Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1657–1661, March 1993
Biochemistry

A selective transcriptional induction system for mammalian cells based on Gal4–estrogen receptor fusion proteins

(estrogen-regulable transcription factors/Gal4-responsive promoter/Fos-mediated transformation/Fos target gene)

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Communicated by Max L. Birnstiel, October 19, 1992

ABSTRACT **Most mammalian cells neither express any Gal4-like activity nor endogenous estrogen receptor, thus rendering estrogen an inert signal for them. For these two reasons we have developed a selective induction system based on the estrogen-regulable transcription factor Gal-ER. Gal-ER consists of the DNA-binding domain of the yeast Gal4 protein fused to the hormone-binding domain of the human estrogen receptor and hence should exclusively regulate a transfected gene under the control of a Gal4-responsive promoter in mammalian cells. Two major improvements of this induction system were made. First, a synthetic Gal4-responsive promoter was constructed which consisted of four Gal4-binding sites, an inverted CCAAT element, a TATA box, and the adenovirus major late initiation region. This promoter showed extremely low basal activity in the absence and high inducibility in the presence of ligand-activated Gal-ER. Second, the transcription factor Gal-ER was rendered more potent and less susceptible to cell type-specific variation by fusing the strong activating domain of the herpesvirus protein VP16 onto its C terminus. In response to estrogen, Gal-ER-VP16 induced the Gal4-responsive promoter at least 100-fold in transiently transfected NIH 3T3 and P19 cells. Rat fibroblast cell lines expressing integrated Gal-ER and Gal4-responsive *fos* genes were transformed in a strictly estrogen-dependent manner. The exogenous *fos* gene was rapidly induced to maximal levels within 1–2 hr of estrogen addition. Elevated Fos activity in turn stimulated transcription of the endogenous *fra-1* gene. These data demonstrate the utility of the Gal-ER induction system as a powerful genetic switch for regulating heterologous genes and, in particular, for identifying Fos targets in mammalian cells.**

Inducible expression systems have proven to be valuable tools for elucidating the biological function of a wide variety of genes in bacteria, yeast, and *Drosophila*. The most commonly used induction systems for mammalian cells consist of promoters that are regulated by endogenous transcription factors in response to glucocorticoid hormone (1, 2), heat shock (3), heavy metal ions (4), or interferon (5). These promoters generally suffer from two disadvantages: their basal activity is often relatively high, and the physiological inducers used trigger an endogenous program of gene expression which may interfere with or mask the specific effects elicted by the gene product of interest.

These problems have been addressed by the recent development of induction systems which make use of prokaryotic, yeast, or *Drosophila* transcription factors to regulate the expression of transfected genes in mammalian cells (6–12). The choice of transcription factors is based on the following criteria. First, mammalian cells should not express any activity that is homologous or related to the exogenous transcription factor used. Second, the inducer of this transcription factor should be an inert signal for the mammalian cells used. Third, the binding site for this transcription factor should be complex and therefore unlikely to occur by chance in the control region of a mammalian gene. As a consequence, high selectivity of induction is achieved in mammalian cells since the exogenous transcription factor is only able to transactivate the promoter of a transfected gene containing its specific recognition sequence.

Steroid receptors belong to a family of ligand-inducible transcription factors with separable DNA- and hormone-binding domains (13). The hormone-binding region of the human estrogen receptor contains a ligand-dependent transactivation (14) and dimerization (15) domain as well as a general "protein inactivation" function which, in the absence of hormone, can repress other activities on the same polypeptide chain through interaction with the abundant heat shock protein hsp90 (16). Taking advantage of the inducible transactivation domain, Webster *et al.* (14) have constructed chimeric receptors with novel DNA-binding specificities. The protein Gal-ER, consisting of the DNA-binding domain of the yeast transcription factor Gal4 joined to the C-terminal region of the human estrogen receptor, was shown to stimulate transcription from Gal4-responsive reporter genes in a hormone-dependent manner (14).

Here we describe a transcriptional induction system based on the hybrid transcription factor Gal-ER. This estrogen-dependent induction system is highly selective, as most mammalian tissue culture cells express neither endogenous estrogen receptor (17) nor any Gal4-like activity (10, 18, 19) with the complex sequence requirement of the yeast Gal4 protein (20). We have made several important improvements of the Gal-ER system. Optimal Gal4-responsive promoters with low basal activity and high inducibility by Gal-ER were constructed. Moreover, the transactivation potential of Gal-ER was increased and rendered cell type-independent by incorporation of the strong transactivation domain of the herpes simplex viral protein VP16. Gal-ER was used to conditionally express a Gal4-responsive c-*fos* gene in rat fibroblasts, leading to estrogen-dependent transformation of these cells. This clearly demonstrates the potential of this induction system for regulating heterologous genes in tissue culture cells.

MATERIALS AND METHODS

Plasmid Constructions. The vector pBS-CAT, containing the chloramphenicol acetyltransferase (CAT) gene in pBluescript (Stratagene), was used to synthesize Gal4-responsive promoters (see Fig. 2*C*) by oligonucleotide insertion into the polylinker. The CAT gene of pCAT-4 was replaced in pfos-1 by a 2800-bp *Nae* I–*Bam*HI fragment (mouse c-*fos* gene) of pSVfos (21). pAγ-3′E was generated by cloning the 3.3-kb *Hin*dIII fragment of the human $^{A}\gamma$-globin gene into a pSP64

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Abbreviation: CAT, chloramphenicol acetyltransferase.
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vector containing the simian virus 40 enhancer in its *Eco*RI site. A 1.3-kb *Eco*RI fragment of the Gal-ER fusion gene (74/282; ref. 14) was inserted downstream of the cytomegalovirus (CMV) promoter of the expression vector pRK7 (D. Goeddel, unpublished data) and into the retroviral vector pMV-7 (22) to obtain plasmids pCMV-GalER and pMV-GalER, respectively. A *Sal* I fragment encoding amino acid residues 416–487 of VP16 (23) was cloned into the *Bsm* I site of pCMV-GalER to generate pCMV-GalER-VP16.

Transient Transfections and CAT Assays. These were performed exactly as described (24).

S1 Nuclease Analysis. Fifteen micrograms of cytoplasmic RNA and 15,000 cpm of each labeled DNA probe were used for S1 nuclease analysis as described (24). The γ-globin, *fos* and *fra-1* S1 DNA probes were 5′ end-labeled at the *Bam*HI site in the second exon of the γ-globin gene, at the *Eag* I site in the first exon of c-*fos* (pfos-3), and at the *Sty* I site of rat *fra-1* cDNA, respectively.

Stable Cell Lines. Rat-1A cells were infected with viral supernatant of GP+E-86 cells stably transfected with pMV-GalER. G418-resistant colonies were selected, expanded, and analyzed for Gal-ER mRNA levels by S1 mapping to generate the cell line Rat1A-GalER. The cell line Rat1A-GalER/Fos was obtained by cotransfection of a pool of G418-resistant Rat-1A cells with plasmid pfos-4 (10 μg) and the hygromycin-resistance vector pY3 (1 μg; ref. 25) by the calcium phosphate precipitation method. Pools of doubly resistant cells were selected for growth in soft agar in the presence of estrogen (24). Individual soft-agar colonies were picked and expanded in estrogen-free medium.

RESULTS

Construction of a Synthetic Gal4-Responsive Promoter. Our original interest in developing a selective transcriptional induction system was to introduce conditional Fos activity into rodent fibroblasts with the aim of identifying Fos target genes. For this purpose we have chosen the estrogen-regulable transcription factor Gal-ER (74/282; ref. 14), which consists of the DNA-binding domain of the yeast Gal4 protein (the first 74 amino acids) linked to the hormone-binding domain of the human estrogen receptor (amino acids 282–595; see Fig. 5). The DNA-binding specificity of this hybrid transcription factor is thus provided by Gal4 and the hormone-inducible transactivation function (TAF-2) by the estrogen receptor (14, 26). An important prerequisite for a Gal4-dependent induction system is the availability of an optimal Gal4-responsive promoter which is characterized by low basal activity in the absence and high inducibility in the presence of ligand-activated Gal-ER. The currently available Gal4-responsive genes were obtained by introducing multiple Gal4-binding sites upstream of known promoters which often show, however, elevated basal activity in the absence of Gal4 transactivator (ref. 14; see below). We therefore decided to construct a synthetic Gal4-responsive promoter (Fig. 1) tailor-made for Gal-ER, which is known to homosynergize and to further depend for full activity on cooperation with other upstream transcription factors (26). For this purpose we cloned the initiation region and the TATA box of the adenovirus major late transcription unit (from −39 to +15) upstream of the CAT gene (pCAT-1). We next introduced four palindromic Gal4-binding sites upstream of the TATA box (pCAT-2) and then inserted between these elements the CCAAT box of the murine class II major histocompatibility complex gene *Ea* or the CACCC motif of the mouse β-major-globin gene in both orientations (pCAT-3 to -6). These CAT genes were transiently transfected into NIH 3T3 cells together with a Gal-ER expression plasmid and a β-galactosidase gene used for normalization of transfection efficiencies. The best promoter construct (pCAT-4) was inducible by a factor of 22, reaching 70% of the activity of the simian virus 40 early control region (pSV2cat). Most importantly, the presence of one CCAAT or CACCC box did not increase basal promoter activity in the absence of estrogen. In contrast, a markedly elevated basal activity was observed for a complex β-globin promoter consisting of a CCAAT box and two CACCC motifs linked to two Gal4 binding sites (17M2-G.CAT; ref. 14).

Construction of a Gal4-Responsive *fos* Gene. We next replaced the CAT gene of construct pCAT-4 with a DNA fragment of the mouse c-*fos* gene comprising all four coding exons but lacking the mRNA-destabilizing 3′ noncoding sequences. The plasmid pfos-1 was transiently transfected into NIH 3T3 cells together with the Gal-ER expression plasmid and a γ-globin reference gene, and cytoplasmic RNA was analyzed for *fos* and γ-globin transcripts by quantitative S1 nuclease mapping (Fig. 2). Estrogen clearly stimulated correct transcription initiation of the exogenous *fos* gene by a factor of ≈20, in agreement with the CAT experiments of Fig. 1. However, the plasmid pfos-1 also gave rise to additional prominent transcripts (labeled x and y) which mapped within the leader sequence of the *fos* gene. These RNAs could not be detected by reverse transcription with a *fos*-specific primer (S.B., unpublished results), suggesting that they originated from upstream transcripts by splicing into the *fos* leader sequence. In agreement with this hypothesis, the 5′ noncoding region of c-*fos* contains several AG dinucleotides that are preceded by a polypyrimidine tract and may therefore serve as potential 3′ splice sites (Fig. 2*C*). The spliced upstream transcripts were eliminated by introducing a synthetic poly(A) site (SPA; ref. 27) upstream of the Gal4-responsive promoter in plasmids pfos-2 to -5 (Fig. 2 *A*

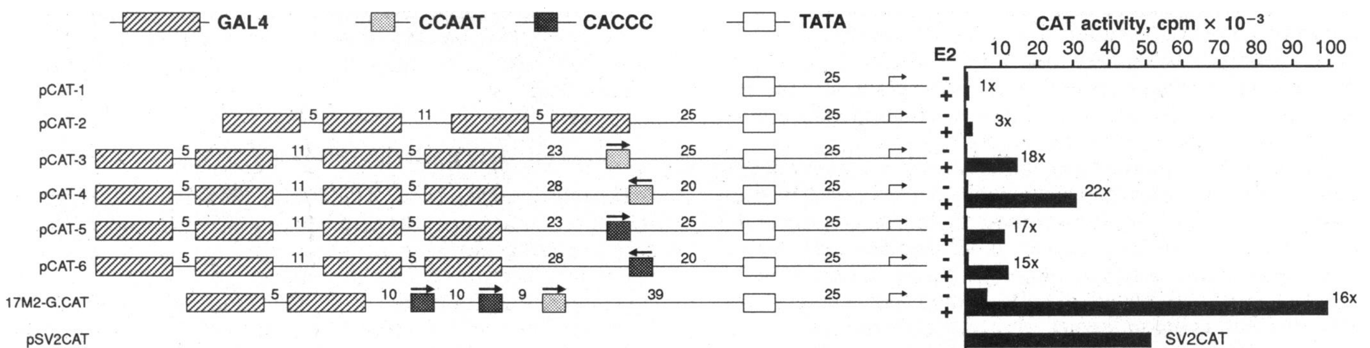

FIG. 1. Construction of Gal4-responsive promoters. Transactivation of synthetic Gal4-responsive promoters by Gal-ER was analyzed by transient transfection of NIH 3T3 tk⁻ cells (thymidine kinase-deficient murine fibroblast line) and CAT assay as described (24). 17β-Estradiol (E2) (1 μM) was added to the medium, where indicated (+). Fold induction by estrogen is indicated. A schematic diagram of the promoters tested is shown at left. DNA sequence motifs are indicated by boxes and the distances between them by the number of intervening base pairs.

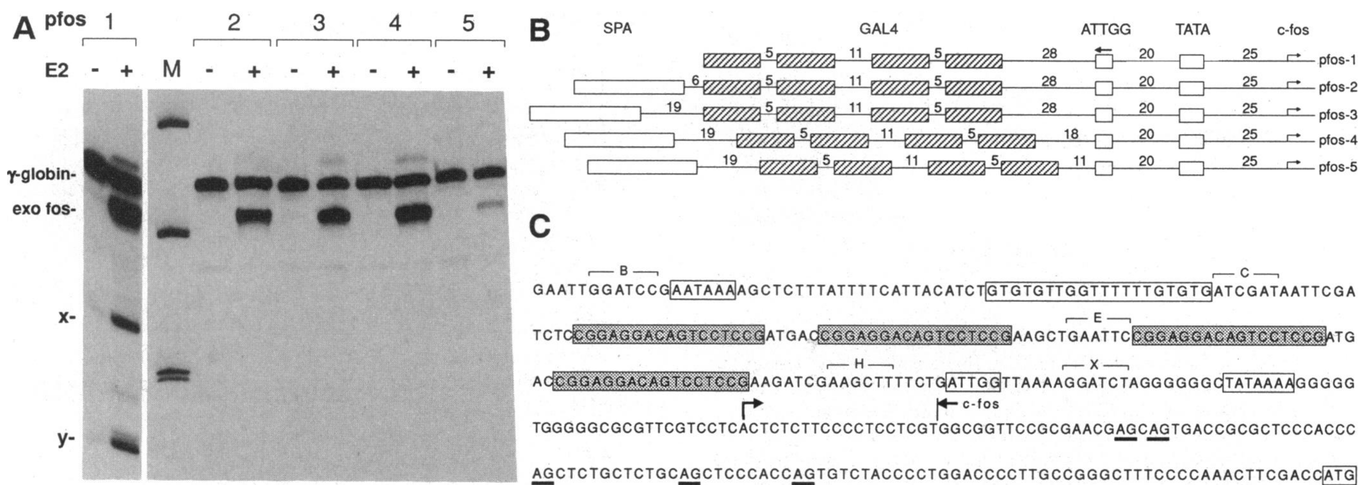

FIG. 2. Construction of a Gal4-responsive c-*fos* gene. (*A*) Estrogen-dependent transactivation of Gal4-responsive *fos* genes by Gal-ER. The plasmids pCMV-GalER (1 μg), pfos (5 μg), and pAγ-3′E (3 μg) were transiently transfected into NIH 3T3 tk⁻ cells and their expression was detected by S1 nuclease analysis. 17β-Estradiol (E2) (1 μM) was added to the medium, where indicated (+). Lane M, DNA size markers (242, 190, and 147 bp). exo, Exogenous. (*B*) Schematic diagram of the Gal4-responsive promoters used to drive transcription of the mouse c-fos gene. SPA, synthetic poly(A) site. Distances are given in base pairs. (*C*) Promoter and leader sequences of the Gal4-responsive c-*fos* gene of pfos-4. The translation initiation codon and the sequence motifs shown in *B* are boxed, the AG dinucleotides of potential 3′ splice sites are underlined, and the transcription initiation site is denoted by an arrow. B, *Bam*HI; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; X, *Xho* II.

and *B*). In addition, the distance between the Gal4-binding sites and the inverted CCAAT box was shortened by one turn or almost two turns of the DNA helix in plasmids pfos-4 and -5, respectively. The Gal4-responsive promoter of pfos-4 consistently displayed the highest induction ratio of all constructs (Fig. 2*A*) and was therefore used for all subsequent experiments.

Transformation of Rat Fibroblasts by Estrogen-Inducible *fos* Expression. As overexpression of the mouse c-Fos protein leads to transformation of rat fibroblasts (24), we were interested to see whether conditional transformation of these cells could be obtained with the Gal-ER induction system. For this purpose we have chosen Rat-1A cells, which do not express any endogenous estrogen receptor (S.B., unpublished data). These cells were infected with the retroviral vector pMV-GalER, expressing both Gal-ER and a neomycin-resistance gene. A pool of neomycin-resistant cells was then cotransfected with the Gal4-responsive *fos* gene of pfos-4 and a hygromycin-resistance gene (25). Doubly resistant cells were selected for estrogen-dependent growth in soft agar and individual cell lines (referred to as Rat1A-GalER/Fos) were established by expanding single soft-agar colonies (see *Materials and Methods*). Fig. 3 presents the analysis of one of these cell lines together with a Gal-ER-expressing control cell line. Estrogen had no effect on the morphological appearance or soft-agar growth of the control cell line, indicating that Gal-ER alone has no intrinsic transforming potential. In contrast, estrogen treatment of the cell line Rat1A-GalER/Fos resulted in overt morphological transformation and growth in soft agar, which is similar to that observed with a Rat-1A cell line constitutively expressing c-Fos (Fig. 3). Previous work established a posttranslational Fos induction system by fusing the c-Fos protein at its C terminus to the hormone-binding of the human estrogen receptor. The transforming activity of this Fos-ER fusion protein was shown to be subject to strict estrogen-dependent control (24). As shown in Fig. 3, both the transcriptional Gal-ER/Fos and the posttranslational Fos-ER induction systems are comparable with regard to estrogen-dependent transformation of Rat-1A fibroblasts (see *Discussion*).

Rapid Induction of the Exogenous *fos* and Endogenous *fra-1* Genes. The induction kinetics of the Gal4-responsive fos gene in Rat1A-GalER/Fos cells were analyzed by S1 nuclease mapping (Fig. 4*A*). Exogenous *fos* mRNA rapidly accumulated within 30 min after estrogen addition and reached a maximal level between 1 and 2 hr. The transfected *fos* gene was expressed at only a low level in untreated cells and was at least 20-fold induced by estrogen treatment. The maximal mRNA expression level of the exogenous *fos* gene was comparable to that of the endogenous c-*fos* gene in serum-stimulated Rat-1A cells (S.B., unpublished data).

By taking advantage of the alternative Fos-ER induction system, it was recently discovered that ligand-induced Fos-ER activity leads to transcriptional stimulation of the endogenous *fra-1* gene (G. Bergers and M.B., unpublished data). A detailed description of these findings will be pub-

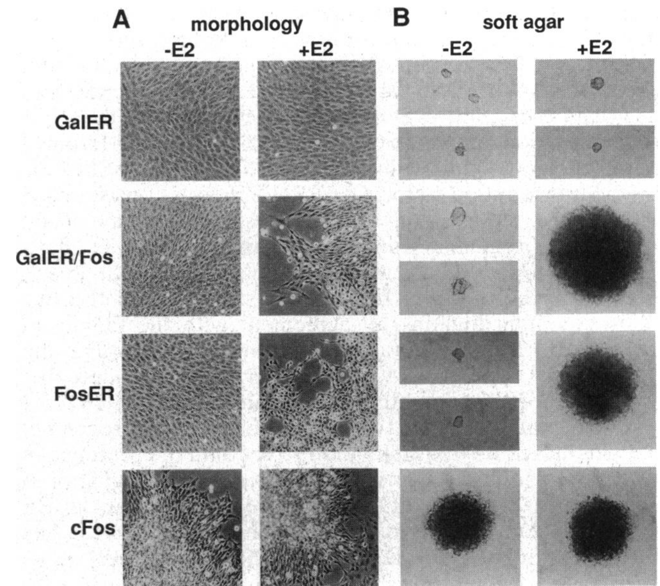

FIG. 3. Estrogen-dependent transformation of Rat-1A fibroblasts by a Gal-ER-responsive c-*fos* gene. (*A*) Morphological transformation. Cells of stable Rat-1A lines constitutively expressing Gal-ER, *fos*-ER, or *fos* genes were plated with or without 1 μM 17β-estradiol (E2). The cell line Rat1A-GalER/Fos contains the Gal4-responsive *fos* gene (pfos-4) in addition to the Gal-ER gene. Photographs were taken after 5 days. (*B*) Growth of the same cell lines in soft agar. Representative colonies were photographed 20 days after seeding.

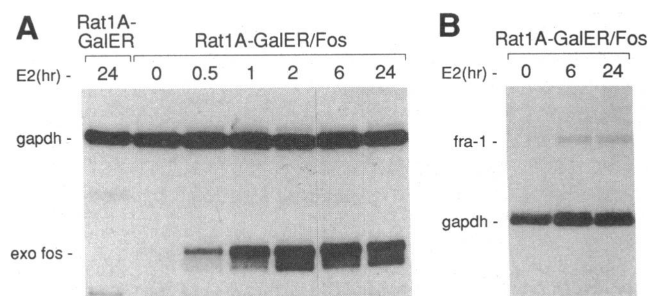

FIG. 4. Kinetics of induction of the exogenous (exo) *fos* and endogenous *fra-1* genes in Rat1A-GalER/Fos cells. (*A*) Rapid induction of the Gal4-responsive *fos* gene. Rat1A-GalER/Fos cells were treated with 1 μM 17β-estradiol (E2) for the indicated time and expression was monitored by S1 nuclease analysis. Expression of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene was monitored as a control. (*B*) Induction of the endogenous *fra-1* gene. The same RNAs were mapped with *fra-1* and gapdh S1 probes.

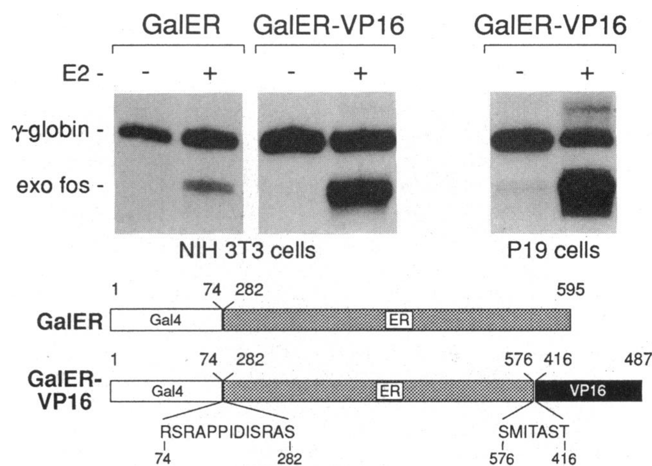

FIG. 5. Transcriptional regulation by the chimeric transcription factor Gal-ER-VP16. The transcription factors GalER and GalER-VP16 are schematically diagrammed with numbers referring to amino acid positions within the different proteins. pCMV-GalER or pCMV-GalER-VP16 (0.2 μg) was transiently transfected together with pfos-4 (5 μg) and pAγ-3′E (3 μg) into NIH 3T3 tk⁻ or P19 cells. 17β-Estradiol (E2) (1 μM) was added to the medium, where indicated (+). Expression of the γ-globin gene and exogenous (exo) *fos* gene was detected by S1 nuclease analysis.

lished elsewhere. In Fig. 4*B*, we show by S1 nuclease analysis that estrogen induction of exogenous fos expression in Rat1A-GalER/Fos cells also resulted in an increase of endogenous *fra-1* gene transcription. These data confirm, therefore, the importance of c-Fos/AP-1 for *fra-1* gene regulation and demonstrate the feasibility of the Gal-ER induction system for identifying Fos target genes.

Gal-ER-VP16: A Cell Type-Independent Transcription Factor. The human estrogen receptor is a modular enhancer factor with two independent nonacidic transactivation functions (TAF-1 and TAF-2) each of which exhibits some cell type specificity (26, 28). The presence of two distinct activation domains therefore increases the potential of the estrogen receptor to regulate transcription in different cellular backgrounds. Gal-ER contains only the hormone-inducible transactivation domain TAF-2, which was shown to be almost inactive in chicken embryo fibroblasts (26). To render Gal-ER less cell type-dependent, we have modified this transcription factor by grafting onto it the transactivation domain of the herpesvirus protein VP16 (23). This VP16 domain encodes one of the strongest acidic activation functions (29), does not transcriptionally interfere with TAF-2 activity (28), functions in species from yeast to man (30), and, like TAF-2, is able to homosynergize and cooperate with upstream transcription factors (26). We have fused the VP16 transactivation domain to Gal-ER at position 576, C-terminal to the hormone-binding domain (amino acids 302–552). This hybrid transcription factor, Gal-ER-VP16, was consistently 5 times more potent than Gal-ER in stimulating the Gal4-responsive *fos* gene in transiently transfected NIH 3T3 cells (Fig. 5). This relatively modest increase appears to result from an additive rather than synergistic effect of the two transactivation domains, in agreement with the finding of Tora *et al.* (26) that the activating functions of TAF-2 and VP16 do not synergize with each other. However, note that grafting the VP16 transactivation domain onto Gal-ER did not lead to elevated basal activity in the absence of estrogen but only increased its transactivation strength in the presence of estrogen, resulting in transcriptional induction by a factor of 100. During the course of this work we realized that Gal-ER was only weakly active in the embryonal carcinoma cell line P19 (P.G., unpublished result). Gal-ER-VP16 proved, however, to be a strong transactivator of the Gal4-responsive fos gene in these cells (Fig. 5), thus indicating that incorporation of the VP16 transactivation domain turned Gal-ER into a more versatile, cell type-independent transcription factor.

DISCUSSION

This study describes a selective transcriptional induction system for mammalian cells. Central to this system is the use of the hybrid transcription factor Gal-ER (74/282; ref. 14), which is composed of the DNA-binding domain of the yeast Gal4 protein and of the hormone-binding domain of the human estrogen receptor. Both the DNA-binding and transactivation functions of this transcription factor are under strict hormonal control. A characteristic feature of Gal-ER is its exceptional target-gene specificity, which is determined by the inducer (estrogen) as well as by the Gal4 DNA-binding domain. Most mammalian cell types do not express endogenous estrogen receptor (17). Estrogen is therefore an inert signal for these cells and is able to stimulate only the exogenous Gal-ER transcription factor. Further, mammalian cells lack any Gal4-like activity (10, 18, 19). In addition, the yeast Gal4 protein recognizes a complex palindromic sequence of 17 bp (20) which is unlikely to occur by chance in a mammalian gene promoter. Indeed, the Gal4 recognition sequence was not found by computer search in the mammalian sequences of the GenBank and EMBL data libraries. Based on these considerations, Gal-ER should exclusively regulate a transfected Gal4-responsive gene in most mammalian cells.

Tora *et al.* (26) demonstrated that the hormone-inducible transactivation function TAF-2 of Gal-ER is strongly stimulated by homosynergism and by cooperation with other upstream transcription factors. According to these findings we synthesized a Gal4-responsive promoter which was optimized for low basal activity in the absence and high inducibility in the presence of ligand-activated Gal-ER. Our best promoter construct consisted of four Gal4-binding sites, an inverted CCAAT element, a TATA box, and the adenovirus major late transcription initiation region. In NIH 3T3 cells this promoter was consistently stimulated 20-fold by Gal-ER and 100-fold by Gal-ER-VP16. The utility of the Gal-ER induction system was further demonstrated in rat fibroblasts by using the mouse c-*fos* gene as a physiological reporter linked to the Gal4-responsive promoter. Rat-1A cell lines containing both the exogenous c-*fos* and Gal-ER genes were untransformed in the absence of estrogen but clearly transformed in the presence of estrogen. The induction kinetics of the exogenous c-*fos* gene were rapid, reaching maximal levels within 1–2 hr of estrogen treatment and were therefore similar

to those of many immediate early genes in response to signal transduction (31).

Previous work established a posttranslational Fos induction system by fusing the c-Fos protein at its C terminus to the hormone-binding domain of the human estrogen receptor. Three activities of c-Fos—i.e., AP1-dependent transactivation, repression of the endogenous *c-fos* promoter, and cellular transformation—were shown to be subject to hormonal control in this Fos-ER fusion protein (24). Both the transcriptional Gal-ER/Fos and the posttranslational Fos-ER induction systems are equally well suited for regulating Fos activity in rat fibroblasts. From a practical point of view, the Fos-ER system appears to be simpler, as it requires transfer of only a single gene into mammalian cells. Moreover, the fusion gene is expressed from a constitutive rather than a regulated promoter. However, the hormone-binding domain of steroid receptors harbors many different functions, including a potent transactivation domain (13) which, upon fusion, may modify the activity of another transcription factor. Furthermore, hormone regulation critically depends on the topology of fusion (16) and has so far been demonstrated for only a few transcription factors (refs. 16 and 24 and references therein). In contrast, the more general Gal-ER induction system allows regulated expression of native protein from any gene that is cloned downstream of the Gal4-responsive promoter. In this respect it is important to note that induction of Fos activity by either the Fos-ER or the Gal-ER/Fos system (Fig. 4) resulted in transcriptional stimulation of the endogenous *fra-1* gene in rat fibroblasts, thus demonstrating the utility of both induction systems for identifying Fos target genes.

The efficacy of the Gal-ER induction system is dependent on the cellular background, as the transactivation function TAF-2 of Gal-ER displays different degrees of activity in different cell lines (26). Gal-ER was modified by incorporation of a second, qualitatively different transactivation function of the herpesvirus protein VP16. The strong acidic activation domain of VP16 functions in a wide spectrum of cells (23, 28–30) and hence rendered the transcription factor Gal-ER-VP16 not only more potent, but also less susceptible to cell type-specific variation (Fig. 5). A constitutively DNA-binding derivative of the Gal-ER-VP16 protein was only weakly active in the absence of hormone, indicating that the VP16 transactivation function, like TAF-2, was also subject to hormonal regulation (S.B., unpublished results). Gal-ER-VP16 is therefore a tightly regulated transcription factor, as four of its essential activities—i.e., dimerization, DNA binding, and both transactivation functions—are under strict hormonal control.

How does the Gal-ER induction system compare with other transcriptional induction systems? The *lac* repressor–operator system of *Escherichia coli* was previously used to regulate heterologous genes in mammalian cells (6–8). A modified version of this system combines the advantage of high target-gene specificity with very high induction ratios (≈1000-fold; ref. 9). In comparison, the best induction ratio (100-fold) obtained with Gal-ER-VP16 is 1 order of magnitude lower but still high enough to allow stringent transcriptional regulation. More importantly, the induction kinetics of the Gal-ER system (1–2 hr) are much faster than those of the *E. coli lac* repressor–operator system, which reaches maximal induction levels only after 24 hr (8, 9). Furthermore, the inducer of the *lac* system, isopropyl β-D-thiogalactopyranoside, is known to be toxic at high concentrations for mammalian cells (7), which further limits the utility of the *lac* repressor–operator system. These disadvantages have been overcome by the recent development of a tightly regulated induction system based on the *E. coli* tetracycline repressor (12), which appears to be comparable to our Gal-ER induction system with regard to its induction kinetics.

We thank P. Chambon for the Gal-ER (74/282) gene and 17M2-G.CAT plasmid, D. Goeddel for pRK7, G. Schaffner for oligonucleotide synthesis and DNA sequencing, T. Kisliakova for suggesting the P19 experiment, H. Tkadletz for graphical work, and M. Cotten for critical reading of the manuscript. This work was supported by the Institute of Molecular Pathology and, in part, by a grant from the Austrian Industrial Research Promotion Fund.

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