Hoppe, *J. Mol. Biol.* **78**, 581 (1972).
18. P. B. Moore, J. A. Langer, J. A. Engelman, D. M. Engelman, *J. Appl. Cryst.* **11**, 479 (1978); R. May, thesis, Technical University, Munich (1978); P. Stockel *et al.*, *J. Appl. Cryst.* **12**, 176 (1979).
19. P. B. Moore, E. Weinstein, *J. Appl. Cryst.* **12**, 321 (1979); V. Ramakrishnan and P. B. Moore, *J. Mol. Biol.* **174**, 719 (1981).
20. M. S. Capel, M. Kjeldgaard, D. M. Engelman, P. B. Moore, *J. Mol. Biol.*, in press.
21. K. R. Leonard and J. A. Lake, *ibid.* **129**, 155 (1979).
22. P. B. Moore and M. S. Capel, *Annu. Rev. Biophys. Chem.*, in press.
23. We thank G. Sun for her able technical assistance, the Brookhaven National Laboratory, the Office of Health and Environmental Research of the Department of Energy, the Institut Laue-Langevin, and G. Stoeffler and M. Stoeffler-Meilicke for supplying us with the photographs in Fig. 2. Supported by the National Science Foundation and NIH AI-20466 (D.M.E. and P.M.B.) and NIH AI-09167 (P.B.M.).

15 May 1987; accepted 9 September 1987

Decreased TRH Receptor mRNA Activity Precedes Homologous Downregulation: Assay in Oocytes

YORAM ORON,* RICHARD E. STRAUB, PAULA TRAKTMAN, MARVIN C. GERSHENGORN

Ligand-induced decrease in cell-surface receptor number (homologous downregulation) is often due to rapid receptor internalization. Thyrotropin-releasing hormone (TRH), however, causes a slow downregulation of TRH receptors (TRH-Rs), with a half-time of approximately 12 hours, in GH_3 rat pituitary cells. The mechanism of TRH-R downregulation was studied by monitoring TRH-evoked depolarizing currents in *Xenopus* oocytes injected with GH_3 cell RNA as a bioassay for TRH-R messenger RNA (mRNA) activity. In GH_3 cells, TRH caused a rapid decrease in TRH-R mRNA activity to 15 percent of control within 3 hours. Because the half-life of TRH-R mRNA activity in control cells was approximately 3 hours, the rapid decrease in mRNA activity was not due to inhibition of mRNA synthesis alone and may represent a post-transcriptional effect.

MODULATION OF CELL-SURFACE receptor number is an important mechanism of cell regulation. The number of available receptors on the cell surface may be decreased by ligand occupancy (homologous downregulation) that in many instances is caused by rapid receptor internalization (*1*). Thyrotropin-releasing hormone (TRH) downregulates the number of TRH receptors (TRH-Rs) on anterior pituitary cells (*2, 3*). The molecular mechanism that mediates regulation of TRH-R number is unknown but does not appear to involve rapid receptor internalization (*4*). The intracellular messengers that mediate TRH stimulation of hormone secretion, which include 1,2-diacylglycerol, inositol 1,4,5-trisphosphate, and calcium ion [for review, see (*5*)], may mediate TRH-R regulation. GH_3 rat pituitary tumor cells are an excellent model for study of TRH-R regulation, and variant clones that have few, if any, TRH-Rs (*4*) are available.

In *Xenopus* oocytes, muscarinic agonists cause membrane electrical responses consisting mainly of depolarizing chloride currents [for review, see (*6*)]. Muscarinic agonist stimulation of chloride currents is mediated by an elevation of intracellular calcium caused by inositol 1,4,5-trisphosphate (*7, 8*). Hence, muscarinic responses in *Xenopus* oocytes and TRH responses in GH_3 cells appear to be mediated by identical mechanisms. We reasoned, therefore, that RNA from GH_3 cells containing messenger RNA (mRNA) for TRH-Rs could be injected into *Xenopus* oocytes and translated, and when the TRH-Rs are inserted into the surface membrane, they could be monitored as acquired responsiveness to TRH. Injected oocytes could be used as a bioassay of TRH-R mRNA activity to study the mechanism of TRH-induced receptor downregulation.

In oocytes injected with total cytosolic RNA from GH_3 cells (120 to 400 ng per oocyte) and maintained under voltage clamp, TRH caused a marked membrane electrical response. Control oocytes (uninjected) did not respond to TRH (42 oocytes from ten frogs) but did respond to acetylcholine (ACh) (*9, 10*). The response to TRH was often observed by 8 to 12 hours and was maximal 30 to 72 hours after RNA injection. A typical response to 1 μM TRH (Fig. 1A) consisted of a very large (range

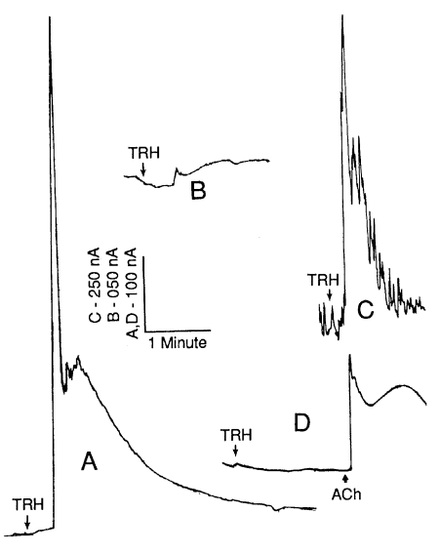

Fig. 1. Typical responses to TRH in *Xenopus* oocytes injected with RNA from GH_3 or GH-Y cells. (**A**) Oocyte injected with 320 ng of GH_3 cell RNA. (**B**) Oocyte injected with 20 ng of GH_3 cell RNA. (**C**) Oocyte injected with 20 ng of poly(A)$^+$ GH_3 cell RNA and 100 ng of carrier transfer RNA (*Escherichia coli*, Boehringer Mannheim). (**D**) Oocyte injected with 140 ng of RNA from GH-Y cells. Assays were performed 48 hours after injection. TRH (1 μM, light arrow) or 0.1 μM ACh (heavy arrow) was added for 1.5 minutes. For the preparation of oocytes, denuded oocytes were prepared as described by Dascal *et al.* (*12*). Oocytes were maintained at 20°C in 70% L-15 medium containing penicillin G (50 μg/ml) and 0.5 mM theophylline. For the preparation of total cytosolic RNA, cells were harvested with 0.02% EDTA, and washed three times with ice cold BSS (NaCl, 135 mM; KCl, 4.5 mM; glucose, 5.6 mM; $CaCl_2$, 1.5 mM; $MgCl_2$, 0.5 mM; and Hepes, 25 mM, *p*H 7.4). The cell pellet was resuspended in ten volumes of hypotonic lysis buffer (NaCl, 10 mM; $MgCl_2$, 1 mM; and tris-HCl, 10 mM, *p*H 7.4), kept on ice for 20 minutes, and vortexed three times for 1 minute at 5-minute intervals. After nuclei and debris were removed by centrifugation at 2000*g* for 10 minutes, the supernatant was diluted with four volumes of a solution containing 4M guanidinium thiocyanate, 0.5% *n*-lauroylsarcosine, 100 mM mercaptoethanol, and 25 mM sodium citrate, *p*H 7, layered onto a 3-ml cushion consisting of 5.7M CsCl and 100 mM EDTA, and centrifuged for 12 hours at 15°C in an SW41 rotor at 35,000 rpm. The RNA pellet was washed with cold 70% ethanol, dissolved in water, precipitated with ethanol, and again dissolved in water. Poly(A)$^+$-enriched RNA was purified by oligo(dT) affinity chromatography (*19*). For the intracellular injections, RNA (120 to 400 ng in 20 to 50 nl) was injected. Electrophysiological measurements were performed on oocytes that were voltage-clamped at −60 to −80 mV (*20*) as described (*10, 13*). In all experiments with GH_3 cell RNA, more than 90% of injected oocytes responded to TRH.

Y. Oron, R. E. Straub, M. C. Gershengorn, Division of Endocrinology and Metabolism, Department of Medicine, Cornell University Medical College, and New York Hospital, New York, NY 10021.
P. Traktman, Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021.

*Permanent address: Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Israel.

300 to 2500 nA), rapid, and transient depolarizing current that developed 20 to 30 seconds after the beginning of exposure to TRH (denoted as $D1_T$ by analogy to the D1 response caused by ACh). This response was followed by a prolonged depolarizing current of much smaller amplitude, often accompanied by very large superimposed current fluctuations. The response to TRH was similar to the responses to serotonin and ACh in oocytes injected with mRNA from brain or heart (*11, 12*). Because the second depolarizing current was variable and was often obscured by current fluctuations, all quantitation was performed on the $D1_T$ component of the response. The magnitude of the response to TRH was dependent on the amount of RNA injected. Threshold responses (approximately 10 to 20 nA) were obtained when 20 ng of RNA was injected (Fig. 1B). Progressively larger responses were observed when the amount of RNA was increased from 20 to 320 ng; no increase was observed when more than 320 ng RNA was injected (Fig. 2). Responses similar to those observed in oocytes injected with total cytosolic RNA were observed in oocytes injected with RNA enriched for polyadenylated [poly(A)$^+$] species from GH_3 cells (Fig. 1C). These data demonstrate the validity of the oocyte system for quantitative assay of TRH receptor mRNA activity.

We tested a variant GH_3 cell clone (GH-Y) (*4*) that had no detectable TRH-Rs. In oocytes injected with cytosolic or poly(A)$^+$ RNA from GH-Y cells, there was no response to TRH (three experiments on two separate RNA preparations); oocytes injected with GH-Y cell mRNA showed the usual intrinsic response to ACh (Fig. 1D). These data suggest that GH-Y cells lack TRH-Rs because they are deficient in mRNA activity that encodes a functional TRH-R.

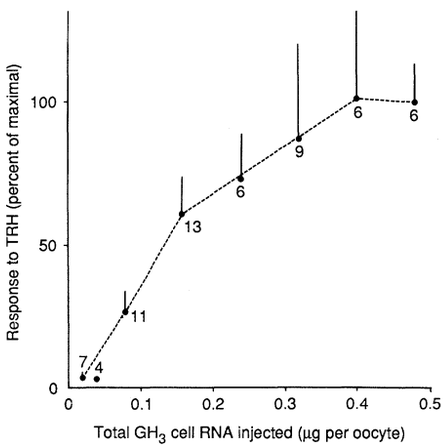

Fig. 2. Effect of injecting increasing amounts of cytosolic GH_3 cell RNA into *Xenopus* oocytes. Oocytes were injected with 20 to 480 ng of GH_3 cell RNA and assayed as described in Fig. 1. Results were normalized as percent of maximal response (602 and 1015 nA in two experiments). Bars represent the SEM. The numbers denote the number of oocytes per data point.

The oocyte system was used to study homologous downregulation of TRH-R number. GH_3 cells were grown for 1 to 48 hours in the presence of 1 μ*M* TRH. TRH-R number was measured in GH_3 cells, and TRH-R mRNA activity was assayed in oocytes. For these experiments, 130 to 150 ng of cytosolic RNA was injected into oocytes of the same batch to monitor changes in mRNA activity most sensitively (Fig. 2). As described (*2*), TRH caused a time-dependent decrease in TRH-R number in GH_3 cells that was half-maximal after 12 hours and reached a new steady state (approximately 30% of control) after 30 hours (Fig. 3). In contrast to the continuous decrease in TRH-R number until a new steady state was attained, the effect of TRH on TRH-R mRNA activity was complex. TRH caused a rapid decrease in TRH-R mRNA activity, which reached a nadir of approximately 15% of control between 3 and 6 hours. This was followed by an increase to approximately 50% of control mRNA activity by 12 hours, and this level was maintained for at least 48 hours (Fig. 3). Hence, TRH caused a rapid decrease in TRH-R mRNA activity that preceded the decrease in receptor number.

To determine if the initial rapid decrease in TRH-R mRNA activity was due to inhibition of transcription, we incubated GH_3 cells with actinomycin D (1 μg/ml), which inhibited RNA synthesis by more than 99%. Incubation of cells with actinomycin D for 3 and 6 hours reduced TRH-R mRNA activity to 55 ± 11% (n = 5) and 26 ± 4% (n = 4) of control (mean ± SEM), respectively. These results indicate that the half-life of TRH-R mRNA activity is approximately 3 hours. In contrast, at 3 and 6 hours of exposure to TRH, TRH-R mRNA activity was 15 ± 6% (n = 7) and 11 ± 5% (n = 3) of control, respectively. At 3 and 6 hours, the effect of TRH was greater than that of actinomycin D ($P < 0.001$ and $P < 0.025$, respectively). Hence, because virtually complete arrest of transcription did not mimic the effect of TRH, the rapid decrease in TRH-R mRNA activity appears to be mediated post-transcriptionally, at least in part.

In summary, these data demonstrate that

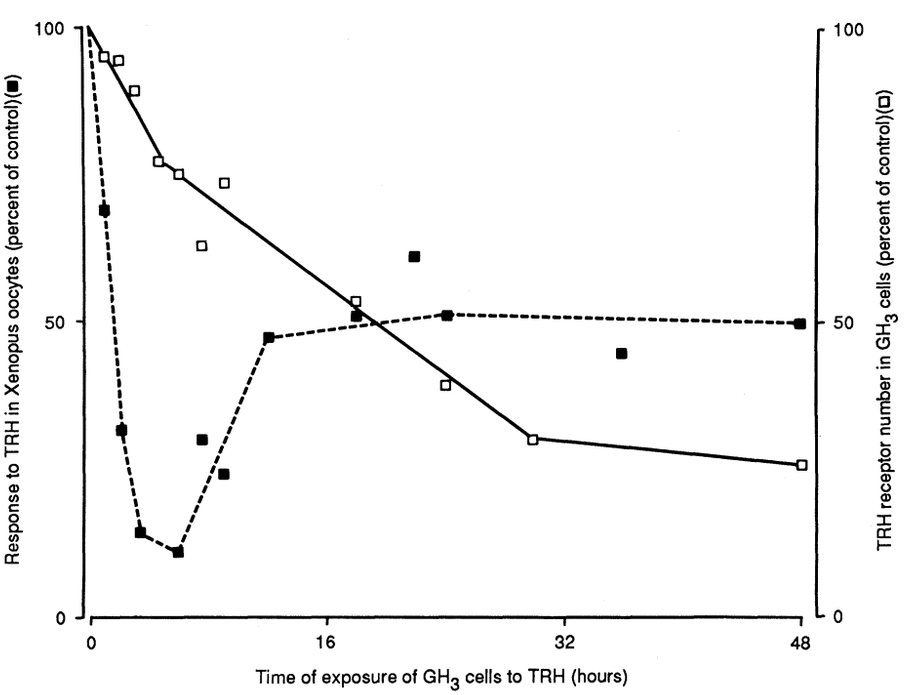

Fig. 3. Effects of TRH on the number of TRH receptors on GH_3 cells and on receptor mRNA activity assayed in *Xenopus* oocytes. For incubation of GH_3 cells, cells (1.8×10^8) from the same passage were distributed equally into six flasks. TRH (1 μ*M*) was added for the indicated times before harvesting. For measurement of TRH receptor number, 10^6 cells were used, and the binding assay was performed with 1 n*M* [^{3}H]methyl-His-TRH (New England Nuclear) (*2*). Nonspecific binding, less than 5% of total, was determined with 0.5 μ*M* unlabeled TRH. Receptor number could be assayed with one concentration of [^{3}H]methyl-His-TRH because TRH decreases receptor number without affecting affinity (*2, 3*). Assays for the measurement of mRNA activity were performed 48 to 72 hours after injection of 130 to 150 ng of RNA as described in Fig. 1. Results from four binding and eight receptor mRNA activity assay experiments were pooled and are presented as percent of control. The mean SEMs for the measurement of receptor binding and receptor mRNA activity were 3.2 ± 0.67% and 12 ± 1.6%, respectively.

the downregulation of TRH-R number by TRH is complex and involves at least two effects on TRH-R mRNA activity. TRH caused a rapid effect that appeared to involve post-transcriptional modification of TRH-R mRNA and a long-term effect that led to a new steady-state level of mRNA activity. This contrasts with reports in which homologous regulation of plasma membrane receptors for interleukin-2 (*13*) and epidermal growth factor (*14*) were associated with increased levels of receptor mRNA, even when receptor number was downregulated (*15*). At least two explanations for the mechanism of the TRH-induced decrease in TRH-R mRNA activity are possible. TRH may increase the rate of TRH-R mRNA degradation. This possibility cannot be assessed directly because a complementary DNA (cDNA) probe for TRH-R mRNA is not available. Alternatively, TRH may cause a modification of TRH-R mRNA so that its activity is decreased; the modification would most likely be covalent because it persists during the RNA preparation, which would almost certainly dissociate any noncovalently attached regulatory factor. The long-term effect of TRH on receptor mRNA activity may be mediated by a different mechanism and may involve transcriptional or post-transcriptional effects, or both.

These results show that receptor mRNA obtained from a defined and well-characterized cell line can be translated, and the receptor can then be incorporated into the oocyte plasma membrane and coupled to a measurable biological response. Although mRNA obtained from a variety of tissues (*16, 17*) or from cDNA vectors (*18*) have been expressed in the oocyte, our experiments demonstrate expression of a plasma membrane receptor mRNA from a permanent, cloned cell line. This approach may be extended to the study of regulation of mRNA for other cell-surface receptors in other cell lines when probes for receptor mRNA are not available.

REFERENCES AND NOTES

1. A. C. King and P. Cuatrecasas, *N. Engl. J. Med.* **305**, 77 (1981).
2. P. M. Hinkle and A. H. Tashjian, Jr., *Biochemistry* **14**, 3845 (1975).
3. M. C. Gershengorn, *J. Clin. Invest.* **62**, 937 (1978).
4. J. Halpern and P. M. Hinkle, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 587 (1981).
5. M. C. Gershengorn, *Annu. Rev. Physiol.* **48**, 515 (1986).
6. Y. Oron and Y. Lass, *Rev. Clin. Basic Pharmacol. Suppl.* **5**, 15s (1985).
7. Y. Oron, N. Dascal, E. Nadler, M. Lupu, *Nature (London)* **313**, 141 (1985).
8. E. Nadler, B. Gillo, Y. Lass, Y. Oron, *FEBS Lett.* **199**, 208 (1986).
9. K. Kusano, R. Miledi, J. Stinnakre, *Nature (London)* **270**, 739 (1977).
10. N. Dascal and E. M. Landau, *Life Sci.* **27**, 1423 (1980).
11. C. B. Gunderson, R. Miledi, I. Parker, *Nature (London)* **308**, 421 (1984).
12. N. Dascal *et al.*, *Mol. Brain Res.* **1**, 201 (1986).
13. J. M. Depper *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4230 (1985).
14. A. J. L. Clark, *ibid.*, p. 8374.
15. G. Carpenter and S. Cohen, *Annu. Rev. Biochem.* **48**, 193 (1979).
16. C. D. Lane, *Curr. Top. Dev. Biol.* **18**, 89 (1983).
17. H. Soreq, *Crit. Rev. Biochem.* **18**, 199 (1985).
18. T. Kubo *et al.*, *Nature (London)* **323**, 411 (1986).
19. T. Maniatis, E. F. Fritch, J. Sambrook, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1985).
20. Y. Oron, B. Gillo, R. E. Straub, M. C. Gershengorn, *Mol Endocrinol.*, in press.

3 June 1987; accepted 24 August 1987

Epidermal Growth Factor–Dependent Transformation by a Human EGF Receptor Proto-Oncogene

THIERRY J. VELU, LAURA BEGUINOT,* WILLIAM C. VASS, MARK C. WILLINGHAM, GLENN T. MERLINO, IRA PASTAN, DOUGLAS R. LOWY

The epidermal growth factor (EGF) receptor gene *EGFR* has been placed in a retrovirus vector to examine the growth properties of cells that experimentally overproduce a full-length EGF receptor. NIH 3T3 cells transfected with the viral DNA or infected with the corresponding rescued retrovirus developed a fully transformed phenotype in vitro that required both functional *EGFR* expression and the presence of EGF in the growth medium. Cells expressing 4×10^5 EGF receptors formed tumors in nude mice, while control cells did not. Therefore, the *EGFR* retrovirus, which had a titer on NIH 3T3 cells that was greater than 10^7 focus-forming units per milliliter, can efficiently transfer and express this gene, and increased numbers of EGF receptors can contribute to the transformed phenotype.

MOST ONCOGENES ARE ALTERED versions of evolutionarily conserved cellular genes that apparently subserve important functions in normal growth and development (*1*). These normal genes are often called proto-oncogenes to emphasize their genetic relationship to oncogenes. Oncogenes typically induce cellular transformation in vitro with high efficiency, while the proto-oncogenes from which they were derived are usually much less active. Many studies have demonstrated the tumorigenic potential of oncogenes, but the possible role of proto-oncogenes in tumor formation has been less completely documented.

The epidermal growth factor (EGF) receptor, which is the product of a proto-oncogene, enables cells to undergo DNA synthesis in response to either of the known physiologic EGF receptor ligands, EGF and TGF-α (*2, 3*). The v-*erb*B oncogene of avian erythroblastosis virus encodes a truncated version of the EGF receptor (*4*). The oncogenic properties of v-*erb*B demonstrate that an altered EGF receptor can contribute directly to tumorigenesis. A potential role for normal EGF receptor in tumor formation has also been proposed, because increased numbers of apparently normal EGF receptors may be present in various human tumors and tumor cell lines, including the epidermoid carcinoma A431 cell line (*5*). However, the oncogenic potential of a full-length EGF receptor has not been demon-

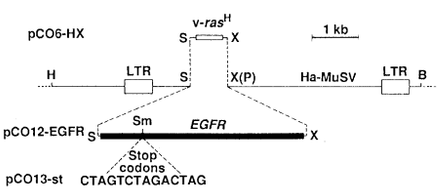

Fig. 1. Construction of the human EGF receptor retroviral genome. Restriction endonuclease sites: H, Hind III; S, Sac II; Sm, Sma I; P, Pst I; X, Xho I; and B, Bam HI. The *EGFR* retroviral vector (pCO12-EGFR) was derived from pCO6-HX, a construct in which the full-length Harvey murine sarcoma virus (Ha-MuSV) genome was cloned in pBR322 (*11*); the v-*ras*^{H} gene is flanked by the long terminal repeat (LTR) and by 5′ and 3′ untranslated regions. In pCO6-HX, the viral Pst I site at nucleotide 1759 (*20*) has been replaced with an Xho I linker (*21*). To insert the h*EGFR* cDNA, the entire coding region of p21 *ras* (Sac II–Xho I) was removed and replaced by the 4.2-kb fragment coding for the full-length hEGF receptor, derived from a pMMTV *EGFR* plasmid (*6*). The pCO13-st was derived from pCO12 by insertion of a universal translation terminator linker at a Sma I site at codon 220 in the *EGFR* cDNA; this construct therefore encodes a nonfunctional protein.

T. J. Velu, W. C. Vass, D. R. Lowy, Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.
L. Beguinot, M. C. Willingham, G. T. Merlino, I. Pastan, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

*Present address: University Microbiology Institute, Øster Farigmagsgade 2A, 1353 Copenhagen, Denmark.