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Alternative Initiation of Translation Accounts for a 67/45 kDa Dimorphism of the Human Estrogen Receptor ERα

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The estrogen receptor protein, in the nuclear receptor superfamily, carries two transactivator domains designated AF1 and AF2. The activity of AF2, localized in the carboxy-terminal region, is ligand-dependent, whereas AF1 (amino-terminal) seems to be activated via the MAPKkinase pathway. Uterine and mammary cells exhibiting large amounts of ERα were the first estrogen target organs demonstrated. The response intensity in these tissues is related to the affinity of the receptor and to the number of sites occupied by its ligand. Certain physiological and pharmacological phenomena of estrogen resistance associated with a truncated form of ERα (deleted in the AF1 domain) would seem however to challenge this assertion. The 45 kDa truncated form is unable to induce cell proliferation but can still increase the expression of certain genes. In this work we suggest that this 45 kDa ERα form may originate from differential regulation of translation of the mRNA encoding the ERα. *In vitro* translation studies and transient expression in COS-7 cells *in vivo* demonstrated a mechanism of translation regulation that produced from a given mRNA either the wild type ER 67 kDa form or the AF1 deleted ER 45 kDa isoform. Bicistronic vectors were used to demonstrate that the 45 kDa protein originates from translation initiation at AUG 174 induced by an internal ribosome entry.

The estrogen receptor protein (hereafter referred to as ERα) is traditionally involved in the activities of estrogens in their target tissues. It is a member of the nuclear hormone receptor superfamily which increases the transcription of target genes after activation by ligand. Two forms of ERα with apparent molecular masses of about 67 and 45 kDa have been evidenced in rat uterus (1) and mammary tumor cells (2) which seem to coincide with variable states of the target cells to hormone stimulation. It was shown that transcriptional regulation was not involved in this ERα polymorphism but that it could be connected to a translation mechanism (3). Translation in eukaryotic cells is thought to be initiated by a ribosome scanning from the capped mRNA 5′ end, the choice of initiation codon often being dependent on an AUG codon in a favorable context (4). Examination of the coding sequence of human ERα cDNA revealed two in frame AUGs in a favorable initiation context, 174 codons downstream from the first AUG codon. This codon initiates a 45 kDa protein corresponding to an A/B domain and transactivator domain AF1 deletion but preserves the DNA and hormone binding domains. The hypothesis of an alternative ribosome binding pathway, independent of cap structure, was first proposed in picornavirus studies (5-7) and has also been reported for hepatitis C virus (8), cowpea mosaic virus (9), and Moloney murine leukemia virus (10). It has recently been shown to depend on cellular *trans*-acting factors (10-11) and to involve eukaryotic cellular mRNAs (12-15). More recently, two other functional forms of ERα (66 and 61 kDa) have been shown to be translated from a single mRNA in Xenopus hepatocytes (16). The aim of this work was therefore to determine a potential translation mechanism which might give rise to the 45 kDa isoform of ER found in states of estrogen insensitivity (1-2). It is shown here that the 45 kDa as opposed to the 67 kDa form of ER indeed results from alternative translation initiation controlled by internal ribosome entry and that two in-frame AUG codons of the mRNA may alternately be used as initiation codons.

MATERIALS AND METHODS

Construction of recombinant plasmids. The wild type ER cDNA (WT) cloned in pSG5 vector was a generous gift from P. Chambon. The Not I site at codon 65 was opened, treated with Klenow enzyme and ligated. This mutation induced a frame shift conducive to a stop codon at position 108 and was named ER 45 (Fig. 1).

The AUG 174 and 176 mutations were obtained by two independent PCR amplifications using T7 oligomer as 5′ primer, (5′ TAGCCGAGCTCCCCTTGTCATTG 3′) as 3′ primer and pSG5 HG0 (17) as template for the first amplification, and (5′ AAAAGAGCTCGGCTATAGAATCTGCCAAG 3′) as 5′ primer, T3 oligomer as 3′ primer

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and pKSHG0 as template for the second amplification. The EcoRI-SacI fragment from the first PCR product and the SacI-HindIII fragment from the second PCR product were cloned into the EcoRI and HindIII sites of pSG5 HG0. The resulting plasmid exibited an AUG/UCG mutation at position 174 and an AUG/AUA mutation at position 176. This construction was named ER 67 (Fig. 1).

Hairpin structures at 5′ of the open reading frames (ORF) were then constructed from the KpnI-SacI fragment containing the pKS Bluescript polylinker (Stratagene) that had been ligated to form concatemers, digested by EcoRI then cloned in the EcoRI site of pSG5 HG0 to form WT 1HP and WT 2HP (Fig. 1). This led to insertion either of the inverted repeat of the EcoRI-KpnI fragment (hpK structure) or the inverted repeat of the EcoRI-SacI fragment (hpS structure). A single insertion of hpK formed a hairpin structure of $\Delta G0 = -91.2$ kCal, as predicted by computer program (18) and the double insertion of hpK, hpS (hpKS) formed a structure of $\Delta G0 = -200.3$ kCal (Fig. 1). A control construction was derived from WT 2HP by deletion of the KpnI-SacI fragment, and blunt ligation, to give a nucleotide sequence equivalent to hpK but lacking the hairpin structure. The ER 45 1HP and ER 67 1HP constructions were obtained by similar procedures (Fig. 1).

As the CAT protein had previously been used with success in the bicistronic strategy (10, 15), various chimeric cDNAs were also constructed which contained the CAT encoding sequence as the first ORF and a sequence coding for a chimeric protein of CAT fused to the ER carboxy-terminal end, as the second (Fig. 2).

ER-CAT fusions were produced by generating two PCR fragments with pSG5 HG0 as template, T7 oligomer as 5′ primer and two 3′ primers (5′ AAGGGCCCATTGCACAGTAGCGAGTCTC 3′), (5′ TTTGGATCCTCGAGGGGCCCGTGACTGTGGCAGGGAAACC 3′), matching the ER sequence at positions 558-541 and 1786-1768. The CAT ORF was obtained from earlier constructions (15). The EcoRI-ApaI fragment of the ER coding sequence and the Apa I-Bgl II CAT ORF containing fragment were cloned into the EcoR I and Bgl II sites of pSG5. The fused ER-CAT sequence was then subcloned into various constructions using the Hind III site of the ER sequence and the Xho I site of pSG5. The first cistron 5′ CAT ORF, with and without the hairpin structure of $\Delta G0 = -40$ kCal, was obtained from previous constructions (15) and inserted upstream from the ER-CAT chimeras between the Sal I and Sac I sites. The resulting intercistronic sequence was 152 bp long and devoid of in frame AUG (Figs. 3 & 4).

In vitro translation. The plasmids were linearized downstream from the 3′ end of the ER or ER-CAT coding sequences. Capped RNAs were generated *in vitro* with T7 RNA polymerase according to the manufacturer's instructions (Promega) and by adding m^7GpppG (0.5 mM) to the transcription reaction mixture. RNA transcripts were calibrated by staining with ethidium bromide on agarose gel and verifying their integrity. In vitro translation was performed in rabbit reticulocyte lysate in the presence of [^{35}S]-methionine, as specified by the manufacturer (Promega) with certain modifications. The translation assays (20 μl) were incubated for 1 h at 37°C (instead of 30°C) and in a 50% (instead of 70%) diluted lysate. The translation products were analyzed by electrophoresis in sodium dodecyl sulfate as 8 or 12% polyacrylamide gels and the dried gels subjected to autoradiography.

DNA transfection and Western immunoblotting. COS-7 monkey cells, maintained in 5% calf serum and Dulbecco's Modified Earle Medium (DMEM, 5% CS), were transfected with DEAE-dextran (19): 1 μg/ml of each plasmid and 1 μg of β-galactosidase expressing plasmid pCH 110 were incubated with the cells for 10 min at 37°C in the presence of 1 mg/ml DEAE-dextran. Chloroquine was added (40 μg/ml) and further incubated for 4 h. The DNA-containing medium was then removed, and the cells incubated in 10% dimethyl sulfoxide for 2 min. The cells were then washed and incubated in DMEM, 5% CS. Cell lysates were prepared 48 h later by scraping the cell monolayers,freezing-thawing the resulting cell pellets their resuspension in Tris pH 7.8, containing aprotinine 2 mg/ml and PMSF 1 mM, and sonication. The total protein content was quantified by Bio-Rad assay and the normalized cell lysates quantified for β-galactosidase activity. Normalized extracts were heated for 2 min at 95°C in sodium dodecyl sulfate and sample buffer containing dithiothreitol before being separated on suitable polyacrylamide gels and transferred to a nitrocellulose membrane for immunodetection. The ER-CAT chimeric proteins were detected with a rabbit polyclonal anti-CAT antibody prepared in our laboratory and the ER proteins with either the 1D5 monoclonal antibody developed in our lab (20) and directed against the N terminal domain of ER or the Sigma rabbit polyclonal antibody directed against the (578-595) carboxy domain of ER (E 0646). They were visualized with an enhanced chemoluminescence kit (Amersham).

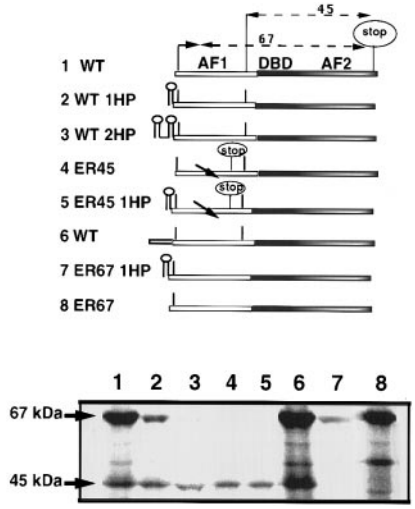

FIG. 1. *In vitro* translation. Transcribed mRNA of corresponding structure shown at the top of the figure. Translation, ^{35}S labeling and electrophoresis were carried out as described in Materials and Methods. Wild type ER (67 kDa) and the 45 kDa ER isoform are indicated with arrows.

RESULTS

Translation of the ER ORF mRNA

Cell free translation. Either one or two high energy RNA structures (hairpins) were introduced into the 5′ untranslated region (UTR) of the RNA to determine usage of the AUG codon in translation initiation (Fig. 1). An increase in the 5′ structure (lanes 1 to 3) led to a decrease in the *in vitro* expression of the 67 kDa form (Fig. 1). Analyses showed that the 45 kDa form was not affected to the same extent by the RNA 5′ structure (lanes 1 to 3), thereby demonstrating its independent production compared to that of the 67 kDa form. A frame shift in lanes 4 and 5, introduced a stop codon at position 108 and led to disappearance of the 67 kDa while production of the 45 kDa form remained unchanged. These results clearly show that the 45 kDa form was not a proteolytic fragment of the 67 kDa and that the 5′RNA structure did not regulate the translation efficiency of the 45 kDa protein. In lane 6, the 5′ UTR sequence which had the same inverted nucleotide sequence as the preceding hairpin structure but a

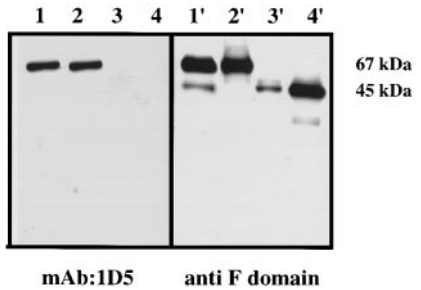

FIG. 2. Western-Blot analysis of transient ER cDNA expressed in COS-7 cells. Estrogen receptor protein was detected after electrophoresis by either (left) monoclonal antibody (1D5) directed against the N-terminal of ER or (right) polyclonal antibody against the carboxy-terminal domain. Ten μg of proteins from COS-7 cells transiently transfected by, wild type ER cDNA lanes 1 and 1′, ER67 cDNA lanes 2,2′ , AUG/UCG mutation at codon 174 and AUG/AUA at 176 of ER45 cDNA in lanes 3,3′, pSG5-HE19 was used as a 45kDa size marker of ER in lanes 4,4′. The different plasmids were cotransfected with pCH110 DNA, encoding a β-galactosidase protein to give an internal transfection standard. The proteins loaded on each lane were calibrated in relation to the β-galactosidase activity.

$\Delta G \neq 0$ kcal, had no effect on mRNA translation. Analysis of lanes 7 and 8 confirmed that translation of the ER 67 (lane 8) was sensitive to 5′ structuration of the mRNA (lane7) and unable to produce a 45 kDa form when translation initiation was prevented by mutation of the 174-176 AUG codons. These findings suggest that the 67 kDa protein in this cell free system was produced from the usual cap-dependent initiation of translation process, and that the 45 kDa protein was produced from a different initiation of translation mechanism.

In vivo studies. Wild type ER (lanes 1 and 1′), AUG 174-176 mutated ER 67 cDNA (lanes 2 and 2′), ER45 (lanes 3 and 3′) and, HE 19 (21) (a generous gift from P. Chambon, used as size marker of the 45 kDa form, lanes 4 and 4′) were transfected in COS-7 cells as described in Materials and Methods. Proteins were analyzed by Western immunoblotting. As shown in Fig. 2, wild type ER cDNA produced the 67kDa protein recognized by both anti ER antibodies (lanes 1 and 1′) although a 45kDa band was only evidenced by the ER-anti-carboxy terminal antibody. This protein was assumed to be the 45 kDa form because of its similar electrophoretic migration to that of HE 19 (lane 4′) and its disappearance in ER 67 transfection experiments (lane 2′) which proved that its presence resulted from a regulation of translation and not from proteolytic cleavage. The ER-1D5 monoclonal antibody, directed against the AF1 domain of ER (20), was unable to recognize either the 45 kDa protein or HE 19 (lanes 3,4) although the 67 kDa was evidenced (lanes 1,2).

Expression of the Chimeric ER-CAT Constructs

A further series of experiments was carried out using the previously described bicistronic strategy (10, 15) to differentiate between the possibility of leaky scanning and an internal ribosome entry on the ER mRNA. Bicistronic mRNA constructs were produced to determine the possible translation, *in vitro* and *in vivo,* of two ORF carried by a single mRNA. The principle of this strategy is based on the fact that, in eukaryotic cells, the second ORF cannot be translated in the absence of an internal ribosome entry between the first and second ORFs (5-6).

In vitro studies. The presence of a CAT encoding sequence as the first ORF (ORF 1) considerably diminished the 67/45 expression ratio by inhibiting the expression of 67 kDa without changing that of 45 kDa (Fig. 3, lanes 1 and 2). CAT (ORF 1) expression clearly decreased in the presence of the 5′ hairpin (Fig. 3, lane 3), whereas the efficiency of 45 kDa expression remained unchanged. Frame shift inhibition of the production of the 67 kDa form (lane 4) confirmed that the presence of the CAT ORF1 was unable to influence production of the 45 kDa form.

In vivo studies. Western blot analyses of the same constructs transfected, with an anti-CAT antibody, in COS 7 cells are shown in Fig. 4. The band corresponding to the protein initiate at AUG 1 (lane 2) was decreased by the presence of the first ORF1 (lane 3). Moreover a 5′ UTR structure (lane 4) enhanced the effect of ORF1 on translation initiation at AUG 1 more efficiently in *in vivo* than in *in vitro* studies. In contrast the band corresponding to a translation initiation at AUG 174 was unaffected either by ORF1 or the 5′ hairpin structure (lanes 2, 3 and 4). This latter observation was confirmed, in the absence of production of the 67 kDa ERα form (lanes 5-6) thereby exhibiting the non-effect of ORF1 on the initiation of translation at AUG 174. The initiation of translation at

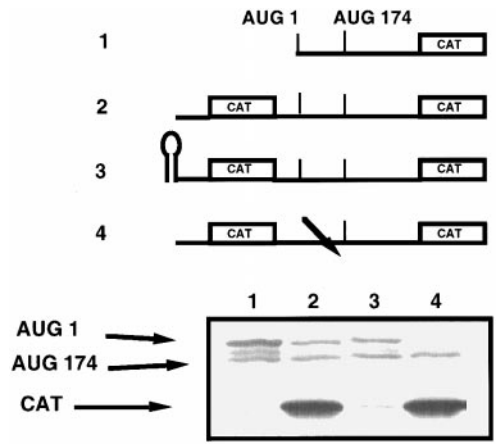

FIG. 3. *In vitro* translation of ER-CAT fusion proteins. Monocistronic RNA lanes 1 and 6; bi-cistronic RNA lanes 2, 3, 4, 5, 7, 8. The first ORF was preceded by a hairpin structure in lanes 3 and 8, the translation starting at AUG 1 was aborted by a frame shift in lane 4, AUG 174 and 176 were mutated in lane 5; the ER-AF1 domain was fused with CAT in lanes 6, 7, 8.

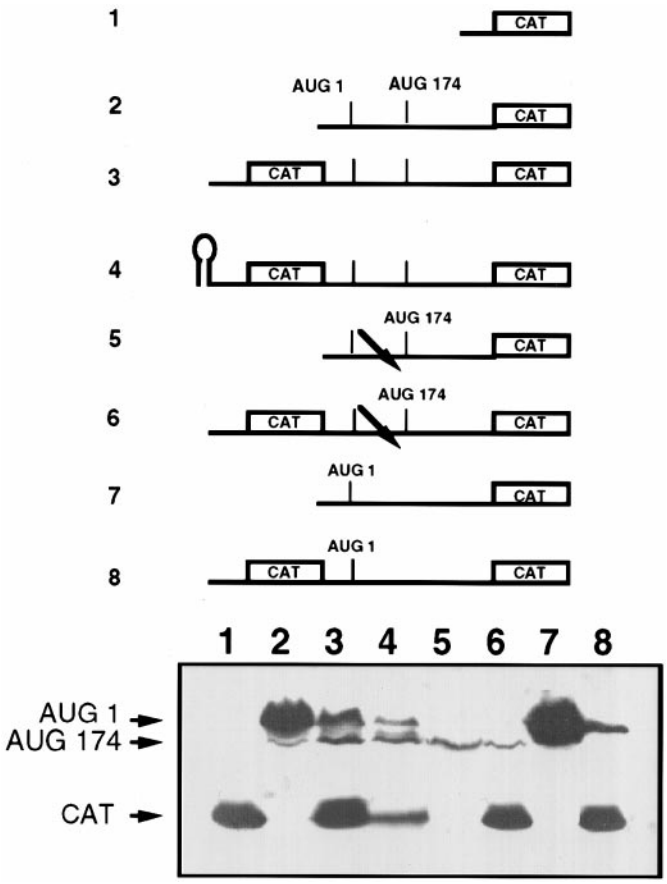

FIG. 4. Western blot analysis of ER-CAT chimeric proteins. COS-7 cells were transiently transfected with cDNA constructs inserted in the pSG5 vector. Ten μg of the total proteins from the transfection of CAT pSG5 DNA were used as reference (lane 1). As in Fig. 2, the β-galactosidase activity was used as an internal transfection standard, loaded proteins were calibrated as a function of the β-galactosidase activity in the lanes 2 to 8. Arrows indicate: the CAT at 27 kDa, the wild type ER in fusion with CAT at 94 kDa,and the AF1 deleted ER isoform at 72 kDa. Each lane corresponds to the cDNA construct at the top of the figure.

AUG 1 of ER was again highly repressed by ORF1 (lanes 7-8) even in the absence of AUG 174.

DISCUSSION

Regulation of the translation of eukaryotic mRNA has recently been shown to be the origin of several proteins and thus to constitute a further mechanism of cell biological control. We show here that two proteins arise from an alternative usage of the translation initiation sites present in ERα mRNA. As previously discussed by Vagner et *al.* for human FGF-2 mRNA (11) and for Moloney murine leukemia virus gag mRNA (10) translation of the second cistron of the bicistronic CAT mRNA is strictly dependent on the integrity of the two AUG 174-176 as shown in functional analyses of mutants both *in vitro* and *in vivo.* Use of the bicistronic strategy showed that the smallest ER 45 kDa form was produced by a cap independent process which included an internal ribosome entry site between the AUG1 and AUG174 codons. The cis and trans factors which regulate this process of translation initiation now need to be characterized. Further initiation sites may even be characterized in the future since other labeled or immunoreactive bands were observed after overexposing the gels or blots from our experiments (data not shown) and the sequences coding for the different and independent ER domains (22) are known to contain potential initiation codons of translation such as AUG.

The two ER forms generated by this process, differ in a sequence of 173 amino acids at the N terminal of the A/B domain involved in the AF1 transactive function. The 45kDa form has already been shown to exert specific transactivation activities in comparison with the wild type receptor (23). Bunone et al have recently shown that the N-terminal portion of ER is essential for its steroid-independent activation via the MAPkinase pathway. The translational regulation of ER expression would thus have an important physiological role since as it could by-pass EGF activation of the unliganded ER (24) while keeping the other estrogen activities intact. The implication of these observations in the previously reported mechanism of estrogen non-responsiveness remains to be characterized.

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