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Expression of human estrogen receptor mutants in *Xenopus* oocytes: correlation between transcriptional activity and ability to form protein - DNA complexes

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The human estrogen receptor (hER) is a *trans*-acting regulatory protein composed of a series of discrete functional domains. We have microinjected an hER expression vector (HEO) into *Xenopus* oocyte nuclei and demonstrate, using Western blot assay, that the hER is synthesized. When nuclear extracts from oocytes were prepared and incubated in the presence of a 2.7 kb DNA fragment comprising the 5′ end of the vitellogenin gene B2, formation of estrogen-dependent complexes could be visualized by electron microscopy over the estrogen responsive element (ERE). Of crucial importance is the observation that the complex formation is inhibited by the estrogen antagonist tamoxifen, is restored by the addition of the hormone and does not take place with extracts from control oocytes injected with the expression vector lacking the sequences encoding the receptor. The presence of the biologically active hER is confirmed in co-injection experiments, in which HEO is co-introduced with a CAT reporter gene under the control of a vitellogenin promoter containing or lacking the ERE. CAT assays and primer extensions analyses reveal that both the receptor and the ERE are essential for estrogen induced stimulation of transcription. The same approach was used to analyze selective hER mutants. We find that the DNA binding domain (region C) is essential for protein - DNA complex formation at the ERE but is not sufficient by itself to activate transcription from the reporter gene. In addition to region C, both the hormone binding (region E) and amino terminal (region A/B) domains are needed for an efficient transcription activation. Taken together, these data strongly suggest that the complexes we detect with the electron microscope result from the binding of the hER to the ERE.

Key words: Xenopus oocyte microinjection/human estrogen receptor/protein - DNA complexes/vitellogenin/estrogen responsive element

Introduction

Regulation of gene transcription in eukaryotes requires the co-ordinate action of both general and specific transcription factors which interact with DNA control elements to activate, stimulate or repress the expression of a particular gene (Brown, 1984; Dynan and Tjian, 1985; Serfling *et al.*, 1985; Ptashne, 1986). Steroid-controlled genes represent valuable model systems to analyze these interactions (Yamamoto, 1985).

In glucocorticoid regulated gene systems there is good evidence from experiments performed with purified receptor that the hormone - receptor complex binds directly to the glucocorticoid responsive element (Scheideret *et al.*, 1983, 1986). So far there is no equivalent direct demonstration (i.e. DNase I footprint, gel retardation, etc.) that the estrogen receptor (ER) binds directly to the estrogen responsive element (ERE) but nevertheless several lines of evidence suggest that it is involved in protein - DNA complex formation at this site. By using extracts of nuclei from estrogen-induced frog liver, stable protein - DNA complexes have been assembled *in vitro* and visualized by electron microscopy (EM) at the 5′ end of *Xenopus* vitellogenin genes (ten Heggeler-Bordier *et al.*, 1987). It was shown that the 13 bp core palindromic element of the ERE is involved in the formation of a complex whose appearance is reduced by tamoxifen, an estrogen antagonist. Furthermore this latter effect is reversed by adding an excess of the hormone suggesting that the estrogen receptor is part of the complex. The interaction of the receptor with the ERE was confirmed by a DNA cellulose competition assay (Klein-Hitpass *et al.*, 1988). Since there is a striking homology between the DNA binding region of both the glucocorticoid receptor (GR) and the ER containing 'zinc-finger'-like motifs, shown to be involved directly in DNA binding (for reviews, see Berg, 1986; Gehring, 1987; Klug and Rhodes, 1987), it is likely that the receptor is directly implicated in the binding to the ERE.

The human estrogen receptor (hER) has been cloned in an expression vector (Green *et al.*, 1986; Greene *et al.*, 1986) and can, after transfection, confer hormone responsiveness to mammalian cell lines which do not normally express the hER (Druege *et al.*, 1986). We extended this analysis by demonstrating that the hER can be synthesized in amphibian cells, namely *Xenopus* oocytes. These exceptionally large cells are particularly suited to injection techniques (Kressman and Birnstiel, 1980) and have been used for numerous studies in gene expression. Moreover they have proven useful in biochemical complementation experiments in which the role of RNAs or proteins was analyzed, in some cases in conjunction with defined genes (Coleman, 1984; Galli *et al.*, 1983; Knowland *et al.*, 1984). In this paper, we show that biologically active estrogen receptor is made in the oocytes where the controlled expression of vitellogenin genes coinjected with the hER expression vector is reconstituted. We used *vit B1 - CAT* chimeric genes containing or lacking the ERE (Seiler-Tuyns *et al.*, 1986; Martinez *et al.*, 1987) as test genes because the CAT enzymatic assay is very sensitive in the oocytes (Theulaz, 1987).

Additionally, we show that nuclear extracts from oocytes

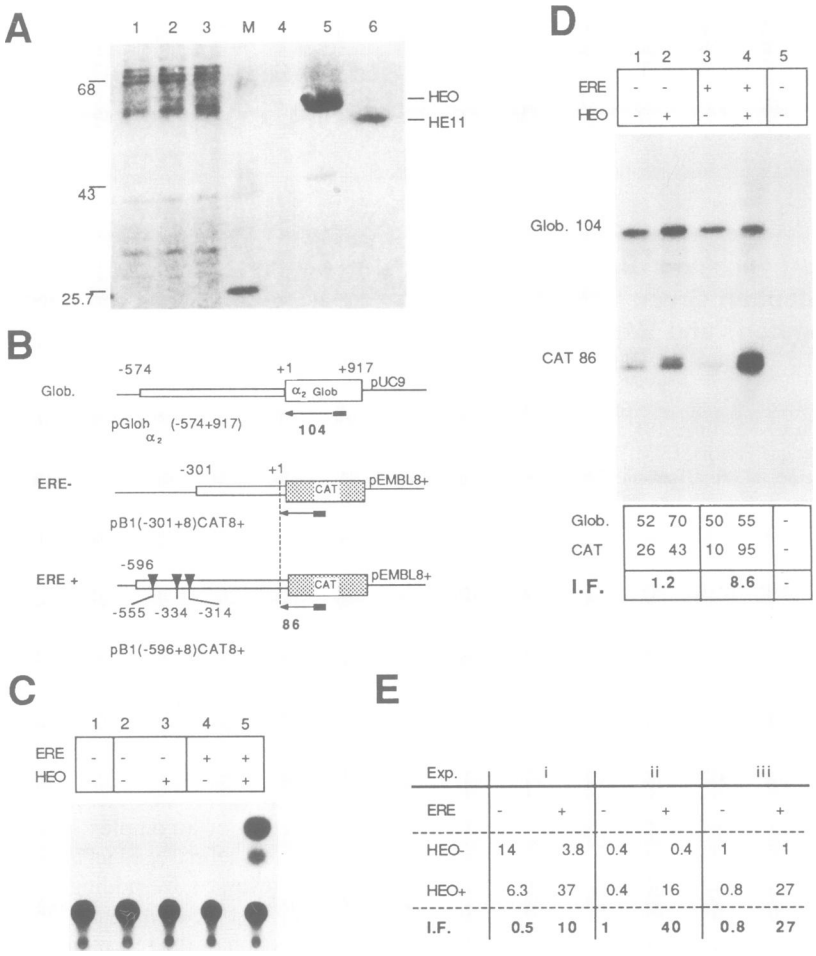

Fig. 1. Expression and activity of the hER in oocytes. (**A**) Expression of the hER in *Xenopus* oocytes. Defolliculated oocytes were maintained for 4−12 h in MBS supplemented with 1 μM 17-β estradiol before injection. They were injected with 6 × 10^8 copies of pKCR2 (**lanes 1** and **4**), HEO (**lanes 2** and **5**) and HE11 (**lanes 3** and **6**, see also Figure 3) and incubated with [^{35}S]Met and [^{35}S]Cys for 40 h in the presence of 1 μM 17-β estradiol. Nuclear extracts were prepared as for EM analysis. Labeled proteins were analyzed directly (lanes 1−3, amount of extract equivalent to one nucleus) or after immunoprecipitation (lanes 4−6, equivalent to 50 nuclei) with monoclonal antibodies (H222, H226; King and Greene, 1984) against hER on a 12% polyacrylamide gel. The positions of HEO (66 kd) and HE11 (~60 kd) are indicated as well as those of the ^{14}C-labeled marker proteins (**lane M**); bovine serum albumin (68 kd), ovalbumin (43 kd) and α-chymotrypsinogen (25.7 kd). (**B**) Schematic diagrams showing the templates used for functional characterization of the hER. pB1(5′/−596+8)CAT8$^+$ contains the ERE identified in MCF-7 cells (Seiler-Tuyns *et al.*, 1986), and the symbol ▼ indicates the position of the 13 bp elements (Walker *et al.*, 1984) involved in the response to estrogen. This region has been deleted in pB1(5′/−301+8)CAT8$^+$. The position of the oligonucleotide primer is given under the CAT sequences by a solid line, and the expected elongation product due to correct initiation is shown by a dotted line. pGlob(−574+917) consists of a 1419 bp *PstI* genomic fragment of the human α-2 globin gene subcloned in pUC9 (A.Seiler-Tuyns, unpublished data). (**C**) CAT activity of injected oocytes. Oocytes were coinjected with 1 × 10^8 molecules of either the hER expression vector (HEO$^+$) or the expression vector alone pKCR2 (HEO$^-$) and 1 × 10^7 molecules of pB1(−596+8)CAT8$^+$ containing the estrogen responsive element (ERE$^+$) or pB1(−301+8)CAT8$^+$ lacking it (ERE$^-$) and incubated with hormone. The equivalent of five oocytes from a pool of 30 was analyzed for CAT activity. **Lane 1** shows the background with uninjected oocytes. This experiment is quantitated in E(ii). (**D**) Sepcific enhancement of the transcriptional activity of the test gene by the hER. As an internal standard, 1 × 10^7 molecules of pGlob(+574-+917) (Figure 2A) were added to the co-injections described above. RNA was prepared from co-injected oocytes (Knowland *et al.*, 1984) and primer extension was performed as described (Seiler-Tuyns *et al.*, 1986), except that 10^5 c.p.m. (Cerenkov) of primer were hybridized with 20 μg of total RNA. Half of the final reaction was analyzed on a 6% acrylamide−8.3 M urea sequencing gel. **Lane 5**, uninjected control oocytes. The relative amounts of transcripts in each band was quantitated by densitometric scanning of the autoradiogram. The values are given in arbitrary units and the induction factors (I.F.) of the CAT transcripts are standardized to the amount of the α-globin transcripts. (**E**) Transcription activation by the hER. In experiment (**i**) 5 × 10^6 copies of the test genes (ERE$^-$ or ERE$^+$) were cointroduced with the hER expression vector (HEO$^+$) or the expression vector pKCR2 without hER sequences (HEO$^-$), 1 × 10^7 copies were injected in (**ii**) and (**iii**). The induction factor (I.F.) represents the transcriptional enhancement by the hER relative to the test gene activity with HEO$^-$.

injected with the cloned hER can induce protein−DNA complex formation at the vitellogenin ERE and that accurate transcription of chimeric genes containing this ERE is stimulated by the newly synthesized hER. The use of this approach combining *in vivo* production of the hER, visualization of DNA-binding complexes by EM and functional assays provide an excellent possibility to analyze a set of hER mutants. We discuss their structure−function properties with respect to their binding and *trans*-activating capacity on the vitellogenin promoter.

Results

Oocytes synthesize the hER from a microinjected cDNA expression vector

To determine whether *Xenopus* oocytes could express the

hER, we first injected the ER expression vector, HEO (Green *et al.*, 1986), and analyzed the nuclear proteins after 48 h of incubation. Electrophoretic analysis of immunoprecipitation reaction products shows that the receptor is made and has the expected mol. wt (Figure 1A, compare lanes 1 and 4 with lanes 2 and 5). Furthermore, the synthesis of the mutant receptor HE11 (Kumar *et al.*, 1986), lacking the putative DNA-binding domain, was confirmed using the same protocol (Figure 1, lane 6). Indeed, it was essential to demonstrate that this mutant was expressed for an unequivocal interpretation of the data, correlating ER structure and *trans*-activation of the vitellogenin promoter (see Discussion).

The hER confers estrogen responsiveness to co-injected vitellogenin–CAT chimeric genes

The data shown above demonstrate that the injection of the hER expression vector into oocytes results in the synthesis of receptor molecules. To test if the newly synthesized receptor is functional and capable of interacting correctly with the oocyte transcriptional system, we coinjected HEO with estrogen-responsive reporter genes. Two *vit–CAT* constructs were used, both of which contain the 5′ flanking region of the *Xenopus* vitellogenin gene B1 linked to the bacterial *CAT* coding sequences (Seiler-Tuyns *et al.*, 1986; Figure 1B). One, pB1 $(-596+8)CAT8^+$, contains the vitellogenin ERE (ERE^+), whilst in the other ($pB1(-301+8)CAT8^+$) it has been deleted (ERE^-), thus rendering the latter uninducible (Seiler-Tuyns *et al.*, 1986). Injection of $\sim 10^8$ copies of the hER expression vector per nucleus yielded maximal CAT activity (Theulaz, 1987) using 10^6-10^7 copies per nucleus of the reporter genes in the $CAT8^+$ vector (Seiler-Tuyns *et al.*, 1986) or 100 times more of the same constructs in a CAT2-derived vector (Gorman *et al.*, 1982). Stimulation by the hER was only detectable 48 h postinjection and persisted for at least another 24 h. Transcription from the ERE-containing *vit–CAT* gene (ERE^+) was strongly stimulated when coinjected with HEO (Figure 1C, HEO^+, lane 5) but not with the parental expression vector (pKCR2; Breathnach and Harris, 1983) lacking the hER sequences (HEO^-, lane 4). The (ERE^-) *vit–CAT* gene was at best weakly expressed with either HEO^+ or pKCR2 (Figure 1C; lanes 3 and 2). Thus stimulation is dependent upon the presence of both the ERE and the hER expression vector. Identical results have been observed using the ERE in front of the HSV thymidine kinase (tk) promoter (Theulaz, 1987).

We next determined whether the stimulation of CAT activity by the hER corresponds to an elevated level of correctly initiated CAT transcripts. Either HEO or pKCR2 were injected with the *vit–CAT* constructs into the oocytes together with an internal control gene for human α-2 globin, which is not estrogen regulated (Figure 1B). After 48 h of incubation, the levels of CAT and globin mRNAs were measured by primer extension (Figure 1D). The *vit–CAT* and globin transcripts initiate at the start site used in the vitellogenin and globin producing cells (Germond *et al.*, 1983; Liebhaber *et al.*, 1980). In addition, the level of RNA synthesized from the template containing the ERE is induced 8- to 9-fold after coinjection with the HEO (Figure 1D; compare lanes 3 and 4). In contrast, no stimulation of CAT RNA was seen when using the *vit–CAT* construct lacking the ERE (Figure 1D, lanes 1 and 2). Thus transcripts initiate correctly from these genes in oocytes and furthermore the increased CAT activity found in the presence of hER, the ERE and estrogen correlates with an increase in *vit–CAT* RNA.

In three independent experiments, estradiol stimulated *vit–CAT* activity 10- to 40-fold (Figure 1E). We observe some variation between experiments and believe that it represents differences between oocytes from different frogs. Nevertheless, the level of induction is significant and comparable to that obtained in transient expression assays in a variety of cell lines (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Green and Chambon, 1987; Kumar *et al.*, 1987). Thus a human *trans*-regulatory factor can control the activity of a *Xenopus* promoter in a *Xenopus* oocyte thereby suggesting that certain aspects of genetic regulation are conserved amongst vertebrates.

Visualization of protein–DNA complexes induced by hER synthesized in Xenopus oocytes

We next attempted to demonstrate the participation of the receptor in the assembly of protein–DNA complexes at the 5′ end of the *Xenopus* vitellogenin gene. Such complexes, formed by incubation with extracts of liver nuclei from *Xenopus*, have been visualized and mapped using EM on the vitellogenin gene B2 (ten Heggeler-Bordier *et al.*, 1987). A different approach was used here to demonstrate the participation of the receptor in the appearance of the complexes observed. Since the hER is accumulated in the nucleus as shown by the results presented above, we were able to prepare extracts from a limited number of manually isolated oocyte nuclei and test their ability to assemble similar complexes. In this respect the EM assay is particularly advantageous in that it requires very small amounts of extract compared to standard biochemical assays. The template utilized for the EM experiments was a 2.7 kb fragment containing the promoter of the vitellogenin gene B2, which was previously used in the EM assay and which is structurally identical to the B1 promoter (Walker *et al.*, 1984) used above. The template was incubated with the extract in the presence of 5 nM 17-β estradiol under conditions in which the ternary transcription complex containing the RNA polymerase II does not form (ten Heggeler and Wahli, 1985). The reactions were processed and prepared for visualization by EM. The micrograph in Figure 2A shows an example of the complexes observed at the 5′ end of the gene B2, using nuclear extracts prepared from oocytes injected with the hER expression vector. Their distribution (Figure 2A, clear histogram) is indistinguishable from that obtained under the same conditions with the liver nuclear extracts (ten Heggeler-Bordier *et al.*, 1987). Two distinct complexes are observed, mapping 330 ± 24 bp upstream of the transcription initiation site at the upstream binding site (UBS) and 350 ± 60 bp downstream of it at the downstream binding site (DBS). These two binding sites on the B2 promoter correspond to those mapped previously in the liver nuclear extracts. The UBS spans the B2 ERE, which can also activate the *CAT* gene in transfection experiments in MCF-7 cells. The DBS, located in the first intron of the B2 gene, shows no homology to the UBS and cannot be hormonally induced in the same assay (ten Heggeler-Bordier *et al.*, 1987). Neither site shows significant binding when incubated in nuclear extracts of oocytes injected with the expression vector lacking hER sequences (pKCR2; Figure 2A, striped histogram). The

appearance of the complex at the UBS decreases to background levels in the presence of tamoxifen (Figure 2B, stippled histogram), while the binding to the DBS is diminished to just above background (Figure 2B, stippled histogram; Figure 3, stippled histograms). Complex formation at both sites reappears quantitatively when an excess of hormone is added with the antagonist (Figure 2B, clear histogram). These results, namely that binding is hormone-dependent, blocked reversibly by tamoxifen, and not seen in control extracts, strongly suggest that the hER is implicated in the formation of the complexes observed by EM.

Correlation between the ability of mutants to form protein–DNA complexes and to activate transcription

Using this same approach we examined the ability of a series of hER mutants (Kumar *et al.*, 1986) to form complexes with the *Xenopus* ERE. The experiments shown in Figure 3 included the dinucleotide ApU, which permits the visualization of the RNA polymerase II-dependent initiation complex on the same template (ten Heggeler and Wahli, 1985). This internal control allows us to compare the amount of complex formation over the ERE using different extracts. Each experiment compared three mutants with the wild-type receptor. The region missing in each of the mutants is depicted in Figure 3, relative to the wild-type receptor (HEO). The histograms show typical profiles for complex formation by each mutant, either in the presence of estradiol (clear histogram) or the antagonist tamoxifen (stippled histogram). HEO shows essentially the same histogram as in Figure 2A, but with the addition of a third peak at the transcription initiation site of the B2 gene, labeled PBS in Figure 3. Note that tamoxifen leads to a 2-fold reduction in the formation of the initiation complex, while it blocks binding at the UBS and diminishes it at the DBS. ER mutant HE19, which lacks the amino terminal domain (Figure 3, regions A and B) and HE12, which has an internal deletion removing most of region D, form complexes that are indistinguishable from the wild-type receptor and can be prevented with tamoxifen (Figure 3). In a functional assay, coinjecting the hER mutants with the B1 ERE$^+$ test gene, HE12 and HE19 show a reduced capacity to enhance transcription compared with HEO (23–60% and 12–30%, respectively; see Table I and Figure 3, transcription activation). Deletion of region C (HE11), which is the putative DNA binding domain, is incapable of forming complexes at the UBS and leads to a small peak at the DBS, and yet a stable mutated receptor protein is expressed in oocytes (Figure 1A). HE11 is also unable to enhance transcription of the test gene in oocytes (Table I).

The ER mutants HE15 and HE21, which lack the hormone-binding domain (see Figure 3), show essentially normal levels of complex formation at all three sites in the presence of estradiol, however, the binding is not altered by the addition of tamoxifen. Moreover, HE15 and HE21 only very weakly stimulate expression of the reporter gene

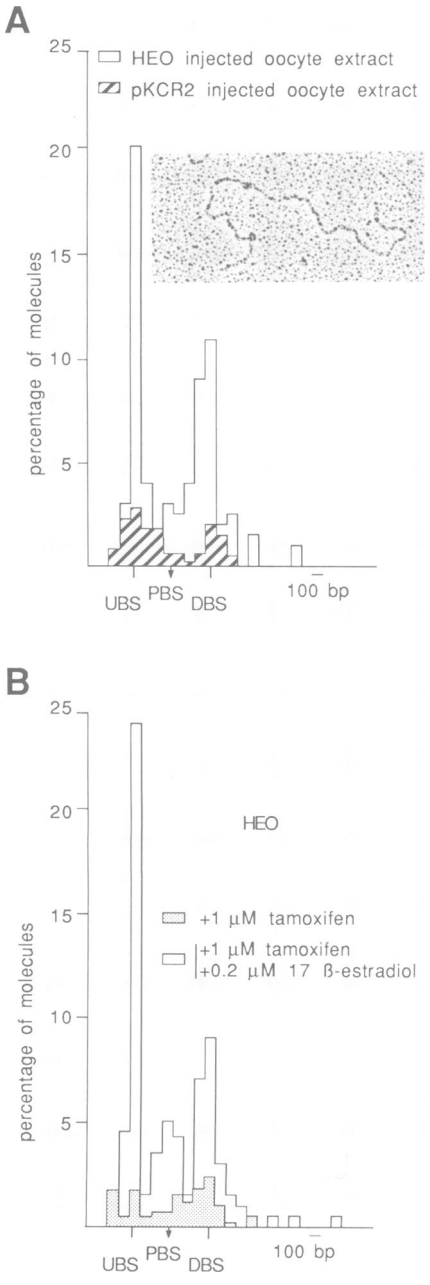

Fig. 2. Distribution of the protein–DNA complexes on the 2.7 kb B2 gene promoter fragment. (**A**) The template DNA, an *Eco*RI fragment extending from position −769 to +1963, relative to the transcription initiation site, was incubated in a nuclear extract from HEO injected oocytes (open histogram) or from oocytes injected with the expression vector without hER coding sequence (pKCR2, striped histogram) in the presence of 5 nM 17-β estradiol. The reactions contained 20% extract (v/v). The arrow below the histogram indicates the transcription initiation site (PBS) of the gene B2 (Germond *et al.*, 1983). The bars correspond to the positions of the UBS and DBS as defined by EM of complexes formed in liver nuclear extracts (ten Heggeler-Bordier *et al.*, 1987). (**B**) Incubation of the B2 promoter fragment with HEO-injected oocyte nuclear extract (20% v/v) in the presence of tamoxifen (stippled histogram) or both tamoxifen and hormone (open histogram). Four hundred molecules were screened for the presence or absence of complexes and the percentage of binding was, in (A), 49% with the HEO injected oocyte extract and 13% in the pKCR2 injected oocyte extract, and, in (B) 14% with 1 µM tamoxifen and 52% with 1 µM tamoxifen and 0.2 µM 17-β estradiol. For each histogram a randomly selected subset, consisting of 50–100 molecules with complexes, was photographed to map the complex position using the criteria previously described (ten Heggeler and Wahli, 1985; ten Heggeler-Bordier *et al.*, 1987). The percentage of molecules in the ordinate takes into account the percentage of total binding calculated from 400 molecules. For example, in (A), with the HEO-injected oocyte extract, complexes were mapped on 72 molecules from a sample of 400 showing 49% binding. The percentages for the ordinate of the histogram were then calculated on the basis that this represents 72/0.49 or 146 molecules in all (100%). The same calculation was used for each histogram.

(see Table I and Figure 3, transcription activation). Table II presents the results of up to three experiments with each mutant as the percentage of molecules containing complexes. In each case the total percentage of binding is given, as is the percentage of complexes over the three specific sites. In general between 40 and 60% of the molecules have complexes, except for HE11. In the case of HEO, HE12 and HE19, tamoxifen significantly lowered the number of complexes mapping at the UBS and DBS and caused a 1.5- to 2-fold reduction in the peak at the PBS. Thus removal of regions A/B or most of region D leads to a receptor that formed complexes like wild-type and which activated transcription at reduced levels, indicating that these regions are important for efficient transcription. It is interesting that both classes of mutant receptor, i.e. lacking either region A/B or regions E/F can form similar complexes in our EM assay. This binding maps over the ERE (UBS) and at a site of unknown function in the first intron (DBS). With HE11 we consistently see 2-fold more complex formation, albeit non-specific, in the presence of tamoxifen as compared to the level with hormone. This could be due to a variety of

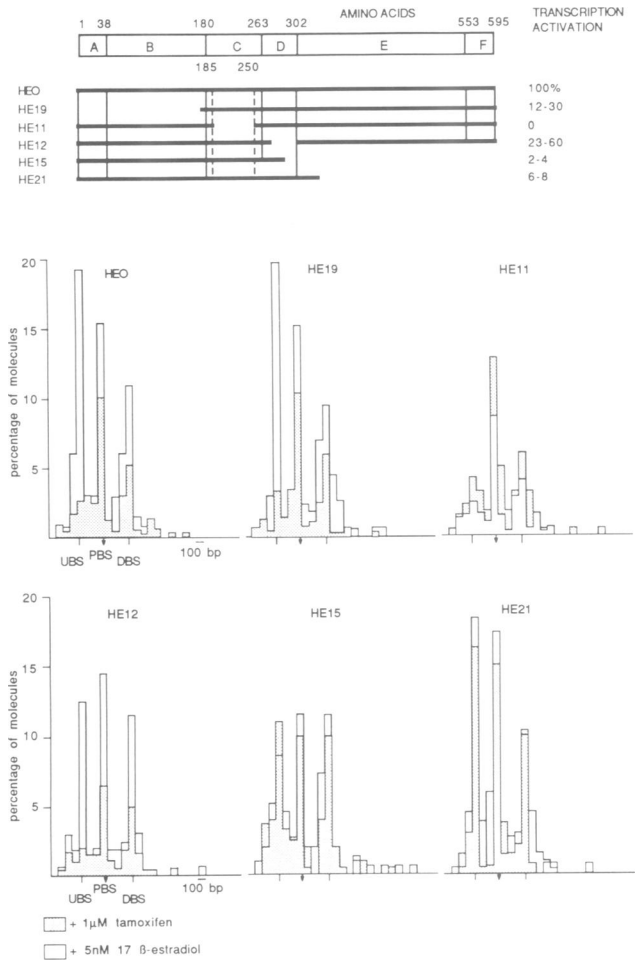

Fig. 3. Effects of selective deletions on the ability to form protein−DNA complexes and on transcriptional enhancement. The coding regions of the deleted hER expression vectors (Kumar *et al.*, 1986) are schematically represented at top of the figure. The exact positions of the deletions are: HE19, amino acids 1−178; HE11, amino acids 185−251; HE12, amino acids 271−300; HE15, amino acids 282−595; and HE21, amino acids 341−595. The experiments were analyzed as described in the other figures, except that the extracts were made 48 h post-injection. In addition, the dinucleotide ApU was included in the reaction to allow us to visualize the ternary transcription complex at the initiation site. In each case the EM experiments were performed with coded extracts. The transcription activation results of the three experiments described in Table I are shown along the right side of the figure as percentages of the wild type activity (100%).

Table I. Transcription activation by the hER mutants

		Experiment			
		1	2	3	4
pKCR2	CAT	2.8	0.3	0.9	ND
	%HEO	8	0.4		
HEO	CAT	35.4	25.2	ND	65.5
	%HEO	100	100		100
HE19	CAT	4.5	3.3	16.4	19.6
	%HEO	13	12		30
HE11	CAT	0.5	0.2	0.5	ND
	%HEO	1	1		
HE12	CAT	8.3	8.0	31.4	40
	%HEO	23	32		61
HE21	CAT	2.7	1.4	1.0	ND
	%HEO	8	6		
HE15	CAT	1.3	0.6	0.3	ND
	%HEO	4	2		

Experiments and quantifications of CAT activity were made as described in Figure 1C. The activity of the hER mutants is given as percentages of acetylated chloramphenicol (CAT) and as percentages of the wild-type activity (HEO%).

Table II. Quantitation of protein−DNA complex formation

% molecular binding	1	2	3
	+H / +T	+H / +T	+H / +T
HEO total	55.2 / 26	41.2 / 13.0	
UBS	19.2 / 2.7	14.0 / 0.8	
PBS	15.4 / 10.3	8.7 / 4.5	
DBS	11.0 / 5.3	8.7 / 1.2	
HE19 total	54.8 / 26.0	43.0 / 11.0	61.2 / 30.4
UBS	19.6 / 3.4	14.0 / 0.6	21.2 / 1.5
PBS	15.2 / 10.5	5.0 / 3.5	19.0 / 9.0
DBS	9.4 / 6.0	13.0 / 1.2	12.6 / 5.5
HE11 total	22.6 / 36.0	14.0 / 30.0	18.0 / 37.4
UBS	2.3 / 4.1	0.8 / 2.9	1.3 / 3.5
PBS	8.5 / 12.8	4.4 / 9.2	5.0 / 12.2
DBS	4.0 / 6.0	2.1 / 2.9	3.0 / 7.9
HE12 total	48.4 / 22.0		
UBS	12.6 / 1.9		
PBS	14.5 / 6.3		
DBS	11.3 / 4.8		
HE21 total	53.2 / 55.2	65.6 / 59.2	
UBS	18.3 / 16.4	23.2 / 20.0	
PBS	17.5 / 15.0	19.2 / 13.0	
DBS	10.0 / 10.4	12.8 / 10.7	
HE15 total	46.3 / 47.2	29.2 / 31.0	46.6 / 45.0
UBS	8.6 / 11.0	8.4 / 10.0	15.2 / 12.1
PBS	11.2 / 10.0	7.6 / 7.5	13.3 / 8.6
DBS	11.3 / 10.0	5.3 / 5.6	8.0 / 7.1

Measurements and determination of the percentage of molecules presenting protein−DNA complexes were made as described in Figure 2. The reactions were performed in presence of hormone (+H) or the antagonist tamoxifen (+T). Up to three independent injection experiments (1−3) have been performed with each receptor. Experiment 1 is presented in the histograms of Figure 3. ND, not determined.

factors, such as different conformational changes in the receptor upon hormone and antagonist binding (Fauque *et al.*, 1985).

Discussion

The present study correlates EM analysis of protein–DNA interaction and measurements of transcriptional activation after expression of a *trans*-acting factor in *Xenopus* oocytes. We show that hER produced in oocytes via microinjection of an hER expression vector promotes the formation of protein–DNA complexes over the ERE of a *vit–CAT* fusion gene and activates its expression. The magnitude of induction was 10–40-fold. Comparable experiments in mammalian cells have given similar results with the hGR and the hER (Giguère *et al.*, 1986; Druege *et al.*, 1986; Green and Chambon, 1987). Here, our first goal was to demonstrate that an important regulatory factor from a different species could be synthesized and function correctly in a *Xenopus* cell by controlling the activity of a *Xenopus* gene promoter. This implies that the newly synthesized receptor is translocated to the nucleus of the oocyte and functions across distant species barriers.

Our results on the hER-dependent formation of protein–DNA complexes at the ERE, as well as those on the hER-dependent specific activation of the *vit–CAT* fusion gene containing the ERE, do not prove a direct interaction of the hER with the ERE. However, it is unlikely that the hER acts indirectly by inducing a *de novo* synthesis of *Xenopus* regulatory factors or alternatively by modifying pre-existing factors. We and others have already shown that functional interactions between the hER and *Xenopus* ERE occur (Klein-Hitpass *et al.*, 1986; Druege *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Green and Chambon, 1987), which is consistent with the high degree of conservation of the ER in vertebrates (Krust *et al.*, 1986; Koike *et al.*, 1987; Weiler *et al.*, 1987). Furthermore, the results discussed below and our previous EM analysis (ten Heggeler-Bordier *et al.*, 1987) together with results from others on the ER-binding activity (Jost *et al.*, 1984; Klein-Hitpass *et al.*, 1988) indicate that the receptor directly participates in the complex observed at the ERE.

We could not test whether the observed transcriptional activation would occur in the presence of the hER but without estradiol since the oocytes contain high amounts of hormone produced by the follicular cells (Fortune, 1983) that cannot be washed out (Knowland *et al.*, 1984). Furthermore, tamoxifen treatment is toxic to oocytes (Theulaz, 1987) and thus cannot be used to compete out the effect of the endogenous estradiol. However, the fact that both the receptor and its ligand are required for induction of ERE-containing chimeric genes in *Xenopus* kidney cells (A.Seiler-Tuyns, unpublished results) and several mammalian cell lines (Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Green and Chambon, 1987; Kumar *et al.*, 1987) suggests that the hormone activation is necessary.

Complex formation using the two mutants lacking the hormone binding domain (HE15 and HE21) is very efficient and is unaffected by the antagonist tamoxifen. However, these two truncated receptors are at least 20-fold less effective than the wild-type receptor in activating transcription in oocytes. Thus removal of the steroid-binding domain does not lead to a hormone-independent activation of the receptor, in contrast to the hGR, for which it has been recently reported that deleting the region E leads to a constitutively active mutant (Hollenberg *et al.*, 1987). The fact that the HE15 and HE21 mutants lack the entire C-terminal sequence (regions E and F) could account for this apparent discrepancy. Alternatively, these mutants might be unable to interact with components of the estrogen-dependent transcription machinery and/or with the polymerase to stabilize initiation complexes, or might have a reduced affinity for the ERE sequence that is not revealed by our assays. These results, correlating DNA binding to biological activity strongly suggest that the hormone binding domain plays a more positive role in achieving hER mediated gene activation than simply just masking the DNA binding or *trans*-activation domain(s) in the unoccupied receptor.

Complete deletion of the A/B domain leads to a mutant protein (HE19) of rather low *trans*-activating activity, but which retains a DNA binding capacity similar to that of the wild-type receptor (Figure 3). It has been suggested in recent studies using human, rat and mouse GR (Hollenberg *et al.*, 1987; Miesfeld *et al.*, 1987; Danielsen *et al.*, 1986) that the A/B domain of these receptors modulates the extent of hormonal responsiveness by facilitating the discrimination between nonspecific and specific DNA. Our EM data reveal that the hER mutant missing the A/B region binds the ERE-containing UBS and the DBS with a specificity similar to that of the wild-type receptor. These results suggest that the function of this domain in the hER might not be equivalent to that of the GR.

Altogether, our results show that the formation of complexes with the ERE only requires half of the receptor molecule, together with region C, which is essential, since the mutant missing region C does not exhibit either complex formation or stimulation of transcription, in spite of its presence in the oocyte nucleus. hER derivatives missing various portions of the protein, except domain C, recognize specific binding sites on the DNA, but fail to activate efficiently the estrogen-dependent vitellogenin promoter. Thus our data clearly dissociate DNA binding from transcription activation function. The loss of the N- and C-terminal domains might modify the ER structure and/or disrupt some cooperative binding with other proteins. The discovery of two domains in hGR, referred to as τ, not involved in the DNA- and hormone-binding, but necessary for full transcriptional activation (Giguère *et al.*, 1987) raises the possibility that similar, but not yet identified, regions exist in the related hER. In addition, one can draw a parallel with the Gal4 system, where DNA binding and *trans*-activation functions are also localized to distinct regions of the polypeptide (Keegan *et al.*, 1986).

We noticed above that the co-injection of the B1 *vit–CAT* reporter gene and the HE19 clone into oocytes gives rise to a moderate level of expression of the promoter, in contrast to what happens in HeLa cells where co-transfection of A2 *vit–tkCAT* and HE19 plasmids generate a wild-type activity (Kumar *et al.*, 1987). The ERE of the A2 gene is a single perfect 13 bp palindromic structure and is by itself sufficient for the enhancer activity (Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988), whereas the ERE of the B1 gene requires for its function two imperfect palindromic elements acting cooperatively (Martinez *et al.*, 1987). If the observed co-operative effect is based upon a stabilizing protein–protein interaction between receptor molecules binding to

lower affinity sites, the HE19 mutant depleted of the A/B domain could possibly not be capable of such interactions, thus explaining its reduced activation ability. Interestingly, it has also been observed that the human estrogen responsive pS2 gene that does not contain the perfect palindromic sequence present in the A2 vitellogenin gene, is not efficiently activated by HE19 in cotransfected HeLa cells (Kumar *et al.*, 1987). These results favor the idea that the nature of the promoter might also play a not yet understood role in the activation process by the ER.

Materials and methods

DNA manipulations

The construction of HEO, previously called pKCR2-pOR8 (Green *et al.*, 1986) and mutants thereof (Kumar *et al.*, 1986, 1987), the expression vector pKCR2 (Breathnach and Harris, 1983), pB1(5′/−596+8)CAT8$^+$ and pB1(5′/−301+8)CAT8$^+$ (Seiler-Tuyns *et al.*, 1986) have been described. pGlob[−574+917] consists of a 1419 bp *PstI* genomic fragment of the human α-2 globin gene subcloned in pUC9 (A.Seiler-Tuyns, unpublished data).

All plasmids used for microinjection were purified on two CsCl/EtBr equilibrium gradients and then either by gel filtration through Biogel A-50 or on NaCl gradients. For all injections, the DNA was resuspended in double distilled H_2O at concentrations ranging from 5 to 500 μg/ml.

Injection of DNA into oocyte nuclei

To prepare oocytes, ovaries from *Xenopus laevis* mature females were incubated in Modified Barth's saline (MBS medium; Coleman, 1984), lacking Ca^{2+} but containing 0.2% collagenase (Sigma), at 20°C until the ovarian tissue was dissociated and the follicle cells removed. The defolliculated oocytes were maintained in MBS supplemented with 1 μM 17-β estradiol at 20°C.

Micropipettes were pulled from glass capillaries with a Narishige microelectrode puller (Narishige, Tokyo) to give tips ~10 μm in diameter. The micropipettes were calibrated to deliver sequential microinjections of 20 nl via an automatic injection apparatus (Inject+Matic, Geneva, Switzerland).

Extract preparation and electron microscopy

Oocytes (100) were injected into the nucleus with 6×10^8 copies of pKCR2, HEO or mutants thereof, and then incubated for 48 or 72 h. Germinal vesicles were isolated manually on ice as described (Hipskind and Reeder, 1980) in 0.1 M NaCl, 20 mM Tris−HCl (pH 8.0), 20% glycerol, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF. The nuclei were homogenized by pipetting 10 times using a Gilson pipettor and an intact tip, and 5 M NaCl was added to a final concentration of 0.42 M. The homogenate was stored on ice for 30 min with frequent vortexing and then centrifuged for 10 min in an Eppendorf centrifuge in the cold room. The supernatant was dialyzed against 1000× volume of 20 mM Hepes (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT and 0.5 mM PMSF for 6 h at 4°C. The precipitated proteins were spun out as above, then aliquots of the supernatant were frozen in liquid nitrogen and stored at −70°C. The resulting 20−30 μl of extract contained 2−7 mg/ml protein, as determined using the Biorad protein assay with BSA as the standard. The formation of protein−DNA complexes and their analysis (mapping of the complexes, orientation of the DNA molecules) were performed as described by ten Heggeler-Bordier *et al.* (1987).

CAT assays

Groups of 30 injected oocytes were homogenized in 10 μl/oocyte of 0.25 M Tris−HCl (pH 8.0) and the equivalent of five oocytes (i.e. 50 μl of homogenate) was analyzed for CAT activity as described by Gorman *et al.* (1982).

RNA preparation and primer extension

RNA was prepared according to Probst *et al.* (1979) and primer extension was performed as described by Seiler-Tuyns *et al.* (1986), except that 10^5 c.p.m. (Cerenkov) of primer were hybridized with 20 μg of total RNA. Half of the final reaction was analyzed on a 6% acrylamide−8.3 M urea sequencing gel.

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