

# Estrogen-controlled gene expression in tissue culture cells by zearalenone

Ulrich E. Mayr

*Institut für Tierernährung, Universität Hohenheim, D-7000 Stuttgart 70, FRG*

Received 23 August 1988

In two estrogen-sensitive cell lines, Le42 and MCF-7, the estrogenic potential of the nonsteroidal mycotoxin zearalenone has been investigated. The chloramphenicol acetyltransferase (CAT) gene expression in Le42 cells is induced by zearalenone after transfection with a CAT-gene construct controlled by an estrogen responsive element [(1986) Cell 46, 1053–1061]. In MCF-7 cells zearalenone induces at least 2 exoproteins (52 and 160 kDa) which are estrogen-specific [(1980) Cell 20, 353–362]. These data suggest that zearalenone acts by activating the estrogen receptor. Due to the high sensitivity of these cell lines for zearalenone both test systems are proposed as assays for a quantitative estimation of the biological (estrogenic) activity of this widespread mycotoxin.

Mycotoxin; Zearalenone; Estrogen receptor activation; Gene induction; Zearalenone bioassay; (MCF-7, Le42 cell)

## 1. INTRODUCTION

The mycotoxin zearalenone is produced by several *Fusarium* species and is one of the frequently occurring contaminants in cereals and other plant products [3]. It exhibits estrogenic effects and presents a problem mainly to agriculture by causing diseases of the reproductive system, impaired fertility, and abnormal fetal development in farm animals [3]. A health risk to man has to be considered as well. Precocious pubertal changes in children reported in Puerto Rico [4], and the high incidence of esophageal cancer in Africa and China seem to be due to zearalenone ingestion [3,4].

Although its chemical structure is nonsteroidal, zearalenone binds to the estrogen receptor (ER), but with relative low affinity (2–5% of 17 $\beta$ -

estradiol) [5–7]. Several estrogenic effects on molecular level, such as alterations of various hormone concentrations [8], elevation of serum triglyceride levels [9], increased activity of uterine acetylcholine esterase [10] and uterine creatine kinase [5] have been reported. This paper describes the responses of two ER-producing cell lines, MCF-7 [2] and Le42 [11], to zearalenone. Direct evidence is presented that the ER is activated by estrogen and zearalenone basically in the same way, leading to the induction of estrogen-controlled gene products. Furthermore it is shown that estimations of these gene products can serve as highly sensitive bioassays for a quantitative determination of the estrogenic potential of zearalenone. Avoiding animal experiments, these systems present easy to handle alternatives to the in vivo uterotrophic assay performed in rodents [12] which is presently used as the official bioassay for this mycotoxin.

## 2. MATERIALS AND METHODS

### 2.1. Cell culture

MCF-7 cells and Le42 cells were a gift of Dr G. Ryffel, Kern-

*Correspondence address:* U. Mayr, Institut für Tierernährung, Emil-Wolff-Str. 8, Universität Hohenheim, D-7000 Stuttgart 70, FRG

*Abbreviations:* CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; ERE, estrogen responsive element

forschungszentrum Karlsruhe, FRG. Both cell lines were maintained as described [2,11]. 3 days prior to experiments fetal calf serum was replaced by serum treated with dextran coated charcoal (MCF-7 cells) [2] or with Ultrosor SF, Serva (Le42 cells) [11].

## 2.2. Transfection of Le42 cells and CAT assay

The recombinant plasmids pA2(-331/-87)tk-CAT, containing an ERE, and pA2(-260/-87)tk-CAT, without ERE, were provided by Dr G. Ryffel, Karlsruhe, and are described in detail in [1]. Transfections into Le42 cells were performed using the calcium phosphate precipitation technique according to [13]. 10 µg DNA was used per 10 cm petri dish containing  $5 \times 10^5$  cells. After transfection cells were stimulated for 72 h and CAT activity in the lysates was estimated according to [13].

## 2.3. Stimulation of MCF-7 cells and analysis of exoproteins

Stimulation of MCF-7 cells and in vivo labeling with [ $^{35}$ S]methionine were done in microtiter plates (0.8 cm diameter per well) following the protocol described in [2]. Treatment with estradiol and zearalenone continued for 48 or 72 h. Exoproteins were analyzed by SDS-polyacrylamide gel electrophoresis of 25 µl of the medium [15] and subsequent fluorography using Amplify TM (Amersham).

## 2.4. Inactivation and estimation of zearalenone

Zearalenone was inactivated by autoclaving with 2% aqueous ammonia for 30 h. Zearalenone concentration was estimated by high-performance liquid chromatography using a 10 µm Porasil column (Waters), with dichloromethane/acetic acid/ethanol (98:1:1) as the liquid phase, and fluorescence detection (excitation wavelength, 280 nm; emission wavelength, 465 nm) [16].

## 3. RESULTS

To investigate whether the zearalenone/ER complex exhibits the same functional properties as the genuine estrogen/ER complex, a system was used which was developed by Klein-Hitpaß et al. [1] and Druge et al. [11].

This system is composed of ER-producing target cells (Le42) [11] and the recombinant plasmid pA2(-331/-87)tk-CAT [1,11] which is transfected into these cells. Le42 cells are derived from mouse Ltk<sup>-</sup> cells and contain the stable transfected gene of the human ER whose expression is directed by the SV40 'early' promotor [11]. The plasmid used is a derivative of pSV0-CAT [14] containing an ERE from *Xenopus laevis* and bases -150 to +51 of the thymidine kinase promotor of Herpes simplex virus [1]. The CAT gene is transiently expressed after transfection of the plasmid into Le42 cells and subsequent treatment with estradiol [11]. Fig.1 shows the CAT activities after treatment with different concentrations of 17 $\beta$ -estradiol (fig.1a)

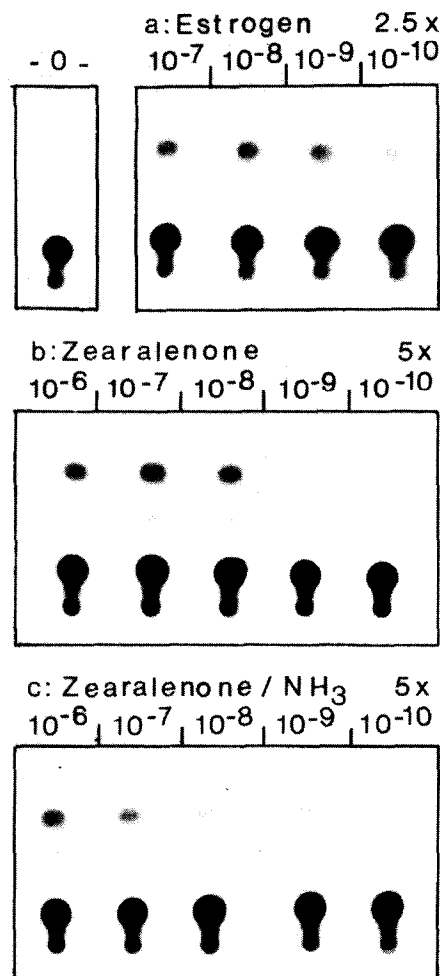


Fig.1. CAT activity in the lysates of  $2.5 \times 10^5$  transfected Le42 cells after treatment with different concentrations of 17 $\beta$ -estradiol (a), zearalenone (b) and ammonia inactivated zearalenone (c). The control (O) represents untreated cells. Concentrations are indicated in molarity (M). The autoradiography of a thin layer chromatogram is shown in which the substrate ([ $^{14}$ C]chloramphenicol, lower spots) is separated from the acetylated forms (the upper spots correspond to 3-acetate chloramphenicol).

and zearalenone (fig.1b). The threshold of induction lies between  $2.5 \times 10^{-10}$  M and  $2.5 \times 10^{-9}$  M with estradiol, while induction with zearalenone requires concentrations between  $5 \times 10^{-9}$  M and  $5 \times 10^{-8}$  M. This result corresponds well with the affinity of zearalenone to the human ER which has been reported to be 20-fold lower than the affinity of 17 $\beta$ -estradiol [7].

The specificity of the system was investigated by zearalenone inactivated by incubation with ammonia (section 2). Ammonia treatment of zearalenone leads to a reduction of its estrogenic potential *in vivo* [17]. The preparation used here contained 5.5% residual, chemically intact, zearalenone. CAT induction with this material is shown in fig.1c. Half-maximal induction was achieved at a concentration of  $5 \times 10^{-7}$  M. Therefore, the capacity of this material is between 10 and 100-fold lower than that of untreated zearalenone which fits well to the concentration of intact zearalenone (5.5%) estimated in this preparation.

In a control experiment Le42 cells were transfected with pA2(-260/-87)tk-CAT, a deletion of pA2(-331/-87)tk-CAT which does not contain the ERE [1]. No induction of CAT activity could be observed after treatment with estradiol or zearalenone (not shown).

These results were substantiated in another system, the MCF-7 cell line, which reconstitutes an established, estrogen-sensitive cell line derived from human breast cancer [2]. Estrogen treatment of these cells leads to the induction of several exoproteins; the most prominent is of 52 kDa, another of 160 kDa [2]. This effect is highly specific, since treatment with other steroid hormones like progesterone or dexamethasone does not lead to the induction of exoprotein synthesis [2].

MCF-7 cells were stimulated with  $17\beta$ -estradiol or zearalenone for 3 days. Exoproteins were labeled *in vivo* with [ $^{35}$ S]methionine, separated by electrophoresis and autoradiographed. Fig.2 shows the results using  $10^{-10}$  M estradiol (lane 2) and zearalenone (lane 3). Induction of a 52 kDa and a 160 kDa protein can clearly be detected after treatment with both compounds. With both estradiol and zearalenone this induction has already reached its maximal level (not shown) at the lowest tested concentration ( $10^{-10}$  M), indicating the high sensitivity of this system. Ammonia-treated zearalenone containing 5.5% intact zearalenone was also tested. Fig.2, lane 4 shows the result using  $10^{-9}$  M of this material. For maximal induction the concentration has to be at least 100-fold higher compared with untreated zearalenone (not shown).

A comparison of the inducing capacities of

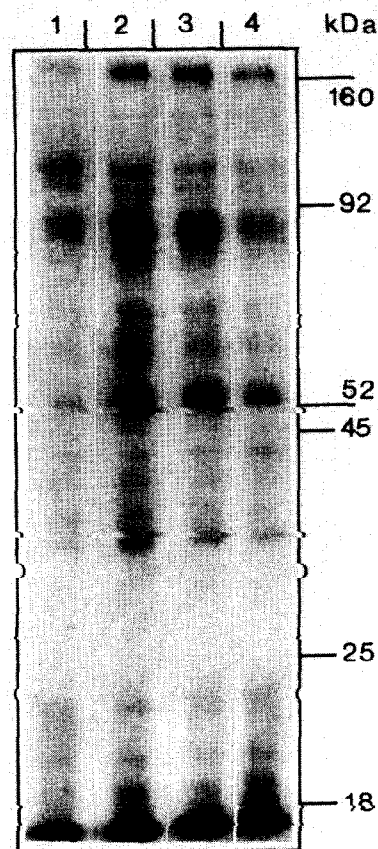


Fig.2. Autoradiography of [ $^{35}$ S]methionine labeled exoproteins produced by MCF-7 cells after 72 h treatment with  $10^{-10}$  M  $17\beta$ -estradiol (lane 2),  $10^{-10}$  M zearalenone (lane 3),  $10^{-9}$  M ammonia inactivated zearalenone (lane 4) and without treatment (lane 1). Proteins were separated by SDS-polyacrylamide gel electrophoresis (15%) using a gel containing 10% acrylamide and 0.27% bisacrylamide.

estradiol and zearalenone (stimulation was for 48 h only; not shown) revealed, that  $17\beta$ -estradiol has an efficiency between 10- and 100-fold higher than zearalenone, a result which is concomitant with the data obtained with transfected Le42 cells.

#### 4. DISCUSSION

The induction of estrogen-controlled genes in estrogen-sensitive tissue culture cell lines by zearalenone supports the idea that this nonsteroidal mycotoxin acts as an agonist by activating the estrogen receptor (ER).

Steroid hormone-dependent gene regulation is not yet understood in full detail. Different models are discussed in [18]. Recent findings concerning glucocorticoid indicate that the steroid free receptor together with hsp90 heat shock protein is part of a heteromeric complex, from which it is released upon addition of the hormone [19]. The steroid hormone ligand seems not to be necessary for specific binding of the receptor to DNA [20,21]. If a similar model holds true for estrogen, the action of zearalenone is the dissociation of a complex containing the ER and a modulating protein.

Gene induction with zearalenone in MCF-7 cells and transfected Le42 cells requires between 10- and 100-fold higher concentrations as compared with  $17\beta$ -estradiol. This is concomitant with the estimation of the affinity of zearalenone to the human ER which in MCF-7 cells has been demonstrated to be 5% as compared with  $17\beta$ -estradiol [7]. It should be noted that the estrogenic potency of zearalenone in living animals, using different assays, is between 100- and 1000-fold lower than that of  $17\beta$ -estradiol [3,5]. An important feature of the described systems is the fact that they should be useful tools in zearalenone analysis: they exhibit high sensitivity, high specificity, and are easy to handle. The most common bioassay for zearalenone is the estimation of the uterotrophic effect in rodents [12], a laborious procedure involving numerous animal experiments. A quantitative estimation of the biological (estrogenic) effect is almost impossible using this method. The systems described here combine the possibilities of detecting the biological activity of zearalenone and its quantitative estimation. Other estrogenic active compounds are not discriminated, but this may even be advantageous, e.g. in assessing the quality of foodstuffs.

The Le42 system can further be improved by the isolation of Le42 clones containing the stable transfected plasmid pA2(-331/-87)tk-CAT. A screening for stably transfected cell lines exhibiting a higher sensitivity towards estrogens or estrogenic active compounds provides an additional interesting aspect.

*Acknowledgements:* Recombinant plasmids, Le42 and MCF-7 cells were a generous gift of Dr G. Ryffel, Kernforschungszentrum Karlsruhe, FRG. The experiments were performed in the Department of Genetics, Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, FRG. I wish to thank Professor J. Collins for the permission to work in his department, Dr G. Gross, Dr H.-J. Hauser, Dr H. Mayer, I. Hollatz and M. Ausmeier for support and advice during this study. The critical reading of the manuscript by Dr G. Gross is gratefully acknowledged.

## REFERENCES

- [1] Klein-Hitpaß, L., Schorpp, M., Wagner, U. and Ryffel, G.U. (1986) *Cell* 46, 1053-1061.
- [2] Westley, B. and Rochefort, H. (1980) *Cell* 20, 353-362.
- [3] Kuiper-Goodman, T., Scott, P.M. and Watanabe, H. (1987) *Regul. Toxicol. Pharmacol.* 7, 253-306.
- [4] Rodriguez, C.A., Bougiovanni, A.M. and Conde de Borrego, L. (1985) *J. Pediatr.* 107, 393-396.
- [5] Katzenellenbogen, B.S., Katzenellenbogen, J.A. and Mordecai, D. (1979) *Endocrinology* 105, 33-40.
- [6] Powell-Jones, W., Ræford, S. and Lucier, G.W. (1981) *Mol. Pharmacol.* 20, 35-42.
- [7] Martin, P.M., Horwitz, K.B., Ryan, D.S. and McGuire, W.L. (1978) *Endocrinology* 103, 1860-1876.
- [8] Hobson, W., Bailey, J. and Fuller, G.B. (1977) *J. Environ. Health* 3, 43-57.
- [9] Mastry, C., Mistry, P. and Lucier, G.W. (1985) *J. Steroid Biochem.* 23, 279-289.
- [10] Nassar, A.Y., Mrgalla, S.E. and Hafez, A.H. (1987) *Mycopathologia* 97, 173-178.
- [11] Druge, P.M., Klein-Hitpaß, L., Green, S., Stack, G., Chambon, P. and Ryffel, G.U. (1986) *Nucleic Acids Res.* 14, 9329-9337.
- [12] Thigpen, J.E., Li, L.A., Richter, C.B., Lebetkin, E.H. and Jameson, C.W. (1987) *Lab. Anim. Sci.* 37, 596-601.
- [13] Graham, F.L. and Van der Eb, A.J. (1973) *Virology* 52, 456-467.
- [14] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell Biol.* 2, 1044-1051.
- [15] Laemmli, U.K. (1970) *Nature* 227, 680-685.
- [16] Olsen, M., Pettersson, H.I., Sandholm, K.A. and Kiessling, K.C. (1985) *J. Assoc. Off. Anal. Chem.* 68, 632-635.
- [17] Lasztity, R., Tamas, K. and Wöller, Ö. (1977) *Ann. Nutr. Aliment.* 31, 495-498.
- [18] Beato, M., Arnemann, G., Chalepakidis, G., Slater, E. and Willmann, T. (1987) *J. Steroid Biochem.* 27, 9-14.
- [19] Denis, M., Poellinger, L., Wikstrom, A.C. and Gustafsson, J.A. (1988) *Nature* 333, 686-688.
- [20] Willmann, T. and Beato, M. (1986) *Nature* 324, 688-691.
- [21] Bailly, A., Le Page, C., Rauch, M. and Milgram, E. (1986) *EMBO J.* 5, 3235-3241.