Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera

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ABSTRACT A highly fluorescent mutant form of the green fluorescent protein (GFP) has been fused to the rat glucocorticoid receptor (GR). When GFP–GR is expressed in living mouse cells, it is competent for normal transactivation of the GR-responsive mouse mammary tumor virus promoter. The unliganded GFP–GR resides in the cytoplasm and translocates to the nucleus in a hormone-dependent manner with ligand specificity similar to that of the native GR receptor. Due to the resistance of the mutant GFP to photobleaching, the translocation process can be studied by time-lapse video microscopy. Confocal laser scanning microscopy showed nuclear accumulation in a discrete series of foci, excluding nucleoli. Complete receptor translocation is induced with RU486 (a ligand with little agonist activity), although concentration into nuclear foci is not observed. This reproducible pattern of transactivation-competent GR reveals a previously undescribed intranuclear architecture of GR target sites.

Steroid receptors are hormone-dependent activators of gene expression. It is generally accepted that the unliganded glucocorticoid receptor (GR) resides in the cytoplasm and that hormone activation leads to both nuclear accumulation and gene activation (see refs. 1–6 and references therein). However, the mechanisms involved in nuclear translocation and targeting of steroid receptors to regulatory sites in chromatin are poorly understood. It has been difficult to discriminate between the ability of a given receptor mutant, or a given receptor–ligand combination, to participate in the separate processes of receptor activation, nuclear translocation, sequence-specific DNA binding, and promoter activation.

The paucity of information on these issues stems in part from the lack of appropriate technology to study the various stages in nuclear targeting. Because knowledge of these steps is essential for understanding the mechanism of steroid hormone action, we have taken the approach of tagging GR with a chromophore, allowing us to visualize in vivo, with the least perturbation, the changes in receptor subcellular localization upon exposure to activating ligand.

Recent characterization of a chromophore, the green fluorescent protein (GFP), provides a general method to label proteins in living cells. Chimeras formed with a highly efficient variant of GFP (7) afford a unique opportunity to examine the organization of proteins of interest within various cellular compartments (8–10). We have prepared a fusion between GFP and the rat GR. This chimera functions normally in cytoplasmic/nuclear translocation and gene activation. Using this reagent, we have monitored subcellular trafficking of GR in living cells. We find that nuclear translocation can be characterized as a process separate and distinct from localization on target genes. Furthermore, we have observed what appears to be direct localization of GR on target genes. Finally, three-dimensional reconstruction of nuclear GR binding sites suggests a reproducible nuclear organization of target sites. These findings show that GFP–GR fusions provide a powerful approach to the study of receptor function and indicate that ligand-dependent changes in receptor activity can now be addressed in single cells over extended time periods to follow receptor activation, translocation, target site selection, inactivation, and cycling between the nucleus and cytoplasm. In addition, it is clear the GFP–GR will be invaluable in exploring the organization and architecture of the interphase nucleus.

MATERIALS AND METHODS

Cell Lines and Plasmids. Cell line 1471.1 contains multiple copies of a bovine papilloma virus–mouse mammary tumor virus (MMTV) long terminal repeat (LTR)–chlamydomal acetyltransferase (CAT) fusion in the murine adenocarcinoma C127 cell (11). pCI-nGFP-C656G was derived from pCINH6HA-C656G and pZA69. pCINH6HA-C656G DNA expresses rat GR with the C656G mutation from the cytomegalovirus promoter/enhancer, and is tagged at the N terminus with (His)6 and hemagglutinin epitope (12). A 768-bp GFP cDNA fragment with the S65T mutation was inserted between the two tags and GR. Other plasmids used in this study are as follows: pLTRLuc (full-length MMTV LTR driving the expression of a luciferase gene) (13), pCMVIL2R (interleukin 2 receptor (IL-2 R) expression plasmid) (14), and pUC18 (Life Technologies, Grand Island, NY).

Transfection. Plasmid DNA was transiently introduced into 1471.1 cells either by calcium phosphate coprecipitation using a N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES)-based buffer (15) or by electroporation. For calcium phosphate coprecipitation, semiconfluent cells were resuspended at 7 × 10^6 cells per ml in DMEM supplemented with charcoal/dextran-treated fetal bovine serum (HyClone), dispensed as 1 ml into a 2 × 2 cm² Lab-Tek Chamber Slide (Nunc) or as 10 ml into a 100-mm Petri dish layered with 24.5-mm diameter coverslips. Cells were grown at 37°C overnight, medium was replaced in the morning, and in the afternoon, cells were transfected with 1 ml of transfection mixture containing 20 μg of plasmid DNA per 10 ml of cells. Cells were left 12–16 hr, medium was replaced, and cells were allowed to recover for 2 hr before further treatment and imaging. For calcium depletion.

Abbreviations: GFP, green fluorescent protein; GR, glucocorticoid receptor; IL-2 R, interleukin-2 receptor; MMTV, mouse mammary tumor virus; CAT, chlamydomal acetyltransferase; LTR, long terminal repeat.

Data deposition: The sequence of the GFP-GR expression plasmid pCI-nGFP-C656G has been deposited in the GenBank data base (accession no. U53602).

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tion experiments, cells were electroporated with 5–20 μg of pCI-nGFP-C656G DNA for 2 x 10^7 cells in 0.2 ml of cold DMEM at 250 V and 800 μF in a 0.4-em electrode gap electroporation chamber, left to recover on ice for 5 min, and then diluted in DMEM supplemented with dextran/charcoal-treated fetal bovine serum before plating. Cells were then grown for 12–16 hr, and medium was replaced before treatment and imaging.

Enrichment of Transfected Cells and Analysis of Cytosolic Extracts. Cells that took up exogenous DNA were enriched by cotransfection with pCMVII2R, an IL-2 R expression plasmid, and selection for IL-2 R* cells using magnetic beads (Dynal, Lake Success, NY) coated with mouse anti-human IL-2 R antibody (Boehringer Mannheim, clone 3G10), as described (14). Extracts from the IL-2 R* and IL-2 R− cells were assayed for the amount of luciferase activity in a MicroLumat LB96P as recommended by the manufacturer (EG & G/Berthold/Wallac, Gaithersburg, MD) and for CAT activity as described (16).

Determination of Intracellular Calcium. Intracellular free calcium concentrations were determined in single cells by measuring the signal from the calcium-sensitive indicator Fura-2, according to Tsien and Harootunian (17). Intracellular calcium content was measured in three independent experiments—least 20 cells in each experiment. Ratio imaging was performed using IMAGE 1 software (Universal Imaging, West Chester, PA), using 340-nm and 380-nm excitation, 510-nm emission, and 490-nm dichroic barrier filters, a Zeiss Photomicroscope III microscope, enclosed in a temperature-controlled incubator, and an intensified (VideoScope, Dallas) charge-coupled device camera (Dage, model 72, Michigan City, IN), and optical disc recorder (Panasonic). Intracellular free calcium concentrations in cells with calcium-supplemented buffer were 350 ± 183 nM, whereas in calcium-free buffer, the concentrations were 60 ± 11 nM.

Image Acquisition and Analysis. For time course studies, cells were placed into a Dvorak-Stotler chamber and perfused at 37°C with assay buffer for 3 min, then with the same buffer containing 1 nM dexamethasone (dex) for 2 hr at 10 ml/hr flow rates (using a perfusion system from Adams & List, Westbury, NY). Samples were evaluated using a Leica confocal microscope equipped with an incubator and equipped for epifluorescence with illumination from a xenon lamp, 490-nm excitation and 525-nm emission, and 505-nm dichroic barrier filters (Chroma Technology, Brattleboro, VT). Images were acquired every 15 sec with a Coolsnap HQ (Roper Scientific) cooled charge-coupled device camera (model P1020) on a BioRad MRC-600 confocal laser scanning unit, using a 40X oil-immersion objective. From living cells expressing GFP-GR, serial 0.5-μm optical sections were collected. Three-dimensional image rendering, analysis, and reconstruction were carried out with the ANALYZE software from the Mayo Clinic, Rochester, MN.

RESULTS

Tagging of GR with GFP. To develop a highly efficient, fluorescent version of GR, we generated a GFP-GR chimera with a 27-kDa GFP variant fused in frame to the second amino acid of rat GR (see Materials and Methods) (Fig. 1A). The GFP variant, from the jellyfish Aequorea victoria, contains a serine-to-threonine substitution at amino acid 65 (S65T mutation), which makes the resulting chromophore 6-fold more fluorescent than the wild-type GFP (7).

Since GR is ubiquitously present in all mouse cells, we used a variant having a higher affinity for its ligand than the endogenous receptor. S65T GFP was fused to a rat GR that contains a cysteine-to-glycine mutation at position 656 of the steroid-binding domain (19). This point mutation, C656G, increases the affinity of the receptor 9-fold for its ligand. The presence of this mutation permits selective activation of the transfected chimeric receptor without activation of the endogenous receptor.

Transcriptional Competence of GFP-GR. When the GFP-GR chimera is introduced into cultured mouse cells, a fusion polypeptide with the predicted molecular mass of 118 kDa is produced (Fig. 1B). When these cells are stimulated with 1 nM dexamethasone, a cotransfected reporter construct (pLTRLuc) containing the luciferase reporter gene under the control of MMTV LTR–CAT sequences present in the 1471.1 cells (11). Cells were treated for 4 hr with the indicated ligand, then harvested, and levels of CAT activity were determined.

FIG. 1. Construction and characterization of GFP-GR. (A) Plasmid pCI-nGFP-C656G contains the GFP fused to the C656G mutant GR. (B) Dexamethasone (dex)-dependent stimulation of MMTV-pLTRLuc (13) is shown for GFP-GR transfected cells. Solid bars represent the IL-2 R* selected population activated for 2 hr with 1 nM dexamethasone, and the open bar depicts activation of the endogenous receptor with 100 nM dexamethasone. The Western blot below the panel shows detection of endogenous GR and transfected GFP-GR present in extracts from parallel transfections with the BuGR 2 monoclonal antibody (18) by enhanced chemiluminescence (ECL; Amersham). (C) Ligand specificity is presented for activation of endogenous MMTV LTR–CAT sequences present in the 1471.1 cells (11). Cells were treated for 4 hr with the indicated ligand, then harvested, and levels of CAT activity were determined.
amethasone-treated cells without added GFP-GR, indicating that 1 nM dexamethasone activated the GFP-GR chimeric protein but not the endogenous GR. With increasing amount of the GFP-GR expression plasmid, luciferase activity in the 1 nM dexamethasone-treated IL-2 R+ (see Materials and Methods) cells reaches the same level as that in 100 nM dexamethasone-treated IL-2 R+ cells lacking GFP-GR expression plasmid (compare 2 µg of pCI-nGFP-C656G/1 nM dexamethasone with 0 µg pCI-nGFP-C656G/100 nM dexamethasone). Because the 100 nM dexamethasone treatment gives complete activation of the endogenous GR in the latter case, we conclude that the GFP-GR chimeric receptor is fully functional in dexamethasone-mediated transcriptional activation of the transiently introduced reporter plasmid DNA.

When activated by dexamethasone, GFP-GR is competent to induce not only the transiently introduced MMTV LTR-luciferase reporter DNA, as mentioned above (Fig. 1B), but also the multicopy MMTV LTR-CAT reporter genes present in 1471.1 cells (1 nM and 10 nM dexamethasone, Fig. 1C). In contrast, treatment with 10 nM RU486, an antagonist with little GR agonist activity, or progesterone, a poor agonist, results in little activation of the MMTV LTR-CAT reporter; 17β-estradiol, a steroid that shows no affinity for GR, fails to activate the LTR. Thus, transcriptional activation of the MMTV LTR target genes by GFP-GR maintains the ligand specificity characteristic of GR (refs. 19–22; also see references in ref. 2).

Visualization of GFP-GR Cytoplasm-to-Nucleus Translocation in a Single Metabolically Active Cell. Because the S65T variant of the GFP chimeroscope is resistant to photobleaching (7), it was possible to use confocal and time-lapse video microscopy to observe GFP-GR over extended periods. Using computer-controlled high-resolution video and confocal laser scanning microscopy, we examined transfected samples for subcellular localization of the chimeric GFP-GR protein (Fig. 2). We observed significant fluorescence in the cytoplasm of ~10% of total cells, approximately the fraction that typically acquires transfected DNA. Thus, the GFP was functional as a chromophore in essentially all of the expressing cells in this mammalian system.

Upon exposure to dexamethasone, translocation of GFP-GR occurs in 100% of fluorescing cells, with the rate of cytoplasm-to-nuclear translocation dependent on the concentration of hormone. At 10 nM, complete translocation was induced within 10 min at 37°C, with half-maximal nuclear accumulation at 5 min; this rate is consistent with previous findings (23). The rate of translocation is decreased with 1 nM dexamethasone (complete translocation over 30 min with half maximum at 9–10 min) and further reduced with 0.1 nM dexamethasone (complete translocation within 2 hr with half maximum at 1 hr).

Analysis of a time-lapse series revealed that GFP-GR accumulated along fibrillar structures (Fig. 2A and 3A) and in the perinuclear region very rapidly after hormone addition (Fig. 2B), probably within seconds. With real-time imaging, perinuclear accumulation was observed in a pulsatile pattern with 1 to 2-sec intervals between brightness changes. After 3 min with 1 nM dexamethasone, GFP-GR was noticeably present in the nucleus, but not in the nucleoli (Fig. 2C). When approximately one-third of the protein had been translocated (9–10 min), a punctate pattern appeared (Fig. 2D), and translocation was complete after 30 min (Fig. 2F). During translocation, the cells frequently became rounded and moved along the long axis of the cell. We observed reduction of the cell surface, as well as the nuclear volume, during the translocation. One hour after hormone treatment, the cells reattach and regain a more flattened shape.

Ligand Specificity of Cytoplasm-to-Nucleus Translocation. Whereas complete translocation of GFP-GR was observed in all fluorescing cells treated with dexamethasone (Fig. 3C), other classes of steroid hormones induced GFP-GR translocation to various extent reflective of the affinity for GR. The glucocorticoid antagonist RU486, known to have a high affinity for GR (19), was as potent as dexamethasone for induction of translocation (Fig. 3D). Progesterone, a weak GR agonist, required a concentration 100-fold higher than dexamethasone for translocation; however, approximately one-half of the GFP-GR remained in the cytoplasm (data not shown). In contrast, 17β-estradiol, a steroid hormone that does not bind GR, did not cause intranuclear GFP-GR accumulation (10 nM, Fig. 3B). Thus, GFP-GR maintained ligand-dependent cytoplasm-to-nuclear translocation, with analog specificity identical to that for the untagged GR with the C656G point mutation (19). Furthermore, whereas ligand binding may suffice to trigger efficient cytoplasm-to-nuclear translocation, the receptor, once in the nucleus, shows differing degrees in its ability to activate the LTR (compare Fig. 3 with Fig. 1C).

Role of Intracellular Free Calcium and Energy in GFP-GR Translocation. We also addressed two important issues concerning the nuclear import of proteins, the role of Ca²⁺ and the energy requirement for translocation. Intracellular stores of Ca²⁺ were depurated by incubating the cells with the endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin and the calcium ionophore ionomycin in calcium-free media. Intracellular free calcium content was measured with ratio imaging in Fura-2-loaded cells (see Materials and Methods). The cytoplasmic pattern of GFP-GR was not significantly altered by calcium depletion (Fig. 4A). When Ca²⁺-depleted cells were subsequently exposed to dexamethasone in Ca²⁺-free media, the hormone induced complete translocation of GFP-GR (Fig. 4B).

To study the energy dependence of ligand binding, cells were exposed to dexamethasone at 4°C (Fig. 4C); then hormone was removed and the cells were warmed to 37°C under continuous monitoring with video microscopy. At 4°C, translocation was

**Fig. 2.** Time-dependent translocation of GFP-GR. Murine adenocarcinoma cells (1471.1) were cultured on coverslips and transfected with GFP-GR fusion chimera 1 day before microscopy. Cells were placed into a Dvorak-Stotler chamber and perfused at 37°C with assay buffer for 3 min (A), then with buffer containing 1 nM dexamethasone for 2 hr. Perinuclear accumulation was evident at 1 min (B), nuclear entry at 3 min (C), target site binding at 7 min (D), half-maximal accumulation at 9 min (E), and complete translocation at 30 min (F). (Bar = 10 μm.)
completely arrested. Rewarming led to complete translocation and reappearance of the focal GFP-GR localization (Fig. 4D). This experiment indicates that hormone binding to GR in living cells does not require energy, in contrast to the energy-dependent step of translocation.

**Focal Accumulation of Nuclear GFP-GR Correlates with Transcriptional Activation.** When the intranuclear accumulation of GFP-GR is examined in detail, it is readily apparent that the receptor localizes most prominently at specific foci within the nucleus (note the bright spots in Figs. 3C, 4B, and 4D). In addition, there is a low level of accumulation in a diffuse reticular pattern, forming the basis for the nuclear background fluorescence (Fig. 3C). The presence of these focal accumulations is unique to dexamethasone-treated cells and is not observed in 17β-estradiol- (Fig. 3B) or progesterone-exposed cells (data not shown). In RU486-treated cells, although a few focal points may be present, they are not readily discernible. Instead, GFP-GR accumulates in a diffuse pattern with regions of condensation in a reticular pattern (Fig. 3D).

Depleting intracellular Ca\(^{2+}\) did not affect the dexamethasone-mediated formation of intranuclear foci (Fig. 4B), but it decreased the amount of diffuse GFP-GR in the nucleus. The ability of agonist to induce focal accumulation of GFP-GR correlated strongly with its ability to activate transcription (Fig. 1C). The striking accumulation of dexamethasone-activated GFP-GR into intranuclear foci immediately suggests that a specific architecture may underlie this distribution.

**Organized Architecture of Interphase Nuclei As Revealed by GFP-GR.** Three-dimensional image analysis of the points of GR accumulation in dexamethasone-treated cells reveals a nonrandom distribution of GFP-GR accumulation. Most strikingly, comparison of adjacent cells demonstrates a reproducible pattern of intranuclear structure for GFP-GR accumulation. This intranuclear architecture is shown in Fig. 5 (Upper) for a typical pair of nuclei. A predominance of GFP-GR-accumulating foci is observed in the quadrant of the nucleus adjacent to the glass attachment surface of the cell, whereas a group of large patches of GFP-GR-containing foci is observed in the top half. Nucleolar structures were always devoid of GFP-GR. The nuclear pattern of RU486-treated cells was again strikingly different from dexamethasone-treated cells (Fig. 5, Lower; note also Fig. 3D). Although essentially all of the GFP-GR is translocated, intranuclear RU486-ligated receptor is distributed throughout the nucleus in a reticular pattern that excludes nucleoli.

**DISCUSSION**

In the present study, we have shown the usefulness of GFP in monitoring the activity of a steroid hormone receptor. The ability to directly observe living cells has allowed us to follow
in real time the process of cytoplasm-to-nucleus translocation, and it has revealed differences in GR intranuclear accumulation pattern dependent on the type of activating ligand. Furthermore, the patterns of GR accumulation are remarkably similar between adjacent cells, suggesting an order in the organization of the interphase nucleus.

Because the use of GFP as a tag involves fusing a rather large protein (27 kDa) to GR, we thought it essential to examine whether any GR activity has been compromised by GFP. To this end, we fused GFP to a rat GR with the C656G point mutation, which can be selectively activated without activating the endogenous receptor. By a number of criteria, GFP-GR functions very much like GR. In particular, the tagged receptor resides in the cytoplasm until activated by a ligand, then it translocates into the nucleus at a rate comparable to that previously reported (23). The rate and extent of GFP-GR translocation show a dependence on the concentration of the activating ligand as well as a ligand specificity reflective of the native receptor (19). Furthermore, because both dexamethasone treatment and RU486 treatment lead to complete translocation of GFP-GR from the cytoplasm to the nucleus in all cells, essentially all of the GFP-GR molecules exist in a conformation competent for both ligand binding and nuclear translocation (Fig. 3C). Once in the nucleus, GFP-GR’s ability to activate the transcription of a MMTV LTR reporter gene depends on the type of activating ligand, consistent with previous results for GR (summarized in ref. 2). In the case of a potent agonist, dexamethasone, less GFP-GR is required for activation of transiently introduced MMTV LTR-luciferase reporter gene than for the endogenous GR, indicating that even with respect to transactivation potential, the presence of GFP has not altered the transcriptional potency ascribed to the C656G point mutation (19). Thus, in all aspects, the presence of GFP appears not to have affected normal GR function.

Because the S65T variant of GFP used here is highly excitable at 488-nm wavelength and is resistant to photo-bleaching, we have been able to follow the course of cytoplasm-to-nucleus translocation of GR in a single living cell for an extended period of time (Fig. 2). Upon binding to dexamethasone, GFP–GR moves vectorially toward the nucleus. Inhibition of import by chilling indicates that this transport is facilitated. Accumulation of GFP–GR along fibrillar structures before dexamethasone addition (Fig. 2A and 3A) and in the perinuclear region after dexamethasone addition, suggests that the cytoskeleton is involved in the transport process (Fig. 2B; refs. 6 and 24–26). Finally, the pulsatile brightness changes in the perinuclear region support an energy- and microtubule-dependent active transport process.

In our system, depleting endoplasmic reticulum calcium stores and removing extracellular calcium did not prevent GFP–GR import into the nucleus. Previous studies, using isolated nuclei or permeabilized cell systems, indicated that calcium is required for both passive diffusion and signal-mediated transport through the nuclear pore (27, 28). Whereas we cannot exclude the possibility that other intracellular calcium pools exist that may contribute to the GFP–GR translocation, differences could also be attributed to protein-specific transport mechanisms or to the involvement of soluble cytoplasmic factors.

The rate of translocation of GFP–GR was dependent on hormone concentration, reflecting the dose and time dependence of GR action. This suggests that the rate of translocation contributes significantly to GR function. Recently, immuno-philin ligand FK506 was shown to enhance both the rate of translocation and extent of transactivation by GR (29). Our
studies indicate that GFP–GR will be a useful model to study reagents that modify rates of nuclear translocation, in addition to those that affect rate of nuclear export (30).

Shortly after GFP–GR enters the nucleus, foci of bright fluorescence appear for the dexamethasone-induced cells (Fig. 2D). These bright foci of dexamethasone-induced GFP–GR appear to be organized in a reproducible three-dimensional architecture. Because the presence of these localized foci correlates with transcriptional activation of the amplified MMTV LTR–CAT fusion chimeras present in this cell, we argue that the foci of bright fluorescence reflect the activation of target genes by the dexamethasone-activated GFP–GR; this hypothesis can now be subjected to direct test. The focal accumulation of GFP–GR in these centers introduces obvious opportunities to study colocalization of other activities needed for GR activation from target genes organized in chromatin.

In contrast to dexamethasone, RU486-activated GFP–GR accumulates almost exclusively in a reticular pattern found throughout the nucleus but excluding nucleoli; this pattern could result from association with the nuclear matrix (31, 32). Using immunofluorescence in fixed cells, van Steensel et al. recently reported (33) that GR accumulates in clusters within the nucleus, but they found no difference between agonist- and RU486-activated receptor; furthermore, they suggested that these clusters are not directly involved in active transcription.

In contrast, hepatocyte nuclear factor 4 (HNF-4) was shown to be generally distributed in nuclei when dephosphorylated, but localized in subnuclear domains when expressed in the phosphorylated, transcriptionally competent form (34). These latter findings are in agreement with our observation that only transcriptionally competent GR localizes in the nuclear foci.

Finally, it has been argued that DNA in the nucleus is localized in a nonrandom fashion (35–38). The reproducible pattern of agonist-induced GFP–GR accumulation may reflect an inherent order of the interphase nucleus; we observed the asymmetric nuclear accumulation not only in nuclei from neighboring cells but also in individual random cells. We note that Lawrence and coworkers (39) reported an asymmetric distribution of poly(A)-associated transcription domains, also in the lower portion of nuclei of human diploid fibroblasts. The availability of functional GFP fusions should advance our ability to evaluate the potential existence of a nuclear architecture.

In conclusion, the use of GFP–GR allowed us to visualize steroid receptor translocation in real time in living cells. It has led to the discovery of an intranuclear architecture of GR target sites. The GFP–GR should serve as an invaluable tool for understanding further details of receptor activation, the translocation process, interaction of receptors with components of the eukaryotic interphase nuclei, and mechanisms of transcriptional activation by steroid receptors. In addition, GFP–GR will surely be invaluable in helping shape our understanding of the architecture and dynamics of the nucleus.

Note Added in Proof. While this paper was in press, Ogawa et al. (40) described a fusion between a fragment of human GR and wild-type GFP. These authors reported a requirement for lower temperature preincubation (30°C versus 37°C) to obtain both GFP as well as GR activity. In addition, they did not observe subnuclear localization of GR.