7. M. D. Garrett, A. J. Self, C. van Oers, A. Hall, *ibid.*, p. 10.
8. P. G. Polakis, B. Rubinfeld, T. Evans, F. McCormick, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 239 (1991).
9. B. Rubinfeld *et al.*, *Cell* **65**, 1033 (1991).
10. G. F. Xu *et al.*, *ibid.* **63**, 835 (1990); R. Ballester *et al.*, *ibid.*, p. 851.
11. G. A. Martin *et al.*, *ibid.*, p. 843.
12. M. Frech *et al.*, *Science* **249**, 169 (1990).
13. K. Zhang *et al.*, *Nature* **346**, 754 (1990); M. Nori, U. S. Vogel, J. B. Gibbs, M. J. Weber, *Mol. Cell. Biol.* **11**, 2812 (1991); J. E. DeClue, K. Zhang, P. Redford, W. C. Vass, D. R. Lowy, *ibid.*, p. 2819.
14. J. B. Gibbs, M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, U. S. Vogel, *J. Biol. Chem.* **265**, 20437 (1990).
15. C. Calès, J. F. Hancock, C. Marshall, A. Hall, *Nature* **332**, 548 (1988).
16. H. Adari, D. R. Lowy, B. M. Willumsen, C. J. Der, F. McCormick, *Science* **240**, 518 (1988).
17. J. B. Gibbs, M. D. Schaber, T. L. Schofield, E. M. Scolnick, I. S. Sigal, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6630 (1989); A. Hall, *Cell* **61**, 921 (1990); A. Yatani *et al.*, *ibid.*, p. 769; F. McCormick, *Mol. Carcinog.* **3**, 185 (1990); C. L. Farnsworth, M. S. Marshall, J. B. Gibbs, D. W. Stacey, L. A. Feig, *Cell* **64**, 625 (1991).
18. M. D. Schaber *et al.*, *Proteins Struct. Funct. Genet.* **6**, 306 (1989).
19. K. Zhang, M. Noda, W. C. Vass, A. G. Papageorge, D. R. Lowy, *Science* **249**, 162 (1990).
20. Each chimera is identified by a three-letter name followed by two numbers in parentheses. For each chimera, the letter designation means that the chimera has only one segment encoded by that gene; the numbers following refer to the NH_2-terminal and COOH-terminal amino acids encoded by that segment.
21. S. K. Srivastava, A. Di Donato, J. C. Lacal, *Mol. Cell Biol.* **9**, 1779 (1989).
22. P. A. Hart and C. J. Marshall, *Oncogene* **5**, 1099 (1990).
23. E. F. Pai *et al.*, *EMBO J.* **9**, 2351 (1990).
24. O. Fasano *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4008 (1984).
25. M. V. Milburn *et al.*, *Science* **247**, 939 (1990); I. Schlichting *et al.*, *Nature* **345**, 309 (1990).
26. K. Zhang *et al.*, unpublished data.
27. J. Gibbs, M. D. Schaber, M. S. Marshall, E. M. Scolnick, I. S. Sigal, *J. Biol. Chem.* **262**, 10426 (1987); T. Satoh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7926 (1990).
28. J. E. DeClue, B. D. Cohen, D. R. Lowy, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9914 (1991).
28a. H. Maruta, J. Holden, A. Sizeland, G. Dabaco, *J. Biol. Chem.* **266**, 11661 (1991).
29. Chimeric genes were made from the plasmids Krev-1 (a synthetic *rap*1A gene), v-*ras*-AT, pK39, and pK40, which have been described (*19*). Each of these genes contain unique Hind III (at codon 5), Pst I (at codon 60), and Xho I 3′ from the stop codon sites. A c-ras^H gene with a Pst I site at codon 60 was made by site-directed mutagenesis. Each chimera was constructed by first making the appropriate Pst I to Xho I fragment that would encode all amino acids COOH-terminal to residue 60. Plasmids pK39 and pK40 were used for *rap*(61–184) and *rap*(61–109), respectively. The fragments containing this Pst I to Xho I segment of *ras*(1–74), *ras*(1–65), and *ras*(1–65)T were synthesized from Krev-1 by means of the polymerase chain reaction (PCR). The partially homologous oligonucleotide used as the 5′ primer contained the desired coding sequence from codons 60 to 80 and the 3′ primer was oligonucleotide complementary to the 3′ noncoding region including the Xho I site. The fragments containing this segment of *rap*(61–74) and *rap*(61–65) were made from c-ras^H as above (oligonucleotide sequences are available on request). The Pst I and Xho I fragments containing these chimeric COOH-terminal regions were used to replace the COOH-terminal region of c-ras^H, v-*ras*-AT in a retroviral vector, pBW1423 as described (*30*). Chimeric genes encoding Ras(61–189), Ras(61–74), and Ras(61–65) were constructed by replacing the region encoding the COOH-terminal region of Rap1A with the Pst I to Xho I fragments encoding Ras, Ras(1–74) and Ras(1–65), respectively. To express chimeric proteins in bacteria, the Hind III to Xho I fragments encoding the amino acids from 5 through the COOH-terminus and the 3′ noncoding region were inserted into the Hind III and Sal I sites of bacterial expression vector 166A3 (*34*), which encodes amino acids 1 to 4 of Ras and contains a λ p1 promoter.
30. B. M. Willumsen *et al.*, *Mol. Cell. Biol.* **6**, 2646 (1986).
31. M. E. Furth, L. J. Davis, B. Fleurdelys, E. M. Scolnick, *J. Virol.* **43**, 294 (1982).
32. J. E. DeClue *et al.*, *Mol. Cell. Biol.* **11**, 3132 (1991).
33. The cells were washed with 2 ml of phosphate-free Dulbecco's minimal essential medium (DMEM; Gibco) on [^{32}P]orthophosphate (0.5 mCi/ml; Amersham, PBS11A) in phosphate-free DMEM supplemented with dialyzed fetal calf serum (10%). After 12 hours, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and disrupted in 0.8 ml of lysis buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, NP40 (0.5%), aprotinin (1%)]. The nuclei were removed by centrifugation for 5 min in 1.5-ml plastic tubes. Monoclonal antibody Y13-238 (*31*) was added to the supernatant of lysates from cells transfected with constructs encoding v-Ras, c-Ras, Rap(61–109), Rap(61–74), and Rap(61–65), and a rabbit polyclonal antibody to Rap was added to lysates from cells transfected with constructs encoding Ras(1–74), Ras(1–65), and Ras(1–65). Fifty microliters of protein A–Sepharose (40%) were then added, and the mixtures were rotated at 4°C for 1 hour. The precipitate was washed three times with lysis buffer and resuspended in 15 μl of 10 mM tris-HCl (pH 7.5), SDS (1%), 20 mM EDTA, 10 μM GTP, and 10 μM GDP. The suspension was heated at 65°C for 5 min and centrifuged briefly. Five microliters of the supernatant were spotted on a polyethyleneimine (PEI) cellulose thin-layer plate, and developed in 1.3 M LiCl. The guanine nucleotides were visualized by exposure to x-ray film for 2 to 5 days. The radioactivity of GTP and GDP was quantified with an AMBIS Radioanalytic Image System. The percentage of nucleotide bound that was GTP was calculated on the basis of the radioactivity. All guanine nucleotides were assumed to be labeled uniformly.
34. J. C. Lacal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5305 (1984).
35. We thank B. Willumsen for helpful discussions and a critical reading of the manuscript.

25 May 1991; accepted 9 September 1991

Cloning and Expression of the cDNA for Human γ-Glutamyl Carboxylase

SHEUE-MEI WU, WING-FAI CHEUNG, DAN FRAZIER, DARREL W. STAFFORD*

The cDNA for human γ-glutamyl carboxylase, which accomplishes the post-translational modification required for the activity of all of the vitamin K–dependent proteins, was cloned. The enzyme is a 758-residue integral membrane protein and appears to have three transmembrane domains near its amino terminus. The hydrophilic COOH-terminal half of the carboxylase has 19.3 percent identity with soybean seed lipoxygenase. Expression of the cloned cDNA resulted in an increase in carboxylase activity in microsomes of transfected cells compared to mock-transfected cells.

THE POST-TRANSLATIONAL MODIFICATION of glutamic acid to γ-carboxyglutamic acid accomplished by γ-glutamyl carboxylase is essential for the activity of all of the "vitamin K–dependent" proteins. These include some of the blood coagulation and anti-coagulation proteins as well as bone gla (γ carboxyglutamic acid) protein and bone matrix protein (*1*). Carboxylase is an integral membrane protein that was recently purified to near homogeneity (*2*). We now report the cloning, sequencing, and expression of the cDNA for the carboxylase. Knowledge of this sequence allows a better understanding of the mechanism of carboxylation, and availability of the expressed enzyme may allow production of fully functional carboxylated blood coagulation proteins in large amounts from cell culture.

The primary sequence of nine different tryptic peptides from the purified bovine γ-glutamyl carboxylase was obtained (Fig. 1). The longest contiguous amino acid sequence determined was 37 residues; part of this sequence was used for polymerase chain reaction (PCR) amplification of an 86-nucleotide (nt) fragment of carboxylase cDNA. The 55-nt sequence between the two PCR primers (Fig. 1) was used to generate a unique probe for screening a bovine liver cDNA library (*3*). This screening yielded three different partial clones, one of which, λ ZAP bGC1.6, contained the longest carboxylase cDNA insert and included six of the tryptic fragments of carboxylase. The entire λ ZAP bGC1.6 insert was used to screen a human liver cDNA library (*3*). More than 15 positive clones were identified; however, all of the cDNA inserts were small and were located in the 3′ end of λ ZAP bGC1.6. Subsequently, an Eco RI–Bgl II fragment [about 280 base pairs (bp)] from the 5′-end of λ ZAP bGC1.6 was used to screen a human erythroleukemia (HEL) cDNA library in λ gt10 (*4*). One cDNA clone, λ gt10.hGC, which contained the

Department of Biology and Center for Thrombosis and Hemostasis, University of North Carolina, Chapel Hill, NC 27599-3280.

*To whom correspondence should be addressed.

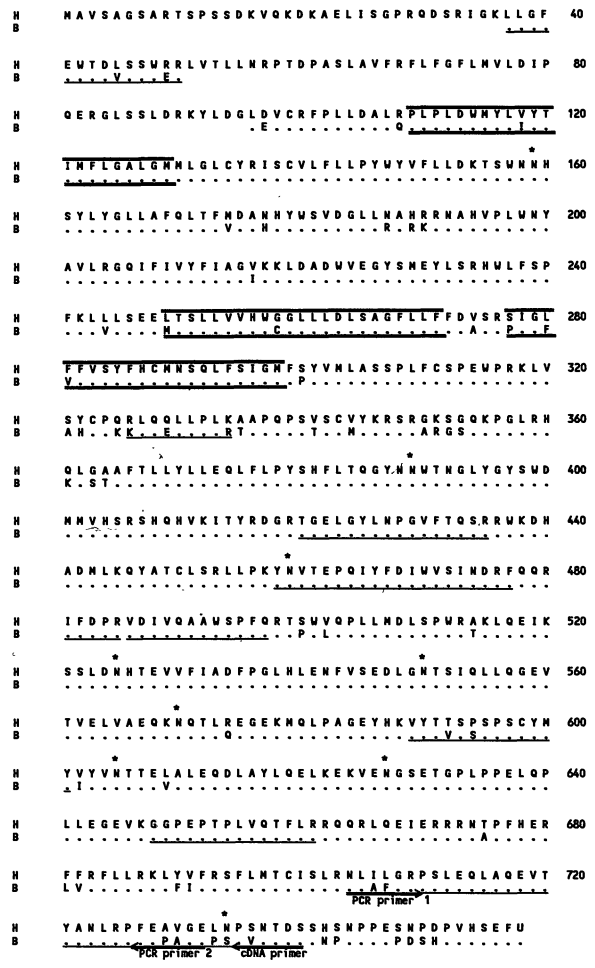

Fig. 1. The deduced amino acid (*11*) sequences of human (H) and bovine (B) γ-glutamyl carboxylase. The numbering is based on the human sequence. *, potential N-glycosylation sites on human carboxylase. Doubled lines indicate transmembrane domains. The purified bovine carboxylase was delipidated (*13*), separated on reducing SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to tryptic digestion; nine fragments (underlined) were sequenced by Edman degradation. The tryptic peptide sequence from residues 704 to 740 was used to generate primers (arrows) for cDNA synthesis and PCR. The cDNA primer was (5′)G/CA/TG/ATCNGTG/ATTNACNGG(3′), PCR primer 1 was (5′)AAFFTG/FGCG/FTTFGGNA/CG(3′), and PCR primer 2 was (5′)TCG/FCCG/FGCG/FGGT/CTCG/AAA(3′). N, a mixture of G,A,T,C; F, 5′-fluorouracil (*14*). The first strand cDNA was synthesized from bovine liver mRNA and then the cDNA was used as the template for two sequential rounds of PCR (94°C, 1 min; 58°C, 2 min; 72°C, 3 min; 30 cycles). All the basic techniques used for DNA manipulation are standard (*15*). The GenBank accession numbers assigned to the human and bovine cDNA sequences are M81592 and M81593, respectively.

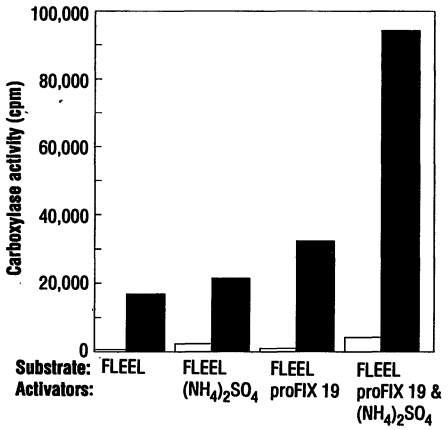

Fig. 2. Comparison of transient expression of carboxylase in kidney 293 cells transfected with pCMV5 or pCMV.hGC$^+$. Purified plasmid DNA was used to transfect (*16*) human kidney 293 cells. Forty-eight hours after transfection, cells from twenty 100-mm dishes were harvested to prepare microsomes (*17*). Carboxylase activity was determined as described (*2*) except that 0.16% phosphatidylcholine, 0.32% CHAPS, and 180-μg microsomal proteins from pCMV5 or pCMV.hGC$^+$–transfected transient expression cells were used. The carboxylase activity of microsomes from mock-transfected cells (unfilled bars) and from cells transfected with pCMV.hGC$^+$ (filled bars) are plotted pairwise. Each pair represents activity under different conditions of assay (indicated below the *x* axis). Carboxylase activity was measured by $^{14}CO_2$ incorporation into the synthetic peptide substrate FLEEL (*11*).

entire coding sequence of carboxylase with an internal Eco RI site, was obtained (Fig. 1). The 5′ 950-bp Eco RI fragment from λ gt10.hGC was then used as the probe to screen the bovine liver library a second time; three additional positive clones were obtained. The sequences from the six bovine partial carboxylase clones were combined (Fig. 1).

The amino terminus of the carboxylase appears to be blocked, which precludes the determination of the amino terminal sequence by Edman degradation. However, several observations indicate that the entire coding sequence is probably contained within this cDNA. (i) The methionine identified as the first amino acid (Fig. 1) is the only in-frame methionine between a stop codon 27 nt upstream and the first tryptic peptide 195 nt downstream. (ii) The open reading frame codes for 758 amino acids and predicts a molecular weight of 87,542 which, if one takes into account that the protein is glycosylated (*5*), agrees with the weight of 94 kD estimated by mobility in SDS–polyacrylamide gel electrophoresis (PAGE). (iii) The cDNA codes for functional carboxylase in mammalian cells.

Bovine carboxylase and human carboxylase share 90% identity over the 663 amino acids where both sequences were determined. The amino acid sequence of carboxylase from human liver and HEL cells is identical over the 353 amino acid residues that have been compared; although two silent DNA sequence polymorphisms were identified.

There is no amino-terminal signal peptide; the first 50 residues are very hydrophilic. Three hydrophobic regions (Fig. 1) near the amino terminus of the carboxylase are above the minimum criterion (20 kcal/mole) for transmembrane domains suggested by Engelman *et al.* (*6*). Eight out of nine potential N-glycosylation sites on human carboxylase are located in the hydrophilic, COOH-terminal half of the carboxylase; we do not know which sites are glycosylated but carboxylase does bind to a concanavalin A column (*5*). The presence of a glycosylated residue within a given sequence suggests that the sequence resides in the lumen of the endoplasmic reticulum (*7*). Carboxylase activity is found in the lumen of the endoplasmic reticulum (*8*); therefore, the enzymatic domain is probably in the carboxyl terminus of the carboxylase. The organization of the domains suggests that active, water soluble carboxylase could be produced by deleting the hydrophobic sequence from the amino terminus.

We have found several potential homologies in the Swissprot and GenBank databases (*9*). Carboxylase and soybean seed lipoxygenase share 19.3% identity over a span of 198 amino acids, from residues 468 to 666 of carboxylase. This is interesting because the carboxylase acts as an oxygenase on the cofactor vitamin K–hydroquinone, and the similarity occurs in that region of the carboxylase likely to have enzymatic function. NADH–ubiquinone oxidoreductase chain 2 and cytochrome b share 17.8% and 16% identity over 219 amino acids and 300 amino acids, respectively. Both are mitochondrial integral membrane proteins, and the similarities are in the apparent transmembrane domains of carboxylase.

We expressed the isolated human carboxylase cDNA in kidney 293 cells. Microsomal carboxylase activity was compared from cells transfected with pCMV5 (*10*) to that of cells transfected with the entire human carboxylase cDNA from λ gt10.hGC subcloned into the Eco RI site of pCMV5 (pCMV.hGC+) (Fig. 2). Increases in carboxylase activity of 27-, 9-, 35-, and 23-fold were observed in the presence of the peptide substrate

FLEEL (*11*) alone, FLEEL with activator ammonium sulfate, FLEEL with activator proFIX 19 (*12*), and FLEEL with both activators, respectively. The increases in carboxylase activity in response to different activators were consistently reproduced in separate transient-expression experiments. Microsomes isolated from permanent cell lines expressing the exogenous carboxylase showed a twofold increased carboxylase activity over the transiently expressing cells.

REFERENCES AND NOTES

1. P. A. Price, *Ann. Rev. Nutr.* **8**, 565 (1988).
2. S.-M. Wu, D. P. Morris, D. W. Stafford, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2236 (1991).
3. The bovine and human liver cDNA libraries were obtained from Stratagene.
4. The λ gt10 library was made from HEL cells which had been selected for large cells with characteristics of megakaryocytes.
5. T. Brody and J. W. Suttie, *Biochim. Biophys. Acta.* **923**, 1 (1987).
6. D. M. Engelman, T. A. Steitz, A. Goldman, *Ann. Rev. Biophys. Biophys. Chem.* **15**, 321 (1986).
7. S. J. Singer, *Annu. Rev. Cell Biol.* **6**, 247 (1990).
8. T. L. Carlisle and J. W. Suttie, *Biochemistry* **19**, 1161 (1980).
9. W. R. Pearson and D. J. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 (1988).
10. S. Andersson, D. L. Davis, H. Dahlback, H. Jornvall, D. W. Russell, *J. Biol. Chem.* **264**, 8222 (1989).
11. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
12. proFIX 19 is an analog of the propeptide of human FIX and has the amino acid sequence AVFLDHENANKILNRPKRY.
13. A. Hara and N. S. Radin, *Anal. Biochem.* **90**, 420 (1978).
14. J. F. Habener *et al.*, *Proc. Natl. Acad. Sci. USA* **85**, 1735 (1988).
15. T. Maniatis, E. Fritsch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
16. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
17. J. M. Girardot, *J. Biol. Chem.* **257**, 15008 (1982).
18. We thank J. Ware for the human erythroleukemia cDNA library.

13 September 1991; accepted 25 October 1991

Dopaminergic and Ligand-Independent Activation of Steroid Hormone Receptors

RONAN F. POWER, SHAILA K. MANI, JUAN CODINA, ORLA M. CONNEELY, BERT W. O'MALLEY*

The current view of how steroid hormone receptors affect gene transcription is that these receptors, on binding ligand, change to a state in which they can interact with chromatin and regulate transcription of target genes. Receptor activation is believed to be dependent only on this ligand-binding event. Selected steroid hormone receptors can be activated in a ligand-independent manner by a membrane receptor agonist, the neurotransmitter dopamine. In vitro, dopamine faithfully mimicked the effect of progesterone by causing a translocation of chicken progesterone receptor (cPR) from cytoplasm to nucleus. Dual activation by progesterone and dopamine was dissociable, and a serine residue in the cPR was identified that is not necessary for progesterone-dependent activation of cPR, but is essential for dopamine activation of this receptor.

STEROID HORMONE RECEPTORS ARE ligand-inducible members of a superfamily of transcription factors that also includes receptors for thyroid hormone, retinoic acid, and vitamin D_3 (*1*). Steroid hormone action involves entry of hormone into a cell where the hormone binds to and induces a conformational change in its cognate intracellular receptor protein (*2*), leading to nuclear translocation and modulation of gene expression (*3*). These events are believed to be dependent exclusively on hormone binding.

Steroids also modulate gene expression in neuronal tissue. Examples of steroid action in brain include the induction of steroid aromatase by testosterone in preoptic neurons and the induction of receptors for progesterone and oxytocin by estradiol in the ventromedial hypothalamus (*4*). The presence of receptors for sex steroid hormones in brain raises certain biological questions. For example, what function can be ascribed to the progesterone receptors in male brain (*5*) or to the androgen receptors in the cerebral cortex of female brain (*6*)? In addition, rapid effects of steroids in neuronal tissue occur (*7*) that are inconsistent with the existing pathway of steroid hormone action. A derivative of adenosine 3′,5′-monophosphate (cAMP), dibutyryl cAMP, can substitute for progesterone in eliciting mating behavior in female rats (*8*). This suggests some steroid pathways in brain may not be regulated exclusively by classical steroid-receptor pathways but also by modified intracellular second messengers. In support of this hypothesis, 8-bromo–cAMP (8-Br–cAMP) has been demonstrated to mediate PR-dependent transcription in the absence of progesterone (*9*). Phosphorylation of the oncogenic derivative of the thyroid hormone receptor, erbA, is required for its function as an oncogene (*10*). Furthermore, the chicken ovalbumin upstream promoter (COUP) transcription factor, a member of the steroid receptor superfamily (*11*), can be activated by the catecholamine neurotransmitter dopamine but does not bind this compound (*12*). We therefore examined whether more classical members of the steroid receptor superfamily can be activated via cell membrane mechanisms such as dopaminergic or adrenergic receptor systems.

Progesterone receptor–negative monkey kidney (CV_1) cells were cotransfected with a chicken progesterone receptor A form (cPR_A) expression vector (*13*) and a reporter plasmid (PREtKCAT) (*14*) that contained a progesterone-glucocorticoid response element (PRE-GRE) upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. Treatment of these cells with progesterone or dopamine increased PR_A-mediated transcription to an equivalent degree (Fig. 1A). The response to dopamine was maximal at a concentration of 100 μM, which is the concentration of this compound required to stimulate adenylyl cyclase activity maximally in these cells. Dopamine had no effect on CAT gene expression in cells transfected with the parent expression vector, p91023(B) and PREtKCAT (*12*). Dopamine does not bind to the PR (*15*), and there are no significant homologies between the PR sequence and published sequences for dopamine receptors. In addition, okadaic acid, an inhibitor of protein phosphatases 1 and 2A, potently stimulated PR-mediated transcription in this assay system, which implicates cellular phosphorylation pathways as a ligand-independent activation mechanism for PR (*9*) (Fig. 1A). The cPR_B protein (*13*) also activated CAT gene expression in cells treated with progesterone, dopamine, or okadaic acid (*16*).

The human estrogen receptor (hER) was also activated by dopamine and okadaic acid (Fig. 1B). Cells were cotransfected with an ER expression construct pSVMT-wER and an ER-responsive reporter plasmid ERE-E1bCAT (*17*). Dopamine induced ER-dependent transcription to a level equal to that induced by the natural ligand, estradiol. Our results with other receptors indicate that the human vitamin D receptor (hVDR) and the human thyroid hormone receptor–β (hTRβ) also activate transcription from tar-

Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

*To whom correspondence should be addressed.