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Metabolically Independent and Accurately Adjustable *Aspergillus* sp. Expression System

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Filamentous fungi are well-established expression hosts often used to produce extracellular proteins of use in the food and pharmaceutical industries. The expression systems presently used in *Aspergillus* species rely on either strong constitutive promoters, e.g., that for glyceraldehyde-3-phosphate dehydrogenase, or inducible systems derived from metabolic pathways, e.g., *glaA* (glucoamylase) or *alc* (alcohol dehydrogenase). We describe for *Aspergillus nidulans* and *Aspergillus niger* a novel expression system that utilizes the transcriptional activation of the human estrogen receptor by estrogenic substances. The system functions independently from metabolic signals and therefore can be used with low-cost, complex media. A combination of positive and negative regulatory elements in the promoter drives the expression of a reporter gene, yielding a linear dose response to the inducer. The off status is completely tight, yet the system responds within minutes to induction and reaches a level of expression of up to 15% of total cell protein after 8 h. Both *Aspergillus* species are very sensitive to estrogenic substances, and low-cost inducers function in the picomolar concentration range, at which estrogenic substances also can be found in the environment. Given this high sensitivity to estrogens, *Aspergillus* cells carrying estrogen-responsive units could be used to detect xenoestrogens in food or in the environment.

In their natural environment, fungi use extracellular enzymes to gain access to complex, often water-insoluble carbon and nitrogen sources. Extracellular protein production by filamentous fungi usually is very efficient for homologous proteins, for which levels of grams per liter can be obtained. *Aspergillus* and *Trichoderma* strains have been reported to secrete up to 30 g of homologous proteins per liter in fermentation processes (e.g., glucoamylases) (11). The production of recombinant proteins of mammalian origin can be up to 4 orders of magnitude lower even when the same expression signals are used.

The reason for this difference is not completely understood (2). Along with mRNA stability, codon usage, and translational efficiency, overloading the secretory machinery with a protein that has a "foreign" structure appears to be a critical bottleneck (16, 34). High concentrations of such heterologous proteins can result in incorrect folding—leading to translational feedback inhibition by phosphorylation of the α subunit of eukaryotic initiation factor 2 (23) and subsequent intracellular degradation of the misfolded protein. Thus, strong but highly regulatable expression systems are needed to control the flow of proteins through the secretory pathway.

The use of strong promoters derived from housekeeping genes, such as those of the various fungal glyceraldehyde-3-phosphate dehydrogenase genes, has the disadvantage of continuous, growth-related expression levels (31). This problem may reduce yields of proteins susceptible to degradation or lead to cell death if the overexpressed foreign proteins are toxic to the host cells. Other promoters, e.g., the alcohol dehydrogenase (*alcA*) promoter from *Aspergillus nidulans*, have lower background expression levels and achieve very high expression levels under conditions of induction and glucose deprivation, but this system is not functional in the industrially relevant species *Aspergillus niger* (27).

Most promoters commonly used for industrial *Aspergillus* species are derived from the amylase (36), xylanase (7), and arabinase (12) genes. These genes are repressed by glucose, and carbon catabolite repression also can override induction (10, 46) and lead to the loss of inducibility. Moreover, it is not possible to obtain a linear dose response by altering the inducer concentration. The metabolic origin of these systems dictates their expression levels; when they are highly expressed, the secretory pathway may be overloaded, resulting in the misfolding and intracellular degradation of proteins (13).

In commercial applications, inducer compounds (applied in the millimolar range) are a major part of fermentation costs and can be toxic; e.g., alcohols require special measures for safe handling, storage, and disposal. The type of fermentation medium also affects the secretory capacity of a given expression host. Most importantly, the expression of extracellular proteases, such as those encoded by *pepA*, *pepB*, and *pepF*, which significantly affect secreted protein yield, are regulated by the types of carbon and nitrogen sources and by the pH of the medium (39, 41). Kurzatkowski et al. (22) have presented evidence that glucose also has a negative effect on the development of an efficient secretory apparatus. These data indicate that the use of promoters derived from metabolic genes requires a specific fermentation medium; therefore, most low-cost, complex media (containing multiple sugars and various nitrogen sources) cannot be used.

The human estrogen receptor (hERα) is a member of a family of nuclear receptors for small hydrophobic ligands (40) that regulate growth, differentiation, and homeostasis in ver-

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TABLE 1. Strains used in this study

Strain designation	Genotype	Strain description
argB pyrG	*argB2 riboA1 pyrG89*	*A. nidulans* parent strain
hER	*argB2 riboA1*	Parent strain + phERpyr4
ERE-URA-nirA	*riboA1*	hER + pERE-URA-nirA
ERE-URA-RS	*riboA1*	hER + pERE-URA-RS
ERE-RS-nirA	*riboA1*	hER + pERE-RS-nirA
alcA	*riboA1*	hER + pRMalcA
A972[a]	*cspA1 acrA brnA2 pyrG5 niaD2*	*A. niger* parent strain
972 ERE-URA-nirA	*cspA1 acrA1 brnA2 niaD2*	A972 + phERpyr4 and pERE-URA-nirA

[a] From the Fungal Genetic Stock Center (www.fgsc.net).

tebrate cells. hERα activity is regulated allosterically by ligand binding, which activates the protein and promotes nuclear entry, binding to high-affinity sites in chromatin, and subsequently transcriptional modulation (15). Expression systems based on hERα function in yeasts (25) and plants (49), but these organisms are not highly sensitive to estrogenic substances (in the micromolar range); for instance, in *Saccharomyces cerevisiae*, ERα induces high-level expression only when the *PDR5* and *SNQ2* genes encoding the ABC transporters are deleted (26).

In addition to the natural hormone, other compounds also can bind to ERα with a high affinity and either activate transcription or, by acting as antiestrogens, interfere with activation by estrogenic substances. Many plant-derived products contain highly active phytoestrogens, e.g., coumestrol, and many industrial and pharmaceutical compounds with xenohormone activity also are available (1).

The objective of this study was to develop a strong but metabolically independent, highly regulatable expression system for *Aspergillus* species. We show that *Aspergillus* species are highly sensitive to estrogenic compounds and that estrogen-responsive elements (EREs) activate the transcription of a reporter gene with a linear dose response.

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. Strain requests should be directed to B. Shoemaker, Contracts Coordinator, GlycoFi, Inc., 21 Lafayette St., Ste. 200, Lebanon, NH 03766.

Plasmid construction. (i) phERpyr4. A 2,941-bp BglII/PstI fragment from pAN52-1 (GenBank accession no. Z32697), containing the P_{gpdA}-NcoI-BamHI-T_{trpc} cassette, was inserted into pBluescript KS(+) (GenBank accession no. X52331) cut with BamHI/PstI. A 1,819-bp EcoRI fragment (end repaired by Klenow filling in) from plasmid Yep90-HEG0 (29), containing the complete coding sequence of hERα, was subsequently cloned into the NcoI site (end repaired by T4 polymerase) within the P_{gpdA}-T_{trpc} cassette. The P_{gpd}-hERα-T_{trpc} cassette was released as a SpeI/ClaI fragment and cloned into a pBluescript KS(+) derivative cut with Spe/ClaI and already containing the *pyr4* gene of *Trichoderma reesei* as a selectable marker. The *pyr4* gene is located on a SalI fragment originating from pFG1 (14).

(ii) pRM2085. A 274-bp PCR fragment containing the 3×ERE-P_{URA3} sequence (the 1× ERE sequence is 5′-GGTCACAGTGACC-3′) was amplified with primers (underlined sequences in primers represent bases introduced for cloning purposes) ERE-FW (5′-CGAATTCAGATCTCCATGCAGTTGGAC G-3′) and URA3-RV (5′-TGGCAGCAACAGGACTAGGAT-3′) and with genomic DNA prepared from yeast strain YYM8 (24) as a template. A 108-bp *nirA* promoter fragment was amplified with primers NirA-RV (5′-TGGATCCATG GTAAATCAAGCCCAGACAGA-3′) and NirA-99 (5′-TCTACTGCAGGGAA ACACGCCGAGC-3′) and with *A. nidulans* genomic DNA as a template. *nirA* codes for a transcriptional regulator mediating nitrate induction and is constitutively expressed at extremely low levels not detectable by Northern analysis (5). The 3×ERE-P_{URA3} sequence and the *nirA* promoter fragment were cut with EcoRI/PstI and BamHI/PstI, respectively, and fused at the unique PstI site (3×ERE-P_{URA3}-P_{nirA}) via subcloning in pBluescript KS(+). From the resulting plasmid, pZRM2059 (GenBank accession no. AY663843), a 284-bp BglII/BamHI fragment was released and cloned into the BamHI site of pAN923-42B_{BglII} (31).

(iii) pERE-URA-RS. The 101-bp BamHI/PstI *nirA* promoter fragment within pZRM2059 (3×ERE-P_{URA3}-P_{nirA}) was replaced with a 101-bp random sequence (RS) derived from the open reading frame (ORF) of the ampicillin resistance gene by PCR with primers RSnirA-BamF (5′-AAGGATCCATATCTTTTA CTTTCACCAGCG-3′) and RSnirA-PstR (5′-TGACTGCAGAACATTTCC GTGTCGCCCTTATTC-3′) to obtain p2059-URA-RS (GenBank accession no. AY663844). Again, the chimeric promoter construct (3×ERE-P_{URA3}-RS) was released (BamHI/BglII) and cloned into the BamHI site of pAN923-42B_{BglII}.

(iv) pERE-RS-nirA. The RS derived from the ORF of the ampicillin resistance gene was generated by PCR with primers RSURA3-PstF (5′-ATTCTGCAGA ACCCACTCGTGCACCCAAC-3′) and RSURA3-R (5′-TTTCCGTGTCGCC CTTATTC-3′). The 3×ERE sequence was generated by annealing oligonucleotides ERE-BglII-F (5′-GGTCACTGTGACCGGTCACTGTGACCGGTCAC TGTGACCAGATCTGGA-3′) and ERE-BglII-R (5′-TCCAGATCTGGTCAC AGTGACCGGTCACAGTGACCGGTCACAGTGACC-3′). The two fragments were ligated and inserted into pRM2059 cut with PstI/BglII to replace the 183-bp 3×ERE-P_{URA3} fragment. The chimeric promoter construct (3×ERE-RS-P_{nirA}) was excised with BamHI/BglII from the resulting plasmid, p2059-RS-nirA (GenBank accession no. AY663845), and cloned into the BamHI site of pAN923-42B_{BglII}.

(v) pRMalcA. pRM2085 was partially cut with XhoI and subsequently digested with BamHI. The 420-bp *alcA* promoter fragment was amplified by PCR with primers alcAXhoIF (5′-GTCCTCGAGCAGCTGAAAAAGCTGA-3′) and alcABamHIR (5′-TTGGATCCATTTTGAGGCGAGGTGA-3′), digested with BamHI/XhoI, and ligated into the vector backbone of pRM2085 to obtain vector pRMalcA.

Culture conditions, transformation, and Northern analysis. *Aspergillus* strains were grown for 12 to 14 h at 37°C in liquid minimal medium (30) with appropriate supplements by shaking on a rotary shaker at 180 rpm. Estrogenic compounds were purchased from Sigma (St. Louis, Mo.) (diethylstilbestrol [DES] [catalog no. D-4628], 17-β-estradiol [catalog no. E-225717], and α-zearalanol [catalog no. Z-0292]) or from Fluka (Buchs, Switzerland) (coumestrol [catalog no. 27885]). Different inducer concentrations, types, and nutrients were tested by harvesting cultures by filtration and washing with two culture volumes of a 4°C *Aspergillus* minimal medium salt solution. Aliquots were transferred to fresh medium containing the inducer, and incubation was continued. After incubation, the mycelium was harvested by filtration and frozen in liquid N_2. Transformation of *Aspergillus* strains and Northern analysis were carried out as described previously (5, 38).

Reporter enzyme assays. Sodium phosphate buffer (50 mM Na_3PO_4, 1 mM EDTA [pH 7.0]) and glass beads (0.75 to 1.0 mm) were added to the frozen mycelium, and cells were disrupted by using a RiboLyser (Hybaid, Heidelberg, Germany). Cell debris was separated by centrifugation, and the supernatant was used immediately for the determination of protein concentrations and for β-galactosidase enzyme assays. Protein concentrations were determined by using a bicinchoninic acid assay (Pierce, Dallas, Tex.), and β-galactosidase specific activities were determined by using the protocol included in a protein expression kit from Invitrogen (Carlsbad, Calif.; catalog no. K1710-01). According to this protocol, β-galactosidase enzyme activity is calculated as 300,000 U of β-galactosidase per mg of protein, and the detection limit is ≥5 U.

RESULTS

Expression of hERα from the *gpdA* promoter. We expressed hERα under the control of the strong constitutive *A. nidulans gpdA* promoter and the *trpC* terminator. Construct phERpyr4 carried the *T. reesei pyr4* gene as a selectable marker. Several prototrophic transformants were analyzed by PCR and Southern blot analysis (data not shown). The strain designated hER carried a single copy of construct phERpyr4 in the *A. nidulans pyrG89 argB2* genome. hER carried as a selectable marker *argB*$^-$, which was used for the site-directed integration of a single copy of the reporter construct into the *argB* locus. By comparing only insertions at *argB*, differences in reporter gene expression due to integration into different genomic loci can be

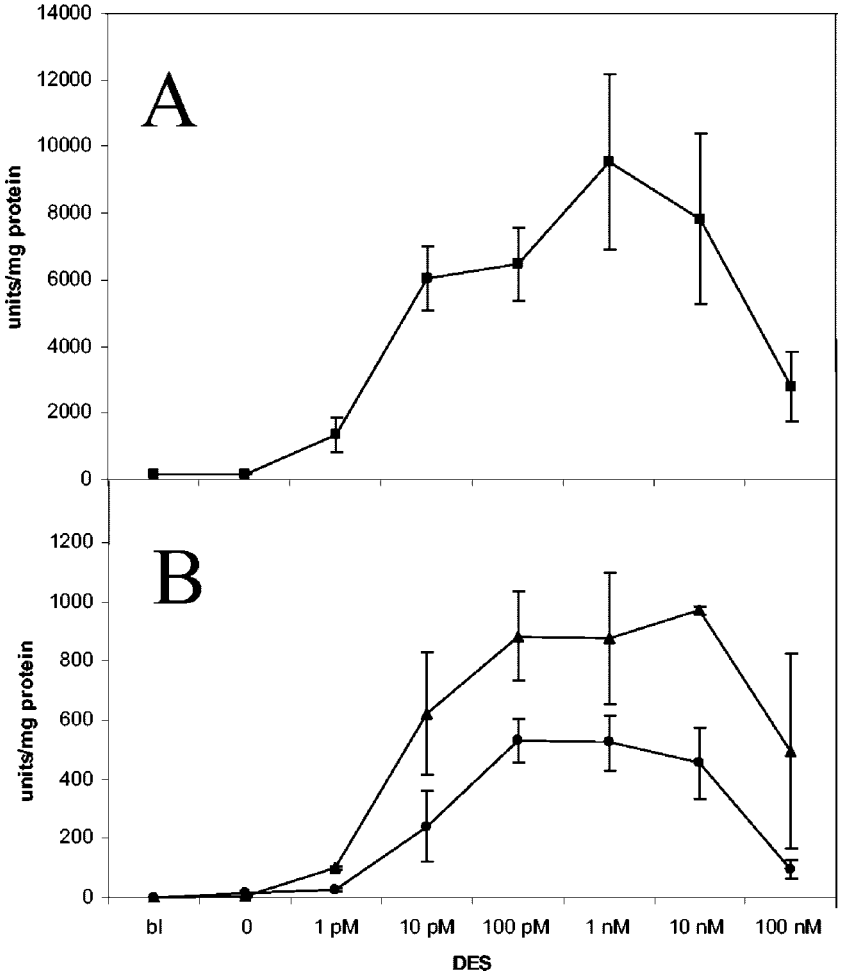

FIG. 1. (A) β-Galactosidase activities of the *A. nidulans* ERE-URA-RS reporter strain at various final concentrations of DES. The strain was grown and shifted to induction medium, and the enzyme activity of the reporter was determined after 8 h. bI, before induction. Error bars indicate standard deviations of three independent experiments. (B) β-Galactosidase activities of the *A. nidulans* ERE-URA-nirA (triangles) and ERE-RS-nirA (circles) reporter strains at various final concentrations of DES. The strains were induced for 8 h, and the enzyme activities were measured.

avoided. When the parent strain was compared with transformants expressing the hERα construct for their responses to the synthetic estrogenic compound DES in the standard minimal medium used in these experiments, we observed that all of the transformants were hypersensitive to DES. At DES concentrations of 100 nM, growth was strongly inhibited, and 1 μM prevented germination and growth. In contrast, growth and germination of the *A. nidulans* or *A. niger* parent strain were not affected by DES at levels of <10 mM (data not shown).

Induction of promoters carrying EREs by DES. We constructed an expression vector based on *A. nidulans* vector pAN923-42$_{BglII}$ (42); the construct carries a mutated *argB* gene as the selectable marker for site-directed integration into the *argB* locus and the *lacZ* gene in front of the *trpC* terminator sequence. We combined a minimal promoter sequence derived from the *S. cerevisiae URA3* gene containing the TATA box with three copies of the human ERE (29). To maintain the approximate distance between the *URA3* TATA element and the ATG of the *lacZ* reporter gene, a 94-bp random "stuffer" fragment (RS) derived from the ORF of the *Escherichia coli* ampicillin resistance gene was introduced to complete the promoter. The final construct, termed pERE-URA-RS, was integrated as a single copy into the *A. nidulans argB* locus or randomly into *A. niger*.

The hERα-ERE system is functional in *Aspergillus* species (Fig. 1A). We tested the effects of various DES concentrations in the medium and found that expression starts at a concentration of 1 pM (~10% of the full level), reaches ~50% of the full level at 10 pM DES, and is highest at 100 pM and 1 nM DES (~10,000 U of β-galactosidase per mg of protein). Increasing the DES concentration to 10 or 100 nM resulted in reduced expression.

The levels of expression induced from this construct are of the same order of magnitude as those from the *alcA-lacZ* construct (~35,000 U of β-galactosidase per mg of protein) transformed into the isogenic strain (also targeted to the *argB* locus). A strain that contains multiple copies of the pERE-URA-RS reporter construct even overrides (~50,000 U of β-galactosidase per mg of protein) the activation potential of the *alcA* construct (data not shown). Thus, the expression level

TABLE 2. Dose responses of various reporter constructs combined with various DES inducer concentrations[a]

Strain designation	DES concn	β-Galactosidase activity	SD
hER	1 nM	ND	NA
ERE-RS-nirA	0 M	ND	NA
	1 pM	25	6
	100 pM	530	74
ERE-URA-nirA	1 pM	100	5
	1 nM	870	220
ERE-URA-RS	0 M	150	19
	1 pM	1,400	530
	2 pM	2,500	1,500
	4 pM	4,100	1,800
	6 pM	5,800	30
	10 pM	6,000	950
	100 pM	6,500	1,100
	1 nM	9,500	2,600
	10 nM	7,800	2,600
	100 nM	2,800	1,100
ERE-URA-RS (multicopy transformant)	1 nM	51,000	9,500

[a] ND, not detectable (detection limit, ≥5 U of β-galactosidase). NA, not applicable. SDs were from three independent experiments.

can be increased by increasing the number of copies of the ERE promoter, and the amount of activated hERα expressed from the *gpdA* promoter is not rate limiting. Background expression without DES in this strain (~150 U of β-galactosidase per mg of protein) is only slightly above the background level seen in the isogenic strain carrying no reporter construct (~20 U of β-galactosidase per mg of protein) and is considerably lower than the background level seen in *A. nidulans* with the noninduced, fully glucose-repressed *alcA* promoter (~300 U of β-galactosidase per mg of protein) (data not shown).

Recognition of different estrogenic compounds by hERα in *Aspergillus* species. We found (data not shown) that 17-β-estradiol was as active as DES. The growth hormone zearalanol (9) resulted in ~40% the activation seen with DES, and coumestrol, a natural phytoestrogen, also showed significant induction at concentrations as low as 1 nM.

Sensitivity of hERα expression to carbon and nitrogen catabolites. In an ideal expression system, there would be no medium constraints on an expression system for high-level protein production. However, most promoters used for protein expression are under the control of carbon catabolite repression and are sensitive to medium composition. Our constructs exhibited no such difficulties. We tested our constructs in a variety of synthetic media (*Aspergillus* minimal medium with various concentrations of glucose, arabinose, xylose, or fructose as the sole carbon source in the presence of ammonia as the sole nitrogen source and nitrate, urea, or ammonia as the sole nitrogen source in the presence of glucose as the sole carbon source) and complete media used for protein expression in *Aspergillus* species. In all media, the system was fully independent of both the nitrogen and the carbon sources, and the levels of expression differed no more than ±5% from those obtained with the standard medium (minimal medium with 1% glucose and 10 mM ammonia as the sole carbon and nitrogen sources, respectively) used in these experiments (data not shown).

Background and control of expression levels. The pERE-URA-nirA construct, when integrated as a single copy into the *argB* locus, reduced background expression to a nondetectable level, while the induction response to DES was maintained (Fig. 1B and Table 2). However, the promoter strength was ~10% that obtained with the original pERE-URA-RS construct, showing that the silencing function of the *nirA* promoter fragment was not fully counteracted by the hERα transactivator. If a longer piece of the *nirA* promoter (287 bp) was inserted instead of the 94-bp fragment, then a dominant repressing effect overrode the activation (data not shown), suggesting that either an additional negative element was introduced or that promoter spacing is critical to the function of the hormone response in *Aspergillus* species. When we compared *A. nidulans* strain ERE-URA-nirA with an *A. niger* strain carrying at least two ectopic integrations of the pERE-URA-nirA reporter construct, we found that absolute expression levels generally were

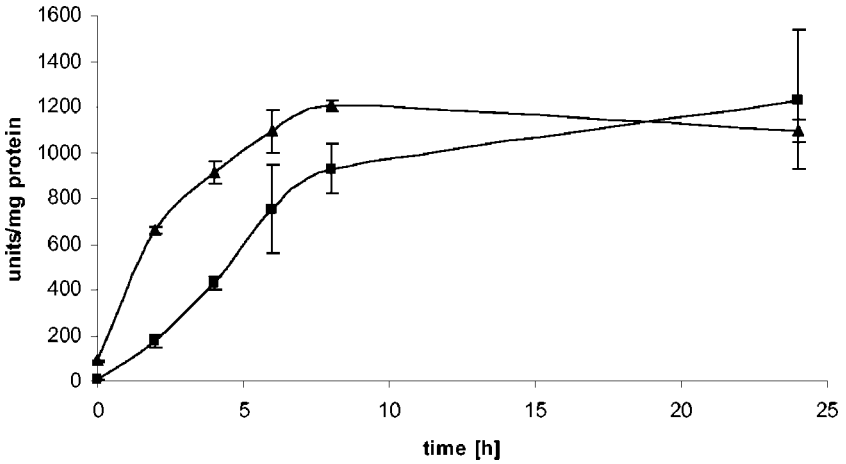

FIG. 2. Comparison of β-galactosidase activities of *A. niger* (triangles) and *A. nidulans* (squares). Both species carry the pERE-URA-nirA reporter construct. The inducer (DES) was added to a concentration of 1 nM (0-h control), and incubation proceeded for 2, 4, 6, 8, and 24 h before the determination of reporter enzyme activities. Error bars indicate standard deviations of three independent experiments.

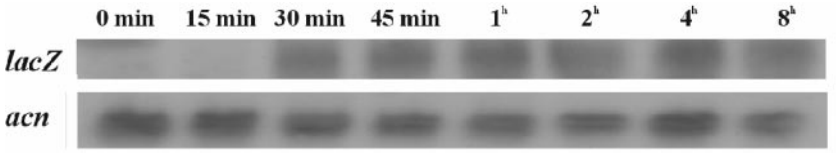

FIG. 3. Northern blot analysis of the *A. nidulans* ERE-URA-nirA reporter strain induced with 1 nM DES. RNA was prepared at the indicated time points. The blot was hybridized once with the *lacZ* gene, stripped, and reprobed with the *A.nidulans acnA* (actin-encoding) gene as a loading control.

higher under noninducing and inducing conditions in *A. niger* than in *A. nidulans* after 8 h (Fig. 2).

Even though it is a repressing element, the *nirA* fragment can serve as a core promoter. We replaced the core *URA3* sequence with an identical length of the ampicillin resistance gene RS, leaving the ERE equidistant from the *nirA* promoter fragment (construct pERE-RS-nirA). This construct has a background level of zero in the absence of the inducer but responds to DES (Fig. 1B). Promoter strength was reduced ~25% by the removal of the *URA3* sequence relative to that seen with pERE-URA-nirA. On the basis of these results, the three functional constructs combined with different concentrations of the inducer DES provided a linear dose response (Table 2) for the expression of a given gene, i.e., for the reporter used here, from 0 U to ~50,000 U of β-galactosidase, the latter corresponding to approximately 15% of the total cellular protein.

Induction kinetics. Induction of the reporter gene transcript was evident as soon as 15 min following the addition of DES and reached the maximum level after 30 min. This level was maintained for at least 8 h (Fig. 3). Reporter enzyme induction lagged by approximately 60 min, and saturation of the intracellular reporter enzyme concentration was reached after 8 h (Fig. 4). Despite continuous transcription, no further increase in intracellular enzyme levels occurred, even when incubation times were extended to 24 h, probably as a result of intracellular reporter protein degradation.

DISCUSSION

We have shown that hERα functions efficiently in *Aspergillus* species. *A. nidulans* and *A. niger* wild-type strains were not affected by <10 mM DES in the medium. Transformants expressing the receptor under the control of the *gpdA* promoter were extremely sensitive to this compound. In *S. cerevisiae*, cells expressing the hERα protein at high levels grow in the presence of 1 μM estrogen (29). In *A. nidulans*, this concentration, at least in the form of DES, prevents colony formation, and growth is impaired at concentrations as low as 10 nM DES in plate assays. It is not clear why *Aspergillus* species are so much more sensitive to large amounts of activated hERα, but the toxic effect could result from the ability of hERα to recruit coactivators (28) that can modify local nucleosome positioning or alter large-scale chromatin structure. Alternatively, high concentrations of activated hERα in *Aspergillus* species could bind, via the AF-1 and AF-2 domains, to the TATA-binding protein (35) and/or to TATA-binding protein-associated factors (17, 18). Such binding would prevent these proteins from participating in RNA polymerase II complexes and polymerase II-dependent transcription. Finally, *Aspergillus* species might take up estrogens more efficiently and/or have less capacity to extrude the compounds via efflux pumps such as ABC transporters. Consistent with this explanation is the toxicity of 100 μM DES for *S. cerevisiae* Δ*PDR5* Δ*SNQ2* cells lacking these transporters, whereas *PDR5 SNQ2*$^+$ strains are viable at this DES concentration. Toxicity for these yeast cells is independent of whether or not they express hERα (26; our unpublished observations). The high sensitivity of *Aspergillus* hERα-expressing strains to DES could enable these strains to be used as novel genetic screening tools.

There are concerns about using DES in large quantities in industrial fermentations. Therefore, we tested the responses of the expression modules to other estrogenic compounds. For instance, the nonsteroidal mycotoxin zearalenone induces the *Aspergillus* hERα-ERE promoter at a concentration of 10 nM (data not shown). This amount of zearalenone is ~20-fold lower than the guideline level for zearalenone concentrations

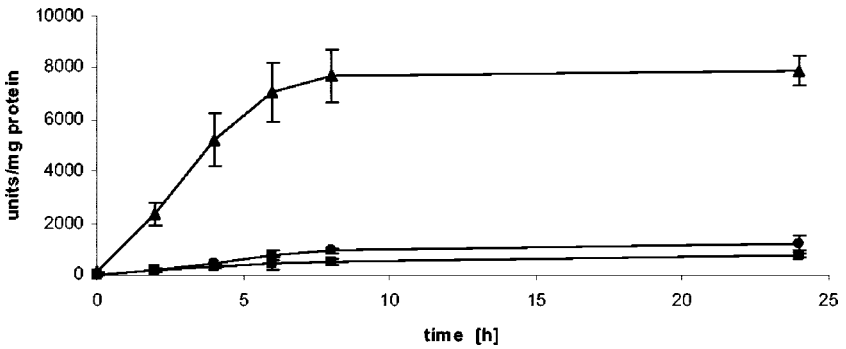

FIG. 4. Saturation of intracellular reporter enzyme activity. β-Galactosidase was measured after induction with 1 nM DES for various times in ERE construct strains ERE-URA-RS (triangles), ERE-URA-nirA (circles), and ERE-RS-nirA (squares). Error bars indicate standard deviations of three independent experiments.

in wheat (200 nmol/kg) intended for human consumption in Austria (26). Zearalanol, a derivative of zearalenone marketed as Ralgro, is an anabolic growth-promoting compound used for finishing cattle in feed lots in the United States. Zearalanol has been found at up to 5 μg/liter (15 nM) in urine samples (21, 48) or up to 0.1 μg/kg in beef tissue samples (6). Zearalanol activates the *Aspergillus* system to about 40% the DES induction capacity. Based on these numbers, a concentration of 0.5 nM in beef tissue samples would be readily detected by an *Aspergillus* reporter strain (calculated as 800 U of β-galactosidase at 1 pM zearalanol). 17-β-Estradiol, an active ingredient in oral contraceptives, is fully equivalent to DES in activation potential; i.e., transcriptional induction can be seen at 1 pM. These concentrations (several nanograms per liter) commonly are found in surface waters or effluents of sewage treatment plants (3). Other endocrine disrupters with estrogenic capabilities, e.g., 4-nonylphenol, which were not tested in our assays, might be present in environmental samples at levels of up to several hundred nanograms per liter.

For the formulation of a fermentation medium, a potentially interesting natural estrogen is coumestrol, which can activate the *Aspergillus* system at nanomolar concentrations. This phytoestrogen is present at levels of milligrams per kilogram in soy sprouts (32), and the addition of soy flour in the range of parts per billion would lead to a fully functional induction medium. It is clear that the extremely high sensitivity of *Aspergillus* cells to synthetic or natural estrogens makes the expression modules suitable for biotechnological applications and the transformed strains potential bioreporting microorganisms for detecting estrogenic compounds in food or environmental samples.

A panoply of expression systems have been described for various organisms; they include common metabolic signals, metal-induced systems (45), antibiotic-induced systems (4), and light-induced gene switches (37). For filamentous fungi, no metabolically independent expression systems have yet been developed. Thus, fungal biotechnologists have had to adapt the fermentation medium to conditions under which an expression system functions optimally. For *Aspergillus glaA*, which encodes glucoamylase (43), or *exlA*, which encodes exoxylanase (7), glucose and fructose (the preferred fungal carbon sources) must be absent or present at limiting concentrations, and specific inducers, such as starch, xylan, cellulose, or derivatives of these compounds, must be supplied. Similarly, the regulation and activities of extracellular proteases which are of major importance in yield optimization are both carbon and nitrogen source sensitive (19) and dependent on ambient pH (44). These constraints on medium composition often prevent the use of cost-effective raw materials in commercial fermentations.

In our system, expression from EREs is independent of medium components, and the induction functions equally well in complex media or minimal media with various carbon and nitrogen sources. *Aspergillus* species show a linear dose response to 3 log concentrations of estrogen and as much as 300-fold induction. The possibility of fine-tuning expression levels further through the modular combination of various inducer concentrations is another advantage of this system.

The expression of a heterologous protein in a fungal host usually results in protein yields much lower than those seen for homologous secreted proteins, such as *Aspergillus* glucoamylase or *Trichoderma* cellulase (13, 20). The native enzymes can accumulate in the fermentation medium at rates of up to 30 g/liter; levels of heterologous proteins usually are in the range of milligrams per liter. The fungal secretory pathway is thought to be the bottleneck for the efficient secretion of heterologous proteins due to misfolding or incorrect glycosylation, either or both of which could evoke the unfolded-protein response and induce protein degradation pathways. The overproduction of foldases and chaperones in some cases can (33) and in others cannot (8) compensate for the lack of appropriate posttranslational modifications. Thus, to achieve optimum expression levels for the secretion of foreign proteins, the secretory pathway should not be overloaded through the hyperexpression of "your favorite gene" (47). The ability to adjust expression levels in *Aspergillus* species with the hERα-ERE system from 0 to ~15% of total cell protein provides a means for systematically addressing optimal expression levels in heterologous protein expression.

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