Compartmentalization of androgen receptor protein–protein interactions in living cells

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Steroid receptors regulate gene expression in a ligand-dependent manner by binding specific DNA sequences. Ligand binding also changes the conformation of the ligand binding domain (LBD), allowing interaction with coregulators via LxxLL motifs. Androgen receptors (ARs) preferentially interact with coregulators containing LxxLL-related FxxLF motifs. The AR is regulated at an extra level by interaction of an FQNLF motif in the N-terminal domain with the C-terminal LBD (N/C interaction). Although it is generally recognized that AR coregulator and N/C interactions are essential for transcription regulation, their spatiotemporal organization is largely unknown.

We performed simultaneous fluorescence resonance energy transfer and fluorescence redistribution after photobleaching measurements in living cells expressing ARs double tagged with yellow and cyan fluorescent proteins. We provide evidence that AR N/C interactions occur predominantly when ARs are mobile, possibly to prevent unfavorable or untimely cofactor interactions. N/C interactions are largely lost when AR transiently binds to DNA, predominantly in foci partly overlapping transcription sites. AR coregulator interactions occur preferentially when ARs are bound to DNA.

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Abbreviations used in this paper: AR, androgen receptor; ARE, androgen response element; BrUTP, 5-bromo-uridine-5′-triphosphate; DBD, DNA binding domain; FRET, fluorescence resonance energy transfer; LBD, ligand binding domain; NTD, N-terminal transactivation domain; SR, steroid receptor.

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transiently bind to immobile elements in the nucleus. This transient immobilization was most likely due to DNA binding, as several non–DNA binding mutants were freely mobile and did not show a detectable immobile fraction (Farla et al., 2004, 2005). In addition, a recent elegant study using ARs double tagged at the N and C termini with the FRET couple CFP and YFP, respectively, has revealed that N/C interactions are initiated promptly after the addition of hormone, before transport to the nucleus (Schaufele et al., 2005). However, questions regarding the spatiotemporal organization of AR in the nuclei of live cells remain unanswered: when, where, and in what order do interactions with coregulators and N/C interaction take place once an AR has entered the nucleus? Does proper regulation of AR function require compartmentalization of such interactions? In this study, we applied innovative combined FRAP and FRET methodology, and ratio imaging, using CFP and YFP tagging of wild-type ARs and AR mutants, to investigate the spatiotemporal regulation of AR N/C interactions and AR coregulator interactions in living cells.

**Results**

**ARs double tagged with CFP and YFP are functional**

We tagged the fluorescent proteins YFP and CFP to the N and C termini of wild-type AR (YFP-AR-CFP) and to two mutant ARs: an N/C interaction–deficient mutant in which the N-terminal FQNL motif is changed into an AQNAA motif (AR[F23,27A/L26A]), and the non–DNA binding mutant carrying a point mutation in the DBD, leading to the inability of this mutant to bind to androgen-regulated promoters (AR[A573D]; Fig. 1 A).

**FRET in double-tagged YFP-AR-CFP represents AR N/C interaction**

We then investigated whether the double-tagged YFP-AR-CFP provided a bona fide tool to study N/C interaction by FRET.

![Figure 1](http://www.jcb.org/cgi/content/full/jcb.200609178/DC1)
The FRET readout system applied was based on photobleaching of the acceptor and measuring the subsequent increase of the donor (abFRET; Bastiaens and Jovin, 1996; Bastiaens et al., 1996; Kenworthy, 2001; Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200609178/DC1). In the presence of R1881, cells with a low expression (Fig. S1) of either the wild-type YFP-AR-CFP or the non–DNA binding mutant YFP-AR(A573D)-CFP showed a considerable increase in CFP fluorescence after acceptor bleaching, whereas only a small increase was observed in the N/C interaction–deficient mutant YFP-AR(F23,27A/L26A)-CFP (Fig. 1 E). In addition, abFRET was not observed in the absence of agonistic ligand (Fig. S2 B). These data indicate that the measured abFRET represents interaction of the FQNLF motif in the AR NTD with the ligand induced groove in the LBD. This was further corroborated by in vitro spectroscopy showing that FRET was strongly reduced by the addition of FQNLF peptide motifs, which compete with the AR N terminus for interaction with the C-terminal LBD, in lysates of cells expressing YFP-AR-CFP (Fig. 1 F). This reduction in FRET signal was not observed when, instead of FQNLF motifs, noncompeting LQNL peptide motifs were added to the lysates (Fig. 1 F), confirming that the observed FRET is due to N/C interaction. Finally, extending previous data (Schaufele et al., 2005), confocal time-lapse microscopy of living cells stably expressing YFP-AR-CFP showed that the YFP/CFP ratio considerably increased immediately after the addition of hormone, followed by efficient translocation to the nucleus (Fig. 1 G). In contrast, the N/C interaction–deficient mutant YFP-AR(F23,27A/L26A)-CFP showed only a small increase in YFP/CFP ratio (Fig. S3). Based on these data, it can be concluded that the FRET measured in the double-tagged YFP-AR-CFP represents N/C interaction.

Simultaneous FRAP and FRET enables analysis of the mobility of interacting molecules

We developed a method based on simultaneous measurement of FRAP and FRET to study the mobility of interacting molecules. In this method, FRET-donor (CFP) and FRET-acceptor (YFP) fluorescence are simultaneously measured at regular time intervals after irreversibly photobleaching the acceptor in a defined subregion of the nucleus. Donor fluorescence increase after acceptor photobleaching and subsequent decrease because of diffusion (donor-FRAP) reflects the mobility of only the interacting molecules (Fig. 2 A). In contrast, acceptor fluorescence redistribution after acceptor bleaching (acceptor-FRAP) reveals the mobility of the total pool of both interacting and noninteracting molecules, similar to a conventional FRAP experiment (Houtsmuller et al., 1999; Houtsmuller and Vermeulen, 2001). Importantly, comparison of donor-FRAP and acceptor-FRAP curves allows us to distinguish the mobility (and immobilization) of the subpopulations of interacting and noninteracting proteins.

First, the method was validated in Hep3B cells expressing either a CFP-YFP fusion protein or separate CFPs and YFPs (Fig. 2, B and C). In brief, a narrow strip spanning the nucleus was scanned at 458 nm excitation with short intervals (100 ms) at low laser power (YFP is sufficiently excited at this wavelength; Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200609178/DC1). Fluorescence intensities of the donor (CFP) and acceptor (YFP) were recorded simultaneously. After 40 scans, a high-intensity, 100-ms bleach pulse at 514 nm was applied to specifically photobleach YFPs inside the strip (CFP was not bleached by the bleach pulse; Fig. S4 B). Subsequently, scanning of the bleached strip was continued at 458 nm at low laser intensity. Acceptor (YFP) fluorescence in the strip was considerably reduced after bleaching and recovered at a velocity expected (Farla et al., 2005) for molecules the size of the fusion proteins (Fig. 2, B and C). In parallel, donor fluorescence in the bleached strip increased immediately after acceptor bleaching and decreased at a similar rate compared with the increase of YFP fluorescence (Fig. 2). The observed CFP increase and...
subsequent decrease was not due to an artifact of YFP or CFP fluorescent properties, as cotransfected separate YFPs and CFPs, as well as ARs tagged with YFP or CFP only, did not show a donor-FRAP signal (Fig. 2 B and Fig. S4).

AR N/C interactions are abolished when ARs are bound to DNA

We performed simultaneous FRAP and FRET experiments to investigate the AR N/C interaction. As a control experiment, we tested an AR tagged at the N terminus with the CFP-YFP fusion protein. FRET will occur in these fusion proteins independent of the N/C interaction, as CFP and YFP are always in proximity. Donor-FRAP and acceptor-FRAP of CFP-YFP-AR both showed the same redistribution kinetics (Fig. 3, A and B), which are slower than that of the CFP-YFP fusion alone (Fig. 2 C) because of transient binding to DNA of wild-type ARs (Farla et al., 2004, 2005; Fig. 3, A and B). In sharp contrast, donor-FRAP of the two-sided double-tagged YFP-AR-CFP (representing solely the mobility of N/C-interacting ARs) was considerably faster than the corresponding acceptor-FRAP (representing the mobility of the total AR pool; Fig. 3 C). The difference between donor-FRAP and acceptor-FRAP was not observed for the double-tagged non–DNA binding AR mutant (YFP-AR[A573D]-CFP; Fig. 3 D). Moreover, the YFP-AR-CFP donor-FRAP curve (Fig. 3 C) showed fast kinetics similar to both donor-FRAP and acceptor-FRAP curves of the non–DNA binding AR mutant (Fig. 3 D). These data strongly suggest that N/C interactions of the wild-type AR occur mainly in the mobile pool and are abolished when ARs are transiently immobilized in a DNA-binding-dependent fashion.

AR N/C interaction is reduced inside speckles

To further explore the observation that N/C interaction is reduced when ARs are transiently immobilized, we determined the spatial distribution of N/C-interacting and non–N/C-interacting ARs by high-resolution confocal ratio imaging of YFP-AR-CFP. Because YFP and CFP are present in the same quantity in cells expressing YFP-AR-CFP protein, these can be analyzed by straightforward ratio imaging. In brief, ratio images of cells expressing YFP-AR-CFP, CFP-YFP-AR, and the non–DNA binding YFP-AR(A537D)-CFP were obtained by calculating for each pixel the ratio between the YFP and CFP emission intensity. Subsequently, the nuclei were divided into three areas based on the mean fluorescence intensity of the entire nuclear area and corresponding standard deviation. In YFP-AR-CFP images, pixels with intensities higher than the mean plus two times the standard deviation (4.1% of total area; Fig. 4 A, red bars) coincided largely with the area that is usually referred to as a speckled or focal pattern, whereas pixels with lower intensities coincided largely with the region outside the speckled pattern (Fig. 4, A and B; for image analysis, see Materials and methods). The mean YFP/CFP ratio in each region was then calculated and expressed relative to the mean ratio in corresponding regions in CFP-YFP-AR with a similar intensity (see Materials and methods). Cells expressing CFP-YFP-AR provide an ideal control to correct for potential imaging artifacts because the ratio should be independent of AR folding and absolute fluorescence intensity. The wild-type YFP-AR-CFP showed a significantly reduced YFP/CFP ratio in the speckles compared with the region outside the speckles (Fig. 4 E; P = 0.0002; see Materials and methods), whereas no correlation is found for the non–DNA binding YFP-AR(A537D)-CFP, which showed a homogeneous distribution (Fig. 4, C, D, and F). Apparently, the concentration of non–N/C-interacting ARs is highest inside speckles.

Figure 3. Simultaneous FRAP and FRET measurements in Hep3B cells expressing CFP-YFP-AR or wild-type or mutant YFP-AR-CFP. (A and B) Donor-FRAP (red line) and acceptor-FRAP (blue line) curves of ARs tagged at the N terminus with the CFP-YFP fusion (CFP-YFP-AR) also show similar redistribution kinetics, but slower than the CFP-YFP fusion (Fig. 2; n = 30). (C) Donor-FRAP (red line) and acceptor-FRAP (blue line) recorded in Hep3B cells expressing YFP-AR-CFP. The donor-FRAP curve (representing the mobility of N/C-interacting ARs only) shows faster recovery than the corresponding acceptor-FRAP curve (representing mobility of the total pool of AR; n = 45). (D) Donor-FRAP (red line) and acceptor-FRAP (blue line) curves of the non–DNA binding YFP-AR(A537D)-CFP are rapid and similar to each other and to the donor-FRAP curve of YFP-AR-CFP (C), suggesting that N/C interactions occur only when ARs are mobile (n = 45). The curves in B, C, and D were normalized by calculating

\[ \text{norm} = \frac{I_{\text{norm}} - I_0}{I_{\text{norm}} - I_{\text{final}}} \]

where \( I_0 \) and \( I_{\text{final}} \) are the fluorescence intensities immediately after the bleach and after complete recovery, respectively.
inside speckles, prompted us to investigate whether the AR speckled pattern is correlated to the distribution of sites of active transcription. Previously, it was shown, using 5-bromo-uridine-5'-triphosphate (BrUTP) incorporation in nascent RNA and immunofluorescence (Jackson et al., 1993; Wansink et al., 1993), that progesterone receptor (Arnett-Mansfield et al., 2007), glucocorticoid receptors (Van Steensel et al., 1995), and several other transcription factors (BRG1, TFIIH, Oct1, and E2F-1; Grande et al., 1997) do not show a complete, but rather a partial, overlap with active sites of transcription (nascent RNA). Using the same approach (see Materials and methods), we were able to detect sites of transcription in Hep3B cells stably expressing GFP-AR at physiological levels (Farla et al., 2005). Newly incorporated BrUTP was detected by immunofluorescence using Cy3, which is excited at 543 nm excitation, and GFP-AR was detected by 488 nm excitation. 60 dual channel images were recorded at a configuration at which no cross talk occurred (Fig. 5, A and B).

Interestingly, visual analysis showed only a partial overlap between the AR speckles and sites of active transcription (Fig. 5, C [right] and D [closed vs. open arrows]). We quantified this observation by image analysis in which AR speckles and areas of active transcription were identified based on the mean fluorescence intensity of the entire nuclear area and corresponding standard deviation. Similar to the ratio imaging analysis (Fig. 4), where we used the same procedure to identify AR speckles (see the previous paragraph), pixels in the GFP-AR image with intensities higher than the mean plus two times the standard deviation coincided largely with AR speckles (Fig. 5 E, left and right). In the Cy3-labeled BrUTP image, pixels with intensities higher than the mean plus two times the standard deviation were defined to be hot spots of transcription (Fig. 5 E, middle and right). The centers of, on average, 110 AR speckles and 130 hot spots of transcription per nucleus were then determined. Subsequently, the distances between each AR speckle and the closest hot spot of transcription were determined and compared with a randomly distributed set consisting of an equal number of spots with the same size distribution as the measured hot spots of transcription, taking care that the random spots were not in the nucleoli or outside the nucleus. The number of AR speckles at relatively short distance (<350 nm; Fig. 5 F, first five columns) to the nearest BrUTP spot was significantly higher compared with what is expected on the basis of a random distribution.

Figure 4. YFP/CFP ratio imaging on Hep3B cells expressing wild-type or mutant YFP-AR: CFP. (A) Fluorescence intensity distributions of nuclei expressing wild-type YFP-AR: CFP. For FRET analysis, the histograms were used to subdivide the nucleus in three areas based on mean intensity (μ) and standard deviation (σ): pixel intensity I < μ + σ (black bars; 81.3% of total area), μ + σ < I < μ + 2σ (green bars; 14.6%), and I > μ + 2σ (red bars; 4.1%). (B, top) Confocal images of the nuclei corresponding to the histograms in A. Bar, 5 μm. (middle) Same nuclei (without background and regions with I > μ + 2σ indicated in red). (bottom) Regions with μ + 2σ < I < μ + 2σ indicated in green. Using the relative intensity threshold μ + 2σ specifically selects high-intensity regions that coincide largely with the well-described nuclear foci that give rise to a speckled pattern (Farla et al., 2005). (C and D) Intensity distribution and confocal image of a nucleus expressing non–DNA binding mutant AR(A573D). Although pixels with an intensity > μ + 2σ are present, these are randomly distributed throughout the nucleus and do not form aggregates or speckles. Contrast and brightness of the AR(A573D) images are digitally enhanced for visualization purposes, not for analysis. (E and F) YFP/CFP ratio of cells expressing wild-type and non–DNA binding mutant YFP-AR(A573D)-CFP in the different relative pixel intensity categories (data are mean ± SEM of 100 and 20 cells measured in three and two independent experiments, respectively). Ratios in each category were normalized to corresponding categories measured in cells expressing CFP-YFP-AR with similar intensity. In wild-type AR (E), a lower YFP/CFP ratio is observed in the regions with higher intensity, indicating the loss of N/C interaction in speckles (*, P = 0.0002). This is not found for the AR(A573D) (F).
distribution (43 ± 5 measured vs. 24 ± 2 random spots; P = 0.00025; Fig. 5 F). Moreover, the largest relative difference between measured and random was highest at the closest detectable distance. In addition, the number of AR speckles that showed overlap with the nearest hot spot of transcription was significantly higher than expected when there would be no correlation between AR and nascent RNA distributions (P = 5.0 × 10⁻⁸; Fig. 5 G).

ARA54 cofactor fragments preferentially interact with DNA-bound ARs

The strongly reduced N/C interaction in the transient immobile AR fraction led us to hypothesize that AR coregulators containing FxxLF motifs may gain access more easily to this fraction, as no competition with the N-terminal AR FQNLF motif is expected to occur. We tested this hypothesis using YFP-tagged fragments of the cofactor ARA54, containing an FNRLF motif. ARA54 and ARA54 fragments containing the FNRLF motif were previously shown to display a strong interaction with the AR LBD (Kang et al., 1999; He et al., 2002; Van de Wijngaart et al., 2006).

In agreement with this hypothesis, abFRET between the single-tagged wild-type AR-CFP and YFP-ARA54 fragments was significantly higher (P = 0.003; see Materials and methods) than that of the non–DNA binding mutant AR(A573D)-CFP with YFP-ARA54, suggesting that interactions between AR and ARA54 fragments are significantly enhanced when ARs are bound to DNA (Fig. 6 A, left). To further test the hypothesis that AR N/C interactions are responsible for blocking coregulator interactions, we performed the same experiment using the N/C interaction-deficient mutant AR(F23,27A/L26A)-CFP. In contrast to wild-type AR, no difference in FRET with the ARA54 fragments was observed for the mutant and its non–DNA binding variant AR(F23,27A/L26A/A573D)-CFP. Moreover, FRET was higher than that of the N/C interaction-proficient wild-type ARs (P = 0.042) and much higher than the N/C interaction-proficient non–DNA binding mutant (Fig. 6 A, left). No FRET was found between any of the AR mutants and free YFP (Fig. 6 A, right). These data are in agreement with a model in which YFP-ARA54 fragments bind preferentially to ARs lacking N/C interaction, i.e., either N/C interaction-deficient AR(F23,27A/L26A) mutants

Figure 5. AR speckles and hot spots of transcription. (A) Distribution of GFP-AR (green) and sites of BrUTP incorporation (red) in stably transfected Hep-3B cells (Farla et al., 2004). Sites of BrUTP incorporation were visualized by immunofluorescence. (B) The fluorescent signals were monitored by sequential imaging of the GFP and Cy3 channels using confocal microscopy at a configuration at which no cross talk of signals occurred. (C) Confocal images of a fixed Hep3B cell that stably expressed GFP-AR (green) and shows incorporated BrUTP staining (red). A partial overlap of the AR speckled pattern with sites of transcription can be seen (right and insets). White lines indicate the position of the line scans in D. Bars, 5 μm. (D) Line scans at the indicated position in C of the AR (green) and the BrUTP signal (red). Some but not all peaks coincided, indicating partial colocalization of some of the AR speckles with sites of transcription. Closed arrows indicate coinciding peaks, and open arrows indicate AR speckles without a colocalized transcription site. (E) Images of AR and BrUTP thresholded similar to YFP-AR-CFP in Fig. 4. In both the GFP-AR (GFP; green) and BrUTP (Cy3; red) channels, regions with an intensity I > μ + 2σ and are indicated (two left panels). A merged image of the selected regions in both channels (right) shows the partial overlap (white) in the regions with an intensity I > μ + 2σ. The insets represent the same regions as in C. (F) Distribution of distances between AR speckles and the nearest BrUTP spot (light gray bars) or randomly distributed spots (dark gray bars; n = 68). The number of AR speckles at relatively short distance (<350 nm) to the nearest BrUTP spot was significantly higher than expected on the basis of random distribution (P = 0.00025) and highest at the closest detectable distance. (G) Mean number of AR speckles overlapping with the nearest UTP spot (n = 68). The number of AR speckles partially overlapping BrUTP spots is larger than expected on the basis of a random distribution (P = 5.0 × 10⁻³).
or wild-type ARs transiently immobilized as a result of DNA binding.

To investigate this more extensively, we repeated the simultaneous FRAP and FRET measurements in living Hep3B cells expressing YFP-AR-CFP, now in the presence of cotransfected YFP-ARA54 fragments. The addition of YFP-ARA54 fragments considerably reduced the kinetics of the donor-FRAP curve compared with YFP-AR-CFP in absence of YFP-ARA54 fragments (Fig. 6 B). This is explained by the fact that in this experimental setup not only the N/C-interacting mobile ARs, but also the non–N/C-interacting immobile ARs, show FRET, now between AR C-terminal domain and the YFP-ARA54 fragments (which binds to the C-terminal domain instead of the YFP-tagged N-terminal domain of immobile YFP-AR-CFP). This indicates that the ARA54 fragments preferably interact with the C terminus of the AR when it is transiently immobilized because of DNA binding when the N-terminal FQNLF motif does not compete for interaction with the C-terminal domain.

In summary, the abFRET data (Fig. 6 A) show that ARA54 fragments interact more frequently with wild-type AR than with the non–DNA binding mutant. The simultaneous FRAP and FRET analysis (Fig. 6 B) suggests that this is because ARA54 fragments gain access more easily to the C-terminal domain of the wild-type ARs when there is no, or less, competition with the NTD. This occurs either when wild-type ARs are transiently immobilized in a DNA binding–dependent manner (Fig. 3 C) or when the N/C interaction is disrupted (Fig. 6 A).

### Discussion

Activity of SRs is not only regulated by ligand binding but also by interacting cofactors. The best-described binding site for SR coregulators is the hydrophobic cleft in the LBD to which LxxLL motifs can bind. The AR LBD is unique in its preference for the interaction with cofactors carrying FxxLF motifs rather than LxxLL motifs (Dubbink et al., 2004; Hur et al., 2004). The AR itself also contains an FQNLF motif in the N-terminal domain, enabling interaction with the LBD (N/C interaction; Doesburg et al., 1997; He et al., 2000). The potential competition between the AR N-terminal FQNLF motif and similar motifs in cofactors for interaction with the LBD raises questions regarding the role of the N/C interaction in orchestrating cofactor interactions. To study AR N/C interactions in living cells, we tagged the AR at the N and C termini with YFP and CFP, respectively, or with CFP alone, and applied FRET and simultaneous FRAP and FRAP experiments. In addition, to investigate cofactor interactions, we tagged ARA54 fragments containing an FNRLF motif with YFP. The presence of the tags had no effect on AR localization and hormone-induced nuclear translocation (Fig. 1, D and G) and only limited effect on the transactivation function of the AR (Fig. 1 C). Acceptor photobleaching FRET assays on living cells and in vitro competition experiments using FxxLF- and LxxLL-peptide motifs demonstrated that FRET represents N/C interaction (Fig. 1, E and F).

Previously, using FRAP assays, we and others have shown that the mobility of ARs is reduced compared with the mobility of non–DNA binding AR(A573D) mutants (Farla et al., 2004), as well as antagonist-bound ARs (Farla et al., 2005). In addition, the observed hormone-induced slow down of AR mobility was always accompanied by the formation of a speckled distribution pattern in the nucleus, suggesting that ARs transiently immobilize in speckles. We have now shown using combined FRET and FRAP analysis that, surprisingly, the mobility of the pool of N/C-interacting ARs is not reduced in the presence of hormone and that, consequently, the pool of non–N/C-interacting ARs is responsible for the observed overall slow down of AR mobility. This suggests that the N/C interaction is largely lost when ARs are transiently immobilized, most likely because of DNA binding (Fig. 3 C). This was confirmed by high-resolution ratio imaging showing that FRET is reduced inside speckles (Fig. 4 E).

The loss of N/C interaction in immobilized ARs suggests that the C-terminal hydrophobic groove, to which FxxLF motifs...
can bind, is optimally accessible for coregulators when the ARs are bound to DNA. Our acceptor bleaching FRET experiments on YFP-tagged FNRLF fragments of the AR cofactor ARA54 and AR-CFP provide evidence that strongly supports this view. First, the experiments indicate that ARA54 fragments interact more frequently with the wild-type AR than with the non–DNA-binding AR mutants (A573D), whereas the non–N/C-interacting mutants of DNA binding and non–DNA binding ARs do not show this difference and interact more frequently than any of the N/C interaction–proficient ARs (Fig. 6 A). Moreover, when YFP-tagged ARA54 fragments are coexpressed with YFP-AR-CFP in a simultaneous FRET and FRAP assay, the mobility of the N/C-interacting pool is reduced (Fig. 6 B). This indicates that on top of the mobile N/C-interacting ARs, the immobile double-tagged ARs now show FRET because of their interaction with the YFP-tagged ARA54 fragments. The observed loss of N/C interaction in immobile ARs and frequent interactions of cofactor fragments with immobile ARs are in line with a scenario in which the AR itself dynamically regulates the time and place of interactions with coregulators by blocking the groove using its N-terminal FQNLNF motif when not associated to DNA and allowing access of coregulators only after DNA binding (Fig. 6 C).

Because our data suggest that DNA binding occurs in speckles, the question arose whether these speckles also represent sites of active transcription. To investigate this, we performed BrUTP incorporation experiments on Hep3B cells stably expressing AR-GFP. Interestingly, visual as well as statistical analysis showed that although speckles are closer to sites of active transcription than expected on the basis of a random distribution, the question of what happens in speckles remains. It has frequently been suggested that many transcription factors, and other nuclear factors involved in DNA metabolism, bind transiently to DNA also at nonspecific sites, thereby scanning the DNA (Phair et al., 2004; M étivier et al., 2006). Possibly the majority of immobile ARs observed in our experiments are involved in such scanning activity. The interaction with cofactors may then play a role in identifying specific binding sites when encountered during scanning. In addition, it is not excluded that part of the speckles represents some sort of storage site. However, as non–DNA-binding mutants do not form speckles and move freely through the nucleus, such a model suggests that the DBD is also involved in storage.

In conclusion, we have used a novel combination of FRAP and FRET to investigate interactions of the AR in living cells and provided evidence that AR N/C interactions are involved in the spatiotemporal regulation of interactions with coregulators. The FRET/FRAP assay provides a novel tool to separately investigate the dynamics of interacting and noninteracting molecules. This opens up a multitude of possibilities to investigate the molecular mechanisms underlying not only the regulation of gene transcription but also that of other DNA transacting systems, such as DNA repair and replication.

Materials and methods

Constructs

The cDNA construct encoding N-terminally YFP-tagged AR was generated by replacing EGFP in pGFP-[GA]_6-AR (Farla et al., 2004) by EYFP-C1 (CLONTECH Laboratories, Inc.). The C-terminally CFP-tagged AR was generated by replacing EGFP by ECFP-N3 in pAR-[GA]_6-EGFP in which two AR fragments from pcDNA-AR0mcs (lacking the AR stop codon; Sui et al., 1999) and pAR0 (B rinkmann et al., 1989), respectively, were sequentially inserted in EGFP-N3 (CLONTECH Laboratories, Inc.) followed by the introduction of a spacer sequence coding for a [Gly-Ala]_5 stretch. The construct code for double-tagged AR [pYFP-[GA]_6-AR-[GA]_6-CFP] was generated by combining a fragment of N-terminally YFP-tagged AR pYFP-[GA]_6-AR with a fragment of C-terminally CFP-tagged AR pAR-[GA]_6-CFP. The F23,27A/L26A variants were generated by QuikChange (Stratagene) mutagenesis using primers 5′-AACCCTAGGAGACCTGTCACTGAAGCTCCACAGGAGCAGCACGACGCGGTAATGCCGTCTCCATCGTCATGTGTGGTAAT-3′ and 5′-TCCGGCAGCTCTGTGCAACATGCTCTGCGCCGCTGAGTCGAGGTG-3′. To generate the A573D variant, the AR DBDs of pYFP-AR-CFP and pAR-[GA]_6-CFP were replaced by a pCFP-AR(A573D) (Farla et al., 2004) fragment containing the AR DBD (A573D) mutation. EYFP in pYFP-[GA]_6-AR was replaced by an ECFP/EYFP fusion to obtain pCFP-EYFP-[GA]_6-AR. The YFP-tagged ARA54 peptide construct was obtained by annealing the primers 5′-GATCAGCCCTGGTACCACGTCCAAGTTCAGTACACCGGCTGTGGTGTTGCAAGCTGAAAAC-3′ and 5′-AATCACCACACTCCTACCATTTTCGATAGACGAAAGTGAAAGCTGGTGGTGG-3′ containing the FNRLF motif and inserting the fragment in pEYFP-C2 (CLONTECH Laboratories, Inc.). Structures of novel constructs were verified by appropriate restriction digests and by sequencing.

Cell culture, transfections, and luciferase assay

2 d before microscopic analyses, Hep3B cells were grown on glass cover slips in 6-well plates in α-MEM (Cambrex) supplemented with 5% FBS (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At least 4 h before transfection, the medium was substituted by medium containing 5% dextan charcoal stripped FBS. Transfections were performed with 1 μg/well AR or CFP-YFP expression constructs or 0.5 μg/well empty vector in FuGENE6 (Roche) transfection medium. In the indicated experiments, YFP-tagged ARA54 peptide expression constructs (0.5 μg/well) were added. 1 h after transfection, the medium was replaced by medium with 5% dextan charcoal stripped FBS with or without 100 nM R1881. Hep3B cells stably expressing AR constructs were subjected to the same medium-replacement schedule.
For the AR transactivation experiments, Hep3B cells were cultured in 24-well plates on a m-MEM supplemented with 5% dextran charcoal stripped FBS in the presence or absence of 100 nM R1881 and transfected using 50 ng AR expression construct and 100 ng (ARE)\textsubscript{3}TATA luc reporter. 24 h after transfection, cells were lysed and luciferase activity was measured in a luminometer (Fluoroscan Ascent FL, Labsystems Oy). Light emission was recorded during 5 s, after a delay of 2 s.

Western blot analysis
Hep3B cells were cultured and transfected in 6-well plates. 24 h after transfection, cells were washed twice in ice-cold PBS and lysed in 200 μL Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 10 mM DTT, and 0.001% Bromophenol blue). After boiling for 5 min, a 5-μL sample was separated on a 10% SDS-polyacrylamide gel and blotted to Nitrocellulose membrane (Protran; Schleicher and Schuell). Blots were incubated with anti-AR (1:2,000; mouse monoclonal F34.4.1) or anti–β-actin (1:10,000; mouse monoclonal anti–β-actin [Sigma-Aldrich]) and subsequently incubated with HRP-conjugated goat anti–mouse antibody (Dako-Cytomation). Proteins were visualized using Super Signal West Pico Luminal solution (Pierce Chemical Co.), followed by exposure to x-ray film.

Confocal imaging and FRET acceptor photobleaching
Live-cell and immunofluorescence imaging was performed using a confocal laser-scanning microscope (LSM510; Carl Zeiss Microimaging, Inc.) equipped with a Plan-Neofluar 40×/1.3 NA oil objective (Carl Zeiss Microimaging, Inc.) at a lateral resolution of 100 nm (FRET acceptor bleaching) or 70 nm (immunofluorescence). An argon laser was used for excitation of CFP, GFP, and YFP at 458, 488, and 514 nm, respectively, and a He/Ne laser was used to excite Cy3 at 543 nm. Interactions between either the N- and C-terminal domain of the YFP-AR-CFP or between AR-CFP and YFP-ARA-S4 were assessed using acceptor photobleaching for this. YFP and CFP images were collected sequentially before photobleaching of the acceptor. CFP was excited at 438 nm at moderate laser power, and emission was detected using a 470–500 nm bandpass emission filter. YFP was excited at 514 nm at low laser power (YFP is sufficiently excited at this wavelength; Fig. S4 A). Fluorescence intensities of the donor (CFP) and acceptor (YFP) were recorded simultaneously using 470–500-nm bandpass and 560-nm longpass filters, respectively. After 40 scans, a high-intensity, 100-ms bleach pulse at 514 nm was applied to specifically photobleach YFPs inside the strip (CFP was not bleached by the bleach pulse; Fig. S4 B). Subsequently, scanning of the bleached strip was continued at 458 nm at low laser intensity. The curves are either normalized by calculating \(I_{\text{final}} = (I_{\text{pre}} - I_{\text{bg}})/(\sigma_{\text{YFP}} - \sigma_{\text{CFP}})\), or to compare donor-FRAP and acceptor-FRAP curves by calculating \(I_{\text{final}} = (I_{\text{pre}} - I_{\text{bg}})/(\sigma_{\text{YFP}} - \sigma_{\text{CFP}})\), where \(I_{\text{pre}}, I_{\text{final}}, \sigma_{\text{YFP}}\) and \(\sigma_{\text{CFP}}\) are the fluorescent intensities before, immediately after the bleach and after complete recovery, respectively, and \(I_{\text{bg}}\) is the background intensity.

YFP/CFP ratio imaging
Because YFP and CFP are present in exactly the same quantity in cells expressing YFP-AR-CFP, ratio imaging can be applied to study the spatial distribution of ARs with and without N/C interaction. Local differences in YFP/CFP ratio within a cell expressing YFP-AR-CFP will only be observed if the ratio between N/C-interacting ARs, showing a relatively high YFP/CFP ratio, and non-N/C-interacting ARs, showing a relatively low YFP/CFP ratio, are different. For high-resolution YFP/CFP ratio imaging, YFP and CFP were imaged simultaneously using a moderate excitation at 458 nm and a 470–500 nm bandpass emission filter for CFP and a 560-nm longpass emission filter for YFP. To reduce noise, eight times line averaging was used. Images were analyzed using the KS-400 image analysis package (Carl Zeiss MicroImaging, Inc.). Ratio images were obtained by calculating an FRET efficiency for each pixel [\(F_{\text{RET}} = (I_{\text{YFP}} - I_{\text{CFP}})/(I_{\text{YFP}} + I_{\text{CFP}})\), where \(I_{\text{YFP}}\) and \(I_{\text{CFP}}\) are the intensities of the YFP and CFP emission, respectively, and \(I_{\text{CFP}}\) is the background intensity. To obtain regions representing successive relative intensity ranges (Fig. 4), the mean of \(I_{\text{YFP}}\) and \(I_{\text{CFP}}\) was calculated for each pixel and \(\mu = (I_{\text{YFP}} + I_{\text{CFP}})/2\). The mean of each nucleus (termed \(\mu\) in Fig. 4) and the standard deviation, \(\sigma\), were then calculated after manual selection of the nuclear area and exclusion of the nucleoli (Fig. 4 B). The mean ratio in areas with pixel intensities \(\mu_{\text{frac}} < \mu + \sigma\), \(\mu + \sigma < \mu_{\text{frac}} < \mu + 2\sigma\), \(\mu + 2\sigma < \mu_{\text{frac}}\), were then first calculated for CFP-YFP-AR expressing cells. Because these molecules emit at a fixed YFP/CFP ratio irrespective of their concentration or local concentration, a constant ratio should be maintained in the three selected areas is due to imaging artifacts. Indeed, CFP/YP ratio increased in CFP-YFP-AR expressing cells with low intensity and decreased in cells with high intensities probably because of the nonlinearity of the detectors. Therefore, data obtained from each cell expressing YFP-AR-CFP and the non–DNA-binding mutant YFP-AR (A573D)-CFP were expressed relative to the mean ratio measured in corresponding areas in seven cells expressing CFP-YFP-AR with similar expression levels. For statistical analysis, the YFP/CFP ratio imaging datasets were tested for normality using the Kolmogorov-Smirnov test, and datasets were compared using the t test.

Immunofluorescent labeling of nascent RNA
Nascent RNA was detected by BrUTP incorporation in permeabilized living Hep3B cells stably expressing GFP-AR (Farla et al., 2004) according to Wansink et al. (1993). Cells were grown overnight on coverslips in medium containing 5% dextran charcoal stripped FBS in the presence of 100 nM R1881. The procedure of BrUTP incorporation has been previously described (Wansink et al., 1993). Cells were permeabilized in glycerol-buffer (20 mM Tris HCl, 0.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 25% glycerol, and 1 mM PMSF) supplemented with 0.05% Triton X-100 and 10 μM RNAsin for 3 min. To allow BrUTP incorporation, permeabilized cells were incubated for 30 min at RT in synthesize buffer (100 mM Tris HCl, 5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 200 mM KCl, 50% glycerol, 0.05 mM SAM, 20 μM RNAsin, and 0.5 mM PMSF) supplemented with 0.5 mM ATP, CTP, GTP, and BrUTP (or UTP as control; Sigma-Aldrich). Next, cells were fixed in 2% formaldehyde in PBS, incubated in 0.5 Triton X-100/PBS for 5 min and in 100 mM glycine/PBS for 10 min, each step followed by two PBS washes. After washing with PBS, cells were permeabilized for 90 min at 4°C with soybean α-mannosidase (SeraLab) at 1:500 in PBS. After four washes with PBS, cells were incubated for 30 min at RT with anti-brachyury mAb (Jackson ImmunoResearch Laboratories) 1:200 in PBS followed by four washes with PBS. The biotinylated antibody was then visualized with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) 1:250 in PBS for 30 min at RT. After extensive washing with PBS and PBS, cells were embedded in Vectashield containing DAPI.

Online supplemental material
Fig. S1 shows YFP-AR-CFP expression analysis of cells used in the acceptor photobleaching FRET experiments and in the simultaneous FRAP and FRET measurements. Fig. S2 presents the validation of FRET measurements by acceptor photobleaching (abFRET) and shows the homogeneity data.
of FRET measured in cells expressing YFP-AR-CFP. Fig. S3 shows the mini-
imal YFP/CFP ratio change after the addition of R1881 in cells expressing
YFP-AR(23,27A/L26A)-CFP variant. Fig. S4 presents the control experi-
ments for donor-FRAP and acceptor-FRAP on cells expressing YFP-AR and
ARC-CP. Online supplemental material is available at http://www.jcb.
org/cgi/content/full/jcb.200609178/DC1.

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