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EXPRESSION OF RAT 5α-REDUCTASE IN *SACCHAROMYCES CEREVISIAE*

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Summary—Dihydrotestosterone (DHT) is the principle androgen in certain tissues such as the prostate. DHT is formed from testosterone by the NADPH-dependent enzyme 5α-reductase (5AR). In this paper we report the expression of catalytically active steroid 5AR from the rat in *Saccharomyces cerevisiae*. A full length cDNA coding for 5AR was isolated from a rat liver cDNA library and fixed in frame to the signal sequence of yeast acid phosphatase. A constitutive short promoter fragment of the acid phosphatase gene (*PHO5*) and the *PHO5* transcriptional terminator were added and the expression cassette ligated into the yeast 2μ vector pDP34. *S. cerevisiae* transformed with the 5AR expression plasmid pDP34/PHO5AR exhibited about 100-fold more activity per gram wet weight than rat prostate.

INTRODUCTION

Steroid 5α-reductase (5AR) is an integral membrane protein which converts testosterone (T) to 5α-dihydrotestosterone (DHT). DHT is the principal androgen in the prostate and is essential in young males for differentiation of the external genitalia [1]. In adult males, DHT has been implicated in the development of benign prostatic hyperplasia [2]. Very little is known about the physical properties of 5AR since it has not yet been purified [3–6]. The cDNAs for rat [7] and human [8] 5AR have been cloned and sequenced and the protein expressed transiently in COS cells [8]. In the rat, the cDNAs encoding prostatic and liver 5AR were identical [7]. Since access to a large quantity of this enzyme would facilitate further characterization of 5AR, a stable expression system is desirable. Yeast is an attractive host since it is easily fermented, has an advanced secretory pathway that allows proper protein folding to attain an active enzyme, and exhibits no endogenous 5AR activity. Other membrane-bound mammalian enzymes have been expressed in yeast [9, 10]. We report here the expression of catalytically active rat 5AR in *Saccharomyces cerevisiae*. Yeast were transformed with an expression plasmid containing the cDNA for 5AR fused to a yeast signal sequence and expressed under the control of a strong, constitutive acid phosphatase promoter fragment.

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MATERIALS AND METHODS

Materials

Restriction enzymes and other DNA modifying enzymes were from Boehringer. Reagents were from Merck, Sigma or Fluka. Peptone, yeast extract and agar were obtained from Difco. Proteinase inhibitors were purchased from Sigma. *N*-(2-methyl-2-propyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide (MK906, finasteride), a known inhibitor of 5AR [11], was synthesized by Dr M. Biollaz (Ciba-Geigy, Ltd., Basel). [^{32}P]dATP, [^{35}S]dATP, [^{14}C]T, [^{14}C]DHT were purchased from Amersham.

Vector construction

The strategy for the preparation of the expression vector containing the 5AR cDNA is indicated in Fig. 1. All recombinant DNA procedures were based on standard protocols [12]. The full length 5AR cDNA was isolated from a rat liver cDNA library (Stratagene) by screening with three oligonucleotides complementary to the reported rat 5AR cDNA sequence. The coding region of the cDNA was fixed in frame to a 51 nucleotide sequence coding for the first 17 amino acids of yeast acid phosphatase (*PHO5*) signal peptide by a double polymerase chain reaction. The full length rat 5AR cDNA

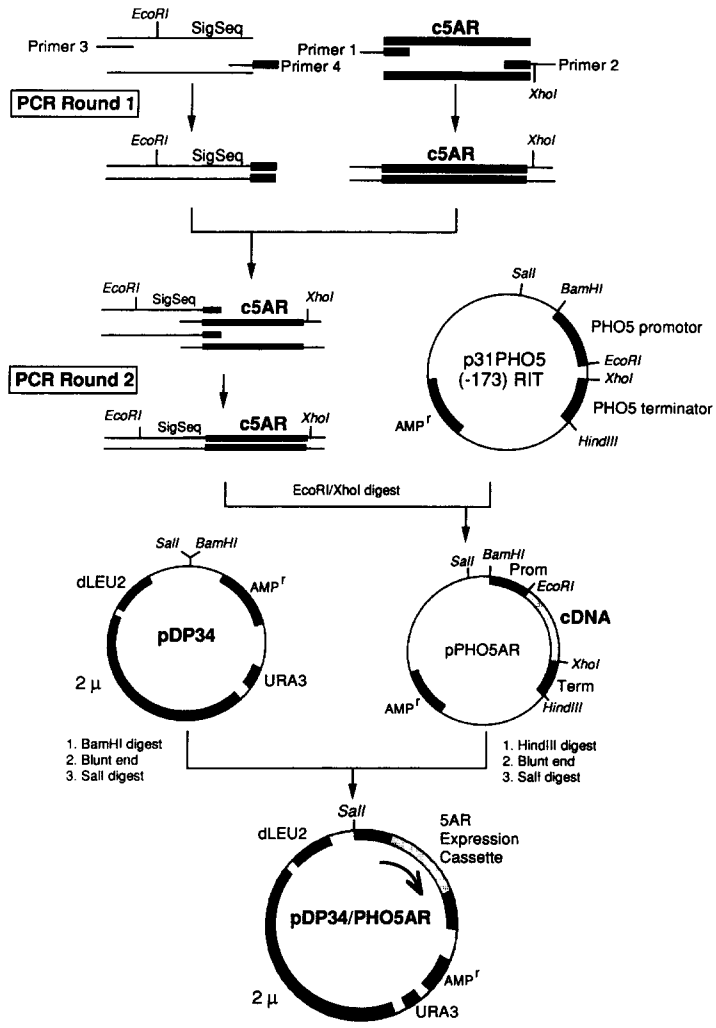

Fig. 1. Construction of the expression vector containing the 5AR cDNA. The coding region of the cDNA for 5AR was modified and amplified by PCR. Primers 1 and 4 contain complementary sequences which allowed the fusion of the *PHO5* signal sequence to the coding sequence by a second PCR. This was then inserted into the EcoRI/XhoI sites of vector p31PHO5(−173)RIT just downstream from a short PHO5 promoter fragment. The 5AR expression cassette was ligated into the yeast-*E. coli* shuttle vector pDP34 and transformed into yeast.

was modified by PCR to remove the initial Met using the upstream primer 1 (5′-GCTTCTTTG-GCCAATGCAGAGTTGGATGAGCTGT-GCCTG-3′) and to add an XhoI site at the 3′ end using primer 2 (5′-ACAGGATCCTC-GAGTTACTAAAGCACAAATGGAATCAG TAT-3′) as the downstream primer. The signal sequence was amplified by PCR using the upstream primer 3 (5′-GGCACTCACACGTG GGAC-3′) and the downstream primer 4 (5′-CCAACTCTGCATTGGCCAAAGAAGC-3′) Primer 1 and 4 contained sequences complementary to the signal sequence and 5AR, respectively. The first round PCR products were combined and reamplified; first 8 cycles alone with a 55°C annealing temperature, then 8 more cycles with a 40°C annealing temperature in the presence of the upstream signal sequence primer and the downstream 5AR primer. This signal sequence-5AR cDNA was restricted with EcoRI and XhoI and subcloned into the EcoRI/XhoI sites of the plasmid Bluescript SK(−) and maintained in *E. coli* XL1-Blue (Stratagene).

Plasmid p31PHO5(−173)RIT contains a short *PHO5* promoter fragment (nucleotide position −173 to −9) acting as a strong, constitutive promoter, followed by an EcoRI linker and a 135 bp XhoI/HindIII fragment with the *PHO5* transcription termination signals cloned in a derivative of pBR322 between BamHI and HindIII. The 821 bp EcoRI–XhoI signal sequence-5AR fragment was inserted into plasmid

p31PHO5(−173)RIT downstream of the short *PHO5* promoter fragment and in front of the *PHO5* transcription terminator. This construct will be referred to as pPHO5AR. Restriction mapping confirmed the construction of the expression cassette and the complete nucleotide sequence of the insert was verified by double stranded DNA sequencing [13].

The expression cassette was introduced into the *E. coli*-yeast shuttle vector pDP34 [14] by restricting pDP34 and pPHO5AR with BamHI and HindIII, respectively. Blunt ends were obtained by T4 DNA polymerase treatment before both fragments were restricted with SalI. After ligation of the SalI/blunt fragments, recombinant DNA was amplified in *E. coli* HB101. Yeast strain YS18cir° (*MATα*, *leu2–3*, *leu2–112*, *his3–11*, *his3–15*, *ura3D5*, [*cir°*]) was transformed with the resulting plasmid pDP34/PHO5AR [16] and colonies selected by growth on minimal media plates lacking leucine. Transformed yeast were maintained in HE17 (0.84% yeast nitrogen base without amino acids, 1% L-asparagine, 0.1% L-histidine, 2% glucose, supplemented with 20 mg/l uracil).

Membrane fractionation

Transformed yeast were grown at 30°C in 20 ml of YPD media (1% yeast extract, 2% peptone and 2% glucose) to a density of 1.4×10^8 cells/ml. Cells were centrifuged for 10 min at 2500 *g* and resuspended in 15 ml of TEKD buffer (0.1 M Tris, pH 8.0, 5 mM EDTA, 100 mM KCl, 5 mM DTT). After a 10 min incubation at 30°C, the cells were again pelleted and resuspended in 10 ml of MES buffer (20 mM MES–Tris, pH 6.0, 1.5 M sorbitol). Spheroplasts were prepared by incubating yeast at 30°C for 1 h in the presence of 24 mg zymolyase and 24 mg cytohelicase. Spheroplasts were washed twice with MES buffer and resuspended in 10 ml of 50 mM sodium phosphate, pH 7.4 containing proteinase inhibitors (1.0 μg/ml) aprotinin and tosyl-arginine methyl ester. After spheroplasting, all fractionation steps were performed at 4°C. Cells were lysed using a teflon homogenizer and centrifuged at 2500 *g* for 15 min to pellet unlysed cells, nuclear components, and cell debris. The supernatant was transferred to a new tube and centrifuged at 12,000 *g*. The 12,000 *g* supernatant was further subjected to a 110,000 *g* centrifugation for 60 min and this final pellet is referred to as the microsomal pellet.

5AR assay

Transformed yeast were precultured in HE17 and used to inoculate YPD to give a starting $o.d._{600}$ of 0.2. After a 48 h incubation at 30°C, 5AR activity was measured either in whole cells, spheroplasts, or sonicated spheroplasts by following the conversion of [^{14}C]T to [^{14}C]DHT. Spheroplasts were prepared according to the previously described method [15] and sonicated twice for 30 s on a Branson Sonifier 250 (30% duty cycle, output = 2). In a standard reaction, the cells were mixed with 1 μM [^{14}C]testosterone in the presence of 0.5 mM NADPH and 50 mM sodium phosphate, pH 7.2, to a final volume of 0.5 ml. After an incubation of 40 min at 37°C, the reaction was stopped with the addition of 2 ml of ethyl acetate. The organic phase, which contained the metabolites was transferred to a new tube and evaporated to dryness in a Speed Vac concentrator (Savant). Samples were resuspended in 100 ml of dichloromethane–diethyl ether (8:2, v/v) and applied onto Merck Silica gel 60 F-254 TLC plates. The metabolites were separated by developing the plates twice using dichloromethane–diethyl ether (8:2, v/v) as the running solvent. Products were then visualized and quantitated using a Berthold Digital Autoradiograph TLC plate reader.

Protein assay

Protein concentration was determined by the BCA method (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard.

RESULTS

The cDNA isolated from the rat liver library contained a coding region which was identical to that reported by Andersson *et al.* [7] except for nucleotide 692 which was adenine instead of guanine. Since this single substitution would change an arginine to a lysine residue, PCR was used to replace the adenine with a guanine.

Yeast transformants carrying the vector pDP34/PHO5AR were assayed for the presence of 5AR activity. No 5AR activity was detected in the culture medium. DHT formation was observed in the lysate of yeast transformed with pDP34/PHO5AR, but not in the lysate of yeast transformed with the pDP34 vector lacking the 5AR cDNA expression cassette (Fig. 2). The activity increased in a concentration and time dependent manner as shown in Fig. 3. To confirm the identity of DHT, the radiolabeled

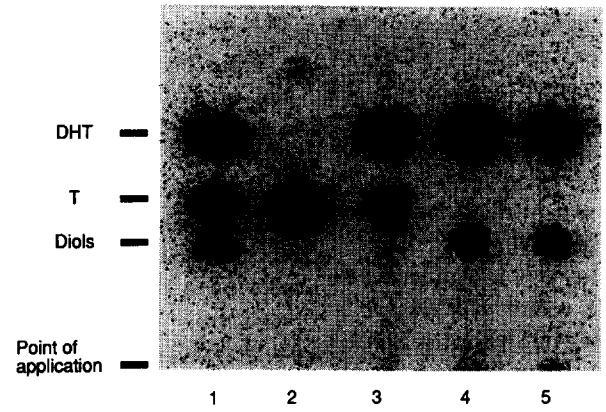

Fig. 2. 5AR activity expressed in transformed yeast. Yeast lysate (6.5 μg) was incubated at 37°C for 40 min with [^{14}C]T in the presence of NADPH. Substrate and metabolites were separated by TLC and visualized on a Berthold autoradiograph. Lane 1: T, DHT and androstanediol standards. Lane 2: Yeast transformed with nonrecombinant pDP34. Lane 3: Yeast transformed with pDP34/PHO5AR expressing 5AR activity. In the last two lanes, equal amounts of lysates from nontransformed (lane 4) and pDP34/PHO5AR transformed (lane 5) yeast were assayed as above except with [^{14}C]DHT as the substrate.

product was extracted from the TLC plate and compared to a DHT standard by rechromatography with dichloromethane–diethylether (8:2, v/v) or with dichloromethane–diethylether (2:8, v/v) as the running solvent. No 5AR activity was observed when MK906, a potent inhibitor of 5AR, was added. A comparison of 5AR from transformed yeast to 5AR from rat prostate revealed about 100-fold more activity per gram wet weight in yeast.

To determine whether T is able to pass through the yeast cell wall, intact cells were checked for their ability to convert T to DHT. Both whole cells and spheroplasts exhibited 5AR activity which was inhibited by MK906. This indicates that both T and the inhibitor MK906 can be absorbed by the yeast. Furthermore, no significant loss of enzyme activity was found when intact yeast, spheroplasts, or lysed yeast were snap frozen and stored at −70 or −20°C.

Yeast lysates containing the expressed enzyme rapidly converted T to DHT. In the presence of excess amounts of enzyme or after prolonged incubation time, a second reaction product appeared which comigrated on the TLC plate with the 5α-androstan-3α(3β)-17β-diols. Incubation of yeast containing the 5AR activity with radiolabeled DHT resulted in a rapid conversion of DHT to this secondary product (Fig. 2). However, yeast which had not been transformed with the 5AR cDNA were also able

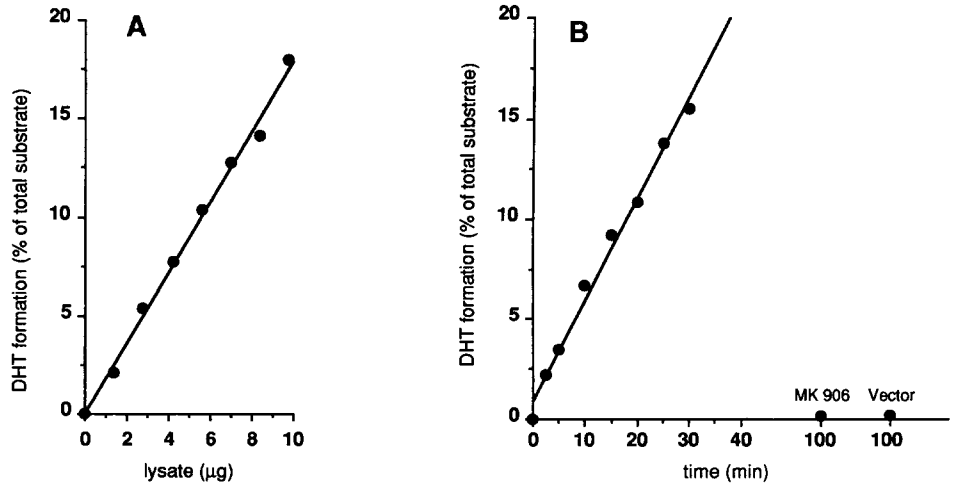

Fig. 3. 5AR activity in lysates of transformed *S. cerevisiae*. (A) DHT formation by increasing amounts of transformed yeast lysate in 15 min at 37°C. The amount of lysate protein added to each assay is indicated. (B) Time course of DHT production by 5.6 μg of lysate protein. Complete inhibition of DHT formation was obtained by the addition of MK 906 (10 μM) and no 5AR activity was observed in yeast transformed with vector alone after a 100 min incubation.

Table 1. Subcellular location of 5AR in yeast

	Homogenate	Pellet (*g*) 2500	12,000	120,000	Cytosol
Total protein per fraction (mg)	11.6	0.57	1.25	1.7	6.6
T conversion (nmol/40 min/fraction)	385	13.75	118.8	59.4	2.64
Specific activity (nmol/40 min/mg)	33.2	24.1	95.0	34.9	0.4

to metabolize DHT. Thus, this metabolism of DHT is not dependent upon expression of 5AR but rather is due to an endogenous enzyme present in yeast.

To understand better the intracellular location of the expressed 5AR in transformed yeast, crude membrane fractions were prepared. The 5AR activity was mainly found in the 12,000 *g* and the microsomal pellet from the 120,000 *g* centrifugation (Table 1). The 12,000 *g* pellet was able to convert 95.0 nmol of T/40 min/mg protein while the microsomal fraction converted 35.0 nmol/40 min/mg protein. Rat prostatic microsomes convert 0.25 nmol of T/40 min/mg total protein.

DISCUSSION

S. cerevisiae transformed with the pDP34/PHO5AR vector expressed catalytically active rat 5AR. The enzyme activity was proportional to the concentration of lysed yeast and to the incubation time. In data not presented here, the presence of the signal sequence was found to be important. A construct containing only the cDNA encoding 5AR but no signal sequence had no 5AR activity. It therefore appears the signal sequence is required for expression of an active enzyme. It is not yet known whether the *PHO5* signal sequence is properly processed or still attached to the expressed protein. Similar levels of activity were obtained when the α-factor leader sequence (prepro-amino acid sequence 1–85) [16] was substituted for the *PHO5* signal sequence. There was no evidence of a soluble or secreted form of the enzyme and the fact that the activity was found associated with the membrane fractions is consistent with 5AR being an integral membrane protein.

While the control yeast do not metabolize T, they do metabolize DHT. This suggests that yeast may produce a steroid converting enzyme with 3α(3β)-hydroxysteroid dehydrogenase activity. *S. cerevisiae* do contain low amounts of 17β-estradiol which is synthesized by the yeast and is identical to the human steroid [17]. In contrast, T is not metabolized under the described assay conditions.

The pDP34/PHO5AR transformed yeast has approx. 100-fold the activity of rat prostate on a wet weight basis and a 1 l culture of yeast provides more 5AR activity than 5000 rat prostates. Due to the high yield and stability upon freezing, the yeast expression system will provide a source for large quantities of 5AR facilitating purification and further characterization.

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REFERENCES

1. Wilson J. D.: Sexual differentiation. *A. Rev. Physiol.* **40** (1978) 279–306.
2. Hudson R. W.: Studies of the nuclear 5 alpha-reductase of human hyperplastic prostatic tissue. *J. Steroid Biochem.* **14** (1981) 579–584.
3. Enderle-Schmitt U., Volck-Badouin E., Schmitt J. and Aumuller G.: Functional characteristics of nuclear 5 alpha-reductase from rat ventral prostate. *J. Steroid Biochem.* **25** (1986) 209–217.
4. Houston B., Chisholm G. D. and Habib F. K.: Solubilization of human prostatic 5 alpha-reductase. *J. Steroid Biochem.* **22** (1985) 461–467.
5. Metcalf B. W., Levy M. A. and Holt D. A.: Inhibitors of steroid 5α-reductase in benign prostatic hyperplasia, male pattern baldness and acne. *TIPS* **10** (1989) 491–495.
6. Liang T., Cascieri M. A., Cheung A. H., Reynolds G. F. and Rasmusson G. H.: Species differences in prostatic steroid 5-alpha-reductases of rat, dog, and human. *Endocrinology* **117** (1985) 571–579.
7. Andersson S., Bishop R. W. and Russell D. W.: Expression cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation. *J. Biol. Chem.* **264** (1989) 16249–16255.
8. Andersson S. and Russell D. W.: Structural and biochemical properties of cloned and expressed human and rat steroid 5α-reductases. *Proc. Natn. Acad. Sci. U.S.A.* **87** (1990) 3640–3644.
9. Oeda K., Sakaki T. and Ohkawa H.: Expression of rat liver cytochrome *P*-450MC cDNA in *Saccharomyces cerevisiae*. *DNA* **4** (1985) 203–210.
10. Toghrol F., Kimura T. and Owens I. S.: Expression of UDP-glucuronosyltransferase cDNA in *Saccharomyces cerevisiae* as a membrane-bound and as a cytosolic form. *Biochemistry* **29** (1990) 2349–2356.
11. Rasmusson G. H., Reynolds G. F., Steinberg N. G., Walton E., Patel G. F., Liang T., Cascieri M. A., Cheung A. H., Brooks J. R. and Berman C.: Azasteroids: structure–activity relationships for inhibition of 5 alpha-reductase and of androgen receptor binding. *J. Med. Chem.* **29** (1986) 2298–2315.

12. Sambrook J., Fritsch E. F. and Maniatis T.: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Lab. Press, New York, 2nd Edn (1989).
13. Chen E. Y. and Seeburg P. H.: Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA* **4** (1985) 165–170.
14. Meyhack B., Hinnen A. and Heim J.: Heterologous gene expression in *Saccharomyces cerevisiae.* In *Genetics and Molecular Biology of Industrial Microorganisms* (Edited by C. L. Hershberger, S. W. Queener and G. Hegeman). ASM, Washington, DC (1989) pp. 311–321.
15. Hinnen A., Hicks J. B. and Fink G. R.: Transformation of yeast. *Proc. Natn. Acad. Sci. U.S.A.* **75** (1978) 1929–1933.
16. Kurjan J. and Herskowitz I.: Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. *Cell* **30** (1982) 933–943.
17. Feldman D., Tökés L., Stathis P., Miller S., Kurz W. and Harvey D.: Identification of 17 beta-estradiol as the estrogenic substance in *Saccharomyces cerevisiae. Proc. Natn. Acad. Sci. U.S.A.* **81** (1984) 4722–4726.