were present in the trigeminal and thalamic nuclei bridging the sensory periphery to the cortex (*11*). Neonatal administration of PCPA (300 mg/kg per day, SC) partly restored the capacity to form cortical barrels. A role for serotonin in barrelfield formation could be considered because, for example, thalamic afferents in the barrels of normal pups express large amounts of 5-HT_{1B} receptors (*22*). Tg8 pup cortices that were stained for the 5-HT_{1B} receptors did not show the barrel pattern that was found in C3H cortices (*11*). It will be interesting to check for the presence of the barrelfield in the progeny of Tg8 mice mated to diverse 5-HT receptor knockouts.

This study shows that MAOA-deficient mouse pups have a dramatically altered serotonin metabolism and severe behavioral alterations, both phenomena being linked. The behavioral traits of adults may be related to persisting defects in monoamine metabolism or to structural alterations such as the one we demonstrated in the cerebral cortex, an issue that pharmacological interventions may help to clarify. The finding that MAOA-deficient males with a C3H/HeJ genetic background display enhanced aggression under standard rearing conditions supports the idea that the particularly aggressive behavior of the few known human males lacking MAOA is not fostered by an unusual genetic background or complex psychosocial stressors but is a more direct consequence of MAOA deficiency.

REFERENCES AND NOTES

1. C. C. Mann, *Science* **264**, 1686 (1994); M. Barinaga, *ibid.*, p. 1690; F. Saudou *et al.*, *ibid.* **265**, 1875 (1994); C. Chen, D. G. Rainnie, R. W. Greene, S. Tonegawa, *ibid.* **266**, 291 (1994).
2. A. W. J. Bach *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4934 (1988); Z.-Y. Chen, J. F. Powell, Y.-P. P. Hsu, X. O. Breakefield, I. W. Craig, *Genomics* **14**, 75 (1992).
3. F. A. Collins *et al.*, *Am. J. Med. Genet.* **42**, 127 (1992).
4. H. G. Brunner, M. Nelen, X. O. Breakefield, H. H. Ropers, B. A. van Oost, *Science* **262**, 578 (1993).
5. The IFN-β minigene is a 1588-bp Acc I–Apa I fragment derived from plasmid pK^b–IFN-β [I. Seif, E. De Maeyer, I. Rivière, J. De Maeyer-Guignard, *J. Virol.* **65**, 664 (1991)]. The Tg8 transgene is identical to the minigene, except for a 64-bp terminal deletion in the herpes virus thymidine kinase (TK) region and a 148-bp deletion in the promoter region from the major histocompatibility gene *H-2K*^{b} (H2).
6. U. Müller *et al.*, *Science* **264**, 1918 (1994).
7. H. Ochman, A. S. Gerber, D. L. Hartl, *Genetics* **120**, 621 (1988). Inverse PCR was done without prior size fractionation and isolation of restriction fragments.
8. This is a region between the *DXHS32* and *A-raf* loci [S. D. M. Brown *et al.*, *Mamm. Genome* **4**, S269 (1993)].
9. T. Kuwahara, S. Takamoto, A. Ito, *Agric. Biol. Chem.* **54**, 253 (1990).
10. J. Grimsby *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3637 (1991); R. K. Wierenga *et al.*, *J. Mol. Biol.* **187**, 101 (1986).
11. O. Cases *et al.*, unpublished data.
12. I. Q. Whishaw, T. Schallert, B. Kolb, *J. Comp. Physiol. Psychol.* **95**, 85 (1981).
13. S. C. Maxson, in *Techniques for the Genetic Analysis of Brain and Behavior: Focus on the Mouse*, D. Goldowitz, D. Wahlstein, R. E. Wimer, Eds. (Elsevier, Amsterdam, 1992), pp. 349–373.
14. R. D. Porsolt, A. Bertin, M. Jalfre, *Arch. Int. Pharmacodyn.* **229**, 327 (1977); F. Borsini and A. Meli, *Psychopharmacology* **94**, 147 (1988).
15. B. Kolb and I. Q. Whishaw, *Can. J. Psychol.* **37**, 211 (1983).
16. W. E. Haefely *et al.*, *Adv. Neurol.* **53**, 505 (1990).
17. S. C. Gerson and R. J. Baldessarini, *Life Sci.* **27**, 1435 (1980).
18. D. Tsang, K. P. Ho, H. L. Wen, *Dev. Neurosci.* **8**, 243 (1986); M. Strolin Benedetti, P. Dostert, K. F. Tipton, *Dev. Pharmacol. Ther.* **18**, 191 (1992); Y. Koide and K. Kobayashi, *Neurochem. Res.* **9**, 595 (1984).
19. W. Lichtensteiger *et al.*, *J. Neurochem.* **14**, 489 (1967); E. Shaskan and S. H. Snyder, *J. Pharmacol. Exp. Ther.* **175**, 404 (1970); H. W. M. Steinbusch, A. A. J. Verhofstad, H. W. J. Joosten, M. Goldstein, in *Cytochemical Methods in Neuroanatomy*, V. Chan-Palay and S. L. Palay, Eds. (Liss, New York, 1982), pp. 407–421; J. A. Wallace *et al.*, *Brain Res. Bull.* **9**, 117 (1982).
20. P. Levitt, J. E. Pintar, X. O. Breakefield, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6385 (1982); K. N. Westlund, R. M. Denney, L. M. Kochersperger, R. M. Rose, C. W. Abell, *Science* **230**, 181 (1985). In the normal brain, most catecholaminergic neurons of the locus coeruleus and nigral complex are rich in MAOA.
21. T. Woolsey and H. Van der Loos, *Brain Res.* **17**, 205 (1970); R. J. D'Amato *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4322 (1987); R. W. Rhoades *et al.*, *J. Comp. Neurol.* **293**, 190 (1990); M. C. Osterheld-Hass *et al.*, *Dev. Brain Res.* **77**, 189 (1994).
22. C. A. Bennett-Clarke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 153 (1993).
23. H.-F. Wu, K. Chen, J. C. Shih, *Mol. Pharmacol.* **43**, 888 (1993).
24. For high-performance liquid chromatography (HPLC) assays, whole brain was homogenized by sonication in 0.1 M $HClO_4$ containing 0.2% $Na_2S_2O_5$. In the first assay, the HPLC system was composed of a 5-μm Ultrosphere column (Beckman, Fullerton, CA) and an LC-2A electrochemical detector (Bioanalytical Systems, West Lafayette, IN), and the mobile phase was an 80:20 mixture (pH 2.9) of 100 mM $Na_2H_2PO_4$ and methanol, with 2.75 mM octane sulphonate, 0.1 mM EDTA, and 0.25 mM triethylamine. In the second assay (to refine NE determination), the HPLC system was a Coulochem II ESA with an HR-80 catecholamine column (ESA, Bedford, MA), and the mobile phase was an 88:6:6 mixture (pH 5.2) of 75 mM $Na_2H_2PO_4$, methanol, and acetonitrile, with 2.75 mM octane sulphonate, 0.02 mM EDTA, and 0.7 mM triethylamine.

25. We thank D. Hervé, J.-P. Tassin, J. Adam, B. Courtier, L. Eusèbe, and D. Haranger for participation; P. Avner for the DNA backcross panel; M. Da Prada for lazabemide; and R. Hen, M. Jouvet, J. P. Changeux, I. Gresser, L. Abbott, E. Lauret, V. Vieillard, R. Zawatzky, and J. De Maeyer-Guignard for discussion. Supported by CNRS, the Agence Nationale de Recherches sur le Sida, the Association pour la Recherche sur le Cancer, and National Institute of Mental Health grants RO1 MH 37020, R37 MH 39085, and K05 MH 00796.

2 November 1994; accepted 6 April 1995

Transcriptional Activation by Tetracyclines in Mammalian Cells

Manfred Gossen,* Sabine Freundlieb, Gabriele Bender, Gerhard Müller, Wolfgang Hillen, Hermann Bujard†

A transcriptional transactivator was developed that fuses the VP16 activation domain with a mutant Tet repressor from *Escherichia coli*. This transactivator requires certain tetracycline (Tc) derivatives for specific DNA binding. Thus, addition of doxycycline to HeLa cells that constitutively synthesized the transactivator and that contained an appropriate, stably integrated reporter unit rapidly induced gene expression more than a thousandfold. The specificity of the Tet repressor–operator–effector interaction and the pharmacological characteristics of Tc's make this regulatory system well suited for the control of gene activities in vivo, such as in transgenic animals and possibly in gene therapy.

The ability to tightly control individual gene activities would greatly facilitate the analysis of gene function, particularly in systems that are not amenable to genetic dissection. The achievement of this goal in more complex eukaryotic cells has been difficult because of a lack of tightness of control or to pleiotropic effects caused by the inducing principle (for example, heat shock, heavy metal ions, or steroid hormones), or both. Systems founded on well-defined regulatory elements from evolutionarily distant species have therefore been expected to be useful. With the use of the *E. coli* lactose and tetracycline (*tet*) resistance operons, several transcription control circuits have been developed and shown to be functional in complex eukaryotic cells (*1*, *2*). One system (*3*) takes advantage of the high specificity of the Tet repressor (TetR)–operator–Tc interaction, the potentcy of the herpes simplex virus (HSV)–VP16 transcription activation domain, and the favorable properties of Tc's. In this system, the Tc-controlled transactivator (tTA) (a fusion between TetR and the activating domain of the VP16 protein) can stimulate the expression of a reporter gene in HeLa cells up to 10^5-fold (*3*). Because Tc prevents tTA from binding to *tet* operators placed upstream of a

M. Gossen, S. Freundlieb, G. Bender, H. Bujard, Zentrum für Molekulare Biologie der Universität Heidelberg, (ZMDH) Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany.
G. Müller and W. Hillen, Institut für Mikrobiologie und Biochemie, Universität Erlangen, Staudtstrasse 5, 91058 Erlangen, Germany.

*To whom correspondence should be addressed.
†Present address: University of California Berkeley, Department of Molecular and Cell Biology, 401 Barker Hall, Berkeley, CA 94720, USA.

minimal promoter, it turns off the tTA-dependent expression unit.

Even though Tc and many Tc derivatives are nontoxic to eukaryotic cells at the low concentrations required to abolish gene expression, their continuous presence is suboptimal in a variety of experimental setups; for example, in the breeding of transgenic animals or in gene therapy. Moreover, the induction of gene expression may be slower when an effector needs to be removed, a problem relevant to studies in which kinetic parameters play a role, such as in developmental processes. To exploit Tc as an inducing effector substance, we envisioned three scenarios: (i) The development of a repression system utilizing the Tet repressor. Limitations of such a system have been outlined (*2*). (ii) The use of fusions between the Tet repressor and certain eukaryotic silencer-repressor proteins. (iii) The modification of TetR or tTA such that Tc induces rather than abolishes binding to the operator. Here we pursued the third concept and developed a transactivator that has reversed DNA binding properties when compared with the wild-type Tet repressor (*4*) or with tTA (*3*). This transactivator requires the presence of Tc for binding to *tet* operator sequences (Fig. 1). Rapid induction and a wide range of regulation of gene activity are achieved with the use of this regulatory system.

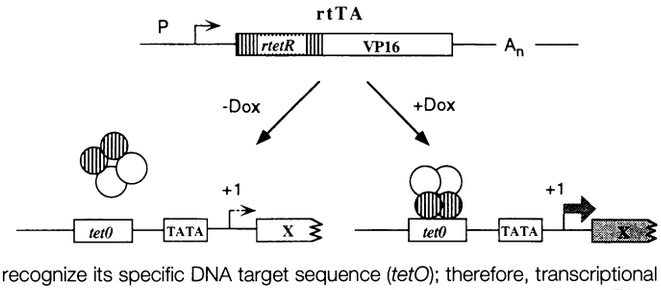

Fig. 1. Schematic representation of the reverse Tet system. The gene encoding rtTA is composed of *rtetR* and the VP16 moiety, driven by an appropriate promoter (P) and followed by a polyadenylate (A_n) site. In the absence of the effector doxycycline (Dox), the transactivator does not recognize its specific DNA target sequence (*tetO*); therefore, transcriptional activation of gene X will not occur (broken arrow). Addition of the effector Dox results in binding of rtTA to *tetO*, which allows the activation of the reporter unit (shaded arrow). The minimal promoter-*tet* operator construct used here is identical to the tTA-responsive promoter P_{hCMV^*-1}, which consists of seven *tet* operators located upstream of a minimal sequence of the CMV IE promoter (*3*).

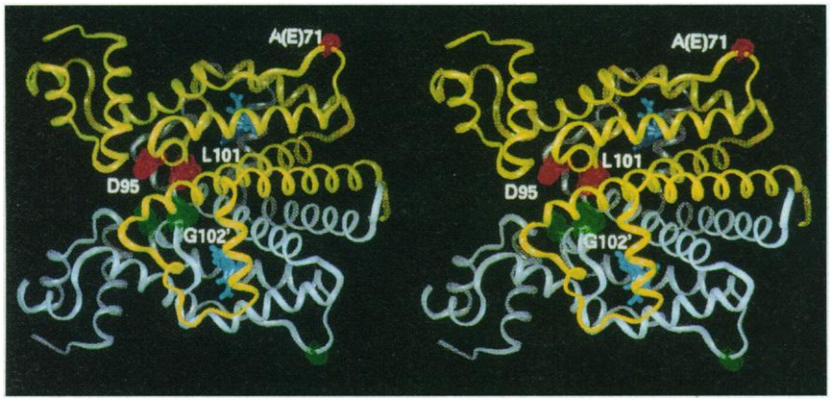

Fig. 2. Stereo plot of the TetR structure showing the backbone of the two subunits (yellow and gray) and the location of the four amino acids altered in the TetR mutant protein exhibiting the reverse phenotype. The van der Waals radii of the wild-type amino acids are indicated in red for the yellow subunit and in green for the gray subunit. For reasons of clarity, the position of Gly^{102} is indicated in the gray subunit (G102′). Ala^{71} (A71) refers to the class B wild-type residue and Glu^{71} (E71) to the class D wild-type residue. Tc is shown as a blue stick model.

The Tn*10-tetR* gene that codes for the class B Tet repressor (*4*) was randomly mutagenized and screened for the Tc dependence of repression in vivo (*5*). One mutant that exhibits a dependence on Tc for repression has four amino acid exchanges in TetR: $Glu^{71} \rightarrow Lys^{71}$, $Asp^{95} \rightarrow Asn^{95}$, $Leu^{101} \rightarrow Ser^{101}$, and $Gly^{102} \rightarrow Asp^{102}$ (*4*). According to the crystal structure of the highly homologous class D Tet repressor (*6*), none of these four amino acids directly contacts Tc. Instead, amino acid 71 is a surface residue, Asp^{95} connects the DNA reading head with the core of the protein, Leu^{101} is involved in

Table 1. Doxycycline-dependent enzyme activity in various double-stable HR5-derived clones. Out of 21 hygromycin-resistant clones obtained after the transfection of HR5 cells with a luciferase reporter unit (*11*), 10 showed inducible luciferase activity on addition of Tc or doxycycline to the culture medium. Four of those clones, as well as one cell line stably expressing an rtTA regulated *lacZ* gene, are shown. Enzyme activities in cell extracts were determined after 60 hours of growth. HR5 clones with a CMV minimal promoter in front of the reporter gene are referred to as HR5-C, and those with the TK minimal promoter are referred to as HR5-T (*11*). L designates the luciferase and Z the β-galactosidase reporter gene. The data show that the CMV-based minimal promoter can be activated to greater amounts than can the HSV-TK–based minimal promoter. However, the TK-derived promoter gives a consistently lower background activity. Luciferase activity was determined as described (*3*). β-Galactosidase activity was measured in a standard ONPG (ortho-nitrophenyl-β-D-galactopyranoside) assay from serial dilutions of cell extracts.

Clone	−Doxycycline	+Doxycycline	Activation factor
	Luciferase activity (relative light units per microgram of protein)		
HR5-CL11	100	165,671	1,660
	142	179,651	1,270
HR5-CL14	43	44,493	1,030
	43	56,274	1,310
Hr5-TL2	56	16,696	298
	40	16,416	410
HR5-TL15	6.8	1,838	270
	6.5	1,688	260
	β-Galactosidase activity per milligram of protein per hour		
HR5-CZ18	0.17	607	3,570
	0.15	801	5,340

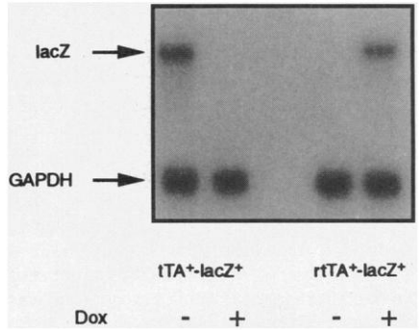

Fig. 3. Northern blot of lacZ RNA from HeLa cells expressing tTA or rtTA. Total RNA (10 mg) from a tTA^+ and an $rtTA^+$ cell line stably expressing the *lacZ* gene was loaded per lane. Dox followed by a plus or minus sign indicates the presence or absence of doxycycline (1 μg/ml) in the culture medium. The blot was hybridized simultaneously with a DNA probe for the *lacZ* gene and, as control for even loading, with a probe of GAPDH (glyceroladehyde phosphate dehydrogenase) complementary DNA.

subunit dimerization, and Gly^{102} is adjacent to an amino acid that contacts Tc (Fig. 2). The latter three amino acids, which are identical in class B and class D repressors, most likely are involved in the conformational change of TetR during induction. Their alteration may therefore generate the observed reverse phenotype. We refer to this mutant as a reverse Tet repressor (rTetR), because repression of an indicator gene under Tet control in *E. coli* is increased about 30-fold in the presence of the inducer doxycycline (*7*). Thus, the effect of Tc is reversed in the mutant as compared with the wild-type repressor.

The reverse Tc-controlled transactivator (rtTA) was obtained by the exchange of the DNA sequences that span the positions of the mutated amino acids between tTA and rTetR (*8*). The gene for rtTA-nls, which encodes a nuclear localization sequence at the 5′ end, was constructed in an analogous experiment (*8*, *9*). Both genes were placed under the control of the human cytomegalovirus IE promoter, which resulted in the construction of plasmids pUHD17-1neo and pUHD172-1neo, which encode rtTA or rtTA-nls, respectively (*9*). When HeLa cells were cotransfected with either one of these plasmids as well as pUHC13-3 [which contains the luciferase gene under the control of a tTA-dependent minimal promoter (*3*)], both rtTA and rtTA-nls showed the expected increase in luciferase activity in the presence of Tc's (*10*).

Stable HeLa cell lines that produced rtTA or rtTA-nls were obtained after transfection with pUHD17-1neo and pUHD172-1neo, respectively, and selection for G418-resistant clones. These clones were screened for expression of a functional transactivator by transient transfection with pUHC13-3. The positive clone HR5, which constitutively produces rtTA-nls, was chosen for stable transfection with reporter units that contained a gene encoding either luciferase or β-galactosidase that was under the control of a tTA-dependent promoter (*11*). Several of the clones that expressed the reporter gene exhibited the expected increase in enzyme activity on addition of doxycycline. More than 1000-fold induction was achieved with these double-stable cell lines (Table 1). Activation of gene expression by doxycycline was also monitored at the level of RNA synthesis (*12*). On addition of the effector, β-galactosidase mRNA increased dramatically in a $rtTA^+$-$lacZ^+$ cell line (Fig. 3). The opposite effect was observed when doxycycline was given to cells that express the *lacZ* gene under control of the authentic transactivator tTA. These data reveal the complementary mode of action of these two transactivators.

Several Tc derivatives were examined for the ability to activate luciferase expression in HR5-CL11 cells. Whereas doxycycline and anhydrotetracycline highly stimulated luciferase activity, Tc, chlortetracycline, and oxytetracycline were less effective (Fig. 4A). The most potent effector, doxycycline, was used in all further experiments. Analysis of the dose-response dependence revealed that doxycycline was apparently ineffective at concentrations below 10 ng/ml. However, when the concentration was raised, an almost linear increase in the expression of luciferase was observed, and maximal activation was achieved at 1 μg/ml (Fig. 4B). Finally, the kinetics of doxycycline-mediated activation of gene expression was measured by monitoring the time course of luciferase activity in HR5-CL11 cells after the addition of doxycycline to the medium. Luciferase activity was induced 100-fold after 5.5 hours and reached fully induced levels in less than 24 hours (Fig. 4C).

We have recently demonstrated the functionality of the previously described tTA system in transgenic plants (*13*) and mice (*14*), and large regulation factors have been monitored in the mouse model (*15*). First experiments with mice transgenic for rtTA reveal comparable ranges of regulation in response to doxycycline (*15*). Thus, the placement of genes under the control of rtTA may lead to the generation of conditional mutants whereby a gene of interest can be kept silent throughout embryonic development in the absence of any effector. The excellent dose-response characteristics of the system would then allow not only a qualitative off-on transition but also a fine tuning of gene expression and the study of quantitative aspects of gene activity. The design of partial induction experiments, particularly in the mouse model, may be facilitated by the use of different Tc derivatives, which activate rtTA to different extents at identical concentrations (Fig. 4A). Finally, the properties of the reverse Tet regulatory system described here appear par-

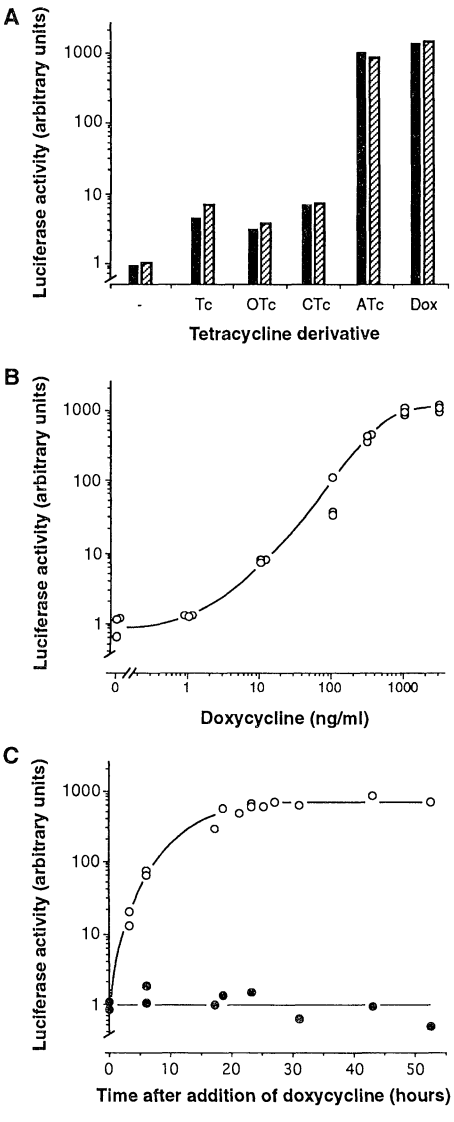

Fig. 4. (**A**) Differential response of HR5-CL11 cells to various Tc's. HR5-CL11 cells were plated at a density of 3×10^4 cells per 35-mm dish. After full attachment of the cells, different Tc's were added to a final concentration of 1 μg/ml [Tc, tetracycline-HCl; OTc, oxytetracycline-HCl; CTc, chlortetracycline; ATc, anhydrotetracycline-HCl; and Dox, doxycycline-HCl. These compounds (Sigma or ACROS Chimica, Nidderau, Germany) were kept in aqueous solution at a concentration of 1 mg/ml]. Control cultures were grown in the absence of antibiotics. After 3 days, the luciferase activity and the protein content were determined in cell extracts. The results of two independent experiments (solid and hatched bars) are shown. Each bar represents the luciferase activity normalized to the protein content of a single culture. The mean of the luciferase activites from two cultures grown without Tc's was defined as 1. (B) and (C) show induction of luciferase activity in HR5-CL11 cells by doxycycline. (**B**) Dose-response analysis of doxycycline on the HR5-CL11 clone. HR5-CL11 cells were plated at a density of 3×10^4 cells per 35-mm dish. After attachment of the cells, doxycycline was added to the final concentrations indicated. (Doxycycline has a slight cytotoxic effect at concentrations greater than 3 μg/ml.) Control cultures were grown without doxycycline. After 3 days, cell extracts were analyzed for luciferase activity and protein content. For each concentration of doxycycline, relative luciferase activities from three independently grown cultures are shown. The mean of the luciferase activities from the cultures grown without doxycycline was defined as 1. (**C**) Kinetics of luciferase induction in HR5-CL11 cells after activation with doxycycline. HR5-CL11 cells were plated at a density of 5×10^4 cells per 35-mm dish. After attachment of the cells, the medium of all cultures was renewed [time (t) = 0], whereby half of the cultures received prewarmed antibiotic-free medium (solid circles) whereas the other half (open circles) received medium supplemented with doxycycline (1 μg/ml). Each point represents the normalized luciferase activity from one of these cultures harvested at the time indicated. Mean luciferase values of the cultures analyzed at t = 0 were defined as 1.

ticularly advantageous for in vivo studies in which the rapid activation of a gene is required, because, in contrast to the authentic tTA system, the kinetics of induction does not depend on the biological half-life of the effector.

REFERENCES AND NOTES

1. G. T. Yarronton, *Curr. Opin. Biotechnol.* **3**, 506 (1992).
2. M. Gossen, A. Bonin, H. Bujard, *Trends Biochem. Sci.* **18**, 471 (1993).
3. M. Gossen and H. Bujard, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547 (1992).
4. W. Hillen and C. Berens, *Annu. Rev. Microbiol.* **48**, 345 (1994).
5. The mutagenesis and selection procedure was described previously [B. Hecht, G. Mller, W. Hillen, *J. Bacteriol.* **175**, 1206 (1993)].
6. W. Hinrichs *et al.*, *Science* **264**, 418 (1994).
7. G. Müller, thesis, Universität Erlangen-Nürnberg, Germany (1995).
8. To transfer the reverse DNA binding behavior of rTetR to tTA (*3*), a 399–base pair Xba I–Eco47 III fragment containing the relevant mutations of the *rtetR* gene was exchanged for the corresponding restriction fragment in pUHD15-1neo (an expression vector for tTA, consisting of a cytomegalovirus (CMV) promoter-enhancer, the gene encoding tTA, and an SV40 polyadenylation site) (*3*). In addition, the vector contains a pSV2neo-derived neomycin resistance cassette [P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982)] to facilitate selection of transfected cells. Stable clones were selected at 500 μg of G418 per milliliter. The resulting expression vector for rtTA was named pUHD17-1neo.
9. A nuclear, localized tTA protein, tTA-nls, was constructed by fusion at the DNA level of a nuclear localization sequence to the NH_2-terminus of tTA. The functionality of the protein with respect to its transactivation capacity and enrichment in the nucleus was demonstrated [A. Kistner, thesis, University of Heidelberg, Germany (1993)]. The actual NH_2-terminal amino acid sequence of this protein is MPKRPRPS . . . (*16*); the underlined serine is amino acid residue 2 of tTA (*3*). The rtTA-nls expression vector pUHD172-1 is a derivative of pUHD152-1 that encodes tTA-nls.
10. M. Gossen, data not shown.
11. The luciferase reporter units used were pUHC13-3 (*3*) and pUHC13-7. In the latter, the CMV minimal promoter of pUHC13-3 is substituted by the HSV–thymidine kinase (TK) promoter truncated at position −37 relative to the transcriptional start site. The β-galactosidase reporter plasmid pUHG16-3 with a CMV minimal promoter has been described [D. Resnitzky, M. Gossen, H. Bujard, S. I. Reed, *Mol. Cell. Biol.* **14**, 1669 (1994)]. Transcription from these reporter units can be stimulated by both tTA and rtTA–rtTA-nls. The reporter plasmids were cotransfected with hygromycin selection markers. Selection was at 300 μg of hygromycin per milliliter.
12. Total RNA was prepared as described [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. Northern (RNA) blot analysis was done according to standard procedures [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989)]. RNA was transferred onto NYTRAN membranes (Schleicher and Schüll, Dassel, Germany) and hybridized at 65°C [G. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)] with probes prepared by the random primer technique [A. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)]. The highest stringency wash was 0.2× sodium chloride sodium citrate buffer (SSC)/0.2% SDS. For quantitation, DNA probes of the *lacZ* gene and a GAPDH complementary DNA were simultaneously hybridized.
13. P. Weinmann, M. Gossen, W. Hillen, H. Bujard, C. Gatz, *Plant J.* **5**, 559 (1994).
14. P. A. Furth *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9302 (1994).
15. Mice doubly transgenic for the luciferase reporter unit (*11*) and for tTA controlled by the CMV IE promoter (*3*) show high luciferase activity in organs where the CMV IE promoter is known to be active. Supply of doxycycline (200 μg/ml) in drinking water given to mice reduces the luciferase activity to background levels. Double-transgenic mice that express rtTA show the reverse phenotype. In both systems, regulatory factors of up to six orders of magnitude can be monitored, depending on the particular organ (A. Kistner and H. Bujard, in preparation).
16. Single-letter abbreviations for the amino acid residues are as follows: K, Lys; M, Met; P, Pro; R, Arg; and S, Ser.

17. We thank W. Hinrichs and W. Saenger for providing the coordinates to produce Fig. 2, S. Reinig for secreterial support in the preparation of the manuscript, and U. Baron for helpful suggestions. Supported by the Deutsche Forschungsgemeinschaft (SFB 229), by the Fonds der Chemischen Industrie Deutschlands, and by the E. C. BRIDGE RTD Programme.

19 December 1994; accepted 23 March 1995

Construction of a Soluble Adenylyl Cyclase Activated by $G_s\alpha$ and Forskolin

Wei-Jen Tang* and Alfred G. Gilman

A soluble adenylyl cyclase was constructed by linkage of portions of the cytosolic domains of the mammalian type I and type II enzymes. The soluble enzyme was stimulated by both forskolin and the α subunit of the heterotrimeric guanine nucleotide–binding protein (G protein) G_s ($G_s\alpha$). Expression of the construct complemented the catabolic defect in a strain of *Escherichia coli* that is deficient in adenylyl cyclase activity. The active, approximately 60-kilodalton enzyme accumulated in the cytoplasmic fraction of *E. coli* to yield activities in excess of 1 nanomole per minute per milligram of protein. The two sets of transmembrane helices of mammalian adenylyl cyclases are thus not necessary for the catalytic or the most characteristic regulatory activities of the enzyme. This system may be useful for both genetic and biochemical analysis of G protein–regulated adenylyl cyclases.

The structures of G protein–regulated adenylyl cyclases are complex, consisting of two intensely hydrophobic domains (M_1 and M_2), each hypothesized to contain six transmembrane helices, and two ~40-kD cytosolic domains (C_1 and C_2). The C_1 and C_2 domains contain sequences (C_{1a} and C_{2a}) that are similar to each other, to the corresponding regions of related adenylyl cyclases, and to the catalytic domains of membrane-bound and soluble guanylyl cyclases (*1*, *2*). Analysis of a series of truncation and alanine-scanning mutants of mammalian adenylyl cyclases indicates that both C_{1a} and C_{2a} (but not C_{1b} and C_{2b}) are necessary for catalytic activity (*3*, *4*). However, by analogy with the guanylyl cyclases, the hydrophobic domains are thought not to be so required. Membrane-bound adenylyl cyclases are expressed in small amounts, and the enzymes are labile and difficult to manipulate in detergent-containing solutions. We have thus attempted to construct a soluble adenylyl cyclase that retains regulatory properties of interest and that is amenable to both biochemical and genetic analysis.

Concurrent expression of the NH_2-terminal half of type I adenylyl cyclase and the COOH-terminal half of type II adenylyl cyclase results in the formation of a noncovalent chimera that remains sensitive to both $G_s\alpha$ and forskolin, despite extremely low basal activity (*4*). We have thus ligated complementary DNAs (cDNAs) that encode C_{1a} from type I adenylyl cyclase and C_{2a} from type II adenylyl cyclase with short linkers between them, resulting in constructs designated IC_1IIC_2-L_1, IC_1IIC_2-L_3, and IC_1IIC_2-L_5 (Fig. 1A) (*5*). To investigate whether these constructs encode functional adenylyl cyclases, we tested complementation of the catabolic defect in *E. coli* Δcya TP2000, which lacks adenylyl cyclase activity (*6*). This deficient bacterial strain cannot use maltose as a carbon source; colonies thus fail to turn red on McConkey agar and do not grow on minimal medium (*7*). To activate adenylyl cyclase in *E. coli*, we coexpressed the cyclase constructs with either wild-type $G_s\alpha$ or a mutant $G_s\alpha$ (in which Gln^{227} is replaced with Leu; designated $G_s\alpha$*) that is deficient in guanosine triphosphatase (GTPase) activity and is thus constitutively active (*8*). *Escherichia coli* TP2000 expressing either $G_s\alpha$ or $G_s\alpha$* remained pale yellow on McConkey agar supplemented with maltose and failed to grow on minimal medium (M63 medium containing arginine and maltose) (Fig. 1B). However, bacteria expressing $G_s\alpha$* and any of the three chimeric adenylyl cyclase constructs turned red on McConkey agar and grew on minimal medium (Fig. 1B). (Wild-

Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235, USA.

*Present address: Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637, USA.