J. Steroid Biochem. Molec. Biol. Vol. 38, No. 4, pp. 423–431, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

HUMAN ESTROGEN RECEPTOR INTRODUCED INTO THE *XENOPUS* OOCYTE REPRESSES EXPRESSION FROM AN ARTIFICIAL FROG ESTROGEN RESPONSE ELEMENT

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(Received 13 August 1990)

Summary—Although the estrogen responsiveness and estrogen receptors of *Xenopus* hepatocytes have been well described, oocytes of this species have not previously been shown to contain estrogen receptors (ER). Recombinant human ER (HER) was expressed in oocytes in a dose dependent fashion as measured by [^{35}S]methionine incorporation into newly synthesized proteins. Chloramphenicol acetyl transferase (CAT) reporter plasmids, driven by a herpes simplex thymidine kinase promotor with or without a 17 base pair estrogen response element (ERE) from the vitellogenin A2 gene, were also injected into oocytes. When injected without the accompanying HER sequences, the construct containing the ERE expressed 10-fold more CAT activity, and this response was saturable as demonstrated by injecting increasing amounts of reporter plasmid. These results suggest either the activity of small amounts of a *Xenopus* ER (measured here by LH-20 assay), or the presence of some endogenous oocyte protein other than the ER that can interact with this ERE. When HER was co-expressed with ERECAT, CAT expression was suppressed over a wide range of HER concentrations. This unexpected repression may be due to displacement of an estrogen receptor or other endogenous oocyte regulatory protein on the ERE. HER's positive regulatory activity may require transcription factors that are lacking or insufficient in the oocyte. Alternatively the simple 17 base pair ERE may not provide DNA binding sites for such transcription factors.

INTRODUCTION

The *Xenopus* oocyte has been used by us and others as a convenient injectable cell for the reconstitution of estrogenic responses. Our previous work has demonstrated that large amounts of the human estrogen–receptor complex alone, synthesized from recombinant plasmids injected into the oocyte, cannot elicit expression from potentially expressible endogenous oocyte genes [1]. We hypothesized that the *Xenopus* oocyte does not provide the other necessary factors for the expression of its dormant vitellogenin genes from their natural promotors or that expression from native chromatin may require auxiliary factors not required for expression of introduced plasmid DNA. Our previous work also supported the conclusion that the oocyte does not possess appreciable estrogen receptors of its own [1]. Although steroid hormones quickly diffused to both cytoplasmic and nuclear compartments of the oocyte, they did not concentrate in the nucleus unless an exogenous receptor was introduced by recombinant-directed synthesis. We also note the lack of any reports from other laboratories that estrogen receptors are a measurable feature of the *Xenopus* oocyte.

Proteins purified from *Xenopus* liver by estradiol affinity chromatography [2] or heparin sepharose chromatography [3] are capable of eliciting expression of endogenous oocyte vitellogenin genes. However, receptor purification by estradiol affinity chromatography results in preparations which contain proteins in addition to the estrogen receptor itself, as observed by silver staining of SDS–polyacrylamide gels from these preparations (Watson and Torres, unpublished observations). Those factors that copurify, perhaps by binding specifically to the receptor (as noted in chromatographic purifications of other steroid receptors [4–6], may be essential for successful expression of the vitellogenin gene directed by the estrogen–receptor complex.

Other investigations have shown that the inability of HER to elicit vitellogenin gene expression is probably not due to species differences, as HER present in MCF-7 breast cancer cells is

capable of causing expression of a transfected frog vitellogenin gene [7, 8]. Also, plasmid constructs consisting of large regions of the vitellogenin A2 or B2 promotors driving a CAT reporter cointroduced with recombinant HER expressing constructs have been expressed in a variety of cell types [8, 9] including the *Xenopus* oocyte [10].

In the present studies, we investigated the hypothesis that expression of large quantities of the HER in the oocyte might either commandeer the oocyte synthetic machinery to the exclusion of any other product, or sequester necessary auxiliary transcription factors onto the excess receptor and thus prevent the formation of an adequate transcription complex. We investigated this possibility by the injection of a wide range of quantities of the HER-producing plasmid into the oocyte to elicit the production of different amounts of the receptor protein. Next we studied the expression in the oocyte of a very simple (17 base pair) ERE-containing CAT reporter construct, driven by a viral thymidine kinase promotor, and the saturation of this response. Finally an unexpected negative regulatory effect of HER on ERECAT expression was examined.

EXPERIMENTAL AND METHODOLOGY

Chemicals and animals

[2,4,6,7,16,17-^{3}H]Estradiol 17β (146 Ci/mmol) and [^{35}S]L-methionine (300 Ci/mmol) were purchased from Amersham International or New England Nuclear. [^{3}H]Estradiol was dried under vacuum or nitrogen and resuspended in absolute ethanol followed by dilution in appropriate buffers before use, or directly resuspended in cytosol samples. Non-radioactive estradiol was purchased from Sigma Chemical Co. (St Louis, Mo.).

Adult female *Xenopus laevis* obtained from NASCO (Ft Akinson, Wisc.) or P. Fraser (Mt Clements, Mich.) were maintained in charcoal filtered, dechlorinated tap water at 19 ± 2°C and fed twice weekly. Animals were anesthetized by immersing in ice water prior to removal of oocytes or other manipulations. Oocytes were surgically removed and prepared for injection by dissection from ovarian tissue; defolliculation was accomplished by incubation in a 0.25 mg/ml collagenase solution (Sigma) for 2 h at room temperature with rotary agitation [11]. Oocytes used for some receptor assays were tweezed apart from ovarian tissues without collagenase treatment. All oocytes were maintained before and after injection in Barth-X culture medium at 19°C as described previously [12]. Exogenous estradiol was not added to the medium because the published work of others demonstrated that oocytes contain high levels of endogenous estradiol [13] that cannot be readily washed out [3].

Plasmid constructs

The expression vector p91023(B) [14] used for the production of HER (human estrogen receptor) in oocytes has been described previously [1, 15]. HER cDNAs [16] in this vector have been expressed to high levels in CV-1 cells [15] and in the *Xenopus* oocyte [1]. This plasmid (pΔHER91023) was injected into the oocyte at a concentration range of 0.01–2 ng DNA/nucleus for various experiments which follow.

Reporter plasmids

The pBLCAT2 plasmid [17] contains a herpes simplex thymidine kinase (tk) promotor preceding the coding sequence for the bacterial chloramphenicol acetyltransferase (CAT) gene, followed by an SV40 polyadenylation signal, and has been expressed previously in CV-1 cells [18]. In the pERECAT constructs the estrogen response element (ERE) from the A2 gene for frog vitellogenin is inserted just prior to the tk sequences. ERE consists of the following sequence: 5′CAGGTCACAGTGACCTG3′. These plasmids were used as "target" constructs to assess the ability of introduced or endogenous oocyte proteins to affect expression from the CAT reporter and were injected into the oocyte at a range of 0.1–5 ng DNA/nucleus for various experiments.

RNA preparation

Liver RNA was prepared by the LiCl-urea precipitation method [19]. Poly(A) RNA (mRNA) was isolated from the total RNA population by oligo-dT cellulose chromatography [20].

Xenopus oocyte injection

Injection needles were positioned with the aid of an Oxford pantograph style micromanipulator (Stoelting, Chicago, Ill.). RNA preparations (in 50 nl) were injected into the oocyte cytoplasmic space. For injection of cDNAs into oocyte nuclei (in 25 nl), oocytes were injected in the center of the animal pole as described in the methodology review by Gurdon and Wakefield [12]. Using this method we were able to score 92% of all injections in the oocyte nucleus as judged by

bromophenol blue or bromocresol green dye detection in 10% trichloroacetic acid (TCA) precipitated dissected oocytes [1]. Single oocytes were injected under a dissecting microscope at 10–12 × magnification with needles pulled from 2 mm diameter glass capillaries (WPI, New Haven, Conn.) on a Narishigi Scientific Instruments (Tokyo, Japan) PE-2 needle puller. Needles were filled with injection fluid by capillary action and oocytes were held in place with 1.3 mm depressions in a lucite injection platform. Precise nanoliter volumes were ejected from a needle by puffs of compressed air delivered through teflon tubing from a Picospritzer (General Valve Corp., Fairfield, N.J.) set at 20 p.s.i. pressure and variable (between 8 and 15 ms) duration of pulse. Each needle was calibrated with a radiolabeled compound over a range of msec duration of air pulse to determine the time required to deliver 25 or 50 nl of water containing nucleic acid, into the nuclear or cytoplasmic compartment of the oocyte respectively. We believe that the precise delivery of volumes and repeatability of such a system is superior to oil or air filled micrometer syringes routinely used in oocyte microinjection.

Incorporation of [^{35}S]methionine into newly synthesized protein

Surviving oocytes (10–15 selected after an overnight incubation in Barth-X) were incubated in 200 μl of Barth-X medium containing antibiotics and 150 μCi of [^{35}S]methionine [2]. After two days of incubation the oocytes were homogenized with a polypropylene pestle fitted for a microfuge tube (Kontes, Vineland, N.J.) in 75 mM Tris, pH 6.8, 1% β-mercaptoethanol, 1% SDS and 1 mM PMSF. The homogenates were then separated by centrifugation at 10,000 *g* into supernatant and yolk platelet (pellet) fractions [21]. An aliquot of the total radioactive proteins from the medium or supernatant fractions was treated with 10% TCA to determine the incorporation of [^{35}S] and amounts of labeled proteins to load on gels [22]. Equivalent cpms of samples were electrophoresed on 7.5% SDS–polyacrylamide, followed by fluorography [22].

Vitellogenin standards were obtained from oocytes which had been injected with female liver mRNA samples (135 ng injected/oocyte) and were secreting this protein into the medium [1, 2]. These oocytes additionally served as a control for the synthetic activity of a given batch of oocytes. The vitellogenin protein is so abundant under these circumstances that immunoprecipitation is not necessary for detecting this labeled protein product [1]. In addition, authentic vitellogenin (prepared as described in [23] was run in adjacent lanes and stained for identification.

Monitoring of expression from CAT reporter plasmids

Plasmids pBLCAT2 and pERECAT were injected into oocyte nuclei in a 25 nl volume which delivered a given ng of plasmid per oocyte nucleus in groups of 15–30 oocytes per experiment. Oocytes were rinsed in a buffer containing 40 mM Tris, pH 7.5, 1 mM EDTA, and 150 mM NaCl and transferred to microfuge tubes. Oocytes were then homogenized in 0.25 M Tris, pH 7.5 buffer (50 μl buffer per oocyte) and centrifuged at 10,000 *g* in a microfuge at 4°C for 10 min. The supernatant was then recentrifuged as before. This extract was used (based on an equivalent amount of protein per assay) in an assay for CAT activity using [^{14}C]chloramphenicol as a substrate. Products of the reaction were ethyl acetate extracted, spotted on a thin layer chromatography plate, and developed in a 5% methanol/95% chloroform solvent system for approximately 2 h. CAT activity was assessed by autoradiography of the plate followed by cutting out the acetylated forms of labeled chloramphenicol for scintillation counting. The data are presented as percentage of total recovered ^{14}C-chloramphenicol acetylated/μg protein. Values are given as fold increase in counts acetylated by various extracts with a designated control or baseline condition, or are normalized around one value per experiment to correct for inter-experiment variability. Any experiment in which extracts were able to acetylate less than 0.1% of the substrate per μg protein were eliminated as being inactive. Experiments in which the % substrate conversion was greater than 50% were also excluded so that the conversion activity was not limited by product inhibition or significant change in substrate concentrations.

Estrogen receptor assays

Animals used to prepare liver cytosol had previously had ovaries removed for 1–3 months prior to liver removal. This was to allow high levels of receptor to be isolated from the cytoplasmic cellular compartment after estrogen withdrawal; *Xenopus* ER is known to have a long period of nuclear occupancy as a result of

hormonal exposure [24]. Animals were anesthetized and pithed before cardiac perfusion with Barth-X saline medium containing 0.1 mg/ml heparin and finally livers were removed. All studies were conducted in accordance with the principles and procedures outlined in "Guidelines for Care and Use of Experimental Animals".

All subsequent procedures were carried out in an ice-bath or in the cold room (4°C). Oocytes were suspended in 2 volumes of homogenization medium containing 0.25 M sucrose, 1 mM dithiothreitol, 20 μg/ml benzamidine, 20 μg/ml PMSF, and 50 mM Hepes, pH 7.4. Livers were rinsed, finely chopped, and the tissue was suspended in two volumes of the homogenization medium. The 100,000 *g* supernatant was prepared as follows. The liver tissue was homogenized with a Tissumizer (Tekmar, Cincinnati, Ohio) in 30 s bursts until homogeneous. Oocytes were homogenized with ~3 strokes of a Dounce glass homogenizer with an A pestle. The homogenates were centrifuged at 10,000 *g* for 10 min and the lipid layer aspirated from the top. The supernatants were then centrifuged at 100,000 *g* for 1 h at 4°C and the lipid layer again aspirated from the top of the resultant supernatant. This preparation was then precipitated for 15 min at 4°C by adding 1.5 volumes of a saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 10,000 *g* for 5 min. The pellet was resuspended in 0.5 M KCl, 50 mM Hepes, pH 7.4, 10% glycerol, 20 μg/ml benzamidine, 20 μg/ml PMSF and 1 mM dithiothreitol (DTT) which then constituted the cytosol fraction for the estrogen receptor assays.

Aliquots (200 μl) of liver cytosol or oocyte cytosol that had been ammonium sulfate precipitated and resuspended were incubated in 5 nM [^{3}H]estradiol 17β ± a 100-fold excess concentration of unlabeled estradiol for 90 min at 10°C. Bound and free hormone were separated by a modification of the Sephadex LH-20 method of Ginsberg *et al.* [25]. We standardized the procedure with 0.7 ml column volumes from which protein bound estradiol could be eluted in a void plus wash volume of 800 μl. An aliquot of this eluant was then added to liquid scintillant and the radioactivity determined. Oocytes were weighed to determine cell number/tissue weight for comparisons to liver tissue. The weight of 50 oocytes was 47.6 ± 0.1 (SE) or 1050 oocytes/g wet weight of oocyte tissue, in agreement with the estimations reported by others [12].

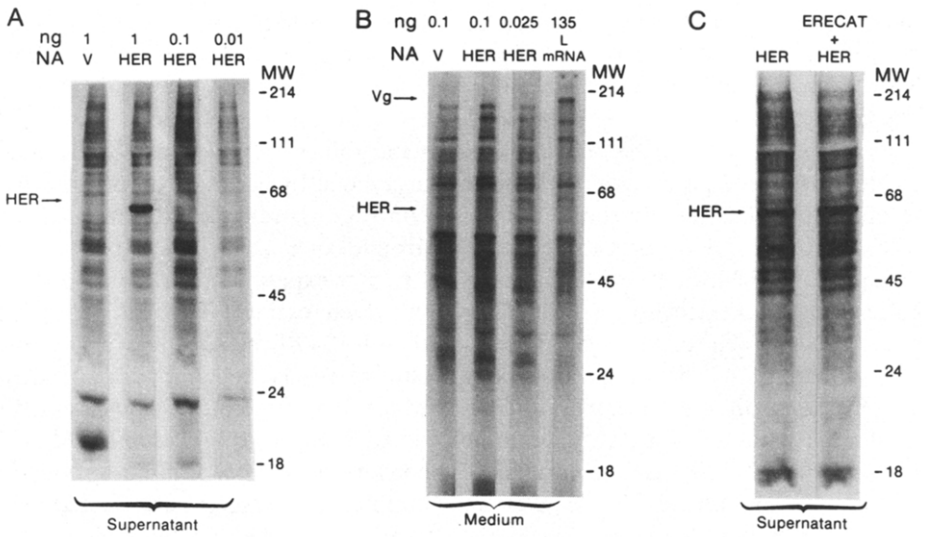

Fig. 1. SDS–PAGE (7.5%) fluorographs of labeled (newly synthesized) oocyte proteins. Forty-eight hours after nucleic acid (NA) injection into the oocyte nucleus in the nanogram (ng) amounts shown, total protein samples were assayed. Panel A: Oocytes injected with 0.01–1 ng of the plasmid pΔHERP91023 (HER lanes) synthesize an [^{35}S]methionine labeled 66,000 Da protein, human estrogen receptor (HER at arrow), that was not produced by p91023 injected oocytes (V or vector lanes). The absence of HER in the medium preparation suggests that these proteins are intracellular. Panel B: Vitellogenin (210,000 mol. wt, Vg at arrow) was synthesized into the medium only in oocytes that were injected with estrogen-stimulated frog liver mRNA (L mRNA). Panel C: Coexpression of the ERECAT reporter construct with the HER expression plasmid (0.5 ng of each injected intranuclearly) did not reduce HER expression over that seen with the HER plasmid alone at the same concentration. Molecular weight (MW) standards (values in kDa) identified by Coomassie blue staining are the following: H-chain myosin (214); phosphorylase B (111); ovalbumin (45); α-chymotrypsinogen (24); and β-lactoglobin (18).

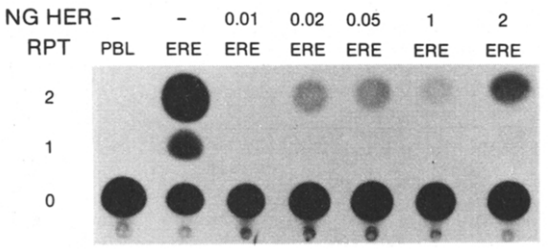

Fig. 2. Thin-layer chromatography autoradiograms of CAT expression in injected oocyte extracts. Reporter plasmids (RPT) alone were injected or reporter plasmids accompanied by HER expression plasmids in the nanogram amounts shown (NG HER). Position 0 represents unacetylated [^{14}C]-chloramphenicol substrate while positions 1 and 2 represent the mono- and di-acetylated forms respectively. The responses shown represent a single batch of oocytes from one frog and therefore show within experiment variability.

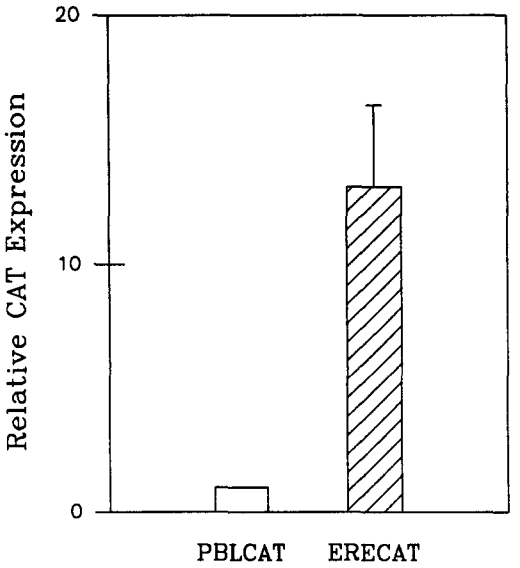

Fig. 3. Expression from CAT reporters in the absence of introduced HER. CAT activity was assessed by autoradiography of the thin layer chromatography plate followed by cutting out of substrate and acetylation products of labeled chloramphenicol for scintillation counting. The data is presented as fold increase in the percentage of total counts recoverable that are acetylated by various extracts. Error bars represent SEM.

RESULTS

SDS–PAGE fluorographs of labeled (newly synthesized) total oocyte proteins (Fig. 1) revealed synthesis of high levels of the human estrogen receptor as a result of injection with plasmid pΔHER91023 as identified by the intense bands of the [^{35}S]methionine containing products at the appropriate molecular weight (66,000 Da), panels A and C). Oocytes injected intranuclearly with 0.01, 0.1, and 1 ng of plasmid pΔHER91023 demonstrated a dose dependent synthesis of HER which could be visualized by fluorography of the radioactive proteins (panel A). As expected, oocytes injected with the parent vector p91023 did not exhibit the 66,000 Da protein. The estrogen receptor was found in the intracellular soluble fraction (supernatant), as shown previously and was not secreted into the surrounding medium (panel B). The injection of lower concentrations of pΔHER91023 did not lead to vitellogenin synthesis and secretion into the oocyte medium (panel B) even when HER synthesis was reduced to levels not visible by fluorography (below 0.1 ng/oocyte nucleus). The position of vitellogenin is known by the expression of this protein in oocytes injected with liver mRNA from an estrogen-stimulated (female) animal. Because the mRNA for vitellogenin is very abundant in such preparations this protein is readily observed as a translation product [1, 21, 22]. The co-expression of an equivalent amount (0.5 ng/oocyte nucleus) of the ERECAT reporter plasmid did not alter the level of expression of HER from its expression vector (panel C).

We first tested the expression levels of our ERE-containing and control reporter constructs in the absence of introduced HER. Unexpectedly, the oocytes injected with the ERECAT construct had far more CAT activity than those injected with the plasmid without the response element for the estrogen receptor. These data are shown in Fig. 2 as an autoradiogram from one such experiment (PBL vs ERE lanes with no HER). The data in Fig. 3 were produced by averaging several such results; measurements of the acetylated forms of [^{14}C]chloramphenicol are expressed as a percentage of total label recovered, when both the product and the unconverted substrate are cut from the thin layer chromatography plates and measured by liquid scintillation spectroscopy. These results suggest the presence of an endogenous oocyte regulator which can act positively upon an ERE. A low level of expression from the construct containing no response element for estrogen (pBLCAT2) was always noted (not detectable by autoradiogram in Fig. 2, but detectable by scintillation counting). Therefore the activity in pBLCAT2-injected oocyte extracts were arbitrarily set to a value of 1 in each experiment and other levels of activity are expressed as a fold increase compared to this value. As oocytes vary seasonally and from batch to batch, it is necessary to normalize the data between experiments in this way.

Because these results suggested that the oocyte contains either an ER or another protein which could substitute for ER in transcriptional

Table 1. Measurements of specific binding in *Xenopus* liver and oocytes. HER plasmid injected oocytes received 0.5 ng of pHER91023/oocyte nucleus. LH-20 single concentration (5 nM [^{3}H]estradiol ± 100-fold excess of unlabeled estradiol) assays were used to determine estrogen receptor levels in oocytes and HER injected oocytes compared to a known receptor-rich tissue, the liver. See text for details of the LH-20 chromatography assay and sites/cell based on cell size. Errors are SEM. Data in other columns are simple mathematical transformations of the data in the dpm/mg tissue column. The number of separate determinations is in parentheses

Tissue	dpm/mg tissue	fmol/g tissue	Sites/cell	Sites/cell based on hepatocyte size
Liver	187 ± 32 (4)	577	2673	2673
Oocyte	24.1 ± 2.6 (4)	74	4.3×10^7	430
HER plasmid injected oocyte	421 ± 115 (2)	1299	7.5×10^8	7500

enhancement, we tried to measure ER in the oocyte. We compared these measurements, using the same reagents, to binding assays performed in frog liver, and HER plasmid injected oocytes (Table 1). Whereas the LH-20 column assay was able to demonstrate the presence of appreciable specific estrogen binding in the frog liver cytosol and HER plasmid injected oocytes, we demonstrated much lower levels of binding in the uninjected *Xenopus* oocyte (8- and 17-fold less, respectively, on a weight of tissue basis).

If such an endogenous oocyte factor binding to the ERE is mediating the expression of this CAT reporter, there should be a finite amount of this factor available in the oocyte. For this reason we delivered different amounts of the ERECAT construct to the oocyte to see if we could detect an upper limit of the response. Figure 4 demonstrates the saturation of this response at a level of 0.5–2 ng of injected plasmid per oocyte nucleus. Data for each point was normalized around a single concentration (1 ng ERE/oocyte nucleus) to account for variability between batches of oocytes.

We next addressed the question of regulatory effects of introduced HER on the ERECAT expression in oocytes that were co-injected with the pΔHER91023 and ERECAT plasmids simultaneously. A range of concentrations of the HER producing plasmid was injected while a constant amount of ERECAT plasmid (0.5 ng/nucleus) was injected; this concentration was chosen for its subsaturating or barely saturating response shown in Fig. 4. Previous results (Fig. 1) indicated that the amount of HER produced could be controlled by altering the amount of plasmid progenitor. Although we expected HER to have an enhancing effect on ERECAT expression, we observed a suppression of ERECAT expression at all concentrations examined (over more than 2 orders of magnitude). Figure 2 shows this result from a single batch of oocytes. Figure 5 is a compendium of 3–13 separate determinations for each concentration with the amount expressed by ERECAT alone in each experiment arbitrarily set to a value of 100% to normalize between batches of oocytes.

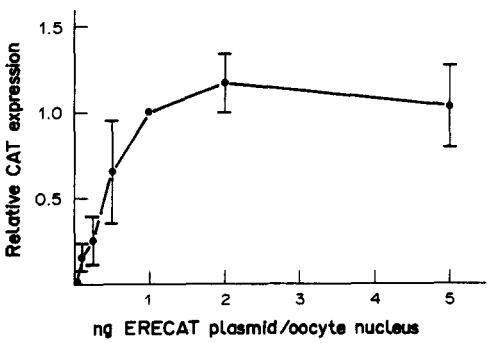

Fig. 4. Saturation of the endogenous oocyte ERE binding factor response. ERECAT plasmid was injected into oocytes in increasing amounts up to 5 ng/oocyte nucleus. CAT expression (% [^{14}C]chloramphenicol acetylated/μg protein) was assessed by scintillation counting as described in text. Data are normalized around the values for 1 ng injected plasmid to adjust for differences between batches of oocytes. These results represent 7 separate experiments (not all concentration points were included in each assay but all were normalized around the 1 ng result). Error bars represent SEM.

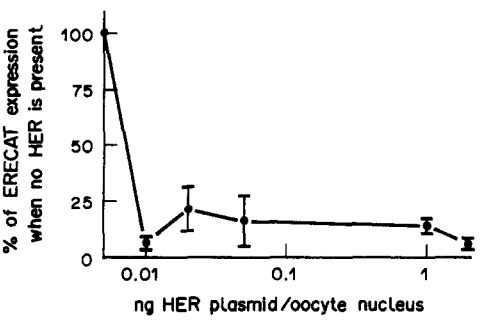

Fig. 5. Effect of HER introduced into the oocyte on ERECAT expression: Oocytes were co-injected with the pΔHER91023 and ERECAT plasmids and processed for CAT activity assays as previously described in text. A range of nanogram (ng) concentrations of the HER producing plasmid was injected while a constant amount of ERECAT plasmid (0.5 ng/nucleus) was injected. All sample values representing no pΔHER91023 injection were normalized to 100% to control for inter-experiment variation. Each concentration represents between 3 and 13 separate determinations; not all concentration points were done for each experiment. Error bars represent SEM.

DISCUSSION

We were surprised to find that oocytes injected with the ERECAT construct alone had far more CAT activity than those injected with the same plasmid minus the estrogen response element. There have been no reports to date of the *Xenopus* oocyte containing any estrogen receptor, and estimates of when ER appears developmentally is in stage 57–62 tadpole liver [26]. Our attempts to measure estrogen receptor in the uninjected oocyte were positive at low levels, but such low concentrations of binding make further characterization difficult. Our previous attempts to measure *Xenopus* estrogen receptor in oocytes [1] (and unpublished observations) were probably unsuccessful due to this low concentration of receptor in oocyte cytosol. Two ammonium sulfate precipitations were used in these experiments; they resulted in concentration of receptor from a large number of oocytes. In addition, the two precipitation steps probably removed other interfering components which might contribute to the high non-specific binding in this tissue as well as the high levels of endogenous free steroid present in these preparations [9, 13]. The oocyte estrogen binding described here is discrepant with our previous negative assessment [1] and the lack of published reports of others, probably because of these modifications to the method of preparation. Specific binding to estrogen in parallel assays of liver cytosol and HER injected oocyte extracts revealed much higher concentrations of receptor. Our estimate of the number of sites per cell in the *Xenopus* liver are in reasonable agreement with the 1000 sites/cell reported by others [24, 27]. When comparing sites per cell to that of the *Xenopus* oocytes one must keep in mind that the oocyte is 100,000 times larger than a *Xenopus* hepatocyte. We base this calculation on information that 1 g of liver contains 1.3×10^8 cells [24] while of 1 g of oocytes contains only 1050 cells. When the cells are compared on an equivalent size basis the oocyte contains 20-fold lower concentration of receptor in our assay than liver cells, and for this reason may not be capable of eliciting expression from endogenous genes. On the other hand, this amount of receptor per endogenous genome is not low. It is presently unclear if receptor concentration as affected by cell volume can explain lack of expression from an endogenous gene. We conclude that either there is a very low but sufficient level of estrogen receptor in the oocyte to enhance expression from ERECAT, or that there is some other non-estrogen binding factor which can substitute for the receptor.

An estimation of the number of molecules of the ERE sequence present in 1 ng of ERECAT plasmid comes to 2.1×10^8. The molecules of *Xenopus* estrogen receptor present in an oocyte by our measurements would equal about 4.4×10^7, not enough to occupy more than 20% of the reporter molecules. It is puzzling to consider that such small occupancy of the target sequence would cause a saturating response and therefore suggests the participation of some other endogenous oocyte protein in eliciting the ERE-linked gene activity. In contrast, the number of HERs measured by binding assay in oocytes injected with 0.5 ng pΔHER91023 is 7.5×10^8, enough to displace the binding of an endogenous factor on injected target sequences. Even when the lowest amount of pΔHER91023 is injected (predicted to yield 1.4×10^7 molecules of receptor), this should effectively displace 1/3 of endogenous ER and occupy ~13% of the injected ERE target sequence. But, it is possible that small amounts of plasmid give rise to more receptor copies/template than do large amounts. Alternatively, the technical difficulties presented by doing estrogen receptor assays in a lipid-rich tissue could cause us to underestimate actual receptor numbers for both introduced HER and endogenous oocyte ER.

The effect of this putative endogenous oocyte factor on expression enhancement of ERECAT appears to be saturable and not diminished by excess target sequence indicating that there is a finite amount of this protein that regulates the expression of the ERE and that it is probably a single limiting factor binding at a single element. If this response were dependent on more than one limiting factor binding independently one would have expected a diminished response beyond the saturation point due to dilution of the factors onto separate plasmid molecules. Although these data do not prove this conclusion, they do demonstrate that multiple limiting factors are not involved in regulation via the ERE sequence. It is also probable that a 17 base pair sequence could not accommodate multiple protein factors bound directly to the DNA. It has been suggested that other transcription factors may stabilize transcription initiation complexes and that various ones binding on adjacent sequences may substitute for each other in this function [28, 29]. Interaction of estrogen receptor (or whatever protein is binding to the ERE

in the oocyte) with other transcription factors of the herpes simplex thymidine kinase promotor is also likely [30, 31].

We explored the dose dependency of HER synthesis in the oocytes because we considered the possibility that maximal expression of HER would commandeer the entire synthetic apparatus of the oocyte and thus sequester needed cellular machinery and raw materials away from synthesis of any other product. The engineering of the p91023B expression vector ensures that the recombinant sequence included will be transcribed and translated with high efficiency [14]. Our success in synthesizing HER is shown by the relative composition of newly synthesized proteins of the oocyte; HER is clearly the most abundant protein produced when 1 ng of pΔHER91023 is injected. Despite lowering the apparent abundance of HER product from the oocyte by injecting less plasmid, we failed to see any synthesis of vitellogenin from endogenous oocyte genes. In addition, the coinjection of moderately high levels of the ERECAT reporter plasmid also did not vitiate the production of recombinant HER, again indicating no inadequacy of the RNA or protein synthetic machinery to accomplish synthesis of both of these proteins from their recombinant templates at these concentrations.

The human estrogen receptor may differ from the frog oocyte receptor in that it is designed to interact with different corroborating transcription factors causing it to abortively sit on the ERE of our construct in the oocyte and therefore suppress transcription from a coinjected ERECAT. The nearly complete inhibition at all concentrations suggests the action of a highly effective regulatory protein and not a general inhibition of other protein synthesis by using up the cellular protein and RNA synthetic machinery, especially in light of this and other demonstrations of a tremendous capacity of the oocyte for macromolecular synthesis. We interpret these findings to mean that human ER can occupy the ERE, but it does not have the appropriate transcription factors available to it in the oocyte to enhance transcription either of our ERECAT construct or the endogenous vitellogenin gene. The unavailability of participating transcription factors also could be due to the unavailability of binding sites for these factors. Theulaz *et al.* have demonstrated the ability of constructs containing a large portion of the vitellogenin B2 gene 5′ flanking regulatory region connected to a CAT reporter construct to activate CAT expression [10]. Where such a large ERE containing region is involved, it is possible that other regulatory elements binding other factors may substitute for or assist the HER in being an adequate positive regulator of the attached sequences [28–31]. Our 17 base pair element probably does not contain any site other than the *Xenopus* estrogen receptor binding site element. Other differences between the studies of Theulaz *et al.* and our work are also evident, such as the use of a *Herpes simplex* virus thymidine kinase (HSV-TK) promotor in our CAT reporter construct. However, this discrepancy is unlikely to account for the observed differences because the authors of the other report state that they also observed a positive response with a construct containing the HSV-TK promotor (although this data was not shown). Comparison of the amounts of HER produced in oocytes by Theulaz *et al.* to our wide range of synthesized HER concentrations suggests that this is also not an explanation for the discrepancy in our reported results. The amount of HER produced in oocytes in our study is likely to have bracketed the single amount produced in the Theulaz studies (by comparison of HER fluorographs).

The nature of the endogenous regulator of ERECAT expression remains to be identified. It may or may not be a scarcely represented estrogen receptor, or bind to estrogen or other steroid hormones. The status of its interaction with endogenous dormant oocyte genes which are potentially estrogen responsive are also of interest. More sensitive assays to measure steroid receptors and their interactions with DNA are being developed in our laboratory to address this question.

Acknowledgements—All expression and reporter plasmids and some helpful experimental suggestions were kindly provided by Dr Ming Jer Tsai of Baylor College of Medicine, Houston, TX. I also thank Dr Mary Thomas and Mr Todd Pappas of this institution for critical reading of my manuscript in preparation for submission. The excellent technical assistance of Ms Teri Torres is gratefully acknowledged. This work was supported by NIH grant HD22746 and by the Pfeiffer Foundation.

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