Bioactive steroids as contaminants of the common carbon source galactose

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Abstract

Most inducible expression vectors for the budding yeast *Saccharomyces cerevisiae* are based on galactose-inducible promoters. Yeast has been increasingly used to study vertebrate steroid receptors because of its powerful genetics. In principle, both regulatory systems are compatible and can be combined in the same strain. However, we found that commercial galactose can be contaminated by bioactive estrogen and progesterone at concentrations that are sufficient to fully activate their cognate receptors. Since steroids can elicit biological responses in pathogenic fungi and possibly other microorganisms, such contaminants in a commonly used fermentable carbon source may need to be screened for. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

1. Introduction

Galactose is commonly used as a fermentable carbon source for a large variety of microorganisms including the budding yeast *Saccharomyces cerevisiae*. Since *S. cerevisiae* prefers glucose over galactose it represses genes needed for galactose utilization in the presence of glucose [1]. The galactose-inducible *GAL1-GAL10* promoters have been exploited for the development of the most widely used regulated expression systems in this species [2]. *S. cerevisiae* has become a genetic test tube to study vertebrate steroid receptors (reviewed in [3]) such as the estrogen [4] and progesterone receptors [5]. Since their cognate

steroid ligands are generally gratuitous signals in budding and fission yeasts, these hormone-dependent transcription factors have provided new tools for regulated expression [6–8]. Here we report on an unexpected difficulty in combining the two inducible expression systems.

2. Materials and methods

2.1. Plasmids

The human estrogen receptor (hER) and the chicken progesterone receptor (cPR) were expressed from plasmids pG/ER(G) and pG/cPR, respectively. These are all based on vector pG-1 [6] which ensures high level constitutive expression independent of car-

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bon source. pG/ER(G) is essentially identical to the previously described plasmid pG/hER [9] except that it encodes wild-type hER (glycine at position 400). pG/cPR was constructed by inserting a *Bam*HI-*Bgl*II fragment from plasmid cPR1 [10], containing the cPR coding sequences and about 80 bp of 3′ flanking sequences from the rabbit β-globin gene, into the *Bam*HI site of pG-1. The reporter plasmid with progesterone/glucocorticoid response elements, pUC△SS-26X, is a pUC derivative of plasmid pSX26.1 [11]. pUC△SS-ERE is a reporter plasmid containing estrogen response element [12].

2.2. β-Galactosidase assays

The strain RMY326 (MAT**a** *his3 leu2-3,112 trp1-1 ura3-52*) (a gift from R. Movva, Sandoz, Basel) was transformed with plasmids encoding the mammalian steroid receptors along with their corresponding β-galactosidase reporter genes by the lithium acetate/polyethyleneglycol method. pUC△SS-26X was used to monitor the activation of cPR. pUC△SS-ERE served to assess the activation of the hER. Transformants were cultured overnight at 30°C in minimal synthetic medium containing 2% glucose as the carbon source, diluted 20-fold to obtain low density cultures and incubated for 12–18 h in the absence or presence of inducing agents. 40% (w/v) stock solutions (= 2.2 M) of galactose preparations were prepared in H_2O. Steroid hormones were added from $1000\times$ stocks in ethanol. β-Galactosidase assays were performed as described [13] and corrected for cell density.

2.3. Extraction of steroids from galactose

1 ml of organic solvent (ethyl acetate, dichloromethane, carbon tetrachloride or ether) was added to 0.5 ml of the galactose stock solution in a microfuge tube. The mixture was thoroughly vortexed. After centrifugation the organic phase was taken and dried in a speed vac and resolubilized in 40 μl ethanol and further diluted in growth medium.

2.4. Steroid measurements

Estrogen and progesterone concentrations were determined by the Laboratoire d'Hormonologie at the Hospital of the University of Geneva on a VIDAS system with the commercially available enzyme linked fluorescent assay kits 'VIDAS Estradiol II' and 'VIDAS Progesterone' (BioMérieux SA).

3. Results and discussion

3.1. A galactose preparation activates steroid receptors

Vertebrate steroid receptors expressed in the budding yeast *S. cerevisiae* are inactive unless they are induced by the addition of cognate steroids to the growth medium. Thus, two different proteins can be expressed independently in a regulated fashion in the same strain using the galactose and the steroid regulatory systems (see for example [12]). To assay steroid receptors in yeast, we introduced expression vectors for cPR and hER together with a β-galactosidase reporter gene into the haploid strain RMY326 (Fig. 1A). We monitored activation of the receptors as a function of hormone concentration by measuring β-galactosidase activities. In the course of such experiments with a particular galactose preparation as the carbon source, we noticed that cPR and hER are fully activated even in the absence of any added steroid hormone (data not shown and Table 1). In similar experiments, we found that this galactose preparation has the same effects in another yeast strain and also induces the human progesterone receptor but not the glucocorticoid receptor (data not shown). Intrigued by this finding, we undertook an analysis of the active principle in our stock of galactose.

Different batches of the same galactose lot gave the same result whereas galactose of higher purity from the same supplier and galactose of similar purity from several other suppliers were negative (see Table 1). This indicated that the inducing agent(s) is not galactose itself but rather a contaminant(s). Indeed, the activation of hER and cPR by small amounts of galactose can also be monitored in the presence of standard amounts (2%) of glucose as the carbon source (Fig. 1B). Under these conditions the physiological galactose response including galactose utilization is completely repressed [1]. The biological activity of the active galactose preparation was ti-
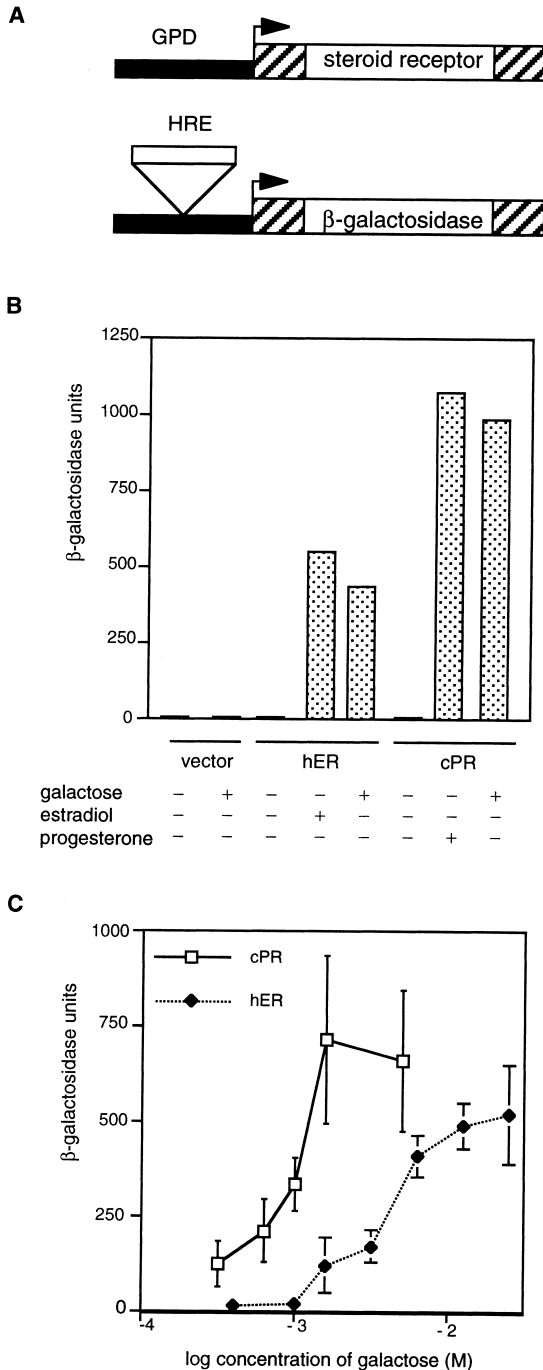
Fig. 1. Induction of steroid receptors in yeast by a commercially available galactose. A: Diagrams of the DNA constructs introduced into yeast for measuring steroid receptor activation. The steroid receptors are constitutively expressed from the glyceraldehyde-3-phosphate dehydrogenase promoter (GPD). The respective reporter genes consist of the corresponding hormone response elements (HRE) upstream of a minimal promoter driving the expression of β-galactosidase. B: Galactose (Fluka, cat. # 48263), estradiol and progesterone were added at 10 mM, 100 nM and 1 μM, respectively, to yeast strains with vector alone or expressing human estrogen receptor (hER) or chicken progesterone receptor (cPR). C: Dose-response curves (average of three independent experiments) with increasing concentrations of the same galactose preparation.
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3.2. Characterization of the active agents

Using steroid receptor mutants and chimeric transcription factors with the hormone binding domains of cPR and hER, we were able to determine that the inducing agent(s) is signaling through the hormone binding domain. Thus, it was conceivable that this galactose contains agonistic ligands for both receptors. As a first step towards the characterization of these putative ligands, we determined conditions for separating the active entity from the galactose. In line with the notion that these agents could be steroids, we found that they can be extracted with several organic solvents including ethyl acetate, dichloromethane, carbon tetrachloride and ether. Like steroids, the extracted chemicals can be dried and solubilized again in ethanol (Table 1). The extraction with carbon tetrachloride is quantitative as the remaining aqueous phase containing the galactose loses all of its activity (data not shown). To identify and quantitate the compounds, representative samples were subjected to an enzyme linked fluorescent assay with specific monoclonal and polyclonal antibodies to progesterone and to the estrogen 17β-estradiol, respectively. This analysis revealed the presence of 17β-estradiol and progesterone in the active galactose preparation as well as in the ethyl acetate extracted fraction (Table 2). Considering the galactose concentrations required for half maximal activation (see above) these would correspond to 0.4 nM 17β-estradiol and 11 pM progesterone. For hER this is compatible with the 17β-estradiol concentration required for half maximal activation [4]

trated in the presence of 2% glucose and was found to yield a typical sigmoidal dose-response curve with half maximal activation at about 5 and 1 mM galactose for hER and cPR, respectively (Fig. 1C).

Table 1
Steroid receptor-inducing activity of galactose preparations

Added reagent[a]	Steroid receptor activation[b]	
	hER	cPR
Glucose	−	−
Galactose (Fluka, cat. # 48263)[c]	+	+
Galactose (Fluka, cat. # 48259)	−	−
Galactose (Fluka, cat. # 48260)	−	−
Galactose (Merck, cat. # 104058	−	−
Galactose (Difco)	−	−
Extractable activity[d]		
Carbon tetrachloride	100%	100%
Ether	30%	n.d.[f]
Ethyl acetate	100%	n.d.
Dichloromethane	50%	n.d.
Whole milk[e]	−	+

[a]Glucose or galactose were added at 2% (111 mM).

[b]− and +, no activation and full activation.

[c]'Biotechnology grade', lot # 353548/1.

[d]Amounts added correspond to those present in 10 mM galactose.

[e]Pasteurized whole milk was diluted 3-fold into minimal medium.

[f]n.d., not determined.

and suggests that 17β-estradiol accounts for most if not all of the estrogenic activity in galactose. The polyclonal anti-17β-estradiol antiserum also weakly crossreacts with estrone, but a significant contribution of this inactive estradiol precursor seems unlikely. The efficiency with which *S. cerevisiae* is able to convert estrone to estradiol [16] would hardly be sufficient. For cPR the measured concentrations of progesterone itself cannot fully account for the activity since half maximal activation is expected at about 100 pM [5]. It is very likely that the value for the progesterone concentration in 0.6 M galactose, as determined by radioimmunoassay, is an underestimate. Moreover, it is possible that a variety of other steroids contributed to the cPR-inducing activity.

3.3. Implications of the steroid contamination of galactose

We have found that a commercially available galactose preparation contains estrogen and progesterone activity. Although we have focused on these two steroids, others may also be present. Our failure to detect glucocorticoid activity may have been due to the relative insensitivity of mammalian glucocorticoid receptor expressed in yeast [7,12,14]. The 'microbiology grade' galactose preparations from different suppliers are devoid of steroid activity, and initially we had no reason to suspect that 'biotechnology grade' galactose would be any different. Interestingly, the galactose with bioactive steroids is manufactured from bovine whey (Fluka, personal communication). Milk, from which whey and thus galactose are purified, is known to contain considerable concentrations of various steroids (for review, see [15]). Indeed, using our yeast bioassay system for steroids, we found that commercially available whole milk can induce cPR (Table 1).

Bacto-peptone, a supplement commonly used in rich medium, has previously been reported to contain significant amounts of the estradiol precursor estrone, which can be converted to estradiol at pM concentrations by *S. cerevisiae* [16]. Thus, medium supplements can be an unexpected source of bioactive steroids. These findings are not only of importance for those wishing to combine steroid and galactose inductions in yeast. The characterization of the growth and differentiation properties of pathogenic microorganisms in basic research or in clinical testing can be adversely affected by contaminating steroids. For example, the pathogenic yeasts *Candida albicans* and *Paracoccidioides brasiliensis* contain estrogen-binding proteins and respond differentially to male and female sex steroids [17–20]. Estrogens have a dramatic effect on *Paracoccidioides brasiliensis* by

Table 2
Quantitation of steroids in galactose by radioimmunoassay

	Galactose 10 mM[1]	Steroids extracted from 10 mM galactose[b]	Steroids extracted from 600 mM galactose[b]
17β-Estradiol	220 pg ml^{-1} (=0.8 nM)	193 pg ml^{-1} (=0.7 nM)	n.d.[c]
Progesterone	undetectable	undetectable	2.1 ng ml^{-1} (=6.7 nM)

[a]Galactose from Fluka, cat. # 48263 (lot # 353548/1).

[b]Extracted with ethyl acetate and diluted back to original concentration.

[c]n.d., not determined.

inhibiting the mycelium to yeast transformation [21]. In analyzing novel pathogenic microorganisms it is particularly important to be able to avoid bioactive contaminants in medium supplements such as the carbon source galactose.

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