Supporting Material

A dynamic model of transcriptional imprinting derived from the vitellogenesis memory effect

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Contents:

Detailed materials and methods

Trout hepatocyte culture
Male rainbow trouts (*Oncorhynchus mykiss*) came from the INRA trout farm of Gournay-sur-Aronde (Oise), France and kept in recycled water in the INRA Poissons, Rennes. After trout stunning and cannulation of the vena porta, hepatocytes were dissociated by collagenase perfusion (260 mg/l in phosphate buffer). The liver was then removed and the cells were dispersed in a Petri dish containing culture medium (Dulbecco's modified Eagle's medium nutrient mixture/F12 (DMEM/F12, 1:l mixture, with L-glutamine and 15 mM HEPES, without phenol red), supplemented with 15 mM TES, 12 mM $NaHCO_3$. 1% (v/v) antibiotic mixture (Penicillin; Streptomycin; Amphotericin B) (Sigma) and with 2% (v/v) serum substitute (Ultroser, SF). Cells in suspension were plated in untreated plastic Petri dishes (Falcon). Aggregates were obtained by constant gyratory shaking at 70 rpm at 18°C. After 3 days, when aggregates had started to form, the culture medium was changed every 2 days. All estrogen treatments, except for Fig. 1, were carried out after 8 days of culture, when the aggregation process is over and gene expression is stable (1). For the in vivo experiment (Fig. 1) one male trout was injected with estradiol and one male was injected with vehicle only, one month before hepatocyte extraction.

Quantification of ER and Vg mRNAs
Total RNA samples were quantified, adjusted to 5 µg by adding yeast tRNA, denatured and spotted onto transfer membrane. After UV-crosslinking, RNA dots were hybridized to radioactive single-stranded ER or Vg cDNA (10^9 dpm/µg). mRNA cell contents were then quantified as pg per µg of total RNA. These values were converted into molecule number per cell, considering that the $rtER_S$ and Vg mRNAs are 3.5 kb-long and 7.2 kb-long respectively, and that a trout hepatocyte contains on average 4.3 pg total RNA, as determined by tissue cell dissociation and counting. For $rtER_S$ mRNA, one obtains 2.5 molecules per cell in hormone-naïve male trouts hepatocytes and 50-65 molecules after estrogen administration.

Quantification of ER protein
The ER protein number was estimated indirectly by counting the high affinity binding sites for estradiol in the cell. Nuclear extracts were prepared at 4°C by Potter homogenization of 1 g liver in 2.5 ml of Tris-HCl 20 mM pH 7.9, $CaCl_2$ 5 mM, $MgCl_2$ 3 mM, sucrose 330 mM, monothioglycerol 10 mM, PMSF 1 mM. After centrigugation of the homogenate 15 min 1000xg, the supernatant was recentrifuged at 90000xg for 30 min and used for hormone binding assays. 3 nM [^{3}H]-17-β-estradiol (Amersham) was incubated with 200 µl nuclear extracts with or without cold hormone for 12 hours at 2°C. Free and bound hormone were then separated by two successive treatments with 1% charcoal - 0.1% Dextran for 5 min at 0°C and centrifuged at 10000xg for 5 min. Radioactivity was measured by liquid scintillation in 4 ml Picofluor 30. Specific binding was determined by subtracting the result obtained with a 100 fold excess of cold hormone. 1 fmole of nuclear ER per gram of trout liver ($200x10^6$ cells), corresponds to 3 molecules per cell, yielding approximately 130 molecules per nucleus for untreated animals and 1250 molecules per nucleus after estrogen treatment.

mRNA degradation
RNAs were extracted from cell aliquots at different time periods after transcription block using actinomycin D, and ER and Vg mRNAs were quantified as described above.

Nuclear run-on analysis
Cultured hepatocytes were harvested, washed and fractionated at 0°C. Transcription completion was then allowed to proceed for 30 min at 26°C after resuspension of nuclei at 10^6 nuclei/10 ml transcription buffer containing [α^{32}P]-UTP. Radioactive RNAs were purified by phenol-chroroform extraction, ethanol precipitation, redissolved in SDS 0.25% and then used as probes and hybridized to linear cDNA spotted on nylon membranes.

Clearance of estradiol-17β in rainbow trout
After injection of 1 mg 17β-estradiol/kg body weight in a male trout, circulating estradiol was measured by radio-immunoassay as described in (2).

β-galactosidase assays in budding yeast
Yeast cells (BJ2168 strain) were stably transformed with 2 µg of rtERs or hER expression plasmids and the β-galactosidase reporter plasmid driven by the estrogen-responsive element of the rtER gene, using the lithium acetate method and selected on minimal medium (0.13% dropout powder without uracil and tryptophan, 0.67% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 1% dextrose). β-galactosidase activity was determined by the transformation of the *0*-nitrophenyl *β*-D-galactopyranoside substrate into a coloured product quantified by spectrophotometry at 420 nm.

Transient expression assays in mammalian cells
After plasmid transfection and transient expression, cells were harvested and the firefly luciferase (F-luc) activity was determined using the luciferase assay system kit and normalized with β-galactosidase activity, expressed from a β-actin-promoter. In the experiment of comparison of the transcriptional strengths of the fish ER gene promoter and the cytomegalovirus (CMV) promoter, these promoters directed the expression of F-luc and were measured by comparison with a thymidine kinase promoter-Renilla luciferase control expression plasmid, using the Dual-Glo Luciferase Assay System (Promega).

Parameter values

TABLE S1. Parameter values used for numerical application. These values were either: I) experimentally approximated, ii) calculated from the measured values, or iii) refer to published data when indicated. Data for hER were used when not available for rtER (DNA-binding and dimerization constants). Parameters without specified units, are unitless. For converting ER dissociation constants (corresponding to hormone binding, dimerization and DNA-binding constants) into molecule copy number per cell, it is assumed that ER is almost exclusively nuclear in fish cells, based on immunocytochemistry experiments. The nuclei of trout hepatocytes resemble to slightly flattened balloons of about 6 µm in their larger dimension, and their volume was approximated to 0.1 picoliter. With this volume, K_{dim} of 0.3 nM corresponds to 18 molecules/cell, and K_{ddim} of 0.4 nM to 24 dimers/cell. The fraction of phosphorylated ER was estimated around 10% based on the relative intensity of the bands corresponding to phosphorylated and non-phosphorylated ER in SDS-polyacrylamide gel electrophoresis. Upon hormone ligation, the dimerization strenght of ER (3) and its DNA-binding efficacy, are multiplied by 3. The maximal transcription rate of Vg (V_V) was calculated as the product of the measured degradation rate and the maximal steady state Vg mRNA cell content $V_V = 0.2\ [mV] = 268$ molecules/day (i.e. 1 productive transcription every 5.3 minutes in our cell culture conditions). Based on comparative run-on analysis (Fig. 4*D*), the maximal transcription rate in presence of hormone (V_{RH}) was estimated about the quarter of V_V, leading to $V_{RH} = 67$ molecules/day. Considering the relative ligand-dependent and ligand-independent activities of $rtER_S$ observed in the LacZ expression assay (Fig. 2*A*), we approximated that phosphorylation-dependent and hormone-dependent maximal transcription initiation rates of DNA-bound ER are similar ($V_R = V_{RH} = V_{RP} = 67$ molecules/day). The Michaelis-Menten formalism for describing Vg expression was replaced by a Hill function $x^n/(K^n+x^n)$, with a Hill coefficient of 4 and $K^n = 10^6$. For validation of the transcription rates, the relative transcriptional activity of the rtER gene promoter was compared to that of the cytomegalovirus promoter (CMV) used as a reference (Fig. 4*E* of the article). Using these values, if Eq. 2 is reduced to a single variable $[m_R]$, the coefficients associated to $[m_R]^2$ are, in the extreme conditions without hormone ($P_H = 0$) or with saturating hormone ($P_H = 1$), 0.01 and 0.1 $(molecules/cell)^{-2}$ respectively.

Parameter	Symbol	Value	
Equilibrium constant of ER dissociation from DNA	K_{dDNA}	0.4 nM	(4)
ER dimer dissociation constant	K_{ddim}	0.3 nM	(5)
ER-hormone binding constant	K_{dH}	1.3 nM	(6)
Rate of removal of ER mRNA in absence of hormone	r_{mR0}	3.3 /day	
Rate of removal of ER mRNA in presence of saturating of hormone	r_{mRH}	1.1 /day	
Rate of removal of Vg mRNA in absence of hormone	r_{mV0}	1.5 /day	

Rate of removal of Vg mRNA in presence of saturating of hormone	r_{mVH}	0.2 /day
In vivo clearance rate of 17-β-estradiol	c	0.084 /day
Maximal transcription rate of Vg gene in presence of saturating of hormone	V_V	268 molecules/day
Maximal transcription rate of ER gene in presence as well as absence of saturating of hormone	$V_{RP} = V_{RH}$	67 molecules/day
RNA content of trout hepatocytes		4.6 pg/cell
Average ER mRNA copy number per cell in absence of hormone	$[m_R]_0$	2.5 molecules/cell
Average ER mRNA copy number per cell after 2 day-hormone treatment	$[m_R]_{H\,max}$	60 molecules/cell
Average ER copy number per cell in presence of hormone	$[R_T]_{H\,max}$	1250 molecules/cell
Average ER protein over mRNA ratio, in presence of hormone	a	21
Average Vg mRNA copy number per cell in absence of hormone	$[m_V]_0$	0 molecule/cell
Average Vg mRNA copy number per cell after 2 day-hormone treatment	$[m_V]_{H\,max}$	1340 molecules/cell
Fraction of phoshorylated ER	P_p	0.1
Hill cooperativity index of ER action on Vg gene promoter	n_H	4
Rainbow trout hepatocyte nucleus volume		0.1 picoliter
Number of hepatocytes per gram of trout liver		$200x10^6$

Bifurcation diagram

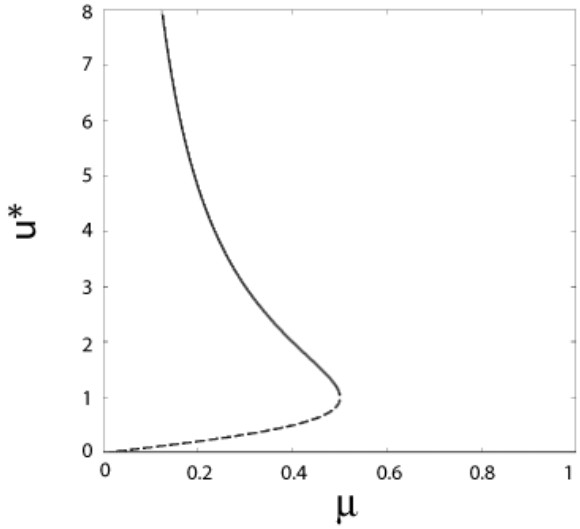

FIGURE S1. Bifurcation diagram of the ER autoregulatory loop in absence of hormone, where the stable and unstable stationary states are represented with dashed and plain lines respectively.

References

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