrough into the FRET channel. If not accounted for, this bleedthrough increases the FRET/donor ratio and results in a false conclusion of interaction. Since the ratios measured are physical parameters of the fluorophores, correctly-calculated FRET measurements are highly consistent between separate experiments provided that all parameters affecting the relative ratios of fluorescence quantification in the donor, acceptor and FRET channels are kept constant. This includes using the same 1) objective lens, 2) dichroic mirror, 3) excitation/emission filters 4) camera and 5) relative integration times for the different channels.

All bleedthrough-corrected measurements were downloaded into Microsoft Excel files and were expressed as a mean +/- standard deviation from multiple nuclei collected from two or more experiments. Statistically significant differences were determined using T-tests. Slopes and y-intercepts were calculated using Excel. 95% confidence intervals in the slopes were calculated using GraphPad (San Diego, CA) Prism software, using only data from the linear range (acceptor/donor amounts <5) and setting the line to have the correct y-intercept of 0.47. Data collected over a wider acceptor/donor range was fit into a single-order interaction plot using GraphPad Prism.
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Image Analysis: FRET Nanoscopy—The amount of FRET at each pixel was determined by applying our calculations directly on each image using Metamorph software. The backgrounds were determined as above, and then subtracted from each pixel within each image using Metamorph arithmetic functions. Also subtracted from each pixel were the bleedthrough contributions of BFP and GFP from matched pixels to the appropriate green, blue and FRET channels. The bleedthrough-corrected images contain the fluorescence values for BFP, GFP and FRET, from which were calculated the FRET/donor and acceptor/donor ratios at each pixel in the image. Since C/EBPα should assume the same relative intranuclear distribution regardless of whether it is linked to BFP- or GFP, the high correlation coefficients observed for BFP and GFP-linked C/EBPα expressed in the same cell confirmed the precise image registration required for this analysis.

The formula $y = mx + b$ was used to calculate the extent of FRET ($m$) at each pixel in every precisely registered image: $y$ is the FRET/donor ratio at a single pixel, $x$ is the acceptor/donor ratio at the same pixel, and $b$ (the $y$-intercept) is the FRET/donor ratio where acceptor/donor = 0 (i.e. the constant determined from the donor alone control). The above FRET nanoscopy analyses were done in a semi-automated fashion by linking the calculations into a Metamorph journal.

Selection of Peri-Centromeric Regions—In the FRET analysis of cells co-expressing BFP and GFP-labeled C/EBPα, there was no counterstaining with the blue fluorescent Hoechst 33342, normally used to identify peri-centromeric regions. Because of the high correlation of C/EBPα location with Hoechst 33342 staining (Fig. 1), peri-centromeric regions within the nuclei were identified as pixels containing more than 1.2 times the average amount of background-subtracted
GFP-tagged C/EBPα fluorescence. Visually and quantitatively, this corresponded well to pericentromeric regions identified by staining with the blue fluorescent Hoechst 33342 (40). FRET and extent of FRET measurements were compared at and away from the marked pericentromeric regions using the co-localization application of Metamorph. The proportions of pixels showing specific extents of FRET were calculated using Metamorph by measuring the number of pixels of progressively higher slope using sequential threshold values.

Estimates of Inter-Fluorophore Distances Calculated from Relative FRET Slopes—FRET efficiency varies with distance according to the relationship first described by Förster (34):

\[ E = (1 + (r/r_o)^6)^{-1} \]

where \( r \) is the distance between two fluorophores, and \( r_o \) is the distance between two fluorophores at which energy transfer is 50% efficient. Other factors including the interaction kinetics and the rotational orientation of the fluorophore contribute to FRET efficiency and are included in more expansive equations (34,36). The relative slopes from the linear portions of the FRET/Donor versus Acceptor/Donor graphs are a close surrogate measurement of the relative FRET efficiencies (37). Determination of the actual FRET efficiency would depend upon equipment calibrations that equate fluorescence amounts with the amounts of each molecule. However, we can compare the relative FRET efficiencies for two different interactions as the ratio of the slopes \( E_1 \) and \( E_2 \):

\[
\frac{E_1}{E_2} = \frac{1 + (r_2/r_o)^6}{1 + (r_1/r_o)^6}
\]

where \( r_2 \) and \( r_1 \) are the BFP to GFP distances under the two conditions being examined; \( r_o \) for FRET from BFP to GFP is 41.4 Angstroms (33). By assuming hypothetical values for \( r_2 \) anywhere between 40 and 100 Angstroms, we calculated the value of \( r_1 \). We then determined the differences between \( r_2 \) and \( r_1 \) distances over a range of values for \( r_2 \). This indicated the range in
average distances separating the fluorophores used to label the proteins under the two different experimental conditions.

RESULTS

C/EBPα localizes to peri-centromeric heterochromatin—C/EBPα, fused at either its amino (GFP-C/EBPα) or carboxy (C/EBPα-GFP) terminus with GFP, was transiently expressed in mouse pituitary progenitor GHFT1-5 cells. GHFT1-5 progenitor cells contain no endogenous C/EBPα (11,38) and have been used extensively in our laboratories to study the effects of C/EBPα expression on pituitary gene transcription and cellular proliferation (11,18,38,40,41).

Western blotting showed the expressed fusion proteins to be of the appropriate molecular weight (11,18,40) and were expressed, on average, at a level comparable to that of the pituitary-specific transcription factor Pit-1 (19), which is present at low levels within GHFT1-5 cells (11,38,42). The expression level of the GFP-C/EBPα and C/EBPα-GFP proteins also was similar to that of endogenous C/EBPα in other cell types (discussed below).

C/EBPα-GFP (Fig. 1A)(18), GFP-C/EBPα (11) and unfused C/EBPα (40) expressed in GHFT1-5 cells all concentrated at discrete locations within the cell nucleus. In all instances, the regions of concentrated C/EBPα coincided with AT-rich DNA stained by the blue fluorescent dye Hoechst 33342 (Fig. 1A)(11,18,40). The Hoechst 33342-stained structures are common to mouse cell lines and represent peri-centromeric heterochromatin (12,13). We previously confirmed in GHFT1-5 cells that the Hoechst 33342-stained structures surround foci that stained with antibodies against the centromeric kinetochore (11). We also determined that the Hoechst 33342-stained structures are relatively devoid of Br-UTP-labeled, nascent transcripts (11).
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Therefore, C/EBPα expressed ectopically in pituitary progenitor GHFT1-5 cells concentrated at a distinct intranuclear structure, the peri-centromeric heterochromatin.

Counterstaining with an antibody against the molecular chaperone Hsp70 demonstrated that GHFT1-5 cells expressing the C/EBPα fusions with GFP and GHFT1-5 cells not expressing C/EBPα both had low amounts and identical intranuclear distributions of Hsp70 (data not shown). This demonstrated that cells expressing C/EBPα remained healthy and did not recognize the accumulation of ectopically expressed C/EBPα in peri-centromeric heterochromatin as a folding defect to be corrected by co-concentrating Hsp70 at those sites. Together with our data demonstrating that C/EBPα sequestration at peri-centromeric heterochromatin is functionally significant and regulated (18-20)(unpublished data), this strongly argued that the observed subnuclear distribution of C/EBPα was not a consequence of protein aggregation and precipitation into inclusion bodies, as is sometimes observed for other cellular factors (43-45).

C/EBPα point mutants that were expressed to the same level as C/EBPα failed to concentrate at the peri-centromeric heterochromatin (B. Wu, F.S., unpublished data). This further suggested that C/EBPα localization to peri-centromeric heterochromatin cells was not an artifact of C/EBPα over-expression in GHFT1-5 cells. Indeed, endogenous C/EBPα expressed in mouse 3T3-L1 cells upon chemical induction of adipocyte differentiation (46,47), and detected by anti-C/EBPα antibody staining, also targeted to peri-centromeric heterochromatin (Fig. 1B)(10). Two different anti-C/EBPα antibodies (see Experimental Procedures), counterstained with rhodamine-linked secondary antibodies, showed the same co-localization of red, rhodamine fluorescence and blue Hoechst 33342 fluorescence in many induced 3T3-L1 cells. No C/EBPα was detected in uninduced 3T3-L1 cells (Fig. 1B). Therefore, C/EBPα expressed in GHFT1-5
cells takes up the same intranuclear distribution of endogenous C/EBPα in another murine cell type.

The similar peri-centromeric targeting of endogenous 3T3-L1 cell C/EBPα, and C/EBPα-GFP or GFP-C/EBPα ectopically expressed in GHFT1-5 cells, also was confirmed by parallel staining with the same anti-C/EBPα antibody, and the subsequent imaging of differentiated 3T3-L1 cells and transfected GHFT1-5 cells under identical collection parameters. For these comparisons, co-localization was quantified by plotting the amount of background-subtracted red fluorescence (C/EBPα, C/EBPα-GFP or GFP-C/EBPα) in each of the thousands of pixels of each image against the amount of blue fluorescence in the corresponding pixel of the matched Hoechst 33342 image (Fig. 1C). Correlation coefficients were calculated that described the degree by which C/EBPα and Hoechst fluorescence at each pixel varied from a perfect correlation of 1.00. Overall, the correlation coefficients averaged 0.78 +/- 0.14 for endogenous C/EBPα expressed in differentiated 3T3-L1 cells (calculated from 205 separate nuclei), 0.69 +/- 0.16 for GFP-C/EBPα expressed in GHFT1-5 cells (n = 94 nuclei) and 0.70 +/- 0.13 for C/EBPα-GFP expressed in GHFT1-5 cells (n = 89 nuclei). The slightly lower correlations for C/EBPα expressed in GHFT1-5 cells likely are due to the low levels of Pit-1 in these cells; we have observed Pit-1 to interact directly with (20) and re-locate (19) C/EBPα from the heterochromatic to the euchromatic regions of the cell nucleus. By comparison, the correlation coefficients measured for red C/EBPα fluorescence and green GFP-C/EBPα or C/EBPα-GFP fluorescence, which should correlate well since the red and green signals both originate from C/EBPα, were 0.85 +/- 0.07 or 0.88 +/- 0.06, respectively.

Plotting the C/EBPα to Hoechst 33342 correlation coefficients calculated for each nucleus against the average rhodamine (C/EBPα) fluorescence intensity of the same nucleus
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(Fig. 1D) demonstrated that C/EBPα co-localization with Hoechst 33342-stained DNA did not vary with the C/EBPα expression level. Low level, rhodamine fluorescence from GHFT1-5 cells sham-transfected with the empty expression vector, or from undifferentiated 3T3-L1 cells, did not correlate with Hoechst 33342 fluorescence (Fig. 1D). Similarly, rhodamine fluorescence from equivalently expressed, mutant C/EBPα proteins that failed to target to peri-centromeric heterochromatin showed correlation coefficients with Hoechst 33342 fluorescence of zero (unpublished data). Finally, C/EBPα was determined to co-localize strongly with Hoechst 33342-stained DNA within the nuclei of most induced 3T3-L1 cells (94.1% of the nuclei that express C/EBPα have a correlation coefficient >0.5) and of GHFT1-5 cells expressing GFP-C/EBPα (88.3%) or C/EBPα-GFP (89.9%). This analysis also demonstrated that the levels of ectopically expressed GFP-C/EBPα and C/EBPα-GFP studied in our experiments were variable, but globally similar to that of endogenous C/EBPα in 3T3-L1 cells. Therefore, C/EBPα concentrated at peri-centromeric heterochromatin when expressed to physiologic levels in GHFT1-5 cells.

C/EBPα dimerization in living cells—C/EBPα is believed to act primarily as a dimer (26,27), although the extent of dimerization in the physiologic environment is unknown. To measure dimerization in living cells, we fused the cDNA for BFP to the carboxy terminus of the cDNA for C/EBPα and quantified if any fluorescence energy was transferred from the donor (C/EBPα-BFP) to the acceptor (C/EBPα-GFP) when co-expressed (31,35-37). C/EBPα-GFP (11,40) and C/EBPα-BFP (unpublished) activated a C/EBPα-responsive promoter when expressed in GHFT1-5 cells.
Images were captured in three different fluorescence channels from each GHFT1-5 cell expressing C/EBPα-GFP or C/EBPα-BFP alone, or in combination (Fig. 2A): 1) the GFP-specific “acceptor” channel (excitation with light of 480-495 nm; emission collected from 500-530 nm), 2) the BFP-specific “donor” channel (excitation: 365-395; emission 435-465) and 3) the “FRET” channel (excitation: 365-395; emission 500-530). The fluorescence contribution of C/EBPα-GFP to the donor channel was quantified from cells expressing only C/EBPα-GFP as a statistically insignificant 0.0040 +/- 0.0145 of the background-subtracted fluorescence detected in the acceptor channel (n = 97 cells). Similarly, the contribution of C/EBPα-BFP to the acceptor channel was 0.0013 +/- 0.0058 that detected in the donor channel (n = 108 cells). To the FRET channel, C/EBPα-GFP consistently contributed 0.0882 +/- 0.0151 the amount of fluorescence measured in the acceptor channel, whereas C/EBPα-BFP contributed 0.4719 +/- 0.0151 the amount of fluorescence measured in the donor channel. These “spectral cross-talk” ratios are constants that reflect the spectral properties of BFP and GFP and the physical properties of the detection equipment. As such, the ratios are the same in cells expressing low and high amounts of C/EBPα-GFP or C/EBPα-BFP (Fig. 2B) and do not vary from experiment to experiment when using the same detection equipment.

The spectral cross-talk constants were used to determine whether there was energy transfer in cells co-expressing C/EBPα-GFP and C/EBPα-BFP (34,36,37). Background-subtracted donor, acceptor, and FRET signals were quantified from multiple cells co-expressing the labeled proteins. The contribution of acceptor to the FRET channel was calculated using the cross-talk ratio and subtracted from the signal in the FRET channel (see Experimental Procedures). The remaining fluorescence in the FRET channel contained the contribution of the donor C/EBPα-BFP (0.4719 of the amount of corrected blue fluorescence) plus any sensitized
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emissions resulting from the transfer of energy from BFP to GFP. If there were no energy
transfer between C/EBPα-BFP and C/EBPα-GFP, the FRET/donor ratios would remain at the
0.4719 +/- 0.0151 value determined for C/EBPα-BFP alone. If there were energy transfer in cells
co-expressing C/EBPα-BFP and C/EBPα-GFP, the amount of fluorescence in the FRET channel
would increase and the amount of C/EBPα-BFP fluorescence in the blue channel would
decrease, so the FRET/donor ratio would increase.

The average FRET/donor ratio for 212 cells co-expressing C/EBPα-BFP and C/EBPα-
GFP was 1.5750 (Table I), indicating energy transfer and C/EBPα dimerization. As controls, the
FRET/donor ratios of cells co-expressing C/EBPα-BFP and GFP-p300 or GFP-CBP, both of
which co-localize with C/EBPα-BFP at peri-centromeric chromatin, (11) were measured as
0.4777 +/- 0.0334 (n = 23) for p300 and 0.4568 +/- 0.0237 (n = 19) for CBP (Table I). Thus,
C/EBPα interacts specifically with other C/EBPα molecules in living cells.

Relative positions of bZIP and transcription activation domains in C/EBPα dimers—Expression
vectors also were constructed in which GFP and BFP were fused to the TA domains at the amino
terminus of C/EBPα (GFP-C/EBPα and BFP-C/EBPα). The four possible pairwise combinations
of C/EBPα tagged with BFP or GFP at its bZIP or TA domains (Figs. 3A-D) were co-expressed
in GHFT1-5 cells. All four combinations showed FRET/donor ratios greater than 0.47 (Table I)
confirming an in vivo interaction between C/EBPα proteins. There was no indication of FRET
between any combination of C/EBPα-BFP or BFP-C/EBPα and the GFP-p300 or GFP-CBP
(Table I). Thus, specific interactions were observed between the carboxy terminal bZIP domains,
between the amino terminal TA domains, and between the bZIP and TA domains in pituitary
progenitor GHFT1-5 cells. Although the interactions observed may occur in dimers or in higher
order multimers, the term “dimer” is used in the remainder of the text for simplicity and because kinetic considerations, discussed later, indicated a bimolecular interaction.

If a transfected cell expresses more acceptor (GFP-linked C/EBPα) than donor (BFP-linked C/EBPα), a greater proportion of BFP-linked C/EBPα will dimerize with GFP-linked C/EBPα than with another BFP-linked C/EBPα. Thus, FRET increases with increasing amounts of acceptor relative to donor (35,37). This was observed when the FRET/donor ratio was plotted against the acceptor/donor ratio from multiple cells for each combination of GFP-linked C/EBPα with BFP-linked C/EBPα (Figs. 4A-4D). These curves flatten towards a plateau provided enough acceptor is present to saturate interactions with donor (Fig. 5A).

The slopes of the FRET/donor versus acceptor/donor graphs, measured within the predominantly linear range for each combination of GFP and BFP-linked C/EBPα (Table I), reflect the relative “extent of FRET” for each combination at equivalent acceptor/donor ratios. The extent of FRET observed for C/EBPα tagged with BFP and GFP at their bZIP domains was significantly higher than the extents of FRET measured for interactions between the TA domains (p = 1 x 10^{-20}) or between the TA and bZIP domains (p = 5 x 10^{-25} and p = 5 x 10^{-34}). This indicated either that the bZIP domains were closer together than were the TA domains in the C/EBPα dimer, or that the bZIP interactions were kinetically more stable (higher on-rate and/or lower off-rate).

Identical kinetics of interaction between the bZIP and transcription activation domains—To distinguish the contributions of fluorophore distance from interaction kinetics to the different levels of FRET, we examined data collected over a wide range of acceptor/donor ratios for the bZIP to bZIP and TA to TA interactions (Fig. 5A). The ability to collect data over a large range
in acceptor/donor ratios is limited by the necessity of maintaining FRET data collection parameters the same for all cells (see Experimental Procedures). However, from the data collected, the curves clearly followed ($r^2 = 0.94$ and 0.75, respectively) first order interaction kinetics. This single-order interaction kinetics indicated a simple bimolecular interaction between C/EBPα monomers or between two interacting units.

Extrapolation of the first-order curves showed that FRET between the bZIP domains saturated at a much higher FRET/donor ratio (7.6 +/- 0.5) than did FRET between the TA domains (2.5 +/- 0.4). This showed that there was more FRET between the bZIP domains than between the TA domains when kinetic differences were minimized at saturation. In contrast, the curves reached saturation at the same rate: the acceptor/donor ratios at half the maximal FRET/donor levels were not statistically different (42 +/- 4 and 43 +/- 11). These similar “kd” values demonstrated that the interactions between the bZIP and between the TA domains were kinetically identical. Identical kd values are expected if the interaction between the fluorophores is governed only by the dimerization of C/EBPα itself. Thus, the different extents of FRET arose primarily from non-kinetic considerations, which would include differences in the distances separating the fluorophores and/or differences in the rotational orientation of fluorophore dipoles that do not radially emit energy (34,36).

Rotational constraints within the C/EBPα dimer—The extent of FRET between the bZIP and TA domains varied significantly ($p = 0.0005$) if the locations of the donor BFP and acceptor GFP at the TA and bZIP domains were reversed (slopes of 0.059 – 0.077 and 0.097 – 0.110, Table I). Since the average distance between the domains and the kinetics of their interactions would not be changed by swapping the fluorophores, the extent of FRET for the two TA and bZIP
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combinations should, at first glance, be the same. However, energy transfer from many fluorophores is not radial, so the extent of energy transfer also depends upon the orientation of non-radial fluorophores (34,36). Symmetry in the TA to bZIP and bZIP to TA FRET would be observed only if both the donor and acceptor fluorophores were rotating freely in space. The asymmetry in the extent of FRET detected for bZIP to TA domain interactions indicated that one, or both, of the bZIP or TA domains were somewhat constrained in the C/EBPα dimer in living cells. Thus, the different level of FRET observed between the bZIP and between the TA domains potentially includes some contribution from rotational constraints on the fluorophores as well as from different domain distances.

Local variations in C/EBPα dimer conformation within the nucleus—The relative positions and rotations of the bZIP and TA domains, determined using the total fluorescence from each nucleus, represent the average conformation of C/EBPα in the nucleus (Fig. 5B). However, the type of C/EBPα dimers formed, and the ability to from dimers, may vary with subnuclear location. To analyze C/EBPα dimerization at different sites within the nucleus, the extent of FRET was calculated at each pixel from the background-subtracted and bleedthrough-corrected acceptor, donor and FRET images (usually 5,000-10,000 pixels/nucleus for 40x magnification)(Figs. 6A-D). This required that pixels measured in the separate acceptor, donor and FRET channels were perfectly matched.

BFP- and GFP-linked C/EBPα should concentrate at the same locations in each nucleus. There was a linear correlation in the amounts of background- and cross-talk-corrected fluorescence emitted from BFP- and GFP-linked C/EBPα in each of thousands of pixels from each nucleus co-expressing both fusion proteins (correlation coefficients of 0.86 +/- 0.07, n =
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374 cells). This correlation demonstrated that the amount of BFP and GFP fluorescence in each channel emitted by a small number of C/EBPα molecules (roughly estimated to average 5-10 molecules/pixel) was the same, and accurately measured, in matched pixels.

With accurate image registration, we were able to determine local variations in C/EBPα conformation by calculating (see Experimental Procedures) the extent of FRET at each pixel within an image (Figs. 6A-D, FRET Extent). The proportion of pixels containing specific extents of FRET was determined in each cell nucleus, then averaged from multiple nuclei (Fig. 7A). For each of the four different interactions mapped, the extent of FRET distributed around a peak that was very similar to the average extent of FRET measured from all the pixels in the cell (Fig. 7B). This suggested that the conformation of C/EBPα within the nucleus was variable around a single preferred conformation. Note the wider distribution in the extent of FRET for interactions between the bZIP domains, which suggests a broader variation in this interaction at different locations in the cell (see below).

Unique conformations of C/EBPα dimers at peri-centromeric regions—The pixel-by-pixel analysis identified a distribution in C/EBPα conformation, but did not associate those variations with any subnuclear structures. We therefore compared C/EBPα conformation at and away from peri-centromeric regions by measuring the extent of FRET at and away from peri-centromeric regions for each combination of BFP and GFP-tagged C/EBPα. Since Hoechst 33342-stained peri-centromeric regions corresponded to the regions of concentrated C/EBPα (Fig. 1), peri-centromeric heterochromatin was marked in cells co-expressing BFP and GFP-tagged C/EBPα as pixels containing more than 1.2 times the average fluorescence intensity of GFP-linked
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C/EBPα within each nucleus (40). On average, those marked pixels had fluorescence intensities 1.74 +/- 0.28 times more than in the remaining pixels (n = 374 cells).

Comparing the extent of FRET at, and away from, the peri-centromeric regions (Table II) showed that the conformations of C/EBPα were different in these subnuclear domains. The extent of FRET between the bZIP domains at peri-centromeric regions was a statistically significant (p = 1 x 10^{-23}) 0.8954 times that measured away from peri-centromeric regions. This showed that the bZIP domains of C/EBPα dimers were in closer contact away from the peri-centromeric chromatin. Comparison of kd and amount of FRET at acceptor/donor saturation calculated at, and away from, peri-centromeric chromatin indicated that the bZIP regions both interacted less well and were further apart at the peri-centromeric chromatin.

The different peri-centromeric conformation of the bZIP domain was confirmed by statistically decreased extents of FRET (p = 0.001) between the bZIP and TA domains at the peri-centromeric regions (Table II). In contrast, the extent of FRET between the fluorophore-tagged TA domains was not statistically different at and away from the peri-centromeric regions. The extents of FRET between the bZIP and TA domains remained asymmetric at both the peri-centromeric and remaining regions of the nucleus, indicating that the torsional constraints on the dimer were present at both locations. The simplest interpretation is that the bZIP domains were further apart and interacting less well at peri-centromeric regions, and that the TA domains have similar orientations and torsional constraints at and away from peri-centromeric regions (Fig. 5B).

*Incubation with phorbol ester alters C/EBPα dimer conformation—*Physiological environment also may affect C/EBPα dimer conformation. Previously, we found that incubation of pituitary
progenitor GHFT1-5 cells with phorbol 12-myristate 13-acetate (PMA) and forskolin enhanced 
C/EBPα activation of the full-length rat growth hormone (GH) promoter (38). We first 
determined that a GH promoter deleted of all sequences except those surrounding the C/EBPα 
binding site (-239 to -209) and the TATA box (-33 to +8) responded to PMA, and not to 
forskolin, in a C/EBPα-dependent fashion (data not shown). This suggested that C/EBPα, or co-
regulatory factors that cooperate with C/EBPα, were a direct target of phorbol ester activation.

We investigated the effects of PMA induction on C/EBPα conformation by comparing 
the extent of FRET for all combinations of GFP- and BFP-linked C/EBPα in sham-treated cells 
and in cells treated with 10^{-8} M PMA (Figs. 8A-D). Pixel-by-pixel analysis showed the 
interactions between the bZIP domains to be identical in sham- and PMA-treated cells (Fig. 8A), 
even when compared for the different interactions at and away from the peri-centromeric regions 
(not shown). This suggested that the overall contact and distances between the bZIP domains 
were similar regardless of PMA incubation. In contrast, PMA incubation resulted in a 
statistically significant decrease in the extent of FRET between the BFP-tagged TA and GFP-
tagged bZIP domains (Fig. 8C, p = 0.03) and between the BFP-tagged TA and GFP-tagged TA 
domains (Fig. 8D, p = 0.04). For both TA to bZIP and TA to TA FRET, the decreased extent of 
FRET was particularly prominent at the peri-centromeric regions (p = 0.01 for both), but less 
consistent away from the peri-centromeric region (p = 0.06 for both). This suggested that the 
PMA induced alteration in conformation was more uniform for C/EBPα dimers at the peri-
centromeric regions than for dimers away from the peri-centromeric regions. The asymmetric 
effect of PMA incubation on the extent of FRET from TA to bZIP (Fig. 8C), but not from bZIP 
to TA (Fig. 8B) or from bZIP to bZIP, indicated that the change in conformation induced by
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PMA included a torsional rotation of the TA domains relative to each other and to the rest of the dimer (Fig. 5B).

DISCUSSION

C/EBPα forms dimers in living cells—FRET between spectral derivatives of the green fluorescent protein fused to C/EBPα defined that C/EBPα is a dimer in living cells (Fig. 2). For BFP and GFP, FRET is 50% efficient when the donor and acceptor are 41.4 Angstroms apart (33) and falls very rapidly, to the sixth power, as the fluorophores are separated (33,34,36). Thus, energy transfer demonstrated that the fluorophores, and therefore C/EBPα, were well within 80 Angstroms of each other within the cell. These interactions were very specific. No FRET was observed between C/EBPα and the co-activators CBP and p300 (Table I) even though GFP-tagged CBP and p300 co-localized with C/EBPα when expressed in GHFT1-5 cells (11). Both CBP and p300 enhanced transcriptional activation by C/EBPα (11,48), but have never been observed to directly interact with C/EBPα (11,48)(unpublished data). However, C/EBPα expression does cause CBP (11) and p300 (unpublished) to redistribute to the intranuclear location of C/EBPα suggesting that C/EBPα forms some sort of complex with CBP and p300 within the cell.

C/EBPα dimers form throughout the nucleus of living cells—We combined the Angstrom-level resolution of FRET with the resolution of light microscopy (250 nm for green light), to map C/EBPα dimer structure throughout the cell (Figs. 4-8). By changing the positions of the fluorophore tags within C/EBPα, this “FRET nanoscopy” technique was used to define, in living cells, the interactions of the bZIP and TA domains in C/EBPα dimers at localized intranuclear
domains and under different cellular conditions. FRET nanoscopy demonstrated that, in virtually all regions of the nucleus, C/EBPα was positioned sufficiently close to another C/EBPα molecule to allow energy to transfer from the attached BFP to GFP. Thus, intermolecular C/EBPα interactions were spread throughout the cell nucleus and were not excluded from any subnuclear structure.

*C/EBPα conformation varies with subnuclear location and with cellular environment*—The amounts of FRET at individual 100 x 100 nm regions within each nucleus were also calculated taking into account the amounts of donor and acceptor at each localized site (Figs. 6-8). This “extent of FRET” closely approximates the FRET efficiency, which is affected by the relative distance between the attached fluorophores, by the on- and off-rates of the interacting C/EBPα, and by constraints on the rotational freedom of asymmetric donor and acceptor fluorophores imposed by their attachment to C/EBPα (34-36). The pixel-by-pixel measurement of FRET extent therefore defined not only whether factors interact within a cell, but also defined the relative quality of that interaction at localized sites. This allowed us to study the relative positioning and rotational constraints of different functional domains of C/EBPα (Fig. 5B) and to define the subcellular conformations of C/EBPα dimers under different cellular conditions.

The lower extent of FRET at peri-centromeric chromatin may have resulted from a skewing of the FRET measurement by nearby pairs of C/EBPα molecules, not dimerizing, but co-concentrating and weakly interacting at the peri-centromeric chromatin. We are confident that this was not the case since 1) this concentration would have led to a higher extent of FRET at the peri-centromeric chromatin rather than the observed lower extent of FRET; 2) the lower extent of FRET was only observed for interactions involving the bZIP domain: interactions between the
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equally concentrated TA domains showed no change in interaction; 3) no FRET was observed for measurements using the similarly concentrated CBP and p300 indicating the absence of near neighbor effects; 4) C/EBPα was only concentrated 1.74 times, on average, at the peri-centromeric chromatin, which is unlikely to be enough to have resulted in a substantive difference in near neighbor effects. Thus, the different extents of FRET represent true conformational differences in the C/EBPα dimers. Local variations in dimer conformation may reflect the presence or absence of specific C/EBPα-interacting co-factors at the peri-centromeric sites (11,40), or the consequences to C/EBPα dimers of interactions with different chromosomal structures.

*Interdomain distances suggested by FRET*—The distance separating the fluorophores will affect the extent of FRET. To provide an indication of the magnitude in the variations that are detected in readily understood terms, we have calculated what the different extents of FRET would connote, assuming that they arose solely as a function of altered distance. Comparing the relative extents of FRET observed between different C/EBPα domains in equations developed by Förster (34) provides an estimation of the relative fluorophore-to-fluorophore distance for each combination of expressed C/EBPα (see Experimental Procedures). These calculations would suggest that the fluorophores attached to the TA domains in the dimers were spaced, on average, 10.4 – 14.9 Angstroms further apart than were the fluorophores attached to the bZIP domains. These calculations also suggest that the fluorophores attached to the bZIP domains were, on average, 1.2 – 1.8 Angstroms further apart at the peri-centromeric regions than elsewhere in the nucleus. Again, this analysis assumes equivalent interaction kinetics and equivalent rotational
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constraints at, and away from, the peri-centromeric chromatin, which is likely not true. However, the calculations provide an indicator of the sensitivity of the FRET measurements.

It should be remembered that FRET is actually measured between the attached fluorophores, which serve as surrogates for the positions of the specific domains of C/EBPα. Since the GFP-derived fluorophores themselves constitute a complete protein domain, actual distances between the domains vary with the stearic requirements of GFP. However, the relative extents of FRET between the donor and acceptor fluorophore remain a good approximation of the relative conformational parameters, including interdomain distance.

_PMA incubation alters C/EBPα dimer structure_— The enhancement of C/EBPα-activated transcription upon incubation of GHFT1-5 cells with PMA was accompanied by an alteration in C/EBPα dimer structure consisting of a change in the position and/or orientation of the TA relative to the bZIP domain (Figs. 5B, 8). The effects of PMA on C/EBPα conformation may be related to PMA activation of protein kinase C, which is known to phosphorylate C/EBPα (49,50). We have seen an identical PMA-induced alteration in the conformation of C/EBPα measured in another cell type using different donor and acceptor fluorophores (CFP and YFP). This specific change in the position and/or orientation of the TA domains of C/EBPα is associated with a single phosphorylation site in C/EBPα (S. Ross, B. Wu, F.S., O.A. MacDougald, unpublished results).

PMA may also influence C/EBPα conformation by regulating other factors that form complexes with C/EBPα (11), by regulating the C/EBPα-induced alteration in histone acetylation at peri-centromeric regions (40), or by regulating dimerization of C/EBPα with other C/EBP family members (46,51). PMA incubation also may indirectly change the environment of
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the peri-centromeric region to affect C/EBPα dimer conformation. Regardless of the underlying basis, the FRET measurements show that C/EBPα dimer conformation was altered when the intracellular environment was changed.

**FRET nanoscopy complements structural analyses**—FRET nanoscopy will be very useful for determining the structural parameters of many interacting molecules at specific intracellular locations. The structural details of C/EBPα, to date, were limited to the assumption that the last 80 amino acids (the bZIP domain) of the 358 amino acid long C/EBPα were similar to coiled-coil structures identified for other bZIP domains (26,27). This predicted that the carboxy termini of C/EBPα should be in very close proximity. Indeed, our FRET measurements demonstrated that the carboxy termini were considerably closer to each other in living cells than were the other domain interactions that were measured.

The structures measured by FRET also may be affected by the requirements of packing of GFP into C/EBPα. Given the close proximities of the carboxy terminal alpha helices in the predicted C/EBPα dimers, it was somewhat surprising that the C/EBPα-GFP fusions remained transcriptionally active (11,40) and competent to block cellular proliferation in GHFT1-5 cells (18). The fusion proteins containing GFP fused to the amino terminal TA domain of C/EBPα also remained competent to block cellular proliferation (18), but were defective in transcriptional activation (40). Packing constraints imposed by the large GFP fluorophore at the amino terminus even may have contributed to the rotational constraint necessary to detect the PMA-induced change in TA domain conformation, at the peri-centromeric regions. Thus, the large size of the GFP fluorophores may have some unexpected stearic advantages, as well as limitations. The
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development of smaller fluorophore tags (52,53) may reduce, but not eliminate, the stearic consequences of fluorophore tags.

FRET nanoscopy is a powerful tool with which to investigate the structural parameters of interacting molecules at localized sites within living cells. We have already applied FRET nanoscopy to investigate interactions between other molecules (unpublished data). In some instances, FRET nanoscopy has permitted us to observe interactions that are limited to a small percentage of sites within the cell. Certainly, techniques such as two-hybrid interactions also may be employed to investigate such interactions, but the extent of FRET uniquely measures the degree to which seemingly similar interactions are different (37). FRET nanoscopy also correlates those interaction nuances with spatial, and potentially temporal, considerations in living cells. We envisage that FRET nanoscopy will become a central technique with which biochemical interactions and structural parameters may be measured directly in the physiologic environment with unprecedented accuracy and detail.

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FIGURE LEGENDS

FIG. 1. **C/EBPα co-localizes with Hoechst 33342-stained DNA.** A, C/EBPα-GFP expressed after transient transfection into mouse GHFT1-5 pituitary progenitor cells. A parallel image of GHFT1-5 cells sham-transfected with the expression vector lacking the C/EBPα-GFP fusion protein is shown as a control. B, Immunofluorescence imaging of endogenous C/EBPα expressed in mouse 3T3-L1 cells seven days after initiating adipocyte differentiation by incubation with insulin, dexamethasone and isobutylmethylxanthine. A parallel image of 3T3-L1 cells not induced is shown as a control. See Experimental Procedures for antibody-staining and fluorescence detection procedures. C, Comparison of the levels of background-subtracted, rhodamine and Hoechst 33342 fluorescence collected in matched pixels from a single differentiated 3T3-L1 cell nucleus. D, Correlation coefficients derived from pixel-by-pixel comparison of anti-C/EBPα and Hoechst 33342 fluorescence for multiple cell nuclei were calculated and plotted against the average anti-C/EBPα fluorescence amount in each pixel.

FIG. 2. **FRET between BFP- and GFP- attached to the carboxy-terminal bZIP domain in full-length C/EBPα and expressed in GHFT1-5 pituitary progenitor cells.** A, Representative images of nuclei in which C/EBPα-GFP and C/EBPα-BFP were expressed alone or were co-expressed. The amounts of nuclear fluorescence were quantified in the green, blue or FRET channels (shown), then corrected (Experimental Procedures) for background fluorescence and acceptor, donor and FRET from acceptor fluorescence using bleedthrough ratios determined from the control cells (see numbers in images). Energy transfer resulted in an increased FRET emission at the expense of donor emission (FRET/donor) in the co-expressing cells relative to
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the donor alone-containing cells (see Table I). Each channel was presented with identical fluorescence scaling to facilitate the visual comparison of intensity. B, Graphing the bleedthrough ratios against the average intensity fluorescence in each cell showed that the ratios were consistently measured over a wide range of fluorescence amounts. Only cells containing fluorescence amounts within this region of accurate measurement were processed for FRET determination.

FIG. 3. Combinations of BFP- and GFP-labeled C/EBPα that were co-expressed to determine the conformation of C/EBPα dimers in living cells. The different combinations depicted, A-D, are presented in the same order in figures 4, 6 and 8, A-D.

FIG. 4. The extent of energy transfer, normalized for the amount of acceptor and donor, varied with the domains in C/EBPα to which BFP and GFP were attached. FRET/donor ratios increased proportionally with the amount of acceptor relative to donor in cells co-expressing full-length C/EBPα fusions of BFP and GFP attached to different domains in C/EBPα (A-D). The relationship of FRET/donor against acceptor/donor was quasi-linear at lower acceptor/donor levels. The slope of the graphs represented the extent of FRET at normalized acceptor/donor levels for each of the four combinations of bZIP and TA-tagged C/EBPα. There was no FRET between BFP-tagged C/EBPα and either CBP or p300 tagged with GFP (shown as X’s in the graphs). 95% confidence intervals in the slopes of these graphs, calculated for acceptor/donor levels <5 and the y-intercept forced through 0.47, are presented in Table I.
FIG. 5. Relative positions of the transcriptional activation (TA) and helical bZIP domains of C/EBPα in living cells. A, The FRET/donor ratio saturated at a higher level for interactions between the bZIP domains than for interactions between the TA domains. This indicated differences in the position and orientation of the fluorophores attached to the TA and bZIP domains in C/EBPα dimers. The kd’s determined from the interaction curves were identical. B, model of the C/EBPα dimer structure in living cells. The bZIP domains were closer together (double-headed arrow) than were the TA domains. The bZIP domains also were slightly further apart, and interacting less well, at peri-centromeric heterochromatin than in the transcriptionally active regions of the nucleus. The TA domain is under a rotational constraint (curved arrow) that is altered upon incubation of the cells with phorbol 12-myristate 13-acetate.

FIG. 6. Representative background-subtracted and bleedthrough-corrected acceptor, donor and FRET images from cells co-expressing BFP and GFP fusions with full-length C/EBPα. Images presented contain similar amounts of acceptor (GFP) and donor (BFP) fluorescence for each co-expression combination (A-D). Fluorescence scaling was identical for each channel to facilitate visual comparisons of intensity. FRET normalized for acceptor and donor amounts (FRET extent) was calculated at matched pixels from each image (see Experimental Procedures).

FIG. 7. Variations in C/EBPα dimer conformation. A. The proportion of pixels within the nuclei containing particular extents of FRET (see Fig. 6) were averaged from multiple cells co-expressing full-length C/EBPα fusions of BFP and GFP attached to different domains in C/EBPα (see Fig. 3A-D). B. Extent of FRET averaged from all of the pixels of the same nuclei. The average extent of FRET was similar to the extent of FRET with the highest proportion of
pixels indicating a relatively normal variation in FRET efficiency for each of the four aspects of C/EBPα dimer conformation investigated.

FIG. 8. **Incubation with the phorbol ester PMA alters the rotational position of the TA domain relative to the rest of the C/EBPα dimer.** PMA incubation had no effect on the extent of FRET measured for A, BFP and GFP fused to the bZIP end or B, BFP fused to the bZIP end and GFP fused to the TA end. In contrast, PMA incubation decreased significantly the extent of FRET C, between GFP fused to the bZIP end and BFP fused to the TA end, and D, between BFP and GFP fused to the TA ends. The asymmetric effect of PMA on TA to bZIP (C,) and TA to TA (D,) FRET indicated that the effect of PMA incubation on C/EBPα dimer conformation included an alteration in the torsional constraints of the donor fluorophore around the TA domain.
C/EBPα dimer conformation varies locally in living cells

### TABLE I. FRET/Donor and Slope Values for Indicated Donor Alone Controls and Combinations of GFP- and BFP-linked C/EBPα (mean +/- sd of n cells)

|                | C/EBPα-BFP alone | BFP-C/EBPα alone | C/EBPα-BFP+ C/EBPα-GFP | BFP-C/EBPα+ C/EBPα-GFP | BFP-C/EBPα+ GFP-C/EBPα |
|----------------|------------------|------------------|-------------------------|-------------------------|-------------------------|
| FRET/Donor     | 0.4719 +/- 0.0151 (n=108) | 0.4698 +/- 0.0289 (n=175) | 1.5750 +/- 0.9558 (n=212) | 0.9317 +/- 0.3749 (n=155) | 0.9150 +/- 0.3042 (n=129) |
| Slope* (95% CI)| 0.165 to 0.189    | 0.059 to 0.077    | 0.097 to 0.110           | 0.061 to 0.075           |                         |

|                | C/EBPα-BFP+ GFP-p300 | C/EBPα-BFP+ GFP-CBP | BFP-C/EBPα+ GFP-p300 | BFP-C/EBPα+ GFP-CBP |
|----------------|----------------------|---------------------|----------------------|---------------------|
| FRET/Donor     | 0.4777 +/- 0.0334 (n=23) | 0.4568 +/- 0.0237 (n=19) | 0.4612 +/- 0.0356 (n=38) | 0.4535 +/- 0.0390 (n=21) |

*Slope calculated with Acceptor/Donor ratios <5 and Acceptor/Donor = 0 as 0.47

### TABLE II. Relative Extents of FRET At, and Away From, Peri-Centromeric Regions (Areas of Concentrated C/EBPα; mean +/- sd of n cells)

|                | C/EBPα-BFP+ C/EBPα-GFP | C/EBPα-BFP+ GFP-C/EBPα | BFP-C/EBPα+ C/EBPα-GFP | BFP-C/EBPα+ GFP-C/EBPα |
|----------------|------------------------|------------------------|------------------------|------------------------|
| Extent of FRET at Concentrated C/EBPα | 0.2046 +/- 0.0515 (n=64) | 0.0758 +/- 0.0339 (n=32) | 0.1054 +/- 0.0314 (n=74) | 0.0847 +/- 0.0327 (n=24) |
| Extent of FRET away from Concentrated C/EBPα | 0.2277 +/- 0.0522 | 0.0802 +/- 0.0315 | 0.1095 +/- 0.0300 | 0.0814 +/- 0.0292 |
| Extent of FRET at / away from Concentrated C/EBPα | 0.8954 +/- 0.0490 * | 0.9330 +/- 0.0833 * | 0.9570 +/- 0.0678 * | 1.0349 +/- 0.0804 |

*p<0.01, paired two-tailed t-tests comparing “at” and “away” extents of FRET
Fig. 1, Schaupfle et al
Fig. 2, Schaufele et al.

Fig. 3, Schaufele et al.
Fig. 4, Schaufele et al
**Fig. 5, Schaufele et al.**

**Fig. 6, Schaufele et al.**
Fig. 7, Schaufele et al
Fig. 8, Schaufele et al
Conformation of CCAAT/enhancer binding protein alpha dimers varies with intranuclear location in living cells
Fred Schaufele, Xia Wang, Xiaowei Liu and Richard N. Day

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