EGF or EGF-like materials may be the source of EGF-CRM in the brain, but whether the blood-brain barrier is permeable to EGF has not been determined. The presence of EGF-CRM only in pallidal structures suggests a neurotransmitter-neuromodulator role for this gut-brain peptide in extrapyramidal motor function. The pallidum receives convergent information from the cortical mantle via the striatum and has divergent outputs to brainstem and to subthalamic, thalamic, and hypothalamic motor- and endocrine-related target areas (*13*). EGF or an EGF-like molecule could then influence extrapyramidal motor function at key nodal points in the pallidum. Further morphological and functional studies are needed to clarify the role of EGF in the central nervous system.

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8. Two of the primary rabbit antisera to EGF were provided by F. Fox. Secondary fluorescein isothiocyanate–coupled IgG directed against rabbit antiserum was purchased from Miles or Antibodies, Incorporated, and was diluted to 1:40 in phosphate-buffered saline with 0.2 percent Triton X-100. Incubation in secondary antiserum was done at room temperature for 1 hour.
9. One percent colchicine (0.5 to 2.0 μl) in saline was injected into the lateral ventricle or other brain structure with a 1- or 5-μl syringe (Unimetrics). The colchicine prevents axoplasmic flow of material (in this case EGF) from the neuron cell body through its axon to the terminals. Neuronal substances such as polypeptides are still produced in the cell body but are not transported. The substance is, therefore, more concentrated in the cell body and is easier to visualize with the immunohistochemical techniques.
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Saccharomyces cerevisiae Produces a Yeast Substance That Exhibits Estrogenic Activity in Mammalian Systems

Abstract. *Partially purified lipid extracts of* Saccharomyces cerevisiae *contain a substance that displaces tritiated estradiol from rat uterine cytosol estrogen receptors. The yeast product induces estrogenic bioresponses in mammalian systems as measured by induction of progesterone receptors in cultured MCF-7 human breast cancer cells and by a uterotrophic response and progesterone receptor induction after administration to ovariectomized mice. The findings raise the possibility that bakers' yeast may be a source of environmental estrogens.*

Saccharomyces cerevisiae contains an estrogen-binding protein (*1, 2*) and produces a lipid-extractable substance capable of displacing [^{3}H]estradiol from the yeast estrogen-binding protein (*1*). We have hypothesized that this substance represents the endogenous ligand for a yeast hormone receptor system. The yeast ligand also displaces [^{3}H]estradiol from rat uterine estrogen receptors (*1*). These findings suggested to us that yeast ligand might exhibit estrogenic activity in higher organisms, including mammals. To evaluate this hypothesis, we used partially purified preparations of ligand and examined them for estrogenic functional bioactivity. We report that yeast ligand exhibits estrogenic activity as measured by induction of progesterone receptors in cultured MCF-7 human breast cancer cells and by a uterotrophic response and uterine progesterone receptor induction after in vivo administration to ovariectomized mice. Estrogen receptor binding activity and reversible inhibition of bioactivity by the antiestrogen tamoxifen both support the hypothesis that yeast ligand acts through the estrogen receptor pathway in the manner of estradiol itself. The determination that *S. cerevisiae* ligand is estrogenic leads us to speculate that bakers' yeast may be a source of environmental estrogens.

Our methods to obtain partially purified yeast ligand from organic solvent extracts of *S. cerevisiae* by a four-step

Table 1. Scheme for partial purification of yeast ligand. Shown are the results of a typical purification experiment in which the decreasing mass required to achieve half-maximal binding or functional responses indicates increasing potency of the preparation. Yeast ligand was partially purified by extracting ~ 500 g of *S. cerevisiae* dried cells (Sigma) with ethanol. After concentration the residue was solubilized in ethanol, washed with water, and reextracted with ethyl acetate. The extract was passed over a 6-cm (outer diameter) column packed with 400 g of silica gel (Kieselgel 60, Merck) and eluted with a stepwise gradient of methylene chloride, ethyl acetate, and methanol (95:5:0, 75:25:0, 50:50:0, 50:45:5, and 50:40:10) (step 1). The active fractions were rechromatographed over a smaller (3.4-cm) silica gel column (160 g) eluted in a similar fashion (step 2). Active fractions were then dried under vacuum and resolubilized in acetonitrile and water (70:30) and rechromatographed on a reversed-phase HPLC semipreparative column (Whatman Partisil-10 C-8, 250 by 9.4 mm) eluted with acetonitrile and water 70:30 (step 3). Step 4 was a series of purifications on an analytic scale HPLC column (Alltech C8, 10 μm, 250 by 4.6 mm) eluted with acetonitrile and water (40:60).

Step	Procedure	Active fraction	
		Binding activity* (μg)	Functional activity† (ng)
1	Silica column	100	
2	Silica column	20	500
3	Semipreparative HPLC	2	50
4	Analytical HPLC	< 1	10

*Binding activity represents the amount of semipurified yeast ligand that displaced ~ 50 percent of [^{3}H]estradiol from MCF-7 cells. †Functional activity represents the amount of semipurified yeast ligand that induced ~ 50 percent of the maximum estradiol-stimulated increment in [^{3}H]R5020 binding sites in MCF-7 cells.

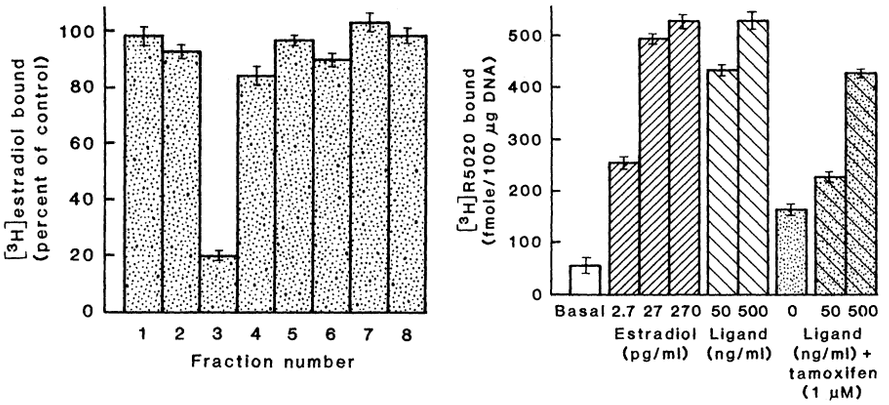

Fig. 1 (left). Potency of [^{3}H]estradiol displacement by HPLC fractions of semipurified *S. cerevisiae* ligand. MCF-7 cells were grown in Eagle's modified minimal essential medium with Earle's salts supplemented with 10 percent calf serum, porcine insulin (6 ng/ml), nonessential amino acids, and 10 m*M* Hepes. Monolayers were trypsinized, washed by centrifugation, and suspended in medium (without serum) at 1×10^6 to 2×10^6 cells per milliliter. The cells were then incubated for 60 minutes at 37°C with 2.6 n*M* [^{3}H]estradiol (47 Ci/mmole; Amersham) and with or without portions of step 3 yeast ligand (10 μg) dissolved in dimethyl sulfoxide. Replicate samples contained various concentrations (2.6 to 26 n*M*) of estradiol (Steraloids) for comparison of binding potency. Corrections were made for nonspecific binding by subtracting binding resistant to the addition of 250-fold molar ratio of unlabeled estradiol (650 n*M*). Cells were rinsed three times with phosphate-buffered saline and the cell pellet was extracted with ethanol. The extracted radioactivity minus the nonspecific binding was taken as specific [^{3}H]estradiol binding. The data are expressed as percentages of control binding so that decreased binding indicates displacement potency. Values are means and ranges for a typical experiment. Fig. 2 (right). Progesterone receptor induction by ligand in MCF-7 cells. Cells were grown as described in the legend to Fig. 1 and processed as described by Horowitz and McGuire (*3*). In brief, 48 hours before the experiments the medium was changed to 5 percent charcoal-treated calf serum. Cells were incubated for 6 days with the indicated concentrations of estradiol (for conversion purposes, 270 pg/ml is approximately 1 n*M*), ligand, or ligand plus tamoxifen (Stuart Pharmaceuticals), with a medium change on day 3. Monolayers were rinsed, harvested by scraping with a rubber policeman, and disrupted by sonication in hypotonic buffer (10 m*M* tris, 1.5 m*M* EDTA, 12 m*M* monothioglycerol, and 10 percent glycerol) (*p*H 7.4), and cytosol was prepared by centrifugation at 204,000*g* for 30 minutes. Progesterone receptors were measured by incubating portions of cytosol (1 to 2 mg of protein per milliliter) with 13 n*M* [^{3}H]R5020 (Promegestone, 87 Ci/mmole; New England Nuclear). A correction was made for nonspecific binding, which was assessed in parallel samples containing 250-fold molar ratio of unlabeled R5020. Protein-bound hormone was removed from free hormone by treatment with dextran-coated charcoal (*4*). DNA was measured by the method of Burton (*5*). Values are means and ranges for two determinations. Multiple experiments were performed with similar results.

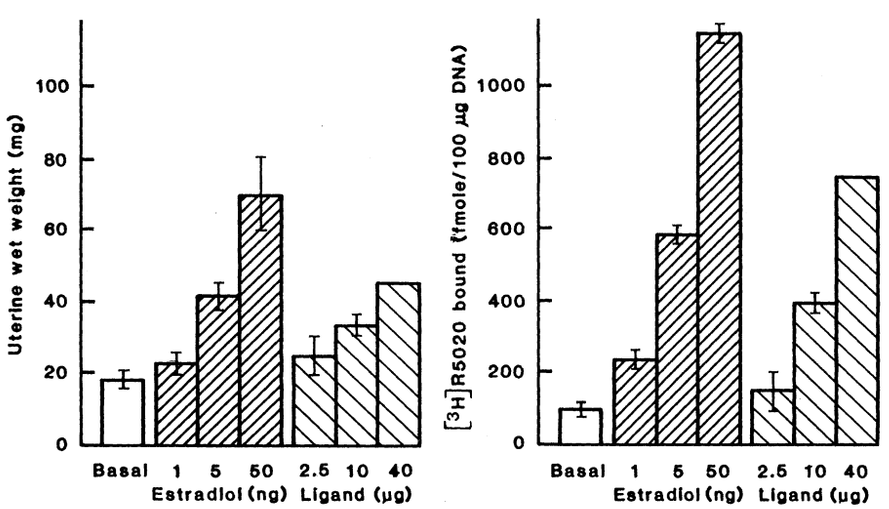

Fig. 3. Estrogenic effects of yeast ligand in ovariectomized mice. Swiss-Webster mice (20 to 25 g) were ovariectomized 3 weeks before the experiments at approximately 6 weeks of age. Vehicle (dimethyl sulfoxide), estradiol, or ligand was injected subcutaneously once a day for 3 days. Animals were killed by cervical dislocation and uteri were removed, cleaned of fat, rinsed with phosphate-buffered saline, and blotted dry. The weight, determined on a sensitive torsion balance, was used to measure uterotrophic activity. The uteri were then homogenized in buffer and specific [^{3}H]R5020 binding was determined as described in the legend to Fig. 2. Values are means ± standard errors for four to six experiments, except for the highest dose of ligand, where $N = 2$.

purification scheme are outlined in the legend to Table 1. Ligand estrogenic activity was initially monitored by [^{3}H]estradiol displacement activity in a whole-cell MCF-7 system. Findings were subsequently confirmed by additional [^{3}H]estradiol displacement experiments with both cytosol estrogen receptors prepared from ovariectomized rat uteri and estrogen-binding protein (EBP) prepared from *S. cerevisiae* α cells (*1, 2*). Active fractions were subsequently tested for functional estrogenic bioactivity both by their ability to induce progesterone receptors in MCF-7 cultured cells (*3*) and by their ability to induce a uterotrophic response and cause a progesterone receptor increase in ovariectomized mice in vivo.

The results of the purification procedure for a batch of dried yeast (Sigma) are summarized in Table 1. Similar results were obtained with other batches of starting material. The decreasing mass of crude ligand required to obtain half-maximal [^{3}H]estradiol displacement or half-maximal bioresponse indicates the increasing potency of the sequentially more purified material. Figure 1 depicts a typical example of the elution profile of one high-performance liquid chromatography (HPLC) purification procedure (step 3) that clearly identified a well-focused peak of [^{3}H]estradiol displacement activity in fraction 3. Step 2 and 4 material showed similar results. Findings in the MCF-7 cell system were mirrored in the rat uterus and in α-cell cytosol experiments.

Fractions exhibiting [^{3}H]estradiol displacement activity were subsequently tested for estrogenic bioactivity, defined as the ability to induce [^{3}H]R5020 binding sites, a measure of progesterone receptors (*3*). MCF-7 cells were treated with estradiol or partially purified ligand, and the results, with step 3 material, are illustrated in Fig. 2. Estradiol exhibited detectable activity at 2.7 pg/ml (0.01 n*M*) and achieved close to a maximum response at 27 pg/ml (0.1 n*M*), inducing an approximately ninefold increase in progesterone receptor sites. Partially purified ligand, at ~ 50 ng/ml, induced an approximately sevenfold increase in progesterone receptors and at ~ 500 ng/ml achieved an approximately ninefold increase in progesterone receptors, an effect equivalent to the maximum response to estradiol. The bioactivity assays proved to be more sensitive than the binding assays and less subject to interference by extraneous substances present in the crude ligand preparation (Table 1).

Addition to the cells of tamoxifen (1

μ*M*), an estrogen-competitive antagonist, elicited a small (about twofold) increase in progesterone receptors. When tamoxifen was included with partially purified ligand (50 ng/ml), the induction of progesterone receptors was reduced from a sevenfold increase by ligand alone to almost the increment seen with tamoxifen alone. On the other hand, when the ligand concentration was raised to 500 ng/ml, the tamoxifen blockade was overcome and near maximal induction of progesterone receptors was achieved.

Numerous other experiments were carried out with ligand preparations at steps 2 and 4 of purification as well as with step 3 material. In various experiments estradiol at 0.1 n*M* induced a 6- to 14-fold increment in progesterone receptors in MCF-7 cells. Responses to 10 to 50 ng of partially purified yeast ligand (step 3) were, in general, equivalent to those seen with 0.01 to 0.1 n*M* estradiol. Fractions of HPLC eluate that did not exhibit [^{3}H]estradiol displacement potency had minimal bioactivity.

We next used ovariectomized mice to ascertain whether the estrogenic activity of partially purified yeast ligand could be demonstrated in vivo. To conserve our limited supply of ligand we used small mice (~ 20 g) and performed only two experiments at the highest ligand dose. As shown in Fig. 3, estradiol, administered on three successive days, elicited a minimal uterotrophic response at a dose of 1 ng per mouse but induced a doubling of uterine weight at 5 ng. Partially purified ligand (step 3 material), at a dose of 2.5 μg per mouse for 3 days, caused a detectable increase in uterine weight. The response was significant at 10 μg and was even more substantial in the two mice that received 40 μg. Induction of progesterone receptors was a more sensitive measure of estrogenic activity than the uterotrophic response. A significant effect could be detected when mice were given 1 ng of estradiol for 3 days, with substantial increments in receptors detected at higher doses. Partially purified yeast ligand, at a dose of 2.5 μg per mouse, caused a minimal increase in progesterone receptors. At 10 μg per mouse the ligand induced a significant response midway between the responses to 1 and 5 ng of estradiol. The response to 40 μg of ligand per mouse was substantial. In all these experiments the ligand preparation used was far from pure. If the active principle in the crude material represented 0.1 percent of the mass of total ligand added, then the ligand would be as potent as estradiol itself.

To determine whether an estrogenic substance might be contaminating the commercially obtained yeast, experiments were performed with various starting materials, including several different batches of dried yeast, bakers' yeast, and α cells grown in our laboratory (*2*) in medium shown not to possess ligand activity. In all cases ligand activity could be demonstrated in extracts prepared from these starting materials, strongly indicating that the ligand was not an extraneous contaminant. We believe that these results demonstrate that a lipid-extractable product of *S. cerevisiae* has estrogenic activity in mammalian systems. The next effort should be to purify this yeast substance and identify its chemical nature and structure.

The presence of an estrogen-binding protein and an estrogenic substance that has [^{3}H]estradiol displacement potency supports our hypothesis that these molecules represent a yeast hormone receptor system (*1*). The functions in the yeast that are regulated by this system remain to be defined.

Substances with estrogenic activity have been described as constituents or contaminants of plants and plant products (*6*). Zearalanones, produced by the fungus *Fusarium*, contaminate feed grain and cause an estrogenic response in swine, apparently acting through the estrogen receptor (*7*). However, since *S. cerevisiae* is the yeast used extensively in the baking and fermentation industries, it is possible that this estrogenic substance may enter the human food supply and affect public health. Our findings indicate that the *S. cerevisiae* ligand is estrogenic in mammalian systems, probably acting through estrogen receptors. Further study will be required to define the structure of this yeast substance, to elucidate its physiological role in *S. cerevisiae*, and to determine whether it is a significant source of environmental estrogens affecting humans.

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Synthesis of a Cyclic Melanotropic Peptide Exhibiting Both Melanin-Concentrating and -Dispersing Activities

Abstract. *A putative melanin-concentrating hormone was synthesized. This peptide, H-Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val-OH, stimulates melanin granule aggregation within teleost melanocytes at nanomolar concentrations as does the natural purified teleost pituitary preparation. In addition, this peptide stimulates melanin granule dispersion within melanocytes of frogs and lizards. The peptide has about one six-hundredth of the activity of α-melanocyte–stimulating hormone on frog and lizard melanocytes and is a full agonist.*

Several melanotropins that stimulate pigment dispersion within integumental melanocytes have been identified within the intermediate lobe of the pituitary and the hypothalamus of vertebrates. These peptides include α-melanocyte–stimulating hormone (α-MSH) as well as other structurally homologous peptides, such as β-MSH, γ-MSH, and ACTH. Evidence suggests that α-MSH is the only physiologically relevant melanotropin that regulates skin coloration in tetrapod vertebrates (*1*). In addition to skin pigmentogenesis, other peripheral and neuroregulatory effects of α-MSH have been reported (*2, 3*). The mechanism of action of α-MSH on melanophores has been elucidated (*4, 5*). After hormone-