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Use of ERE and Reporter Gene Constructs to Assess Putative Estrogenic Activity

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ABSTRACT

Estrogens primarily function through the activation of their receptors, which subsequently function as nuclear transcription factors. There are two estrogen receptor (ER) genes, now designated ERα (the classic ER gene) and ERβ. The key consequence of the activation of either gene product is the regulation of gene transcription. The extent and nature of transcription appear to be regulated by a series of coregulator proteins.

One of the most sensitive assays for detection of potential estrogenic activity is measurement of the ability of a test compound to influence the transcription of reporter genes. In this regard, many investigators use promoter-reporter constructs. To assess putative estrogenic activity, an estrogen-responsive promoter is generally placed upstream of a reporter gene and transiently transfected into a target cell. When exposed to an estrogenic compound, expression of the reporter gene would normally be induced.

We briefly discuss several issues pertinent to the use of these assays and the interpretation of resulting data, including estrogen-responsive, promoter-reporter constructs, reporter genes and measurements of activity, choice of target cell or cell line, transient introduction of promoter-reporter constructs into cells, basic statistical approaches to data analysis, and definitions of agonist, partial agonist, and antagonist.

INTRODUCTION

ESTROGENS AFFECT A WIDE RANGE of biological endpoints and target tissues. Tissues responsive to estrogenic stimuli include bone, uterine, mammary, cardiovascular, and brain. Within many targets, estrogens affect the cellular choice to proliferate, differentiate, or die. This can influence tissue development and differentiation during early life (e.g., *in utero* exposure to estrogens alters mammary gland differentiation) and susceptibility to chemical carcinogenesis in later life (Hilakivi-Clarke et al., 1997). Reduced estrogenicity (e.g., that accompanying menopause in women) contributes significantly to the increased risk of osteoporosis and death from cardiovascular disease. Administration of exogenous estrogens to these women, in the form of hormone replacement therapy, can help maintain bone density and reduce the risk of cardiovascular disease. In contrast, increased estrogenicity is associated with increases in the risks of breast and uterine cancer (Zeleniuch-Jacquotte et al., 1995; Collaborative Group on Hormonal Factors in Breast Cancer, 1997; Berrino et al., 1996). Estrogens also affect the expression of several

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important behaviors, including sexual, aggressive, and depressive behaviors.

Estrogenicity is apparently pervasive in our environment. Plant chemicals with estrogenic activities (phytoestrogens) have been known for some time. These have been implicated in reproductive toxicity in livestock (e.g., clover disease in sheep). Environmental contamination of alligator habitats with manmade chemicals exhibiting estrogenic activity (xenoestrogens) has been associated with effects on the sexual development of alligators. It is not surprising that there is considerable interest in identifying the natural and other sources of estrogenicity and assessing the importance of exposures to such sources on the regulation of these functions. Successful identification of phytoestrogenic and xenoestrogenic activities could have a significant impact on agriculture, the environment, and human health.

There are several experimental approaches for the assessment of estrogenicity. The most common assays measure either effects on uterine wet weight in castrate rodents (estrogens increase uterine weight), ability to stimulate breast cancer cell proliferation *in vitro* (or occasionally *in vivo*), or ability to induce estrogen-regulated gene transcription (generally through use of promoter-reporter constructs). In this report we focus on issues related primarily to the use of promoter-reporter construct assays.

Estrogen receptors

There are two estrogen receptor genes, now called ERα (human chromosome 6) and ERβ (human chromosome 14). Both function as nuclear transcription factors and act primarily by assembling a productive transcription complex at specific locations (estrogen-responsive elements, or EREs) in the promoters of target genes. The ability of the receptors to function appears to be related to their phosphorylation status (Migliaccio et al., 1989) and their ability to recruit a panel of coregulator/cointegrator proteins (Clarke and Brünner, 1996). Although it is primarily ligand activated, there is evidence from several cell systems that ER can experience a ligand-independent activation. Some cells both express ER and have the ability to synthesize ligand. For example, adipose and breast tissues can aromatize adrenal androgens to estrogens (Bulun and Simpson, 1994; Brodie et al., 1997). Local ligand production can be significant in nonovarian tissues; for example, intratumor estrogen concentrations are similar in some breast cancers regardless of menopausal status.

Estrogen-responsive elements

An ERE is generally that sequence, present in the promoter of a directly ER-regulated gene, to which the activated receptor binds as a dimer. The preferred conformation is an ERα/ERβ heterodimer. The "classic ERE," as found in the vitellogenin A2 promoter, comprises the perfect palindromic sequence GGTCAnnnTGACC. The DNA binding domain of each monomer is presumed to bind to each repeat. The number of genes that express this classic sequence seems small in relation to the number of expressing imperfect palindromic variations. Therefore, there is some degree of freedom within this motif.

A further level of complexity is apparent in the ability of unrelated sequences to act as EREs, for example, a subset of the repetitive Alu DNA sequences that appear scattered throughout the genome (Norris et al., 1995). Recent studies have shown that ER can activate transcription through an AP-1 site (Paech et al., 1997). Although this has been demonstrated with the use of promoter-reporter assays, it remains unclear whether it occurs at the promoters of endogenous genes.

Promoter constructs, reporter genes, and measurements of activity

Several constructs have been relatively widely used, and for many investigators it is probably most effective to choose a previously published and characterized construct. Therefore, only some of the more pertinent issues are discussed briefly here. These comments also may be useful for those who are not familiar with these assays but who wish to generate their own constructs.

Promoter-reporter constructs generally contain a basal promoter sequence, such as the TATA box from the thymidine kinase pro-

moter; the appropriate enhancer sequence (ERE), such as the ERE from the vitellogenin gene; the reporter gene, such as chloramphenicol acetyltransferase (CAT), luciferase (firefly, renilla), green fluorescent protein, or β-galactosidase; and appropriate poly A^+ sequences downstream of the reporter gene (and often upstream to eliminate activity of cryptic promoters in flanking DNA sequences).

In experimental systems, where different promoter constructs are placed adjacent to a reporter gene, the relative organization of the promoter appears to be important in determining the potency of transcriptional regulation. For example, the number of EREs in a promoter and their relative spacing may influence the number of active ERs bound (Klinge et al., 1992). The nucleotide sequence of an ERE also may be important (Klinge et al., 1992); it could significantly influence both the affinity of the ERE for ER (El-Ashry et al., 1996) and its effects on the conformation of DNA-bound receptors. The latter could alter the ability of ER to recruit additional cofactors required for regulation of transcription. Thus, the context of the promoter with respect to its gene may influence transcription.

The choice of a reporter gene determines the sensitivity and ease of the assay. CAT has been widely used, the assay being based on the ability of the enzyme to transfer acetyl groups from acetyl coenzyme A to chloramphenicol. The acetylated and nonacetylated forms can be separated by thin-layer chromatography and detected by autoradiography. CAT-based fluorescence and enzyme-linked immunosorbent assays also are available. Although this method is widely used, detection of CAT activity tends to be less sensitive than that of the products of other reporter genes. Perhaps the most useful reporter genes are the luciferases. These produce a bioluminescent reaction (substrate luciferin), and total light emission is proportional to luciferase activity. Light detection with a luminometer can be up to 1000-fold more sensitive than CAT-based assays. The assays also are more quickly and easily performed.

A further advantage of the luciferase reporter system is the presence of two luciferase genes, which can allow for the concurrent assessment of two reporter genes. The plasmid system developed by Promega (Madison, WI) provides the renilla luciferase downstream of a constitutive promoter. Detection of this activity controls for transfection efficiency and any variability produced by cell lysis efficiency. The firefly luciferase is downstream of sites that facilitate introduction of a regulable promoter motif (e.g., an ERE). Luminescence from the firefly luciferase reaction can be quenched while concurrently activating the renilla luciferase reaction (Dual-Luciferase reporter assay system, Promega, Madison, WI).

Transfection

Most investigators perform these assays by transient transfection, rather than by generating stable transfectants. This reflects the potential chromosomal location to influence expression and regulation of the promoter-reported construct. With transient transfection, the plasmid does not integrate into the genome but is retained for several days within the cells. Ultimately, the plasmids are lost from the cell. Because loss can be rapid and unpredictable, most assays are performed within a few days of the transfection, usually 1–4 days (Ausbel et al., 1997).

The choice of transfection method (e.g., calcium phosphate precipitation, electroporation, retroviral vector transduction, use of polycationic compounds) can influence transfection efficiency. We have found calcium phosphate precipitation and retroviral transduction to produce good, stable transfection efficiencies in human breast cancer cells (Clarke et al., 1989; Leonessa et al., 1996). However, for both stable and transient transfections into breast cancer cells, the SuperFect reagent (Qiagen, Valencia, CA) and the low-CO_2 calcium phosphate precipitation methods produce high efficiency transfection with low toxicity. We found electroporation a relatively poor method (because of high toxicity) in these cells, although it is an effective method in other cells.

Choice of target cell or cell line and the importance of cellular context

The choice of target cells in which to assess estrogenicity may influence outcomes. There are basically two choices, either cells that en-

dogenously express ER and respond to estrogenic stimulation or cells that do not naturally express either receptor but into which ER has been introduced by stable or transient transfection. The choice of cell line for endogenous ER expression depends on whether it is necessary or important to know or study a specific receptor (ERα or ERβ). If an investigator wishes merely to assess estrogenic potential, the MCF-7 human breast cancer cell line (Clarke et al., 1996) provides a reasonable first screen. However, it seems likely that the majority of regulation may be mediated through ERα in these cells. This may not be problematic, because most ligands studied to date do not show marked differences in affinity for ERα compared with ERβ (Kuiper et al., 1997; Tong et al., 1999). A notable exception is the soy isoflavone genistein, which can exhibit more than fivefold increased affinity for ERβ (Kuiper et al., 1997). If this receptor selectivity is seen with other important phytoestrogens or xenoestrogens, it may be necessary to identify or develop specific models for studying ERβ-mediated transcriptional regulation when screening compounds for estrogenicity. ER-negative cell lines used by others, after transfection of ERα or ERβ, or both, include HeLa and COS cells.

Cellular context is important in regulating the estrogenic response (Clarke and Brünner, 1996). ER-negative human breast cancer cells transfected with the ER gene exhibit a growth-inhibited phenotype, the opposite of most ER-positive breast cancer cell lines (Jiang and Jordan, 1992). However, this may not affect how a promoter-reporter construct functions in response to estrogens in these cells. This suggests that, although a ligand may produce an apparent estrogenic response in a promoter-reporter assay, this may not predict the biological role of this estrogenicity.

Agonists, partial agonists, and antagonists

When assessing estrogenic gene regulation, it may be important to determine whether the test compound is functioning as an agonist, antagonist, or partial agonist. Making such an assessment requires a workable definition of these properties, but this is not a simple matter. In principle, an agonist (e.g., 17β-estradiol) and an antagonist (e.g., IC 182,780) produce opposite changes in the same endpoint. A partial agonist (e.g., tamoxifen) displays properties of both agonism and antagonism, with these differential properties expressed in a dose-, tissue-, or species-specific manner. Thus, the definition depends on the endpoint and, as seen for the effects of cellular context, data from a promoter-reporter construct assay may not predict regulation of another biological endpoint. An ER ligand that induces transcription of a growth-suppressor gene would be an antagonist for proliferation and an agonist for gene expression. For example, ICI 182,780 can activate ERβ-dependent transcription from an AP-1 site in MCF-7 cells (Paech et al., 1997), but this ER ligand inhibits MCF-7 cell proliferation (Brünner et al., 1997). These observations suggest that, when performing analyses of activity, it is important to define the endpoint clearly. When an assessment of agonism versus antagonism is required, appropriate positive and negative controls must be included. For example, when studying ERα-mediated transcription, 17β-estradiol may be a useful agonist control, whereas ICI 182,780 is a potentially useful control for antagonism (for example, it should antagonize the effects of 17β-estradiol without producing an agonist response when administered alone). Tamoxifen would be a poor choice because of its partial agonist properties in several systems.

Controls

There are several key controls for the adequate performance of promoter-reporter analyses. It is difficult to provide solid guidelines, because some controls depend on the purpose of the experiment and its design and others on the system being used. However, among the most important are those controlling for transfection efficiency. An excellent control has been alluded previously, the use of a dual reporter system. Many constructs do not have an internal control for transfection efficiency and require cotransfection of a further plasmid containing a second, but constitutively expressed, reporter gene. It is assumed that the transfection efficiency is comparable, allowing the data to be "normalized" using the level of expres-

sion of the constitutive gene. This approach has been widely used and appears to work well in most systems. Additional controls (e.g., vectors without the reporter gene or with the regulable element but without the reporter gene) are often used to assess nonspecific gene expression. Where several plasmids may be introduced into cells, in both control and experimental groups, it may be necessary to ensure that comparable amounts of DNA are introduced into all groups. This can be accomplished by varying the amount of an appropriate control plasmid.

Treatment, dose, and pharmacology

A major goal of reporter-promoter assays is to assess a ligand's potency for regulating gene expression. There are several issues that are often overlooked in this regard, particularly when the test is intended to predict biological activity. For example, it is often assumed that biological activity should correlate with affinity, so that 50% of the maximal biological response (an assessment of biological potency) is seen after treatment with concentrations that approximate the Kd of the ligand for the receptor. A major issue with many ER-positive cell lines, and ER-negative cell lines transfected with an ER gene, is the level of ER expression. In cells that express high levels of receptor, the cells possess "spare receptors." Therefore, there is a poor relationship between receptor occupancy and biological activity, with maximal activity seen at concentrations that may saturate a relatively small proportion of available receptors. This is very likely to be the case with MCF-7 cells, which can express ER levels in excess of 400 fmol/mg protein (Martin et al., 1991) and exhibit significant growth stimulation at concentrations of 17β-estradiol more than one log lower than the apparent Kd. An estrogenic ligand may appear more potent in MCF-7 cells than in cells where there are no "spare receptors."

Most studies should use a dose-response design, in which multiple doses over a predetermined range are evaluated in the same endpoint. This raises the issue of how to choose a dose range. Where the Kd for the receptor is known, this concentration could be placed close to the center of the range. An initial study can be performed using several log scale increments above and below this concentration, with a more rational range used in subsequent studies if a better defined dose-response relationship is required. Alternatively, if there are relevant pharmacologic data available, such as tissue or serum concentrations, these may be used to select a concentration from which increasing and decreasing concentrations can be chosen to produce an appropriate range. When there are no guiding data, a range can sometimes be best determined by estimating the most likely highest concentration and then generating a range that decreases from there on a log scale. The highest concentration can be derived from the chemical properties of the test compound (e.g., its solubility in an appropriate nontoxic solvent).

Data analysis

Approaches to data analysis depend on the general study design, and only very general suggestions can be made. Although it is widely used, Student's t test is generally only appropriate for the comparison of pairs of data and would probably be suboptimal or inappropriate for most time course or dose-response analyses. For such studies, where several groups are independently compared with a single appropriate control, Dunnett's t test or a similar approach may be sufficient (Gad and Weil, 1988). Most other study designs compare expression across several experimental and control groups. These are relatively simple and can often be analyzed by appropriate examination of variance techniques (e.g., comparison of three or more groups of continuous data). However, the data also must be independent and normally distributed, and the variances homogeneous. If the number of observations is small and the distribution of the data cannot easily be determined, it may be necessary to transform the data before analysis (e.g., transformation to a log scale). It may not be possible to transform the data so that they are normally distributed, and nonparametric approaches may be necessary. If it is necessary to estimate a median effective dose, this should be obtained by performing appropriate probit

or logit analyses of the dose-response data (Govindarajulu, 1988; Armitage and Berry, 1994). Where investigators are uncertain, it is advisable to consult with a biostatistician.

CONCLUSION

The use of promoter-reporter assays to assess estrogenicity or antiestrogenicity has become a routine approach for the assessment of estrogenicity in many laboratories. It is well suited as a primary screen for estrogenicity because of its relatively good reproducibility, ease (it can often be readily automated for high throughput), and cost efficiency. The assay can be done on a time scale comparable to that of proliferation-based assays and is easily scaled up to a 96-well format for high throughput. Therefore, it is as effective as any assay using proliferation as the endpoint. However, it has the potential advantage of producing more easily interpretable data. For example, an ER-independent mitogenic compound should have a higher probability of being correctly eliminated in the gene transcription assay. When both an appropriate proliferation-based and a transcription-based assay are concurrently performed, a toxic chemical with no activity through ER also should be effectively identified. Such a compound might be interpreted as an antiestrogen in a purely proliferation endpoint assay.

In vivo assays are too time and cost inefficient to provide a useful primary screen for estrogenicity. However, they do provide a level of rigor not evident in any of the *in vitro* assays. Although a ligand may be fully available to enter cells and interact with ER *in vitro*, its absorption, distribution, metabolism, or elimination may mean that it will never reach target tissues in sufficient concentrations to elicit a biological response *in vivo*. Therefore, a final assessment of biological and/or pharmacological relevance may require an *in vivo* screen. Such *in vivo* screens could involve assessment of changes in rodent uterine wet weight; ability to influence the timing of onset of sexual maturation or to alter mammary gland development or the expression of several behaviors (Hilakivi-Clarke, 1997; Hilakivi-Clarke et al., 1997, 1998); or support or inhibition of the growth of estrogen-dependent human mammary tumor cell xenografts (Clarke, 1996). Nonetheless, promoter-reporter assays provide an easy, rapid and sensitive initial screen to identify compounds that can influence the transcriptional regulatory activities of ER.

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