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Estrogen-Dependent in Vitro Transcription from the Vitellogenin Promoter in Liver Nuclear Extracts

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One approach to analyzing the molecular mechanisms of gene expression in vivo is to reconstitute these events in cell-free systems in vitro. Although there is some evidence for tissue-specific transcription in vitro, transcriptionally active extracts that mimic a steroid hormone–dependent enhancement of transcription have not been described. In the study reported here, nuclear extracts of liver from the frog *Xenopus laevis* were capable of estrogen-dependent induction of a homologous vitellogenin promoter that contained the estrogen-responsive element.

IN FEMALE FROGS OF THE SPECIES *Xenopus laevis*, the vitellogenin genes that encode the precursor of the yolk proteins are activated in the liver by estrogen, whereas in the males the vitellogenin genes are normally silent (*1*). However, hormone treatment of males leads to both an increase in estrogen receptor and a strong de novo activation of vitellogenin transcription (*2*). This activation can be mimicked in a variety of cell lines and in *Xenopus* oocytes, provided that both the estrogen receptor and the *Xenopus* target sequences, the estrogen-responsive element (ERE), are present (*3, 4*). Since these earlier studies did not address the regulation of the genes in their natural context, we prepared extracts of nuclei isolated from *Xenopus* liver and assayed their ability in vitro to sustain hormone-dependent transcription from the vitellogenin B1 cloned promoter (*5*).

We first tested a nuclear extract (*6*) prepared from adult female *Xenopus* livers, which express the vitellogenin genes and thus are most likely to yield a transcriptionally active extract. The template consisted of the vitellogenin B1 gene 5′ flanking sequences from −596 to +8 fused to the chloramphenicol acetyltransferase (CAT) reporter gene. This construct is inducible by estrogen after transfer into a hormone-responsive human cell line (*3*). We assayed its transcription by primer extension. The amount of extract and the amount of DNA in the transcription assays were optimized in the presence of 17β-estradiol (Fig. 1). The vitellogenin-specific extension product appeared at a range of concentrations but the optimum was at 1.4 mg of protein per milliliter with three extracts tested (Fig. 1A). Furthermore, optimal transcription was at 1000 ng of specific template (Fig. 1B). Thus the ratio of protein to the DNA template in the reaction must be carefully maintained to obtain efficient transcription, a requirement reported previously for other in vitro transcription systems (*6, 7*). Vitellogenin transcription was inhibited by α-amanitin (0.5 μg/ml) and was thus due to RNA polymerase II.

The crucial question was whether the observed transcription from the vitellogenin promoter was dependent on the estradiol added to the assay. We therefore tested the transcription of the same template as above in the absence or presence of hormone. We also analyzed a second template in which the ERE had been deleted (*3*) (Fig. 2B). As an estrogen-independent internal control, we included a chimeric gene in which the SV40 early promoter was linked to the CAT gene (*8*) (Fig. 2B). Very little transcription of either vitellogenin template occurred in the

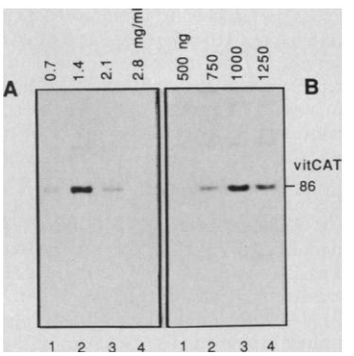

Fig. 1. Optimization of transcription conditions. (**A**) Effect of nuclear extract concentrations on transcription in vitro. Transcriptions were performed in a total reaction volume of 20 μl containing increasing amounts of the extract. Numbers at the top of the figure indicate the final protein concentration in each reaction. The amount of specific DNA template was 750 ng per reaction. The template was the pB1(5′/−596+8)CAT8+ construct (Fig. 2B). The size (86 nucleotides) of the correctly initiated transcripts, measured by primer extension, is indicated. (**B**) Effect of specific template concentrations on transcription in vitro. Assays were performed with increasing amounts of pB1(5′/−596+8) CAT8+ DNA brought to a constant total amount of 1250 ng per reaction with plasmid DNA. The nuclear extract concentration gave a final protein concentration of 1.4 mg/ml in each reaction. The numbers above the lanes indicate the amount of specific template. Transcription was assayed by primer extension. *Xenopus laevis* adults were induced by two injections of 1 mg of 17β-estradiol into the dorsal lymph sac with an interval of 3 days between the two injections, or they were left unstimulated. They were killed 1 day after the final injection. Extracts of liver nuclei were prepared essentially as described (*6*), except that the livers were perfused with 75% phosphate-buffered saline containing 0.5 mg of heparin per milliliter, and the homogenization buffer contained 2.4*M* sucrose and 15 μg of aprotinin per milliliter. Possible residual estradiol in the extracts was eliminated during the dialysis in the final step of the preparation. For in vitro transcription the standard reaction consisted of the following components (final volume, 20 μl): 33 m*M* Hepes (*p*H 7.9), 60 m*M* KCl, 6 m*M* $MgCl_2$, 5 m*M* creatine phosphate, 0.6 m*M* nucleoside triphosphates, 10% glycerol, 0.6 m*M* dithiothreitol, 0.06 m*M* EDTA, 1 μl RNAsin (Promega), and 2.5 n*M* 17β-estradiol when not indicated otherwise. After 45 minutes at 30°C, the reaction was terminated by the addition of 180 μl of STOP-mix [1% SDS, 200 m*M* NaCl, 25 m*M* tris-HCl (*p*H 7.5), 5 m*M* EDTA, and transfer RNA (20 μg/ml)], and nucleic acids were purified by extraction in phenol-chloroform and recovered by precipitation with ethanol. Primer extension analysis was performed as described (*12*), with 80,000 cpm of ^{32}P end-labeled CAT primer per reaction. Half of the reaction product was denatured and analyzed on a 6% polyacrylamide 7.5*M* urea sequencing gel.

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absence of hormone, although the SV40 promoter was active (Fig. 2A, panel 1, lanes 1 and 2). In the presence of estradiol, the ERE-containing template was induced in contrast to the template without ERE (as seen in Fig. 2A, panel 1, lanes 3 and 4), whereas the transcription of the control SV40 template was not affected. A similar induction is revealed if one compares the transcription of the −301 template with that of the −596 construct in the presence of the hormone. Thus, in these extracts, estradiol is necessary and sufficient to activate the ERE-containing vitellogenin promoter. In contrast, we observed that in an extract from HeLa cells, both templates were constitutively expressed, showing that the −301 construct was functional and that our observations reflect a specific effect due to the *Xenopus* liver extracts.

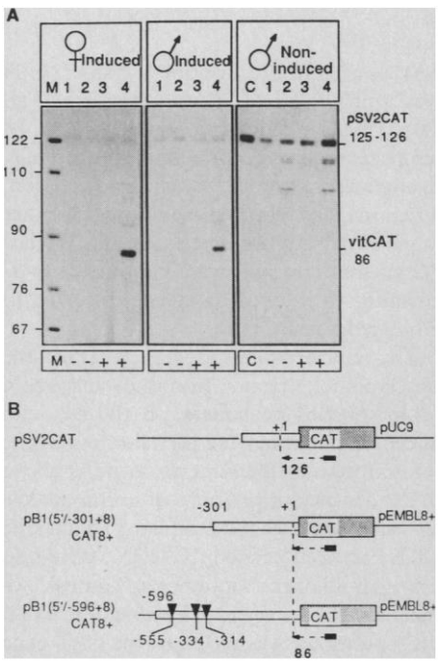

Fig. 2. Transcription in vitro in various *Xenopus* liver nuclear extracts. (**A**) Two vitCAT templates were tested in extracts prepared from estrogen-treated livers of females (panel 1), estrogen-induced livers from males (panel 2), or livers from males not exposed to hormone (panel 3). In each panel, lanes 1 and 2 represent transcription in the absence of hormone (−), and lanes 3 and 4 represent reactions performed in the presence of 2.5 n*M* 17β-estradiol (+). Lanes 1 and 3 contained the transcription product of the template pB1(5′/−301+8)CAT8+, while lanes 2 and 4 contained those of pB1(5′/−596+8)CAT8+. Each reaction contained 1000 ng of the specific vitCAT template and 250 ng of the control template pSV2CAT; the protein concentration was 1.4 mg/ml. In lane C, the vitCAT template was omitted (M, molecular weight standards). Analysis of the transcripts was performed by primer extension. The positions of the transcripts are indicated. The autoradiogram corresponding to experiments with uninduced extracts from males was deliberately overexposed in order to ensure that no specific signal appeared at the position of the extended vitCAT transcript. (**B**) Schematic diagrams of the templates used for transcription. Plasmid constructions have been described (*3*); pB1(5′/−596+8)CAT8+ contains the ERE identified in MCF-7 cells (*3*), and the symbol ▼ indicates the position of the 13-bp elements involved in the response to estrogen, while 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 box; the expected elongation product due to correct initiation is shown by a dotted line, and its size is indicated.

We also tested the transcription capacity of nuclear extracts prepared from livers of *Xenopus* males never exposed to hormone and males injected with estradiol, and compared these extracts to the extracts from livers of females. As one would expect, the extracts from males treated with estradiol behaved similarly to the extracts from females (Fig. 2A, panel 2). Only the transcription of the ERE-containing plasmid was activated by the hormone, whereas the SV40 promoter was equally active in all four lanes. In contrast, no transcription occurred under any conditions from vitellogenin sequences in extracts from uninduced males (Fig. 2A, panel 3), yet the SV40 promoter was active.

In extracts from induced females and males we consistently observed a 20-fold increase in transcription with addition of hormone. If this induction was related to the level of nuclear estrogen receptor, we should have detected a weak signal with extracts from uninduced males since for these animals the liver contains 1/10 as much receptor as that of females (*2*). Furthermore, vitellogenin messenger RNA synthesis can be induced, albeit weakly, in cultured male hepatocytes even when protein synthesis has been inhibited (*9*). Nevertheless, we detected no transcription in extracts from uninduced males, suggesting that the estrogen receptor concentration was not high enough. Alternatively, the extract from males may have contained factors repressing the transcription from the vitellogenin promoter.

If the hormonal induction of vitellogenin transcription detected in our system was mediated by the high-affinity liver estrogen receptor [dissociation constant, 0.5 n*M*; (*10*)], then activation should have been observed at physiological concentrations of estradiol, that is, in the nanomolar range. Therefore, we tested dilutions of the hormone in the extract from females (Fig. 3). Induction was detectable when the hormone concentration was 2.5 n*M*, and increasing the concentration did not significantly affect the signal. In contrast, no vitellogenin transcription above the background level occurred when the hormone concentration was lowered to 0.25 n*M*, although the SV40 internal control remained active. Thus these results are consistent with the involvement of the estrogen receptor in mediating the hormonal response. Since no runoff transcripts were obtained with linearized DNA, hormonal regulation in our assay might require a supercoiled template.

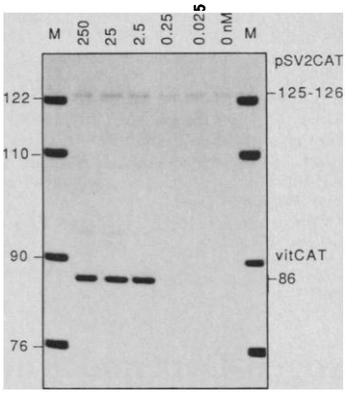

Fig. 3. Hormone concentration–dependent transcription in the nuclear extract of the female liver. Reactions were performed as described in Fig. 2, except that the 17β-estradiol concentration was varied as shown.

From our experiments, we conclude, on the basis of the differential activation of two templates either containing or lacking the ERE, that the liver extracts in vitro mimicked the hormonally modulated expression of the vitellogenin genes in vivo. Thus, the ERE previously identified in a heterologous system is similarly required for hormonal induction in the homologous liver nuclear extracts. Our results also show that the estrogen-inducible enhancement effect occurs at hormone concentrations permitting more than half-saturation of the receptor, strongly suggesting its implication in the induction process. We have visualized hormone-dependent complex formation on this sequence in the electron microscope (*11*). The use of these nuclear extracts whose preparation procedure (*6*) was essential to obtain hormone-responsiveness should offer a means for the biochemical characterization of positive and negative trans-acting factors involved in the tissue-specific and estrogen regulation of the liver genes.

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Germline Transformation Used to Define Key Features of Heat-Shock Response Elements

HUA XIAO AND JOHN T. LIS

The heat-shock consensus element (HSE), CTNGAANNTTCNAG, is found in multiple copies upstream of all heat-shock genes. Here, the sequence requirements for heat-shock induction are tested by *Drosophila* germline transformation with an *hsp70–lacZ* gene fused to a pair of synthetic HSEs. Certain single-base substitutions in either HSE cause a dramatic reduction (fortyfold) in expression. Surprisingly, variations in sequences immediately flanking the HSEs also reduced levels of induction. One such variant that contains two perfect 14–base pair HSEs, which are correctly spaced relative to each other and the TATA box, retained only 7% of wild type–induced expression. These and additional analyses indicate that the heat-shock regulatory element includes sequences beyond the 14–base pair HSE and may be better described as a dimer of a 10–base pair sequence, NTTCNNGAAN.

THE REGULATORY REGIONS OF highly expressed eukaryotic genes usually have multiple short sequence elements that function as binding sites for one or more regulatory proteins (*1*). A typical example is the heat-shock consensus element (HSE), CTNGAANNTTCNAG, which is found in multiple copies upstream of the transcription start of heat-shock genes from a wide variety of organisms (*2*). Sites containing this 14-bp sequence have been shown to bind purified protein, heat-shock factor (*3–5*). A *Drosophila hsp70* gene with an upstream regulatory region that contains two HSEs is expressed at high levels in response to heat shock when introduced into the *Drosophila* genome, whereas the same gene with one HSE is expressed at 1/100 the level (*6, 7*). This led to the hypothesis that two copies of the HSE are absolutely required to achieve high levels of induced expression.

Our study uses germline transformation of *Drosophila melanogaster* (*8*) to examine the specific sequence requirements for heat-shock inducibility of the *hsp70* gene. In germline transformants, an *hsp70–lacZ* fusion gene with regulatory sequences to position −89 shows the same range of inducibility (about 200-fold induction) as the endogenous *hsp70* genes; however, this fusion gene and other related *hsp70* fusion genes show relatively high basal level expression or low induced levels (3- to 20-fold induction) in transient expression assays in transfected cell cultures (*9*). The full range of inducibility in germline transformants provides a more sensitive measure of the effects of mutations on the expression of the *hsp70* gene. *Hsp70–lacZ* hybrid genes with variant regulatory regions were generated, and stable transformants containing single copies of these genes were used to assay heat-induced expression of the transformed gene (*9*). The effect of line-to-line variation was minimized by obtaining an average value of 3 to 8 independent transformant lines for each variant (a total of 94 independent lines that have a single inserted *hsp70–lacZ* gene were examined in this study).

Two synthetic HSEs, which are perfect matches to the 14-bp HSE (*10*) conferred 27% of wild-type induction to an *hsp70–*

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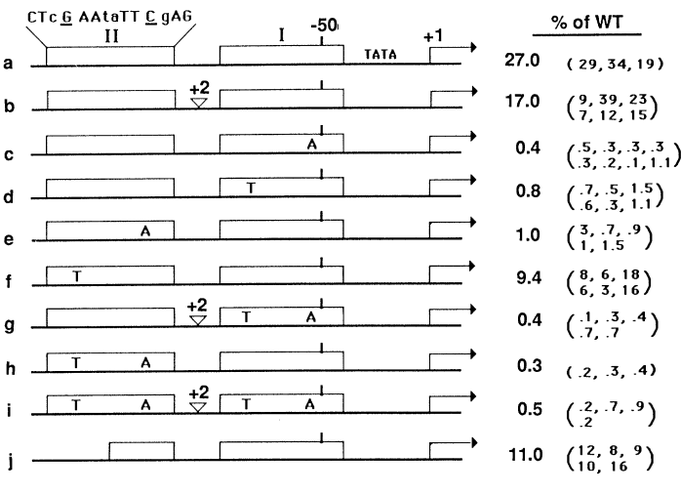

Fig. 1. Heat-shock–induced expression of *hsp70* genes containing mutations in conserved nucleotides of the 14-bp HSE. Diagrams of *hsp70–lacZ* fusion genes shown in (**a** to **j**) represent the synthetic regulatory regions (*13, 14*) that were substituted for the native *hsp70* regulatory region by fusion to position −50 (*15–17*). The transcription start of the genes is designated +1. The TATA homology between site I and the transcription start is shown in the diagrams. The two HSEs of sites I and II are shown as boxes, and the sequence of the synthetic 14-bp HSE is written above site II (see Fig. 3d for the complete sequence of the synthetic regulatory region of this construct). The underlined G and C residues were replaced by the noncomplementary transversions to T and A in the constructs indicated. The cloning to generate the constructs in this figure and in Figs. 3 and 4 was performed by standard methods (*18*). The fusion genes were subcloned into a P-element transformation vector c70T1, a Carnegie 20 derivative that contains the transcription termination sequence from *hsp70* (*9*). Germline transformants with single inserts were backcrossed to the parental strain, Adh^{fn6}, *cn*; ry^{502}. Heat-shock induction and measurements of β-galactosidase activity from adult female progeny by means of the substrate chlorophenol red–β-D-galactopyranoside were performed as described previously (*9*). Each transformant line was assayed in duplicate and the values are reported here and in experiments of Figs. 3 and 4 as the percentage of wild-type activity (% of WT), where wild type is an identical fusion gene containing native *hsp70* regulatory sequences to position −89. In large type are the mean values of all measurements made on three to eight independent transformant lines (shown in parentheses). Since constructs g and i contain an extra 2 bp between the two mutant HSEs, construct b with this same 2-bp insert between two perfect synthetic HSEs is included for comparison.