Differential Subcellular Distribution and Transcriptional Activity of ΣE3, ΣE4, and ΣE3–4 Isoforms of the Rat Estrogen Receptor-α

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ΣE3, ΣE4, and ΣE3–4 are naturally occurring estrogen receptor (ER) isoforms, generated through differential splicing of the ERα primary transcript and abundantly expressed in embryonic rat pituitary. Studies in COS cells transfected with full-length ERα or its three splice variants fused to green fluorescent protein (GFP), revealed a different subcellular localization for each isoform. In the absence of estradiol, full-length ERα-GFP was predominantly nuclear, and ΣE3-GFP and ΣE4-GFP were present both in cytoplasm and nucleus, whereas ΣE3–4-GFP was predominantly cytoplasmic. Upon hormone treatment, a dramatic redistribution of full-length ERα-GFP and ΣE3-GFP, from a diffuse to punctate pattern, occurred within the nucleus. In contrast, the distribution of ΣE4-GFP and ΣE3–4-GFP was unaffected. Nuclear fractionation studies showed that full-length ER-α and ΣE3 displayed the same hormone-induced ability to tether to nuclear matrix, whereas nuclear ΣE4 appeared to remain loosely associated to functional nuclear constituents. When cotransfected with an estrogen-inducible reporter plasmid (VIT-TK-CAT) in ER-negative (CHO k1) and ER-positive pituitary (GH4 C1) cells, ΣE3–4 exhibited a very weak estrogen-dependent transactivation activity, whereas ΣE3 had an inhibitory effect on full-length ER action. Conversely, ΣE4 displayed estrogen-independent transcriptional activity in ER-negative cells, and in ER-positive cells, enhanced the estrogen-induced gene expression as efficiently as full-length ERα. In a gel mobility shift assay, phosphorylated ΣE4 was able to form a specific complex with a consensus ERE, while ΣE3 and ΣE3–4 never did bind by themselves. The observed inhibitory action of ΣE3 on estrogen-dependent transcription would rather involve protein-protein interactions such as formation of heterodimers with full-length ERα, as suggested by immunoprecipitation followed by Western blotting. These data suggest that ΣE3 and ΣE4 may play a physiologically relevant role as negative or constitutively positive modulators of transcription, in the developing rat pituitary. (Molecular Endocrinology 15: 894–908, 2001)

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INTRODUCTION

Estrogens act in a variety of tissues to regulate target gene expression by modulating the activity of specific nuclear receptors. To date, two nuclear estrogen receptors (ERα and -β) have been identified that are encoded by different genes (1–3). ERs belong to the large superfamily of nuclear receptors including steroid hormone, thyroid hormone, retinoic acid, and vitamin D_3 receptors. In addition to these ligand-activated transcription factors, several orphan receptors have been isolated for which a physiological ligand is either unknown or unnecessary (4–7). All members of the nuclear receptor superfamily share a characteristic modular structure with at least three structural and functional domains able to act relatively independently of each other: the ligand-binding domain located at the C-terminal half of the protein, the DNA-binding domain located centrally, and a variable transactivation domain located at the N-terminal end (8).

For both ERα and -β, a number of variant transcripts have been described, particularly in cancer cell lines or tumors (see Refs. 9 and 10 for reviews). While several of these transcripts, created by skipping internal exons, retain the same reading frame as the full-length transcript, the corresponding variant proteins have been rarely detected. In nontumoral tissue, only one ERα variant (TERP1; Refs. 11–14) and one ERβ variant

(15) have been shown to be translated into functional proteins.

We have recently identified in the pituitary gland three naturally occurring ERα isoforms (ΣE3, ΣE4, and ΣE3–4), the expression of which is specifically modulated during development (16). ΣE3 harbors a deletion of exon 3 encoding the second zinc finger of the DNA-binding domain, ΣE4 lacks exon 4 that encodes a nuclear localization signal (NLS) and part of the steroid binding domain, and ΣE3–4 lacks both exons 3 and 4 (Fig. 1). At early stages of pituitary development in the rat, these spliced isoforms are expressed abundantly, at least 4 days earlier than full-length ERα. In addition, ΣE3 and ΣE4 are found essentially in the pituitary intermediate lobe and in the cytoplasm, in contrast to full-length ERα , which is mainly present in the nucleus (16).

The localization of the unliganded nuclear receptors within the cell and the mechanism of their activation after hormone binding have been intensively debated. Contrary to the original proposal (17), ERs appear to be already located in the nucleus before hormone binding (18, 19), which, in fact, would mainly stabilize an active conformation of the receptor. Hormone binding would promote the dissociation of the receptor from chaperone proteins, ultimately triggering the tight binding of the dimerized receptor to cognate sites on estrogen-responsive genes (20, 21). Although full-length ER is located predominantly in the nucleus at steady state, it actually shuttles between the nucleus and the cytoplasm (22), involving an active and continuous relocation of the hormone-receptor complex (23). There is evidence that specific NLSs are responsible for nuclear targeting of proteins. This process includes two distinct steps: binding to the cytoplasmic side of the nuclear pore followed by energy-dependent transfer inside the nucleus (Ref. 24, for review). ERs contain multiple NLS: an estrogen-inducible proto-NLS was found in the hormone binding domain, which, on its own, is not sufficient but which, in the presence of estrogen, can cooperate with three constitutive NLS (25). Although it probably involves interaction with NLS-binding proteins (26), the molecular mechanism of nuclear translocation of the receptors is still unknown.

The peculiar cellular localization of the ΣE3, ΣE4, and ΣE3–4 ER isoforms observed in fetal pituitary tissue, and the fact that they lack certain functional regions, prompted us to investigate whether they might play a specific regulatory function during pituitary development. Using transiently transfected cells,

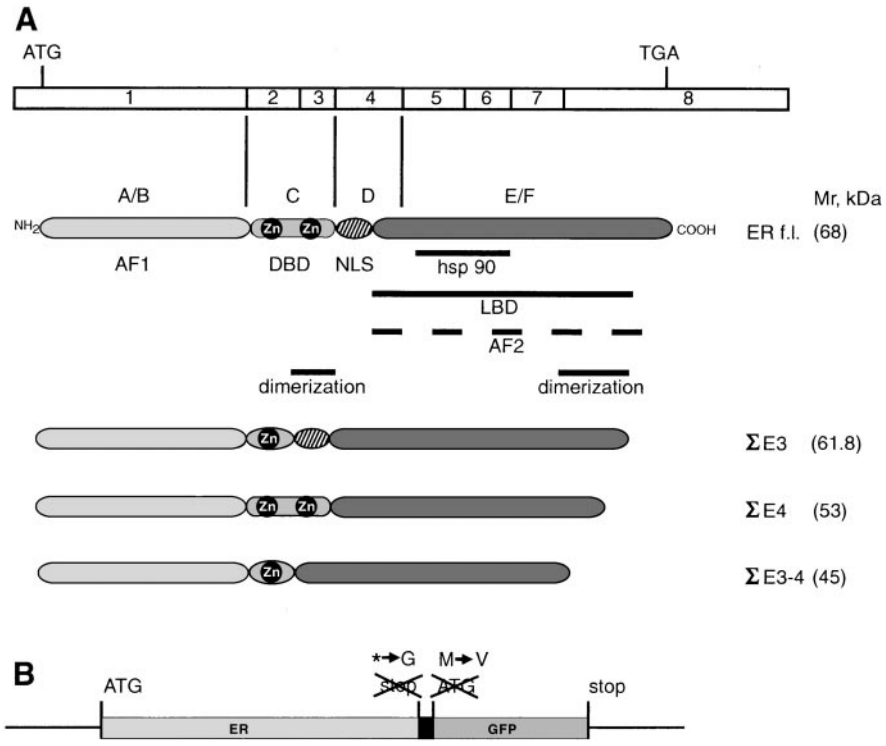

Fig. 1. Schematic Representation of the Structure of ER Isoforms and GFP Constructs Used in This Study

A, Schematic representation of the structure and the diverse functional domains (see text) of full-length ERα and its splice variants. ΣE3, ΣE4 and ΣE3–4 isoforms are created by skipping of either exon 3, exon 4, or both exons 3 and 4 from the ERα primary transcript. Molecular masses of the resulting proteins are indicated in kilodaltons (kDa). B, Construction of ER-GFP and ERΣ-GFP expression plasmids: pEGFPμ plasmid was generated from pEGFP-N1 (CLONTECH Laboratories, Inc.) by mutation of the GFP initiation methionine to a valine. The ERα and ERΣ sequences were modified by mutation of the stop codon to a glycine codon and fused to the 5′-end of the mutated GFP of the pEGFPμ plasmid.

we first studied their precise subcellular distribution, in the presence or absence of hormone. Then, we examined the ability of these short isoforms to modulate transcription of estrogen-target genes, *i.e.* whether they are similarly activated by the hormone, whether they act as real transcription factors, and finally whether and how they affect the transcriptional activity of full-length ERα.

RESULTS

Subcellular Localization of ERα Isoforms in Transfected Cells

To observe the subcellular localization of the ERα and its isoforms in living cells, the C terminus of the four ERα isoforms was fused to green fluorescent protein (ERs-GFP; see *Materials and Methods* and Fig. 1B). COS-1 cells, transfected with the ERs-GFP expression vectors and treated or not with 17β-estradiol (E_2), were analyzed under a fluorescence microscope with a laser scanning confocal imaging system. To avoid problems that may be caused by overexpression of receptors (27), we selected cells expressing relatively low levels of GFP-tagged molecules. In these conditions (Fig. 2), full-length ER-GFP was essentially nuclear in the absence of hormone and appeared evenly distributed throughout the nucleus, excluding the nucleolus. In contrast, ΣE3–4-GFP was present predominantly in cytoplasm, while ΣE3-GFP and ΣE4-GFP were observed both in cytoplasm and nucleus.

Upon E_2 treatment, fluorescence became strictly restricted to the nucleus of COS-1 cells transfected with full-length ER-GFP and ΣE3-GFP, and a dramatic redistribution of both isoforms from a diffuse to a hyperspeckled pattern occurred within the nuclei. By contrast, nuclear ΣE4-GFP remained evenly distributed after hormone treatment, and ΣE3–4-GFP remained cytoplasmic.

Estradiol Effect on the Association of ERα Isoforms with the Different Nuclear Constituents

It has been consistently shown that upon binding of an agonist ligand, ERα undergoes changes in its subnuclear distribution and becomes tightly associated with nuclear matrix (NM) (20, 27–30). We thus qualitatively assessed whether the three deleted isoforms showed the same hormone-induced tethering to the NM.

To identify the subnuclear compartments in which liganded or unliganded ER isoforms would reside, serial extractions were performed on transfected COS-7 cells treated or not with E_2 (1 nM). Cytosolic fractions, as well as nuclear fractions containing detergent-extractable proteins (Triton X-100-supernatant or nucleoplasm), DNase I-extractable proteins (DNase I supernatant or chromatin), and finally, proteins remaining after removal of DNA and its associated proteins (NM), were analyzed by Western blotting using a previously described anti-ERα antiserum (16). As shown in Fig. 3, LDH and histone, used as controls, were only detected in the cytosolic and chromatin fractions, respectively. Still, overexpression of proteins in transfected cells may lead to nuclear leakage, as well as nuclear contamination with perinuclear cytoplasm during cell fractionation procedures. This may account, for instance, for the presence of ΣE3–4 in the nucleoplasm fraction. The distribution of the three other isoforms in the absence of hormone was nevertheless qualitatively similar to that described in our cytological study, with large amounts of full-length ER and ΣE3 in the nucleoplasm, and ΣE4 being located both in the nucleoplasm and the cytosol. The amount of full-length ER and ΣE3 resistant to extractions was significantly increased after treatment of cells with E_2 for 2 h, thus showing their tight association with the chromatin and NM fractions. By contrast, ΣE3–4 distribution was almost unaffected, and the nuclear ΣE4 remained fully soluble in Triton X-100 (nucleoplasm), being unassociated with chromatin or NM. Thus, for all ERα isoforms, the hormone effect on their subcellular distribution reflected the changes in their ability to tightly associate to the functional components of the nucleus.

Transcriptional Activity of Alternatively Spliced Isoforms of ERα

To assess the biological activity of the alternatively spliced receptor forms, expression vectors containing one specific ERα isoform were coexpressed with an estrogen-inducible reporter plasmid (pVit-TK-CAT) in CHO k1 (ERα-negative) and GH4 C1 (ERα-positive) cells.

Transfection of CHO cells with full-length ERα and reporter plasmid resulted in an approximately 4-fold increase in chloramphenicol acetyltransferase (CAT) synthesis in response to E_2 (Fig. 4A). Changes in gene expression were dependent on the expression of an exogenous ERα and not the result of an overall increase in basal transcription. Indeed, when CHO cells transfected with the reporter gene alone were treated with E_2, in no instance was transcription of the pVit-TK-CAT target gene stimulated (Fig. 4A, lanes 1and 2).

Regarding the effects of the ER short isoforms, we found that while ΣE3 alone had no effect on estrogen-dependent transcription, a coexpression of ΣE3 and full-length ERα resulted in a potent ($\sim$60%) inhibition of reporter gene transactivation in comparison to full-length ERα alone. Conversely, ΣE4 strongly stimulated the transcription of the Vit-TK-CAT reporter gene, even in the absence of E_2. ΣE4 thus displayed estrogen-independent constitutive transcriptional activity that was approximately 110% of the activity of the full-length receptor with E_2. Coexpression of full-length ERα and ΣE4 resulted in the same increase in CAT quantity as in the

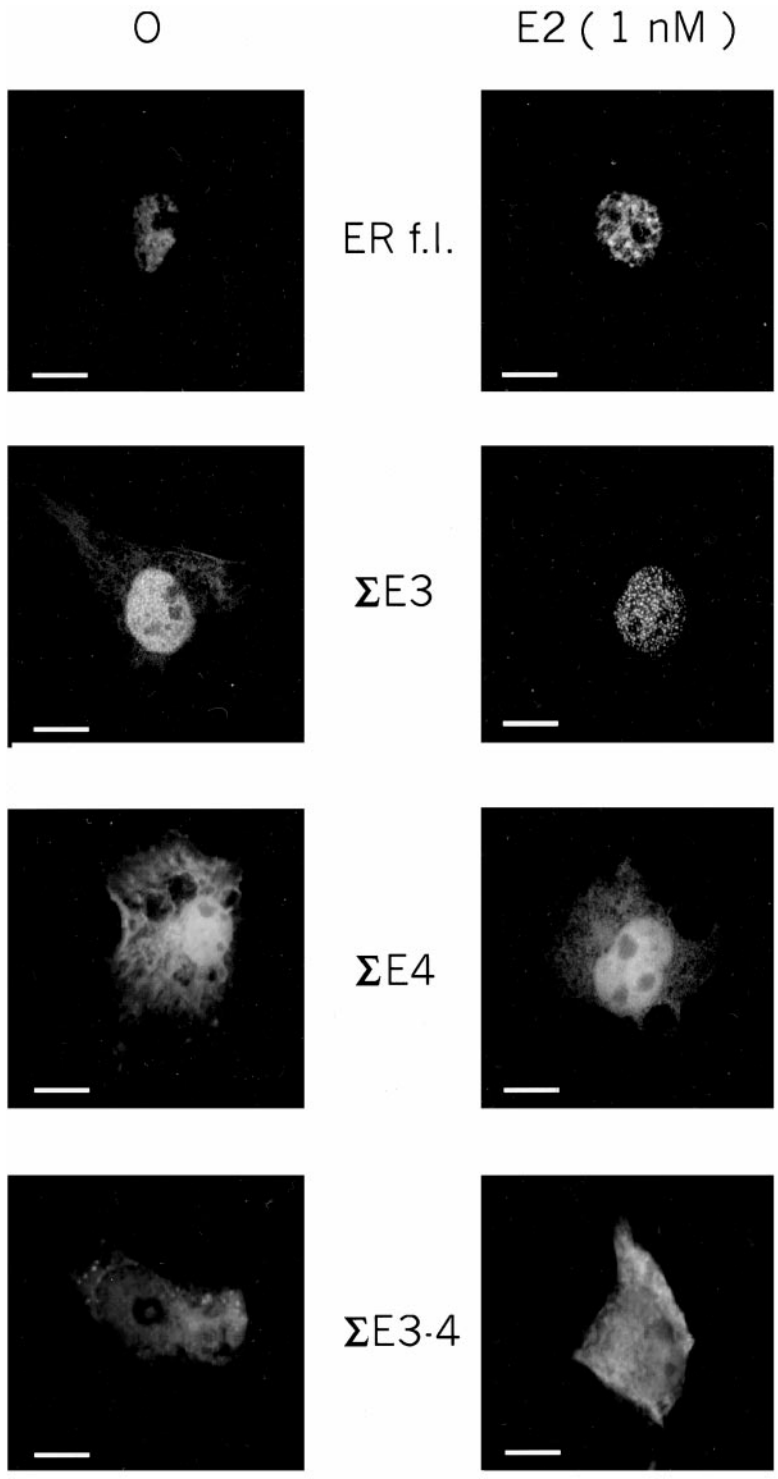

Fig. 2. *In Vitro* Subcellular Localization of ERα and Its Splice Isoforms in the Presence (E_2), or Absence (0) of E_2

COS-1 cells were transfected with plasmid encoding a GFP-tagged isoform of ER, cultured on glass coverslips for 30 h in DMEM without phenol red supplemented with 10% FCS twice stripped by charcoal/dextran, and finally treated or not with 1 nM E_2 for 2 h. Confocal fluorescence microscopy images are presented. *Bars* =10 μm.

absence of full-length ER, but E_2 dependency was recovered. Expression of ΣE3–4 did not significantly stimulate the transcription of the Vit-TK-CAT reporter gene in response to E_2. Coexpression of full-length ERα and ΣE3–4 yielded the same increase in CAT quantity as in the absence of ΣE3–4.

As shown in Fig. 4B, transcription of the CAT target gene was stimulated approximately 4-fold when GH4

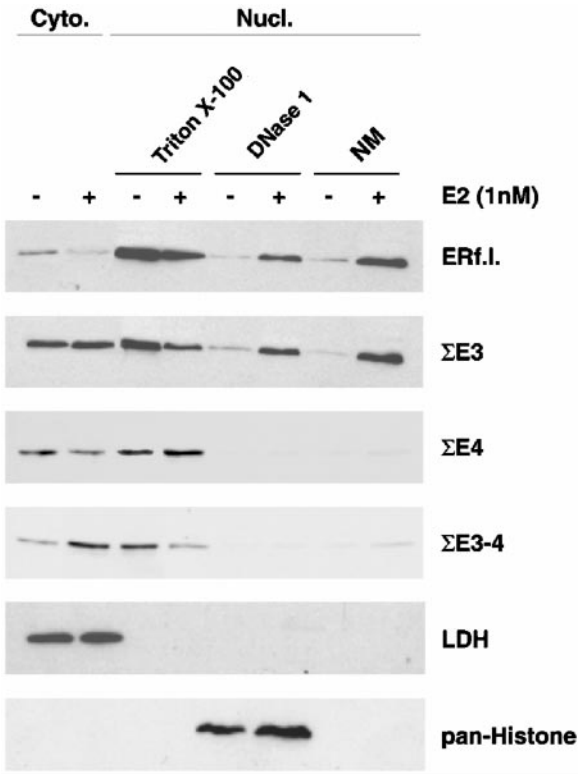

Fig. 3. Subcellular and Subnuclear Distribution of ERα Isoforms, in the Presence or Absence of E_2

COS-7 cells transfected with an ERα isoform and treated (+) or not (−) with E_2 were sequentially fractionated as described in *Materials and Methods*. Cyto., Cytosolic; Nucl., nuclear fractions; Triton X-100, detergent-extractable proteins (nucleoplasm); DNase 1, DNase 1-extractable proteins (DNA-associated proteins or chromatin); NM, nuclear matrix-bound proteins (*i.e*. those remaining after the removal of chromatin). Proteins from each fraction were analyzed by Western blotting, using 1) the anti-ERα polyclonal antiserum, 2) an anti-LDH antibody, and 3) an antipanhistone antibody. Note the highly increased association of full-length ER and ΣE3 to chromatin and NM after ligand binding.

C1 cells transfected with the estrogen response element (ERE)-containing reporter plasmid alone were treated with E_2, confirming the presence of active endogenous ERs. In the same conditions, the activity of the minimal TK-CAT promoter was not stimulated. GH4 C1 cells were used to further analyze the potential interactions between a distinct isoform and the endogenous ER activity. In these cells, overexpression of full-length ERα resulted in an approximately 7-fold increase in CAT quantity in response to E_2. Expression of either ΣE3 or ΣE3–4 did not stimulate CAT synthesis (~3-fold or 5-fold, respectively) above the level obtained by the reporter plasmid alone (VTC) in response to E_2. By contrast, expression of ΣE4 resulted in an approximately 12-fold increase in CAT quantity in response to E_2. Thus, in GH4 C1 cells, ΣE4 appeared to be able to enhance CAT reporter gene expression at least as efficiently as full-length ERα.

Coimmunoprecipitation of Full-Length ERα and ER Isoforms

To investigate whether the effects of ERα isoforms on transcription could involve protein-protein interactions, a series of immunoprecipitations was performed in which the ability of the short variants to form heterodimers with full-length ERα was examined. To differentiate isoforms from full-length ER, we used a full-length ERα tagged with an hemagglutinin epitope (HA).

COS-7 cells were transfected with plasmids encoding HA-tagged full-length ER alone or together with a plasmid encoding one isoform. Western blots prepared from aliquots of cell extracts were used to confirm that the proteins were expressed at equal levels. Blots were reacted either with a monoclonal antibody against HA (data not shown) or with the anti-ERα antiserum (Fig. 5A). The remainder of the extracts were immunoprecipitated with the anti-HA monoclonal antibody, and Western blots of the immunoprecipitated proteins were probed with the anti-ERα polyclonal antiserum. The data presented in Fig. 5B demonstrate that ΣE3 was efficiently coimmunoprecipitated with the HA-tagged full-length ERα, in the presence or absence of hormone. In contrast, ΣE4 and ΣE3–4 were unable to form stable complexes with full-length ERα.

Gel Mobility Shift Assays and DNA Binding of ERα Isoforms

To investigate whether the inhibition or estrogen-independent activation of transcription observed for ERα isoforms is exerted at the level of DNA binding, a series of gel mobility shift assays were carried out. The ERE oligonucleotide used in these assays contained the 13-bp palindromic sequence identified in the *Xenopus* vitellogenin (Vit) A2 promoter (31). The ERα isoforms were produced in COS-7 cells, and reliability of their expression at equal levels was probed by Western blotting. As determined by SDS-PAGE, the apparent molecular mass of full-length ERα, ΣE3, ΣE4, and ΣE3–4 was 67, 61, 53, and 45 kDa, respectively, close to those predicted from DNA sequences.

Aliquots of WCEs containing individual receptors, prepared from cells treated or not with forskolin/IBMX (isobutylmethylxanthine), were incubated with a ^{32}P-labeled double-stranded ERE oligonucleotide and assayed by electrophoresis on a nondenaturing polyacrylamide gel. As expected, specific ER-ERE complexes were formed with full-length ERα (in the basal, or phosphorylated state), and the intensity of the band increased with the amount of WCE (Fig. 6A, lanes 3 to 6). It may be noticed that more than one complex has formed. However, the lower band (marked with an *asterisk*) is likely to be nonspecific,

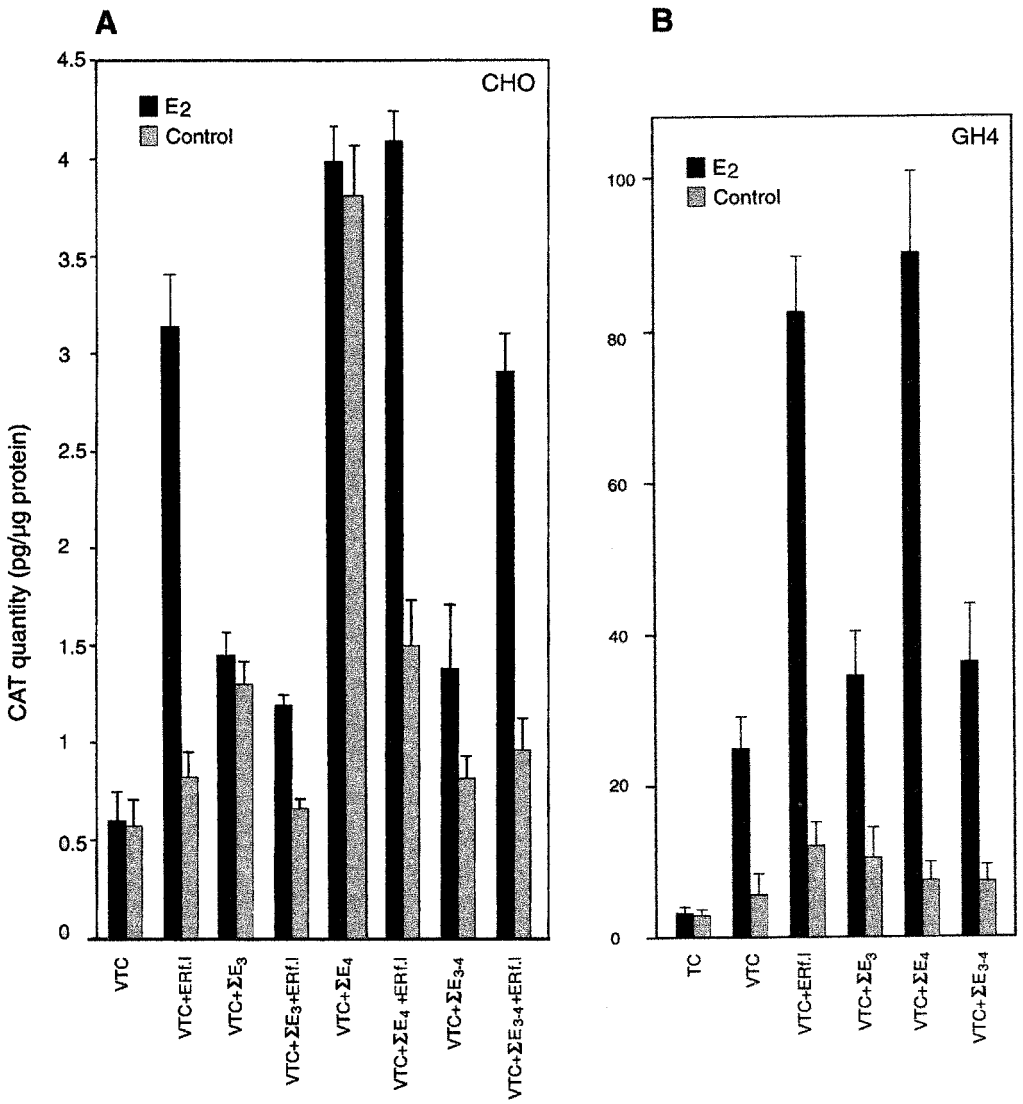

Fig. 4. Transcriptional Activation of a CAT Reporter Gene Containing an ERE (Vit-TK-CAT; VTC) by the Different ERα Isoforms in the Presence or Absence of E_2 (1 nM)

A, CHO (ERα-negative) cells were transfected with CAT plasmid alone (VTC), or together with an ER isoform encoding plasmid, combined or not with full-length ERα plasmid. B, GH4 (ERα-positive) cells were transfected with CAT reporter plasmid (VTC) alone or together with an ER isoform encoding plasmid. Because of the presence of endogenous active ERs, a CAT reporter plasmid containing no ERE was added as negative control (TC). CAT quantities (expressed as picograms CAT/μg total protein) produced 48 h after transfection are calculated as means (*error bars,* SD) from three to six independent experiments.

being present also in the control lane, which contains extracts from COS-7 cells transfected with the empty expression vector.

When prepared from cells that were not previously treated by forskolin/IBMX, ΣE3, ΣE4, and ΣE3–4 isoforms were unable to form any specific complex with the consensus ERE sequence (data not shown). By contrast, after a general activation via a cAMP-dependent phosphorylation, ΣE4 formed a specific complex with the labeled ERE (Fig. 6, A and B), the intensity of which increased with the amount of WCE (Fig. 6A, lanes 7 to 10). The formation of this complex could be competitively inhibited by an excess of unlabeled ERE (Fig. 6 B, compare lanes 6 and 10), and its intensity progressively diminished when an increasing amount of anti-ERα antiserum was added (Fig. 6B, lanes 6 to 9), whereas an anti-BSA (control-) antiserum had no effect (data not shown). This complex was remarkably smaller than that formed between full-length ERα and the ERE probe, and it also behaved very differently in the presence of anti-ERα antibody (Fig. 6B, compare lanes 3, 4, 6, and 8). Indeed, with this antiserum directed against the carboxy terminus of ERα, a supershift was observed for the full-length ER-ERE complex, but not for the ΣE4-ERE complex.

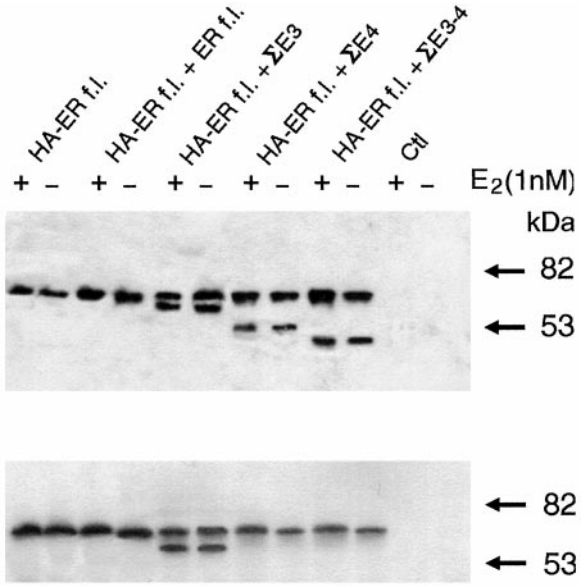

Fig. 5. Coimmunoprecipitation of Full-Length ERα and Its Isoforms

COS-7 cells were transfected with expression plasmids encoding full-length HA-tagged ERα alone (HA-ER f.l.), or together with individual ER isoforms, and cultures were treated (+) or not (−) with 1 nM E_2. Upper panel, Western blot analysis of protein extracts from transfected COS-7 cells. Blots were reacted with antiserum directed against ERα, which recognizes the HA-tagged full-length receptor as well as the truncated isoforms. Note that each isoform is expressed in about equal amounts. Ctl, Control, extracts from COS-7 cells transfected with the empty expression vector. *Lower panel,* Extracts were immunoprecipitated as described in *Materials and Methods* using a monoclonal antibody against the HA tag. Immunoprecipitates were then subjected to Western blot analysis using anti-ERα polyclonal antiserum. Molecular masses (in kilodaltons) are indicated on the *right*. These immunoblots are representative of three independent experiments. Note that *in vivo* ΣE3 heterodimerizes with full-length ERα, in contrast to the other splice variants.

Forskolin-Induced Change in ΣE4-GFP Subcellular Localization

Since ΣE4 expressed in COS cells that were treated with forskolin/IBMX was able to bind a consensus ERE, we tested whether the same treatment could also promote specific morphological changes in the nucleus, suggestive of an activated state for ΣE4, and its tethering to NM.

COS-1 cells transfected with expression plasmids encoding the GFP-tagged ERs, and treated or not with forskolin/IBMX for 15 min, were analyzed under confocal fluorescence microscopy. The distribution of full-length ER-GFP, ΣE3-GFP, or ΣE3–4-GFP was totally unaffected by this treatment (data not shown). On the contrary, as shown in Fig. 7, unlike E_2 treatment (see Fig. 2), forskolin/IBMX treatment appeared to change the diffuse nucleoplasmic pattern of ΣE4-GFP distribution (*left image*) into a partially speckled one and may even induce the complete disappearance of ΣE4-GFP from the cytoplasm (*right image*).

DISCUSSION

We previously identified three short isoforms of ERα mRNA and the corresponding proteins that are expressed in the developing rat pituitary (16). The truncated ERα proteins, lacking certain functional regions, appeared to be localized differentially within the pituitary cells. Here, to characterize their functional properties, we used transiently transfected cells to examine their precise subcellular distribution, as well as their respective ability to modulate gene transcription, in comparison to full-length ERα.

ERs are known to undergo a continuous nucleocytoplasmic shuttling (22, 32, 33). Nevertheless, at any given time, a major fraction of the protein is present in the nucleus even in the absence of hormone (18, 19). The unliganded receptor with a sedimentation coefficient of 9S is loosely associated to nuclear components, and hormone binding causes activation of the receptor, *i.e.* a biochemical transformation to a complex with a sedimentation coefficient of 5S that associates more tightly with the nucleus (20, 28, 34). Recently, using GFP tagging, two groups provided visual descriptions of the unoccupied and the ligand-bound ER. The majority of unliganded GFP-ER appears to be evenly distributed throughout the nucleus. Addition of E_2 dramatically changes the diffuse nucleoplasmic pattern of GFP-ERα to a punctate or hyperspeckled pattern (27, 30). This early intranuclear redistribution of GFP-ERα corresponds to the formation of NM-bound foci of ERα. These foci are probably similar to the so-called matrix-attachment regions (MARs) that involve 1–4% of the genomic DNA, occur at DNase hypersensitive sites near active genes, and contain many transcription factors (35, 36). The NM-bound foci are quite numerous as compared with real sites of transcription starts, and most of the receptor attachment sites are not actively involved in transcription (27, 37). In fact, it is possible that both constitutive and regulatory MARs exist, and that only the latter are transiently attached to transcriptionally active genes and involved in their regulation (38). The formation of these NM-bound foci of ERα is not sufficient for transcription to occur, as both agonist and antagonist ligands may elicit such an event (27). Nevertheless, this early intranuclear redistribution of the receptor and association with NM is a mandatory step to transcription activation that, provided it is induced by an agonist, enables ERs to additionally recruit coactivators and finally trigger transcription (27).

In full agreement with these results, our present studies in COS cells transfected with full-length ERα, fused or not to GFP, showed that, in the absence of ligand, the protein was predominantly present in the nucleus and exhibited a diffuse nucleoplasmic pattern.

In a nuclear fractionation study, unliganded full-length ERα was found most prominently in the Triton X-100 extract. After E_2 treatment, receptor distribution was restricted to the nucleus where it displayed a characteristic speckled pattern, and a substantial amount of this protein was strongly associated with chromatin and NM. It has been pointed out by Stenoien *et al.* (27) that transient expression of ERs in heterologous cells may lead to saturation of the intracellular transport mechanisms, and hence to a misrepresentation of soluble *vs.* NM-bound forms of ER. While this may explain why even after ligand binding, a certain amount of full-length ERα remained in the Triton X-100 soluble fraction, our observations concerning the ligand-induced changes in compartmentalization are in agreement with those described for cells naturally expressing ERs.

In addition, our present study on ERα variants revealed marked differences between full-length receptor and its isoforms with regard to their subcellular localization, solubility partitioning, activation, and biological actions.

ΣE3: An Inhibitor of Estrogen-Target Gene Transcription

In the absence of ligand, the ΣE3 protein was present both in the cytoplasm and the nucleus of transfected cells, as determined either by fluorescence studies employing GFP tagging or by Western blot analysis of subcellular/subnuclear fractions. This isoform retains the ligand binding domain and the NLSs, allowing for hormone binding and translocation to the nucleus. Accordingly, we observed that E_2 treatment clearly altered the partitioning of ΣE3 between cytosol and nucleus, and its intranuclear distribution: in the presence of hormone, ΣE3 was found exclusively in the nucleus, showed a punctate distribution, and was found tightly associated with chromatin and NM, like full-length ERα. Yet, ΣE3 lacks the second zinc finger of the DNA-binding domain, and, as anticipated, our current gel mobility shift assay data show that this protein was unable to bind to a canonical ERE *in vitro* or to activate transcription of a CAT reporter gene containing an ERE *in vivo*. In fact, when cotransfected with full-length ERα, ΣE3 repressed the estrogen-dependent increase in CAT synthesis induced by full-length ERα *in vivo*. Thus, in agreement with previous results obtained in the MCF-7 breast cancer cell line (39), ΣE3 inhibits transcriptional activation by full-length ERα in a dominant negative fashion. This finding is probably related to the fact that ΣE3 inhibits full-length ER binding to a canonical ERE *in vitro,* as demonstrated by Wang and Miksicek (39).

The ΣE3 isoform is thus likely to modulate the activity of full-length ERα by protein-protein interactions. For this inhibition, at least two mechanisms that are not mutually exclusive seem possible. First, ΣE3, which still contains a strong hormone-inducible dimerization motif within the ligand-binding domain, could form inactive heterodimers with full-length ERα. In support of this model, we showed herein that ΣE3, in the presence or absence of E_2, can be coimmunoprecipitated with full-length ER-HA, implying a direct physical association between ΣE3 and full-length ERα. Such heterodimers could then sterically hinder binding of functional receptors. A second mechanism would involve interactions of ΣE3, as a homo- or heterodimer, with a limiting factor required either for receptor function or transcriptional activation. This type of factor, which interact both with enhancer binding proteins and basal transcription factors, can, under some circumstances, be readily depleted via binding to other transcription factors. In this respect, Bollig and Miksicek (40) showed that in the presence of E_2, ΣE3 can bind the nuclear receptor coactivator SRC-1, as does full-length ERα. The coactivators are limiting factors for which ΣE3 and full-length ER-α are probably competing. Such interaction with an adaptor molecule may also account for the tight association of ΣE3 with chromatin or NM that we observed upon addition of E_2.

It has been shown that the amount of ΣE3 transcript is dramatically reduced in primary breast cancers and cancer cell lines, while transfection of ΣE3 into MCF-7 cells leads to a suppression of the transformed phenotype (41). In the light of these latter results and our data, it seems likely that ΣE3 expression in normal tissue may provide a means of decreasing or blocking estrogen responsiveness.

ΣE4: An Estrogen-Independent Activator of Transcription

The ΣE4 isoform lacks amino acids 255–366, corresponding mainly to the hinge region that contains a constitutive NLS. Yet, the present study shows that, in the absence of hormone, a certain amount of this protein was found in the nucleus of transiently transfected COS cells. Lacking also the first 48 amino acids of the ligand binding domain, ΣE4 was expected to be insensitive to estrogens. Accordingly, E_2 promoted no change in the subcellular distribution of ΣE4: it was unable to induce a speckled nuclear distribution pattern for ΣE4-GFP, or a tight association of ΣE4 to NM.

Nevertheless, ΣE4 has an intact DNA-binding domain. Since the DNA-binding domain alone was shown to be sufficient for transcriptional activity, at least for certain nuclear receptors (42, 43), it has been suggested that ΣE4 might play a role as constitutively active or estrogen-independent transcription factor. In support of this hypothesis, we showed that this protein indeed retained some activity as a transcription factor, and that this activity was independent of the hormonal ligand: ΣE4 protein exhibited an estrogen-independent transactivation capacity when expressed in an ER-deficient cell line and enhanced estrogen-dependent transcription activity when transfected into cells naturally expressing full-length ER. Thus, ΣE4 is an effective transcriptional activator of estrogen target

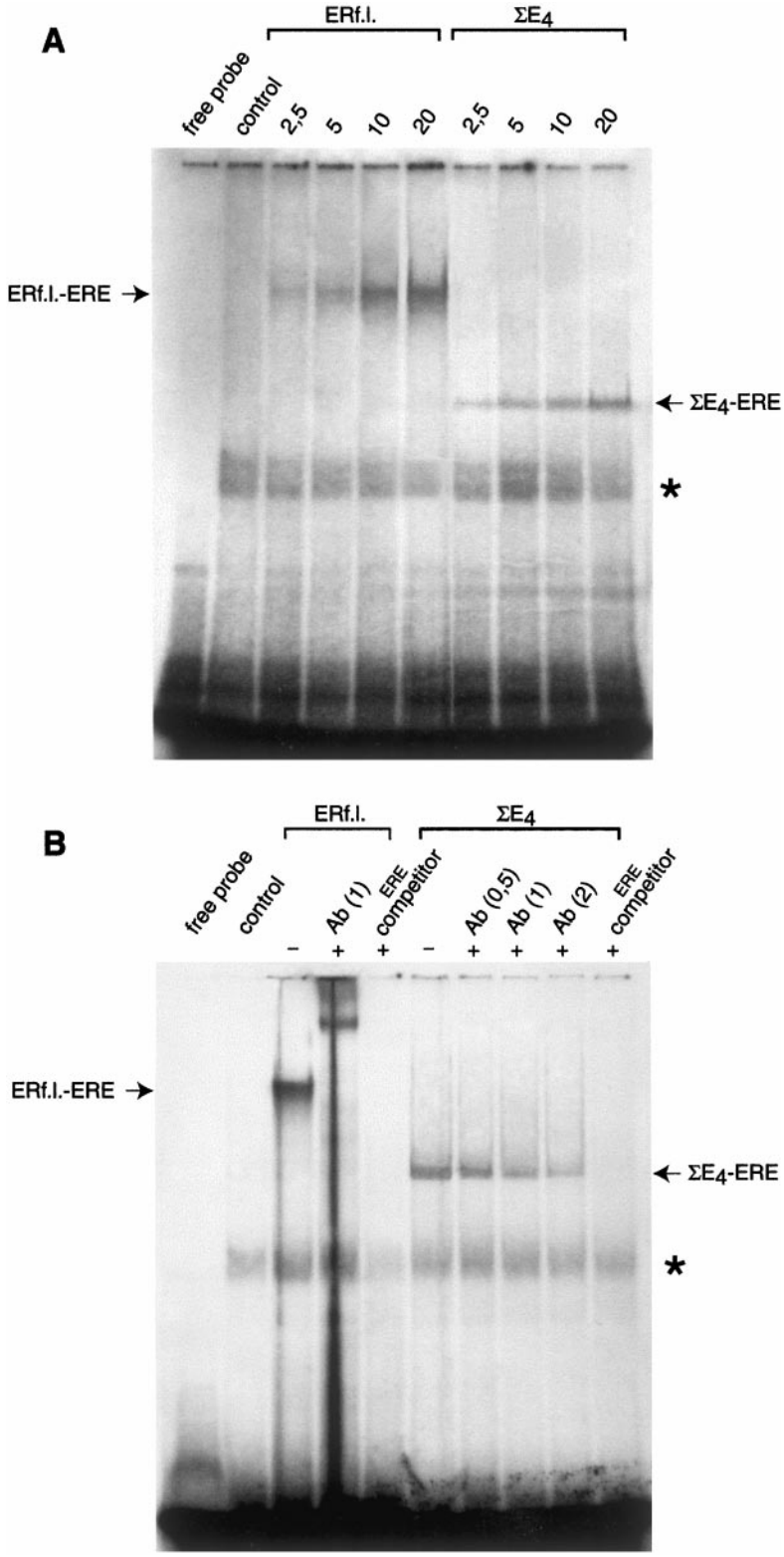

Fig. 6. Electrophoretic Gel Mobility Shift Assays (EMSA), Showing ΣE4 Binding to a Consensus ERE *in Vitro*

WCEs were prepared from COS-7 cells transfected with expression plasmids encoding full-length ER-α (ER f.l.) or ΣE4 and treated 15 min with forskolin-IBMX before harvesting. Aliquots of cell extracts containing the indicated receptors were incubated with a ^{32}P-labeled consensus ERE probe and subjected to EMSA on a 5% polyacrylamide gel as detailed in *Materials and Methods.* A, The amount of complex formed with the ERE increases with increasing amounts of WCEs loaded on the gel (from 2.5 to 20 μg total protein), both for WCEs containing full-length ERα, as for ΣE4. B, A fixed amount (20 μg total protein) of WCEs containing either receptor was incubated with ^{32}P-labeled ERE in the presence of anti-ERα antiserum (Ab; in micrograms), or

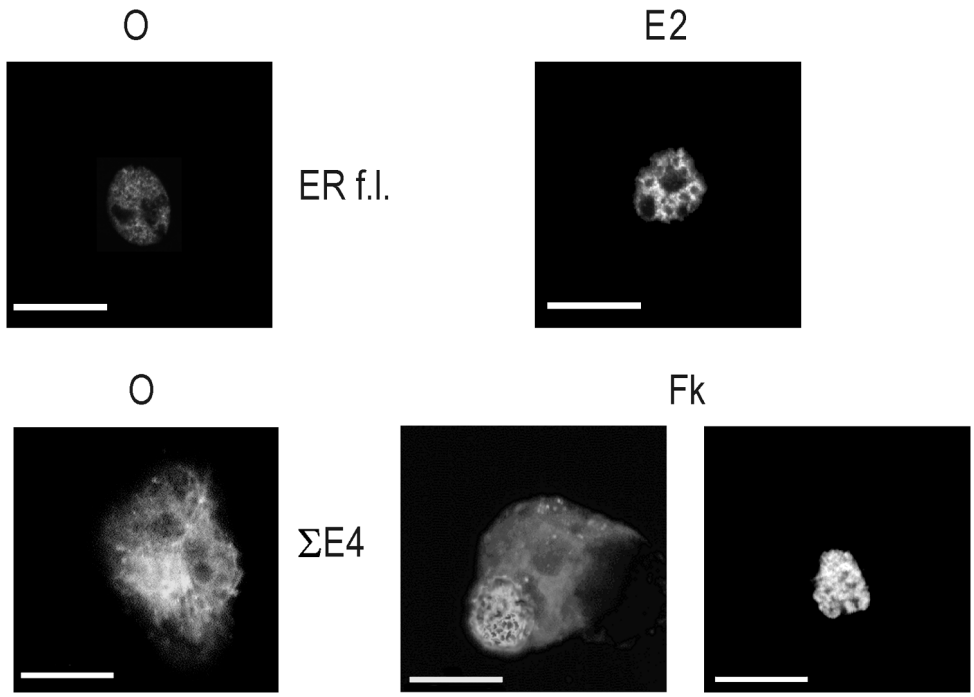

Fig. 7. Forskolin-Induced Changes in ΣE4-GFP Intranuclear Distribution

COS-1 cells were electroporated with an expression plasmid encoding ΣE4-GFP, cultured on glass coverslips for 30 h in DMEM without phenol red supplemented with 10% FCS twice stripped with charcoal/dextran, and finally treated (Fk) or not (0) with forskolin/IBMX for 15 min. For comparison, transfected cells expressing full-length ER-GFP and treated with E_2 are also shown. Cells were analyzed under confocal fluorescence microscopy. *Bars* = 10 μm. Unlike E_2 treatment (compare Fig. 2), Fk treatment appears to change the *diffuse* nucleoplasmic pattern of ΣE4-GFP distribution (*left panel*) into a (more or less pronounced) *speckled pattern* (*central* and *right panels*), suggestive of a phosphorylation-induced tighter association of ΣE4 with the nucleus.

genes. Since it failed to coimmunoprecipitate with full-length ERα, a heterodimerization with full-length ERα will not be involved in this ligand-independent transcriptional activation. In fact, ΣE4 was able, by its own, to bind a consensus ERE *in vitro*, as it formed a complex with the ERE probe in a gel mobility shift assay. This complex was clearly different from that formed between full-length ERα and the ERE probe. First, it was of an obviously smaller size. Second, anti-ERα antiserum did not retard its migration, but inhibited its formation in a dose-dependent manner. According to a recent study from Tyulmenkov and Klinge (44), who compared the impact of various ERα and ERβ antibodies on the interaction of ERs with a consensus ERE, both supershift and inhibition of ER-ERE interaction with a specific antibody are equally reliable as means of detecting an ER isoform in a gel mobility shift assay. Therefore, the observed differences between ΣE4-ERE and full-length ERα-ERE complexes are suggestive of a difference in the action mechanism of these two protein isoforms. In the classical model, the ERs bind, in response to their ligand (E_2), as homodimers to specific estrogen response elements (EREs) within target genes. In fact, we and others have shown that formation of ER homodimers is not the only way for ER to regulate gene transcription. Heterodimers could also be formed and modulate transcription. For instance, heterodimeric complexes can form between ERα and ERβ *in vitro,* with retained DNA-binding ability and specificity (45). ΣE3 and full-length ERα were herein shown to form inactive heterodimers. Furthermore, some other nuclear receptors, such as orphan receptors of the Nur type, have been shown to bind DNA as monomers (46). This may also be the case for ΣE4, which lacks sequences involved in dimerization (47) and was shown here to form a complex with the consensus ERE much smaller than the one formed between full-length ERα and ERE.

of an approximately 50-fold excess of unlabeled ERE (ERE competitor). Note that full-length ERα, but not ΣE4, shows a supershift in the presence of antibody. *Arrows* indicate the specific complexes formed in the presence of full-length ERα or ΣE4. *Asterisks* mark a complex formed between ^{32}P-labeled ERE and an unknown protein present in WCEs. On both gels, a free probe was loaded on lane 1, and an extract of cells transfected with an empty expression vector on lane 2 (controls). In this figure, two autoradiograms are shown of a 12-h exposure at −70 C with an intensifying screen, and both are representative of three independent experiments.

ΣE4 appears not to require E_2 to activate transcription of a reporter gene but needs to be phosphorylated to bind a consensus ERE *in vitro*. In support of this idea, a partially speckled pattern of ΣE4-GFP distribution was observed in the nucleus, after treatment of the transfected cells with forskolin/IBMX. In fact, many studies have shown that, in addition to the conventional hormone-dependent regulation of ER activity, there is substantial cross-talk between signal transduction pathways and steroid receptors. In a number of cases, a modulation of kinase/phosphatase activity in cells leads to activation of steroid receptors in the absence of hormone (48). Thus, an altered phosphorylation of the receptor and/or associated proteins is likely to be a key event in the ligand-independent activation of ERs.

Transcriptional regulation by ERs involves two activation functions (AFs) that reside on opposite ends of the receptors. AF-1 is located at the amino terminus and is constitutively active, whereas AF-2 is situated at the carboxy-terminal end of the ligand- binding domain (49, 50) and is strictly hormone regulated. Although both AF-1 and AF-2 are required to achieve maximal transcriptional activity, the two transactivating regions may either function independently or cooperate, depending on the target gene promoter and the presence of tissue-specific factors (51). When steroid receptors, including ERs, are activated by nonsteroid agents such as growth factors, protein kinase A, phorbol ester, and dopamine (for reviews, see Refs. 52 and 53), which all induce protein phosphorylation, their AF-1 domain appears to be the phosphorylation target. Moreover, it has been shown that the MAP kinase-mediated phosphorylation of the AF-1 domain of ERβ and of SF-1 promotes ligand-independent recruitment of nuclear receptor coactivators (54, 55). In the case of ΣE4, it seems possible that phosphorylation of its AF-1 domain would allow for a recruitment of specific coactivators, and a functional interaction with the transcription machinery, independently of the ligand.

ΣE3–4: A Silent Variant

The third ER isoform that we had detected in the developing pituitary, ΣE3–4, appeared to have no biological activity: In transfected cells, it was found predominantly in the cytoplasm, it exhibited no real ability to enter the nucleus and to associate tightly with NM upon ligand binding, nor did it bind a consensus ERE *in vitro*, dimerize with full-length ERα, or modulate transcription of estrogen-dependent genes.

Conclusions

In summary, our results demonstrate that ΣE3 and ΣE4, two alternatively spliced isoforms of ERα mRNA, may antagonize, or mimic, respectively, the function of the full-length gene product. The functional properties of these variants, as well as their expression time course and relative abundance, suggest that they may well play a physiological role as negative and positive regulators of transcription in the pituitary, at least before birth. The strong expression of ΣE3, which antagonizes full-length ERα, in male embryos during the critical period of development (16), could represent for the pituitary an additional protection mechanism against the potentially deleterious effects of maternal estrogens. Conversely, the ΣE4 isoform, which is early and abundantly expressed in the female embryonic pituitary (16), could represent a hormone-independent, phosphorylation-activated transcription factor, allowing for some intrinsic ER activity regardless of the circulating estrogen levels. The coordinated action during development of differentially active transcription factors created from a single ER gene probably contributes to the differentiation of pituitary cells first in the absence, and then in the presence, of high concentrations of estrogens.

MATERIALS AND METHODS

Construction of the ER Expression Plasmids

RNAs from pituitary gland of male or female rat were extracted in Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) and used for reverse transcription with a mix of oligo-dT and random nonamers using Superscript (Life Technologies, Inc.) according to the manufacturer's protocol. Sense oligonucleotide (GTCTGGTCCTGTGAAGGCCTGCA) and antisense oligonucleotide (TGACGTAGCCAGCAACATGTCAAAG) located upstream exon 3 and downstream exon 6 of the ER-α mRNA, respectively, were used as primers for *Taq* polymerase (Promega Corp., Madison, WI). PCR products corresponding to the three short isoforms deleted from exon 3 (SE3), exon 4 (SE4), and both exons 3 and 4 (SE3–4), respectively, described by Pasqualini *et al.* (16) were separated on agarose gel, cloned in pCRII by the TA cloning method (Invitrogen, San Diego, CA) and sequenced for verification.

The coding sequence of the ERα (gift of Professor Muramatsu) in pUC118 (56) was subcloned in pCDNA3 (Invitrogen). To obtain the complete sequence of the ERα isoforms, the corresponding PCR products inserted in pCRII and ERα in pCDNA3 were digested with *Bsm*I and *Bgl*I and separated on agarose gel. The isoform-specific fragments and the remaining ERα sequence in pCDNA3 were ligated together to form ΣER-pCDNA3 plasmids. All the constructs were checked by full-length sequencing.

Generation of ER-GFP Fusion Plasmid

The plasmid pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA) was modified by mutation of the translation start codon of the GFP to a valine codon, to generate pEGFPμ plasmid. To fuse the C terminus of the various ERα sequences to the mutated EGFP, the ΣER-pCDNA3 and ERα-pCDNA3 plasmids were modified by mutation of the stop codon to a glycine codon and digested by *Eco*RI and *Kpn*I. The fragments coding for full-length ERα and ΣERs were isolated on agarose gel and subcloned in pEGFPμ in the Bsm and *Bgl*II sites to generate the ER-pEGFP and ΣER-pEGFP fusion plasmids (Fig. 1B). The integrity of the fusion was checked by DNA sequencing and direct observation of the fluorescence in transfected cells. Functionality of full-length

ER-GFP as a ligand-dependent transcription factor was assayed on an ERE-containing reporter gene in transient transfections experiments using CHOk1 cells, as described in *Transcriptional Activity*. The activity of full-length ER-GFP was 130 ± 15% that of the native receptor.

Subcellular Localization of GFP-Tagged ER Isoforms in Transfected Cells

COS-1 cells were grown in DMEM (Life Technologies, Inc.) supplemented with 10% FCS. Twenty four hours before transfections, DMEM without phenol red was supplemented with 10% twice charcoal/dextran-stripped FCS. Cells were transfected with 8 μg expression vector ERs-pEGFP, using an electroporator (Bio-Rad Laboratories, Inc., Hercules, CA), and plated on glass coverslips for 30 h in serum-free medium. They were then treated or not with E_2 (1 nM) for 2 h. Cells were washed twice in ice-cold PBS and fixed for 30 min at 4 C in PBS-paraformaldehyde 3%. Before mounting in Mowiol (Polysciences, Inc., Warrington, PA), coverglasses were washed twice for 5 min with PBS and cells were observed under a fluorescence microscope, with a laser scanning confocal imaging system.

Sequential Fractionation of the Nuclear Constituents

Forty-eight hours after electroporation, COS-7 cells transfected with individual isoform and treated or not with 1 nM E_2 as described above, were washed twice with ice-cold PBS, scraped into PBS, and pelleted by centrifugation at 700 × *g* for 5 min at 4 C. They were then gently resuspended in 5 vol. of lysis buffer (Tris, pH 7.5, 10 mM NP-40 0.05%, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, aprotinin, 20 μg/ml, 1 mM orthovanadate, 1 mM 4-[2-aminoethyl]-benzenosulfonyl fluoride (AEBSF), and leupeptin, 10 μg/ml) and centrifuged at 350 × *g* for 5 min at 4 C: the supernatant, corresponding to the cytosol, was collected. It represented approximately 80% of the total cell proteins, as assayed using the Coomassie assay reagent (Pierce Chemical Co., Bezons, France).

The crude nuclear pellet was washed once in lysis buffer and twice in ice-cold CSK buffer (10 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid), pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, aprotinin, 20 μg/ml, 1 mM orthovanadate, 1 mM AEBSF, and leupeptin, 10 μg/ml) by gentle resuspension in 5 vol buffer and centrifugation at 350 × *g* for 5 min at 4 C. Nuclei were then resuspended in CSK buffer containing 1 M sucrose and centrifuged at 1,200 × *g* for 10 min at 4 C. From this purified nuclei pellet, soluble proteins (nucleoplasm; ~20% of nuclear proteins) were extracted by treatment for 5 min at 4 C with CSK buffer containing 0.5% Triton X-100, followed by a centrifugation at 700 × *g* for 5 min at 4 C. Chromatin was then digested with DNase I (700 U/ml) in CSK buffer containing 50 mM NaCl, for 60 min at 4 C. The (digested) chromatin-associated proteins were eluted by slowly adding ammonium sulfate in CSK buffer to a final concentration of 0.25 M. The NM (~20% of nuclear proteins) was pelleted at 1,000 × *g* for 5 min and the chromatin fraction (~ 60% of nuclear proteins) was recovered in the supernatant. All four fractions (cytosol, Triton X-100-soluble fraction, *i.e.* nucleoplasm-, DNase I supernatant, *i.e.* chromatin- and NM), were prepared in Laemmli buffer and subjected to SDS-PAGE and Western blot analysis.

Western Blot Analysis of ER Isoforms in Transfected COS Cells

Quantitation of Expression Level of ER Isoforms. Extracts (5 or 8 μg protein) prepared from COS-1 cells transfected with expression plasmids encoding individual isoforms were compared with a scale of extracts (5–30 μg protein) from ERα-naturally expressing GH4C1 cells. All the extracts were thus loaded and run on a 10% SDS-PAGE. Corresponding blots were reacted with a previously described (16) antiserum directed against ERα (1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by an HRP-coupled secondary antibody (The Jackson Laboratory, Bar Harbor, ME) and enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech, Arlington Heights, IL). As shown in Fig. 8, the level of ER isoforms expression in transfected COS cells is between 1.5 and 3 times that found in GH4C1 cells which naturally express ERα.

Subcellular and Subnuclear Distribution of ER Isoforms. Ten micrograms of protein of each fraction were loaded and run on a 10% SDS-PAGE and transferred onto nitrocellulose membrane. Blots were incubated with an antiserum directed against ERα (1:1,000; Santa Cruz Biotechnology, Inc.), and also with an antibody directed against LDH (1:500; Roche Molecular Biochemicals, Indianapolis, IN), or pan-Histone (1:100; Roche Molecular Biochemicals). They were subsequently reacted with the appropriate HRP-coupled secondary antibody and revealed by chemiluminescence.

Transcriptional Activity

ER-negative CHO k1 cells were grown in DMEM/F12 (1:1) medium supplemented with 10% FCS. Twenty-four hours before transfection, cells were plated in phenol red-free DMEM/F12 medium supplemented with 10% of twice charcoal-stripped FCS.

ER-positive GH4 C1 cells were maintained in MEM supplemented with 2.5% FCS and 10% horse serum. Twenty-four hours before transfection, cells were grown in phenol red-free DMEM supplemented with 2.5 and 10% twice charcoal-stripped FCS and horse serum, respectively, containing RU 58668 (50 nM), an ER antagonist.

Transcriptional effects of individual isoforms were tested using an artificial promoter containing consensus Vit EREs inserted upstream of the thymidine kinase (TK) promoter which drives the expression of the CAT reporter gene. Cells were cotransfected with 10 μg expression vector encoding the different forms of ERα and 10 μg of the CAT reporter plasmid, pVit-TK-CAT (VTC), with or without (10 μg) full-length ER, by electroporation. Estradiol (1 nM) was added immediately after transfection. Twelve hours (for GH4 C1 line), or 48 h (for CHO k1 line) thereafter, cells were harvested, and CAT quantity was measured in cell extracts. The CAT assays were performed by a colorimetric enzyme immuno-

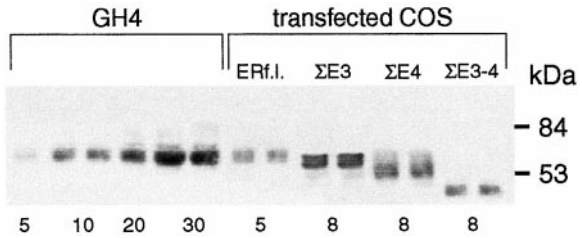

Fig. 8. Comparison of the Level of ERs Expression in Transfected COS-1 Cells *vs.* that of Naturally ERα Expressing GH4C1 Cells

Fixed amounts of protein extracts prepared from COS-1 cells transfected with expression plasmids encoding individual ER isoforms (5 or 8 μg of total protein), and varying amounts of GH4C1 cell extracts (5–30 μg of total protein), were subjected to Western blot analysis. Blots were reacted with antiserum directed against ERα. ER isoform expression in transfected COS-1 cells is slightly higher, but of about the same order as ER expression in GH4C1 cells.

assay (CAT-ELISA kit, Roche Molecular Biochemicals, Meylan, France) and were all normalized to protein concentration. In some experiments, CAT values were also standardized to the β-galactosidase (expression driven by the Rous sarcoma virus promoter) activity, without any qualitative difference.

Immunoprecipitation

COS-7 cells were transiently transfected with an expression plasmid encoding a full-length ERα tagged with a HA epitope (~68 kDa; HA-ER f.l.) alone or together with expression vector encoding the different (nontagged) isoforms of ERα and grown for 48 h in a medium containing or not E_2. Functionality of HA-ERα as a ligand dependent-transcription factor was assayed using CHOk1 cells, as described above (see *Transcriptional Activity*). The activity of HA-ERα was found to be 135 ± 20% that of the native receptor. To determine whether all isoforms were expressed at equal levels in the cells, aliquots of the cellular extracts were analyzed by Western blotting. Blots were reacted either with a monoclonal antibody directed against HA epitope (12CA5, Roche Molecular Biochemicals), or with the antiserum directed against ERα (Santa Cruz Biotechnology, Inc.).

The remainder of the cell extracts (containing 200–400 μg total protein) were incubated with 4 μl monoclonal antibody directed against HA epitope and 20 μl pansorbin for 2 h at 4 C. After four washes with cell lysis buffer, immunoprecipitates were pelleted by centrifugation, released into 15 μl SDS loading buffer by boiling for 5 min, and subjected to electrophoresis on a 12% acrylamide gel. Blots were reacted with an antiserum directed against ERα, followed by the appropriate horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence revelation.

Gel Mobility Shift Assays for *in Vitro* DNA Binding

Preparation of WCEs. WCEs were prepared from COS-7 cells transfected with expression vectors encoding individual isoforms of ERα. Forty-eight hours after electroporation, cells were lysed at 4 C in 500 μl of lysis buffer [20 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 12% glycerol, 0.2 mM EDTA, 0.1% NP-40, 5 mM DTT, 1 mM AEBSF, 0.5 μg/ml leupeptin, 1 μg/ml chymostatin, 1 μg/ml pepstatin, 1 μg/ml aprotinin]. Extracts were then centrifuged at 15,000 × *g* for 10 min at 4 C, and supernatants were collected, aliquoted, and stored at −70 C. In some experiments, 15 min before lysis and preparation of WCEs, the cells were treated with forskolin (10 μM) and IBMX (1 μM) to activate the PKA pathway via an increase in intracellular cAMP levels.

To determine whether the isoforms of ERα are faithfully and equally produced by COS-7 cells, aliquots of extracts were analyzed by Western blotting using a polyclonal anti-ERα antiserum.

DNA Probe. A synthetic oligonucleotide (27-mer) containing a 13-bp perfect palindromic ERE and corresponding to the Vit A2 ERE: 5′-GATCCTAGAGGTCACAGTGACCTACGA-3′ was mixed with an equal molar quantity of the complementary strand and annealed in water by heating at 95 C and cooling slowly to room temperature (4 h). Fifty nanograms of this 27-bp double-stranded fragment was end-labeled with 200 μCi [γ-^{32}P]-dATP using 5–8 U T4 PNK in 20 μl. The radiolabeled DNA was purified on a S200 column (Pharmacia Biotech).

***In Vitro* DNA Binding.** *In vitro* DNA binding was performed as previously described (57). Briefly, aliquots of WCEs containing the receptors were incubated with 1 μg poly(dI.dC) in a reaction buffer (final concentration,10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 80 mM KCl, 5% glycerol) at 4 C for 20 min. Purified ^{32}P-labeled double-stranded ERE oligonucleotide probe (5 × 10^4 cpm) was added, and the reaction was performed at room temperature for 30 min. (For competition experiments, ~50-fold excess radioinert oligonucleotides were added before radiolabeled probe.) The resulting 20 μl reaction mixture was analyzed on a 5–6% nondenaturing polyacrylamide gel. Electrophoresis was carried out at 150 V in 0.5 × Tris-borate-EDTA (TBE) for 2 h. The gel was dried and exposed for autoradiography.

Forskolin-Induced Change in ΣE4-GFP Subcellular Localization

COS-1 cells were electroporated with 8 μg expression plasmid encoding the GFP-tagged ERs, cultured on glass coverslips for 30 h in DMEM without phenol red, supplemented with 10% FCS that had been twice stripped by charcoal/dextran. They were finally treated or not (0) either with estradiol (E_2) for 2 h, or with forskolin-IBMX (Fk) for 15 min. Cells were washed in ice-cold PBS and fixed for 30 min at 4 C in PBS- 3% PFA. Coverslips were then washed twice for 5 min with PBS, mounted in Mowiol, and analyzed under confocal fluorescence microscopy.

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