Ultraspiracle promotes the nuclear localization of ecdysteroid receptor in mammalian cells

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Abstract:

The heterodimer consisting of ecdysteroid receptor (EcR) and ultraspiracle (USP), both of which are members of the nuclear receptor superfamily, is considered to be the functional ecdysteroid receptor. Here we analyzed the subcellular distribution of EcR and USP fused to fluorescent proteins. The experiments were carried out in mammalian COS-7, CHO-K1 and HeLa cells to facilitate investigation of the subcellular trafficking of EcR and USP in the absence of endogenous expression of these two receptors. The distribution of USP tagged with a yellow fluorescent protein (YFP-USP) was almost exclusively nuclear in all cell types analyzed. The nuclear localization remained constant for at least 1 day after the first visible signs of expression. In contrast, the intracellular distribution of EcR tagged with a yellow fluorescent protein (YFP-EcR) varied and was dependent on time and cell type, although YFP-EcR alone was also able to partially translocate into the nuclear compartment. Coexpression of YFP-EcR with USP tagged with a cyan fluorescent protein (CFP-USP) resulted in exclusively nuclear localization of both proteins in all cell types analyzed. The USP-induced nuclear localization of YFP-EcR was stable for at least 20 hours. These experiments suggest that USP has a profound effect on the subcellular distribution of EcR.

Keywords: ecdysone; green fluorescent protein; nuclear receptor; subcellular distribution.

Article:

Introduction

The steroid hormone, 20-hydroxyecdysone (20E), is critical for inducing the molecular events that lead to molting and metamorphosis in insects and crustaceans (Riddiford et al., 2000; Spindler-Barth and Spindler, 2003). The effects of 20E are mediated through a ligand-activated nuclear receptor, the ecdysteroid receptor (EcR) (Koelle et al., 1991). In insect cells the most important partner for EcR is the ultraspiracle (USP) protein (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990), an orthologue of the vertebrate retinoid X nuclear receptor; although other nuclear transcription factors such as seven up can replace USP, whereas DHR 38 can replace EcR (Riddiford et al., 2000; Spindler-Barth and Spindler, 2003). The EcR/ USP heterodimer is a functional unit that is able to bind to ecdysone response elements present in the promoters of 20E-responsive genes in vitro and in vivo (Yao et al., 1992; Thomas et al., 1993) but there are also data to suggest that EcR can bind response elements alone. In particular, it has been demonstrated that EcR itself can activate transcription via the heat shock protein hsp27 element (Riddihough and Pelham, 1987; ˙Ozyhar et al., 1991) in yeast cells without specific ligands or its heterodimeric partner (Cruz and Mak, 1997).

According to data obtained recently for other nuclear receptors and transcription factors (Hager et al., 2001) it is quite possible that the function of the EcR/USP complex may be tuned by dynamic regulation of intracellular distribution of its components. The available data clearly indicate that the distribution-dependent regulation of the activity of the particular receptor is very specific and differs among various family members. Until now the distribution of EcR and USP has been studied partly using classical biochemical and immunohistochemical

techniques in insects (Yund et al., 1978; Maroy et al., 1978; Schaltmann and Pongs, 1982; Turberg et al., 1988; Turberg and Spindler, 1992; Lammerding-Köppel et al., 1998) and crustaceans (Kuppert et al., 1978, 1981; Spindler-Barth et al., 1981; Londershausen and Spindler, 1981; Londershausen et al., 1982; Kuppert and Spindler, 1982). Since many of these approaches may lead to artifacts, we decided to investigate the subcellular distribution of EcR and USP in living mammalian cells utilizing proteins fused with derivatives of green fluorescent protein (GFP). Fluorescent proteins have been demonstrated to be useful tags for monitoring the subcellular distribution and trafficking of various proteins in living cells (Chalfie et al., 1994). Mammalian cells facilitate independent analysis of the subcellular trafficking of EcR and USP under conditions when the heterodimerization partner is absent, which is not the case in insect cells. Second, there are few data concerning subcellular distribution of the EcR/USP complex, although this complex is potentially useful in the regulation of transgene expression in mammalian cell types and transgenic animals (Toniatti et al., 2004).

Results
Characteristics of the fusion proteins
The expression of a full-length EcR (isoform B1; Talbot et al., 1993) and a full-length USP tagged with yellow fluorescent protein (YFP) in CHO-K1 cells was analyzed by Western blotting using anti-GFP antibody. As shown in Figure 1A (lanes 2 and 4), after fusion of the YFP to the C-terminus of EcR or of USP, more than one product could be observed for both proteins, probably resulting from a degradation process. In contrast, the EcR and USP with YFP attached to the N-terminus appear as single bands corresponding to the full-length YFP-EcR and YFP-USP (lanes 1 and 3). Thus, the N-terminal tagged derivatives were chosen for further experiments. The same results were obtained with COS-7 cells (data not shown).

Gel mobility shift experiments revealed that the YFPEcR/GFP-USP complex binds to the 20E responsive element hsp27, even in the absence of muristerone A, which is a 20E agonist (Figure 1 B, lane 1). The intensity of this band was considerably increased by the addition of muristerone A (Figure 1 B, lane 2). The hormone-dependent transactivation capacity of EcR and YFP-EcR was tested with a USP fusion protein (Henrich et al., 2003). Transactivation was slightly reduced in COS-7 cells (69.8±23.3%; n=5) but not in CHO-K1 cells compared to the EcR/USP complex (data not shown).

USP is present exclusively in the nucleus
Initial experiments demonstrated that YFP was equally distributed between the cytoplasm and nucleus in all cell types analyzed (Figure 2A–C). Identical results were obtained for other GFP-derivatives used in this study as fluorescent tags (data not shown). In contrast, YFP-USP was always found exclusively (>90%) in the nuclei of all cell types analyzed (Figure 2D,F,H). This localization was independent of the fluorescent tag used (data not shown). In most instances nuclei were stained homogeneously and nucleoli were never stained (Figure 2D,F,H). Nuclear localization of tagged USP remained constant for at least 1 day after the first visible signs of expression (Figure 3 demonstrates representative images obtained for CHO-K1 cells).

EcR is found in cytoplasm and nuclei
The intracellular distribution of YFP-EcR was not identical in various cell types. Approximately 6 h after transfection, the majority of CHO-K1 cells contained the fusion EcR in the nucleus (Figure 4A,J). Later, the percentage of cells with exclusively nuclear localization decreased continuously (Figure 4J) and a gradual increase in the number of cells containing YFP-EcR in nuclei as well as in cytoplasm (Figure 4G,H) or mainly in the cytoplasm, in the form of clusters (Figure 4I), was observed. In contrast, in COS-7 cells the proportion of YFP-EcR localized exclusively in the nucleus increased with time after an initial decrease (Figure 4K). As observed for USP, nucleoli were not stained in all cell types (Figure 4A–C).

Effect of coexpression of USP on the nuclear localization of EcR
Coexpression of YFP-EcR with USP tagged with cyan fluorescent protein (CFP-USP) resulted in the nuclear colocalization of both proteins in nearly all transfected cells. This pattern was observed for all cell types analyzed (Figure 5, compare panels A and B, D and E, and G and H, respectively). The USP-induced nuclear localization of EcR was stable for at least 20 h after the first visible signs of expression (see Figure 6 for

representative analysis obtained for CHO-K1 cells). This observation contrasts with the results obtained for YFP-EcR expressed alone, in which YFP-EcR exhibited predominantly cytoplasmic localization at the corresponding time point (Figure 4J). In a few cells, YFP-EcR remained in the cytoplasm in the form of clusters (Figure 5K,N) similar to those observed for YFP-EcR expressed alone (Figure 4I). Interestingly, the clusters observed in cells cotransfected with fluorescent derivatives of EcR and USP also contained CFP-USP (Figure 5J,M).
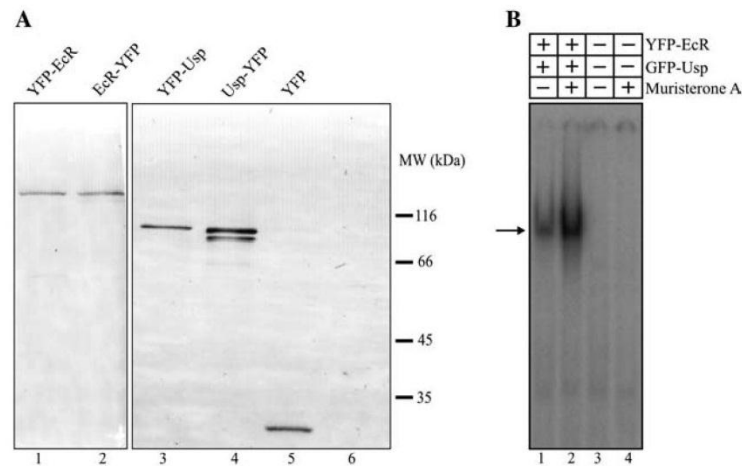

Figure 1 Analysis of expression and DNA binding activities of EcR and USP tagged with fluorescence proteins. (A) Western blot analysis of EcR (lanes 1 and 2) and USP (lanes 3 and 4) tagged with YFP, expressed in CHO-K1 cells. YFP was attached to the N-terminus (lanes 1 and 3) or to the C-terminus (lanes 2 and 4) of EcR or USP. Lane 5 corresponds to YFP alone and lane 6 to the cellular lysate from non-transfected cells. Molecular mass markers were run on a parallel lane (not shown). (B) EMSA was carried out with a ds oligonucleotide containing *hsp27* 20E response element (see the Materials and methods section) and with cell extracts containing YFP-EcR and GFP-USP. Muristerone A was added where indicated (final concentration 10^{-5} M). The arrow indicates the complex of GFP-USP/YFP-EcR with DNA.
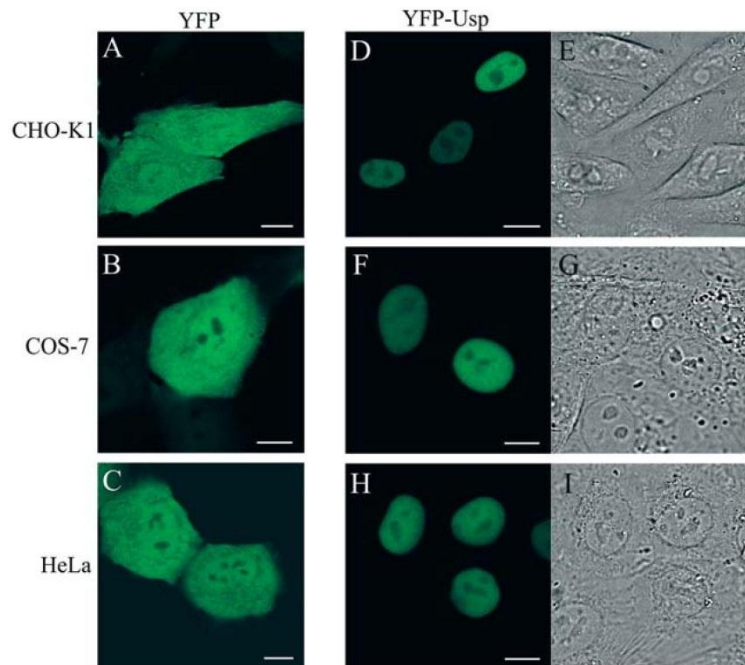

Figure 2 USP tagged with YFP localizes in the cell nucleus, while YFP is localized mostly in the cytoplasm and the nucleus. Cellular localization of YFP and YFP-USP in three different cell lines demonstrated by confocal imaging: CHO-K1 (A,D), COS-7 (B,F) and HeLa (C,H). (E,G,J) Non-confocal transmitted light images of the same fields of view. Independent experiments have demonstrated that the dark spots within the nuclei correspond to nucleoli (not shown). The bar represents 10 μm.

Discussion

Immunocytochemical analysis of the intracellular distribution of nuclear receptors has often produced unclear results because of staining artifacts (Scheller et al., 2000; Yamashita, 2001). We therefore utilized the expression of GFP derivatives fused with USP and EcR. Fluorescent proteins have been shown to be useful tags

that allow exploration of the subcellular distribution and trafficking of other transcription factors, including nuclear receptors. Heterologous expression of EcR and USP separately in mammalian cells facilitates analysis of their individual nuclear localization capabilities, whereas insect tissue typically contains variable amounts of both receptors. Furthermore, to the best of our knowledge, no data are available on the intracellular trafficking of EcR and USP in any cell, although some components of these proteins are used in designing molecular switches for gene therapy (Toniatti et al., 2004).
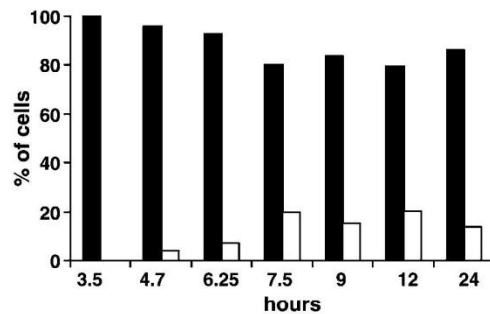

Figure 3 Intracellular localization of GFP-USP in CHO cells. Black bars represent the proportion of cells exhibiting exclusively nuclear localization of the tagged receptor 3.5–24 h after transfection; white bars represent cells with additional faint fluorescence in the cytoplasm.

The transactivation potency of the YFP-EcR/USP heterodimer is slightly reduced compared to the non-tagged proteins. A similar reduction has also been described for the androgen receptor-GFP (Tomura et al., 2001).
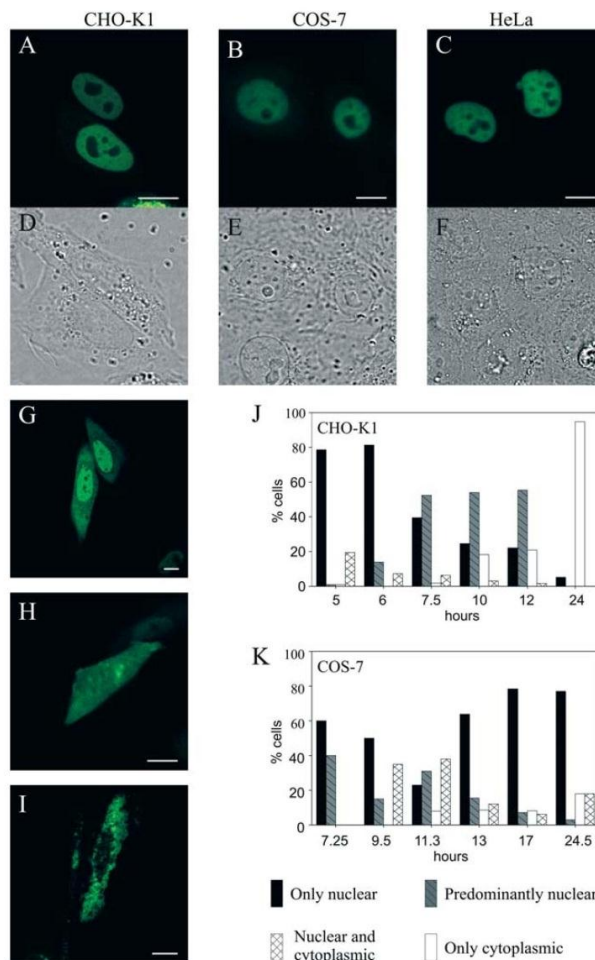

Figure 4 Analysis of the cellular distribution of YFP-EcR. Confocal images of the nuclear localization of YFP-EcR in three different cell lines: CHO-K1 (A,G,H,I), COS-7 (B) and HeLa (C). (D–F) Corresponding transmitted light images. Occasionally, localization patterns of YFP-EcR other than nuclear were encountered (CHO-K1; G,H,J). (J,K) Kinetics of the intracellular distribution of YFP-EcR in CHO-K1 and COS-7 cells after transfection, respectively. The bar represents 10 μm.

Unlike Drosophila USP, for which a single form of mRNA has been identified (Henrich et al., 1990; Shea et al., 1990; Oro et al., 1990), two USP isoforms have been reported in the mosquito Aedes aegypti (Kapitskaya et al., 1996), in the moth Manduca sexta (Jindra et al., 1997) and in the midge Chironomus tentans (Vögtli et al., 1999). These isoforms are most likely derived from the same gene via utilization of alternative promoters (Kapitskaya et al., 1996).
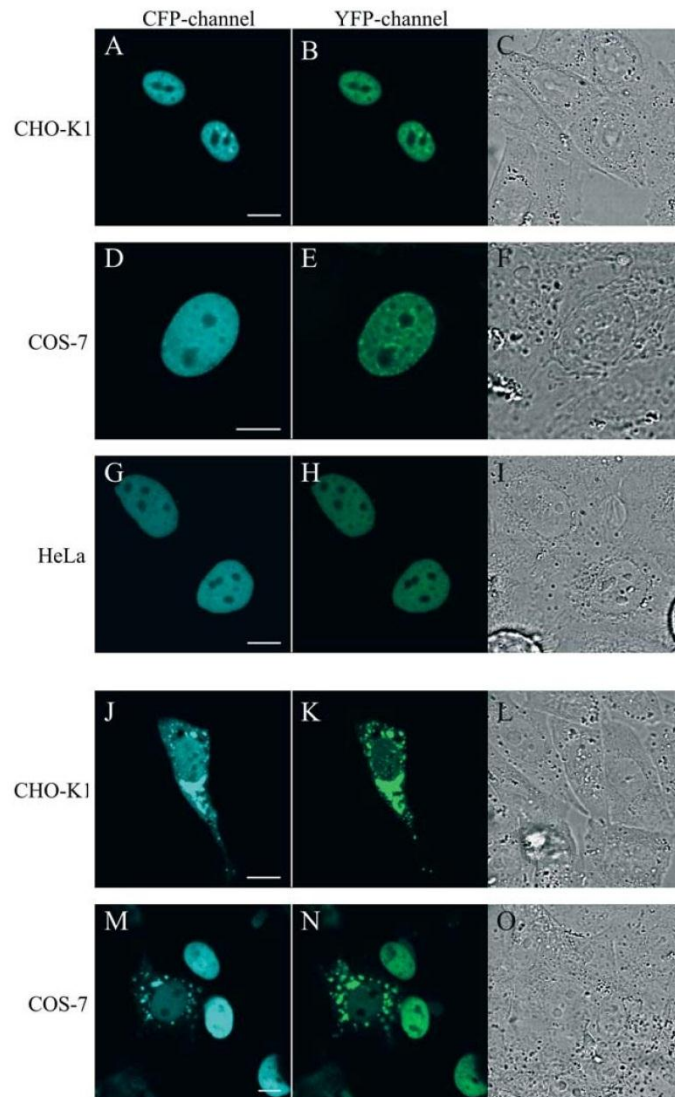

Figure 5 CFP-USP and YFP-EcRco localize in cell nuclei in co-transfected cells.
Confocal images of coexpressed CFP-USP (cyan) and YFP-EcR (green) in CHO-K1 (A,B), COS-7 (D,E) and HeLa (G,H) cells. Occasionally, bright extranuclear regions containing both CFP-USP and YCF-EcR were encountered in CHO-K1 (J,K) and COS-7 (M,N) cells. (C,F,I,L,O) Corresponding transmitted light images. The bar represents 10 μm.

We have no direct evidence for degradation of the C-terminally tagged USP. However, our observations and others concerning recombinant Drosophila USP (Christiansen et al., 1992; Christiansen and Kafatos, 1993; Rymarczyk et al., 2003) or USP expressed in heterologous systems (Vögtli et al., 1998) clearly indicate that this protein is very susceptible to degradation. It has also been shown that the most protease-sensitive region is localized in the N-terminus of USP (Rymarczyk et al., 2003). Thus, it is quite possible that, in contrast to the C-terminally tagged USP that has a free N-terminus, i.e., accessible for proteases, the N-terminus of the N-terminally tagged USP is protected against proteolysis due to the presence of the GFP derivative.

USP is localized exclusively in the nucleus, whereas the intracellular localization of EcR is dependent on time, cell type, and the presence of USP. Importantly, our results demonstrate for the first time that USP is an essential factor that dominates the cellular distribution of EcR – even in the absence of the ligand. Nevertheless, EcR alone is able to localize in the nuclear compartment to a high degree as well. This indicates that there are

active nuclear localization sequences in both proteins, responsible for their nuclear translocation. It also indicates that neither a ligand nor the heterodimerization partner is mandatory for nuclear import of EcR. At the moment we cannot explain the differences in the distribution of EcR within a given population of cells and between different cell types. One of the possible explanations for these phenomena is differences in the content of the putative partner for EcR. The most plausible candidate is retinoid X nuclear receptor, which is a mammalian homologue of USP with predominantly nuclear localization (Prüfer et al., 2000). Other possible reasons could be differences in the expression rate of EcR or the phase within the cell cycle, as well as the intracellular titer of transport proteins. Finally, it is possible that the velocity of EcR import/export varies among different cell types.

In some cases EcR occurs in defined clusters outside the nuclei. It seems that the clusters are formed when the concentration of EcR substantially rises. The clusters are EcR-specific, since they have never been observed for USP-fluorescent derivatives or for YFP alone. The EcR clusters may result from a specific interaction with some cytoplasmic components or concentration-dependent formation of inclusion body-like particles within the cytoplasm. The clusters formed in cells coexpressing CFP-USP contain CFP-USP as well. This may indicate a direct interaction between EcR and USP. Interestingly, this interaction does not require the presence of the ligand. An uneven distribution of fluorescence-labeled nuclear receptors can also be observed in some cases for vitamin D (Racz and Barsony, 1999) and the androgen receptor (Tomura et al., 2001). In the latter case, pronounced clustering has been demonstrated, especially in the nucleus.
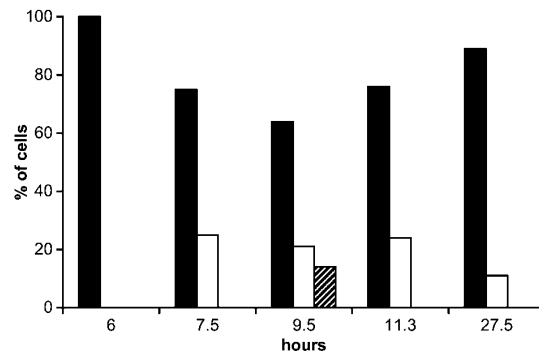

Figure 6 In the presence of USP, EcR is localized predominantly in the nucleus.
Intracellular distribution of YFP-EcR in CHO-K1 cells cotransfected with USP at different times after transfection (symbols as in Figure 4J,K).

The different intracellular localizations of USP and EcR observed in the absence of the corresponding heterodimerization partner may point to separate roles for the two nuclear receptors. This is further substantiated by several indications that USP is functional in the absence of EcR and vice versa, and by the fact that the developmental profiles of EcR and USP do not coincide (Riddiford et al., 2000; Spindler-Barth and Spindler, 2000). In addition, USP is involved in juvenile hormone signaling, as demonstrated in transactivation studies in which mutations in the juvenile hormone binding pocket of USP do not bind juvenile hormone any longer and suppress the juvenile hormone-induced activation of a reporter gene (Xu et al., 2002).

Materials and methods
Plasmid construction
Full-length EcR B1 isoform and USP were amplified by PCR (Saiki et al., 1988) and fused to the fluorescent proteins YFP and CFP by ligation with pECFP-C1, pEYFP-C1, pECFP-N1 or pEYFP-N1 (Clontech, Warszawa, Poland) using EcoRI and SalI (USP) or HindIII and BamHI (EcR) restriction sites. USP constructs were also fused to GFP and BFP by cloning into the expression vectors pQBI25 and pQBI50 using the restriction sites ApaI and NotI (Biogen, Carlsbad, USA). Recombinant plasmid DNA was purified with a Qiagen Plasmid Maxi Kit (Hilden, Germany) and verified by sequencing.

Cell culture and DNA transfection
Chinese hamster ovaricytes CHO-K1 (ATCC CCL-61) were maintained in Ham's F12 medium. African green

monkey kidney fibroblasts COS-7 (ATCC CRL-1651) and human cervix adenocarcinoma HeLa cells (ATCC CCL-2) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 1 % non-essential amino acids (Gibco/Invitrogen, Karlsruhe, Germany and Gibco/Invitrogen, Warszawa, Poland), 1 mM sodium pyruvate and 2% glutamine (Gibco). Both media were supplemented with fetal calf serum (FCS), 5% for CHO-K1 cells and 10% for COS-7 and HeLa cells. Cells were grown at 37°C in a 95% air/5% CO_2 atmosphere. Cells were transfected either with 3 µg DNA/300 000 cells using jetPEI (Quatrum Biogene, Gdansk, Poland) or with 250 ng DNA/10 000 cells with lipofectamine (Invitrogen) according to the manufacturer's instruction. For kinetic studies we used 12-well TC plates applying 1.6 µg/130 000 cells per well.

Transcription activation assay
Transactivation studies with EcR and receptor fusion proteins were performed using the hormone responsive element hsp27 (Riddihough and Pelham, 1987; ˙Ozyhar et al., 1991) coupled to luciferase as a reporter gene as described by Henrich et al. (2003). The influence of hormone was studied after addition of 10^{-5} M (final concentration) muristerone A (Sigma, Deisenhofen, Germany) dissolved in dimethyl sulfoxide.

Microscopy and confocal laser scanning
Before imaging experiments, cells were grown on 0.17-mm-thick round glass coverslips (Mentzel, Braunschweig, Germany) submerged in culture medium in 4-cm-diammeter Petri dishes. The standard culture medium was replaced 18 h after transfection by DMEM/F12 buffered with 15 mM HEPES without phenol red (Sigma, Poznan, Poland). Coverslips with cell cultures were transferred into a steel holder and mounted in a microscope stage microincubator (Life Science Resources, Cambridge, UK). During microscopy studies, the temperature of cell cultures was maintained at 37°C. Images of fluorescently labeled proteins were acquired using an MRC1024 confocal system (Bio-Rad, Hemel Hempstead, UK), built on a Nikon Diaphot 300 inverted microscope (Nikon, Amsterdam, Netherlands), and equipped with a 100-mW argon ion laser (I LT, Salt Lake City, USA). A 60× PlanApo oil-immersion NA 1.4 objective lens was used. CFP and YFP fluorescence was excited using light at 457 and 514 nm, respectively. A z458/514rpc dual primary dichroic filter (Chroma, Rockingham, USA) was used. To separate fluorescence emissions of CFP and YFP a 510DCLP dichroic filter (VHS filter block) and HQ485/30 and HQ540/30 (Chroma) emission filters were used.

Electrophoresis and Western blotting
Cells were solubilized 24 h after transfection in lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1 % Nonidet P-40). The lysate was mixed with SDS 2× gel loading buffer (Laemmli, 1970), boiled for 5 min and centrifuged for 5 min (13 000 g). Proteins were separated by 10% SDS-PAGE and transferred to a Protran Nitrocellulose Transfer Membrane (Schleicher & Schuell GmbH, Dassel, Germany) with a Mini Trans-Blot apparatus (Bio-Rad, München, Germany). The membrane was incubated overnight at 4°C with anti-GFP polyclonal antibody, which cross-reacts with CFP and YFP (1:400; Clontech, Warszawa, Poland). Secondary goat anti-rabbit antibody coupled to horseradish peroxidase (Vector Laboratories, Warszawa, Poland) was added (1:10 000) and incubated for 1 h at room temperature. Blots were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Freiburg, Germany).

Gel mobility shift assay
The oligonucleotides 59-AGC GAC AAG GGT TCA ATG CAC TTG TCC AAT GAA-3 and 59-TTC ATT GGA CAA GTG CAT TGA ACC CTT GTC GCT-39 corresponding to the 20E response element from the hsp27 gene promoter (Riddihough and Pelham, 1987; ˙Ozyhar et al., 1991) were annealed and labeled with [α^{32}P]dCTP in a fill-in reaction with Klenow polymerase. Oligonucleotides were separated from monomers using Mini Quick Spin Oligo Columns (Roche, Mannheim, Germany) and used as probes for binding of the fusion proteins.

The reaction mix contained binding buffer [20 mM HEPES, pH 7.4, 100 mM KCl, 5% (v/v) glycerol, 2 mM dithiothreitol, 0.1 % NP-40], cell extracts with the EcR and USP fusion proteins, 1 µg of non-specific competitor poly(dIdC) and approximately 10fmol of labeled oligonucleotide; 10^{-5} M muristerone A (final concentration, dissolved in ethanol) was added where indicated. The reaction mix was incubated at room

temperature for 30 min and separated at 10 V/cm on a 5% non-denaturing polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.0) for 2 h. Gels were dried, scanned with a phosphor-imager and evaluated with ImageQuant- software (Molecular Dynamics, Sunnyvale, USA).

Kinetics of intracellular distribution

For time course experiments, the intracellular distribution of fluorescence was evaluated with an inverted microscope (Zeiss Axiovert M35). To ensure a randomized approach, various samples from different regions within a well were analyzed, with a total of approximately 100 cells for each time interval. Three independent transfection experiments were performed. The intracellular localization was assigned to one of the arbitrary categories (only nuclear, predominantly nuclear, nuclear and cytoplasmic, or only cytoplasmic).

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