Assessment of aryl hydrocarbon receptor complex interactions using pBEVY plasmids: expression vectors with bi-directional promoters for use in *Saccharomyces cerevisiae*

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ABSTRACT

The pBEVY (bi-directional expression vectors for yeast) plasmids were designed with constitutive and galactose-induced bi-directional promoters to direct the expression of multiple proteins in *Saccharomyces cerevisiae*. Using human estrogen receptor as a test gene, relatively balanced expression levels from each side of a bi-directional promoter were observed. Expression of a functional heterodimeric transcription factor composed of human aryl hydrocarbon receptor (Ahr) and aryl hydrocarbon receptor nuclear translocator (Arnt) proteins was accomplished using a single pBEVY plasmid. Previous studies suggest that inhibitory cross-talk between the estrogen receptor and the Ahr/Arnt complex may occur and that Hsp90–Ahr complex formation is important for Ahr-mediated signal transduction. Evidence for functional interaction among these proteins was investigated using pBEVY plasmids in a yeast system. No inhibitory cross-talk was observed in signaling assays performed with yeast that co-expressed Ahr, Arnt and estrogen receptor. In contrast, Ahr/Arnt-mediated signal transduction was reduced by 80% in a temperature-sensitive Hsp90 strain grown under non-permissive conditions. We conclude that pBEVY plasmids facilitate the examination of multiple protein interactions in yeast model systems.

INTRODUCTION

The ability to genetically manipulate *Saccharomyces cerevisiae* has made this yeast an important tool and model system for contemporary molecular biology (1). For instance, the development of yeast artificial chromosomes (2) and the two-hybrid assay (3) have provided for significant scientific advances. The widely used two-hybrid assay for elucidation of protein function and interactions has a drawback in that construction of chimeric protein derivatives is required. Consequently, some interactions may go undetected and non-specific (false) interactions can be problematic in this system (4). The yeast two-hybrid system is also limited in that interactions between more than two proteins may not be amenable to analysis using this methodology. To circumvent some of these problems, we made a group of plasmids for the expression of intact proteins in yeast. The pBEVY (bi-directional expression vectors for yeast) plasmid family provides a means to alter the genetic background of a yeast strain while allowing for the introduction of additional experimental plasmids. The pBEVY plasmids expand the possibilities for genetic modification of yeast and are particularly useful for the study of proteins which interact or function together in a pathway.

The chief feature of the pBEVY plasmids is the bi-directional promoter, which provides for either constitutive or galactose-induced expression of exogenous genes. The constitutively active bi-directional promoter consists of a fusion between a glyceroldehyde 3-phosphate dehydrogenase (*GPD*) promoter (5) and a fragment of the alcohol dehydrogenase 1 (*ADH1*) promoter (6). The promoter region between the *GAL1* and *GAL10* genes (7,8) serves as a regulatable promoter for the pBEVY-G group of plasmids. The *GAL1,10* promoter is tightly repressed in glucose and displays variable expression in yeast grown on other carbon sources. The Gal4 transcription factor strongly induces expression from the *GAL1,10* promoter in yeast grown in galactose-containing media (9). Some useful features of the pBEVY vectors include unique restriction sites of the bi-directional promoters that allow cDNAs to be easily inserted and four different selectable marker genes for transformation into most strains of yeast. pBEVY plasmids contain a modified pUC19 plasmid region that provides for replication and resistance to ampicillin in *Escherichia coli*. A yeast 2µ plasmid sequence imparts both replication origin and partitioning functions to pBEVY plasmids in yeast (10).

The pBEVY plasmids were created out of a need to simultaneously express several proteins in yeast. We were interested in developing a functional assay to test whether the aryl hydrocarbon receptor complex (AHRC) interacts with other proteins. AHRC is a ligand-activated transcription factor composed of the aryl hydrocarbon receptor (Ahr) and the aryl hydrocarbon receptor nuclear translocator (Arnt) proteins (11). Investigation of these interactions *in vivo* requires co-expression of Ahr, Arnt, a reporter plasmid and a putative interacting protein (e.g. the estrogen

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This manuscript is dedicated to the memory of Michael and Minda Gerber

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receptor) in the same yeast strain. Introduction of four or more plasmids into yeast can prove difficult and can give rise to plasmid instability (10). Additionally, the availability of selectable marker genes for use in plasmid transformation quickly becomes limiting when several proteins must be co-expressed. The availability of pBEVY plasmids makes these types of experiments tractable, because fewer plasmids are needed to express multiple foreign genes in a yeast cell. With these vectors, we reasoned that we could assess stimulatory or inhibitory activity of multiple transcription factors that interact with each other by using reporter gene transactivation assays. In support of our idea, a similar assessment of the effects of transcriptional co-activators on mammalian steroid hormone receptor signaling in yeast has been described (12).

One desirable feature of pBEVY plasmids would be relatively balanced levels of expression from either side of the bi-directional promoter. Below we describe the use of a test gene to assess the activity from each side of the bi-directional promoters. We demonstrate the use of a single pBEVY plasmid to express functional human AHRC, a heterodimeric transcription factor, in yeast. Two cases of reported protein interactions that may involve AHRC were examined in the yeast system using pBEVY plasmids. One set of experiments tested whether interactions between Ahr/Arnt and the estrogen receptor could be identified in this system. In support of this possibility, an antagonistic relationship between the estrogen receptor and the AHRC signal transduction pathways has been reported (13). Inhibitory cross-talk between receptors could account for endocrine disruption effects of aromatic compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (14,15), which is a potent ligand of Ahr. These inhibitory effects may occur at the level of transactivation and the possibility of direct inhibitory interactions between Ahr, Arnt and estrogen receptor exists. Saccharomyces cerevisiae does not naturally express Ahr, Arnt and estrogen receptor homologs and thus it provides a unique cellular background to examine possible interactions between these receptors.

In a second set of experiments, we examined Hsp90–Ahr interactions in yeast. There is strong experimental evidence for a regulatory role of Hsp90 proteins in the folding and tethering of Ahr in the cytoplasm prior to ligand binding (16,17). The regulation of Ahr by Hsp90 proteins appears similar to that of Hsp90-mediated regulation of the glucocorticoid receptor (18,19). The Hsp90 proteins of yeast, Hsp82 and Hsc82, and their associated factors are structurally and functionally conserved among eukaryotes (20,21). Yeast Hsp90 proteins presumably provide good surrogates for their mammalian counterparts, as they have been shown to regulate Ahr (22,23), steroid receptor and tyrosine kinase function in yeast (18,19). Co-expression of Ahr and Arnt proteins using a pBEVY plasmid transformed into yeast along with an AHRC-responsive reporter plasmid provides a genetically tractable system to test for interactions with mutated Hsp90 proteins. If Hsp90 is important for Ahr function, then mutant strains with functionally compromised Hsp90 should show reduced AHRC-mediated signal transduction.

**MATERIALS AND METHODS**

The DH5α strain of E.coli was used for the manipulation and cloning of DNA plasmids (24). The YPH499 (MATα, ade2-101, his3-A200, leu2-Δ1, lys2-801, trp1Δ63, ura3-52), W303a (MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, tryp1-1, ura3-1) and temperature-sensitive iG170D (MATα/a, ade2-1, can1-100, leu2-3,112, trp1-1, Δhsc82::LEU2, Δhsp82::HIS3, ura3-1) strains of yeast were used in assessment of the pBEVY plasmids. The iG170D and W303a strains were provided by S.Lindquist (University of Chicago) (19) and the YPH499 strain was purchased from Stratagene (La Jolla, CA). Yeast were transformed with plasmids using the lithium acetate method (25). Transformants were cultured in a synthetic minimal medium supplemented with amino acids and nucleosides (26). Media components were purchased from Difco and Sigma Chemical Co.

YEpplac195, YEpplac181 and YEpplac112, containing the URA3 (orotidine monophosphate decarboxylase), LEU2 (isopropylmalate dehydrogenase) and TRP1 (phosphoribosylanthranilate isomerase) genes respectively, provide the main vector sequences for generation of the pBEVY plasmid series (27). These plasmids were a gift from R.D.Gietz (University of Manitoba). The pASZ11 vector was kindly provided by P.Linder (Université de Genève) and was used as the source for the ADE2 (phosphoribosylaminomimidazole carboxylase) gene (28). The pGAD424 plasmid was used as a source of the ADH1 promoter and terminator sequences (Clontech, Palo Alto, CA). The bi-directional GAL1,10 promoter was derived from pBM272 (GenBank accession no. U03497). Expression vector p2HG/ER was the source of the GPD promoter (18). The source of the ADH2 terminator sequence, pTTT3, was developed in the laboratory of L.Hyman (Tulane University) (unpublished data). In all cases described below, DNA fragments were purified from low melting point agarose gels following electrophoresis and joined together using T4 phage DNA ligase (24). T4 DNA ligase, restriction endonucleases and other DNA modification enzymes were purchased from New England Biolabs.

Standard PCR methods were used to generate the promoter and terminator sequences of the pBEVY plasmids. To construct the pBEVY-G series of plasmids, the GAL1,10 promoter was amplified by PCR and inserted into the dephosphorylated Smal site within the polylinker of YEpplac181. The GAL1,10 promoter was inserted into the center of the polylinker in an orientation that regenerated a Smal site 3’ of the GAL1 side of the promoter. EcoRI and Hpal were used to remove a portion of the parent plasmid sequence and an ADH2 terminator having an EcoRI overhang and a blunt end was inserted to provide a termination function for transcription from the GAL1 side of the bi-directional promoter. The ADH1 terminator was digested with HindIII and was inserted into the dephosphorylated HindIII site of the plasmid, placing a second terminator 3’ of the GAL10 gene. The resulting plasmid was called pBEVY-GL because of its galactose-induction and the LEU2 selection gene. pBEVY-T and pBEVY-U plasmids were made in a similar manner. pBEVY-A was made by cutting pBEVY-T with AorII and XcmI to remove the TRP1 gene. A fragment containing the ADE2 gene derived from a BglII digest of the pASZ11 plasmid was inserted using blunt ligation to generate pBEVY-A. To make the constitutively expressed pBEVY series, the GAL1,10 promoter was substituted with a constitutively active bi-directional promoter that consisted of a fusion of the GPD promoter and a short derivative of the ADH1 promoter. Thus, four constitutively regulated and four galactose-regulated expression plasmids were generated.

An expression plasmid containing the cDNA of the human estrogen receptor (p2HG/ER) was obtained from K.Yamamoto (University of California at San Francisco) (18). The cDNA encoding the estrogen receptor from p2HG/ER was used to test promoter strengths among the pBEVY plasmids. The estrogen
receptor cDNA was excised from p2HG/ER using a BamHI digest, a Smal digest or a complete XmaI digest with partial SacI cutting. The BamHI estrogen receptor fragments were subcloned into the dephosphorylated BamHI site of pBEVY-T and pBEVY-GT to make pBEVY-T-hERB and pBEVY-GT-hERB, respectively. The XmaI-SacI estrogen receptor fragment was subcloned between the XmaI and SacI sites of pBEVY-GT to make pBEVY-GT-hERS and the Smal fragment was subcloned into the dephosphorylated Smal site of pBEVY-T to generate pBEVY-T-hERS. These pBEVY derivatives express estrogen receptor mRNA from the four promoters of the pBEVY plasmids: GAL1, GAL10, ADH1 and GPD. The lacZ reporter plasmid used for the assessment of estrogen receptor activity, pASSERE, contains the URA3 marker and an estrogen response element from the regulatory region of the vitellogenin gene (18).

Plasmids containing the human Ahr and Arnt cDNAs were obtained from C.Bradfield and O.Hankinson, respectively (29,30). A BamHI fragment containing the human Amt cDNA sequence was excised and ligated into the dephosphorylated BamHI site of pBEVY-GT to create pBEVY-GT-ARNT. Insertion of an XmaI Ahr fragment into the dephosphorylated XmaI site of pBEVY-GT-ARNT produced pBEVY-GT-AHR/ARNT, a plasmid that co-expresses the Ahr and Arnt genes. A LEU2-marked version of this plasmid, pBEVY-GL-AHR/ARNT was made using identical methods.

A TRP1-marked centromeric lacZ reporter plasmid, pRW95-3, was modified by inserting five xenobiotic response elements (XRES) (or dioxin response elements, consensus sequence TNGCGTG) between the BglII and XmaI sites in the polynlinker. This plasmid, called pTXRE5-Z, was used to assess transactivation by the AHR heterodimer as reported by β-galactosidase activity expressed by yeast. A second XRE-containing lacZ reporter plasmid, pDRE23Z, was used in the Hsp90 studies described below. pDRE23Z is a URA3 plasmid that contains a segment from the upstream regulatory region of the murine CYP1AI gene, providing for responsiveness to AHR. Although pDRE23Z contains three XRE consensus matches, it yields ~500-fold lower lacZ activity in comparison with pTXRE5-Z (C.Miller, unpublished). The basis for the large signaling difference between pTXRE5-Z and pDRE23Z is not known.

The use of a reporter plasmid assay provides a simple quantitative test for the degree of transcription factor expression and activation in yeast. In particular, β-galactosidase (lacZ) assays are especially useful in yeast because there is low background activity and a broad signal response range. In conducting these assays, three independently derived transformants were selected and grown overnight at 30°C in a shaking incubator in synthetic glucose medium. The next day 20 µl from each saturated culture was added to 1 ml synthetic medium containing either 2% raffinose or galactose medium. The next day 20 µl from each saturated culture was added to 1 ml synthetic medium containing either 2% raffinose or galactose medium. The overnight culture was used as an inoculum. Ligands were added to a microcentrifuge tube containing 700 µl Z-buffer. The reaction was started by adding 200 µl o-nitrophenol-β-D-galactopyranoside (ONPG) (4 mg/ml solution in Z-buffer). Samples were mixed thoroughly, placed in a 37°C water bath and incubated for 10–60 min as needed to generate a moderate yellow color. Incubation times > 1 h were avoided because β-galactosidase activity declined rapidly over long incubation periods. Four hundred microliters of 1 M sodium carbonate solution was added to stop the reactions and the samples were clarified by centrifugation for 2 min at 14 000 g. The absorbances of the supernatants were read in a spectrophotometer at 405 nm. The activity of β-galactosidase (referred to as Miller or lacZ units) was calculated by the following formula: absorbance at 405 nm × 1000 ÷ (absorbance at 595 nm × ml cell suspension added × min reaction time). Care was taken to be sure readings of cells and enzyme assays were within the linear response range.

RESULTS

General features of pBEVY plasmids

The features of the pBEVY plasmid family are listed in Table 1 and the general structure of a pBEVY plasmid is shown in Figure 1A. The central feature of these vectors is the bi-directional promoter sequence, which allows for expression of two genes from the same plasmid. Figure 1B shows a diagram of the galactose-inducible GAL11,10 promoter of the pBEVY-G series and the constitutively active GPD-ADH1 fusion promoter of the pBEVY series. Two multiple cloning sites (MCS) which flank the bi-directional promoters are shown. The restriction sites in the two MCSs are unique, with the exception that there are multiple XhoI sites in the pBEVY-A and pBEVY-GA plasmids. Each MCS is flanked by a termination sequence. In pBEVY plasmids, the ADH1 promoter is flanked by the ADH2 terminator and the ADH1 terminator flanks the MCS adjacent to the GPD promoter. ADH2 sequences terminate transcription from the GAL1 promoter and ADH1 terminator sequences flank the MCSs of the GAL10 promoter in pBEVY-G plasmids. Both ADH1 and ADH2 terminators function to terminate transcription with high efficiency. Mapping of the 3′-ends of transcripts of both promoters indicates that proper transcriptional termination is conferred by these terminators (data not shown).

Determining relative expression levels from pBEVY promoters

One factor to consider when expressing multiple proteins is that the levels of protein expression from the different promoter

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of estradiol-dependent degree of estrogen receptor expression was indicated by induction pBEVY-GT-hERB and pBEVY-GT-hERS respectively. The relative pBEVY-GT plasmids, creating pBEVY-T-hERB, pBEVY-T-hERS, of the bi-directional promoters of the systematically placed on the promoters in this regard. An estrogen receptor cDNA was human estrogen receptor gene was used to assess the pBEVY indication of how much variation in protein expression there was important in a particular experiment. Thus, we wanted a general would be undesirable if balanced co-expression levels were

the estrogen receptor from either side of the GAL1,10 promoter gave a similar result. Thus, <2-fold differences in lacZ induction were observed from the dual promoter constructs when compared under a given medium condition (Fig. 2A and B). Cells expressing estrogen receptor from the pBEVY-GT (galactose-induced) plasmids displayed a low level of estradiol-dependent activity in cells grown in glucose. The estradiol-induced lacZ activity in glucose may reflect a leakiness of the glucose-repressed GAL1,10 promoter in these particular constructs. Galactose strongly induced β-galactosidase activity (~3000 U) in the presence of the optimal level of estradiol, indicating that signal induction was mediated by the estrogen receptor. We conclude that the levels of estrogen receptor production from the GPD and ADH1 promoters were similar for a given condition and that expression levels from either side of the galactose-regulated bi-directional promoter were almost identical. Thus, the bi-directional promoters of the pBEVY plasmids are capable of producing relatively balanced levels of gene expression.

**Construction and assessment of heterodimeric AHRC expression and function**

In the experiments above we showed that the pBEVY expression vectors can be used to reconstitute a biological activity that is dependent on the expression of a single gene product (estrogen receptor) in yeast. However, many biological pathways involve the interaction of multiple protein components. In our case, we were interested in studying whether a heterodimeric transcription factor, AHRC, could be expressed in a functional form. Furthermore, we
wanted to assess whether other proteins can interact and enhance or interfere with AHRC signaling in yeast. We chose to co-express Ahr and Arnt from a pBEVY-GL plasmid, which affords regulated expression of this heterodimeric transcription factor. Analysis of Ahr and Arnt protein expression levels by isoelectric focusing/SDS two-dimensional gel electrophoresis of whole cell extracts showed two unique spots of ∼97 kDa, pl 6 and 87 kDa, pl 6 from galactose-induced cells that contained pBEVY-GL-AHR/ARNT (data not shown). It is very likely that these two proteins represent induced Ahr and Arnt expression, since they appeared only in cells with the expression plasmid. The staining intensity of Ahr and Arnt protein spots in two-dimensional gels was identical, indicating that Ahr and Arnt were expressed to similar levels. This result is expected, given the well-described equivalent levels of transcriptional output from the bi-directional GAL1,10 promoter (9). Ahr is the ligand-activated component of AHRC and cells containing Ahr and Arnt should respond with enhanced lacZ expression in response to an aromatic hydrocarbon ligand such as β-naphthoflavone (β-NF). Yeast grown in glucose strongly repress the GAL1,10 promoter, in raffinose there is a low level of basal transcription and in galactose there is highly induced expression. These properties were observed in yeast co-expressing Ahr and Arnt from pBEVY-GL-AHR/ARNT (Fig. 3). No detectable response to ligand was seen when these cells were cultured in glucose. Thus, there was no transcriptional leakiness (e.g. reporter activation) of AHRC expressed from pBEVY-GL-AHR/ARNT constructs in cells grown in glucose. Yeast displayed ∼1.8- and 2.5-fold increases in β-galactosidase in response to ligand when grown in raffinose and galactose respectively. The induction in galactose is much greater in magnitude (∼7000 U) than with the other sugars, consistent with the properties of the strongly inducible GAL1,10 promoter. A high background of AHRC activity in the absence of ligand is observed in these experiments and has previously been reported in both yeast and some mammalian cell lines (33,34). The ligand-independent activity could be due to production of an endogenous Ahr ligand or due to other mechanisms that lead to ligand-independent activation of AHRC in yeast. It is clear, however, that there is a ligand-induced component to AHRC activation in the experiments above. Thus, we conclude that this system reflects the expected biology of AHRC, and that we have expressed a functional human heterodimeric transactivator using a single expression vector.

Assessing AHRC–estrogen receptor interactions in yeast

The possibility of interference between the estrogen receptor and Ahr signaling pathways has been reported (13). Since yeast do not naturally express estrogen receptor or AHRC, yeast provide a unique cellular environment to assess whether these proteins interfere directly with each others transactivation. To test the receptor interaction hypothesis, we co-expressed Arnt, Ahr or both in cells that expressed estrogen receptor and the estrogen receptor reporter plasmid pASSERE (Fig. 4). Activation of this simple estrogen response pathway was stimulated by 10 nM estradiol. Cells co-expressing Arnt, Ahr or both along with estrogen receptor were treated with or without the aromatic ligand β-NF. No evidence for transcriptional interference of estrogen receptor signaling was observed, regardless of the presence of Ahr ligand. The converse experiment was also performed. No effect of estrogen receptor (with or without estradiol activation) on AHRC-mediated transcription was observed in this system (data not shown). We also performed experiments designed to under-express AHRC with respect to estrogen receptor levels by reducing Ahr and Arnt copy number via genomic integration. Transactivation by AHRC expressed from single copy genes remained unchanged in cells over-expressing estrogen receptor from a high copy 2µ plasmid in the presence or absence of estradiol (data not shown). Thus, we conclude that there may be no direct modulatory interactions between AHRC and estrogen receptor. If transcriptional interference between these receptors occurs, it may be mediated through an indirect mechanism.

Assessing AHR–HSP90 interactions in yeast

From the results above, it might be concluded that our system is not capable of detecting interactions which affect AHRC function. Hsp90 proteins provide a clear example of a factor that interacts with and imparts function to Ahr. As a positive test for this system, we investigated whether we could detect an interaction between AHRC and Hsp90. Genetically perturbing Hsp90 levels in
We have described the construction of eight plasmids which can be used to modify the genetic composition of most commonly used yeast strains. The chief feature of these plasmids is the bi-directional promoter, which affords simultaneous introduction of two genes into yeast. The GPA/DH1 promoter fusion and the GAL1,10 promoter provide similar levels of expression of a test gene, human estrogen receptor (Fig. 2). Thus, expression from either side of a bi-directional promoter provides for relatively balanced levels of gene expression. Such balanced expression levels are important for proper stoichiometry when testing proteins which are suspected to interact.

Both the GPA/DH1 and the GAL1,10 promoters are known to produce high levels of gene expression and regulation of these promoters under various conditions is well described. Although a transcriptionally weakened fragment of the ADH1 promoter was used in these plasmids, a point that must be considered is that this truncated ADH1 promoter derivative is fused with the GPA promoter. Transactivation from the ADH1 and the GPA promoter sequences is probably a composite of positive and negative contributions of factors which regulate the fused promoter. This 'sharing' of regulatory elements in bi-directional promoters is well described for the GAL1,10 promoter (7,8). The promoter fusion effect may be reflected by the lacZ signal resulting from estrogen receptor expressed from the ADH1 side of the bi-directional promoter of pBEVY-T.

It is important to emphasize that the manner in which a cDNA expression vector is constructed can have dramatic effects on subsequent expression levels. Although there is improved expression by inclusion of specific 5'-untranslated mRNA sequences in yeast expression constructs, the sequence requirements of 5'-untranslated mRNAs are relaxed in yeast relative to that of higher eukaryotes (35). In particular, 5'-untranslated mRNA sequences that contain significant secondary structure can greatly diminish protein translation. For the experiments shown in Figure 2, the estrogen receptor cDNA was subcloned into the first restriction site downstream of the promoter sequence. This minimizes the potential for secondary structure due to folding of polynucleotides sequences in the 5'-untranslated mRNA. The levels of estrogen receptor-induced β-galactosidase activity that were detected in cells expressing pBEVY plasmids (Fig. 2) were ~50% of that observed for another GPA-directed estrogen receptor expression plasmid (p2HG/ER; C. Miller, unpublished data). This result suggests that production of greater amounts of estrogen receptor than that produced from pBEVY plasmids is possible. A refined expression vector design may be needed when maximal amounts of a protein are desired. However, in most cases the level of protein expressed by cDNA insertion into the multiple cloning sites of pBEVY plasmids should be adequate. We expect that the pBEVY plasmids will produce appreciable levels of most proteins, since they have strong promoters and are high copy number plasmids.

The utility of the pBEVY plasmids lies in their ability for expression of multiple proteins in yeast. Co-expression of Ahr and Arnt proteins was accomplished using a single pBEVY plasmid (Fig. 3). The response to β-NF demonstrates that the heterodimeric AHRC is functional in yeast. This specific response was not observed in cells which lacked a component of the complete signal transduction system, e.g. a reporter plasmid lacking XREs or cells lacking either Ahr or Arnt expression (34). The use of carbon sources to regulate expression levels is also demonstrated in Figures 2 and 3. The galactose-induced GAL1,10 promoter gave rise to strong reporter assay signals, indicative of considerable receptor expression under the inducing condition. Variable repression in glucose medium was observed for the GAL1,10 promoter and the leakage of estrogen receptor expression probably resulted in the β-galactosidase activity observed in glucose (Fig. 2B). If small amounts of estrogen...
receptor were made, amplification of the signal through reporter plasmid activation could account for the estradiol-dependent signal observed under repressive conditions. In contrast, the GAL1,10-regulated Ahr/Arnt expression construct displayed very tight glucose repression. Thus, the particular properties of a given construct may affect the resultant expression levels under various conditions.

The utility of the pBEVY plasmids is further demonstrated by analysis of the effects of Ahr, Arnt and AHRC on estrogen receptor signaling and of mutant hsp82 effects on AHRC signaling (Figs 4 and 5). Interference in endocrine pathways by exposure to xenobiotic compounds such as halogenated dioxins and biphenyls, which are Ahr ligands, has been reported as a cause of endocrine disruption (14,15). One possible explanation for the adverse effects of Ahr signaling on the estrogen response pathway is that Ahr and estrogen receptor interact to repress each other (13). We addressed this possibility in our model system. The data in Figure 4 suggest that there is no direct transcriptional interference between Ahr, Arnt, AHRC and estrogen receptor–receptor interference mechanism.

Thus, if there is an antagonism between these two signaling pathways, it appears complex and may not be due to a direct receptor–receptor interference mechanism.

We obtained positive evidence for an effect of Hsp90 mutations on AHRC signaling in yeast, which may be indicative of an Ahr–HSP90 interaction (Fig. 5). Hsp90 proteins are immuno-precipitated as a complex with Ahr, and cellular fractionation studies show that a dimer of Hsp90 proteins along with an ~40 kDa protein exists in a complex with Ahr prior to ligand binding (16,37,38). Additionally, genetic studies in yeast show that chimeric AHR proteins are affected by the level of Hsp90 protein in yeast (22,23). Thus, there is good biochemical and genetic evidence supporting an interaction between Ahr and Hsp90 proteins. Our system differs from the yeast systems previously described in that we expressed intact Ahr and Arnt rather than a single chimeric Ahr protein, and XREs were used as the response elements in our reporter assays. An 80% reduction in transactivation by AHRC with ligand at a non-permissive temperature in the hsp90 mutant strain. Previous experiments show that specific receptor expression levels and the capacity of the general transcription and translational machinery are not affected at non-permissive conditions for hsp82 strains (19,22,23). Thus, the effect we observed was likely to be due to the lack of an effective interaction between Ahr and the temperature-sensitive yeast Hsp90 protein. Previous studies with a chimeric Arnt protein assessed in a yeast system suggest that Arnt does not require an association with Hsp90 (yeast Hsp82) to achieve a functional state (22). When this result is considered in conjunction with our experiments, it suggests that Ahr, rather than Arnt, is the component of AHRC that is affected in the Hsp82 mutant strain.

In conclusion, we have described a family of plasmids that are useful for protein expression in yeast. We speculate that pBEVY plasmids could be employed in a variety of instances (e.g. altering the genetic background while conducting two-hybrid assays) when there is a need to express multiple proteins in yeast. The pBEVY vectors are particularly well suited for the molecular dissection of interactions and pathways using intact proteins expressed in yeast.

**References**