vector (pSTC-tkGST) (*7*), and transfected by the calcium phosphate method into 293 cells. In vitro kinase assays with immobilized proteins were done as described in (*6*).

15. COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%). Subconfluent cells were transfected by the DEAE method with pcDNA3-based expression vectors (Invitrogen). The amount of plasmid DNA was kept constant by the addition of empty vector when necessary (2 μg per 10-cm plate). After 24 hours, cells were deprived of serum for 24 hours and subsequently treated with inhibitors or stimulated before lysis [in 20 mM Hepes (pH 7.5), 10 mM EGTA, 1% NP-40, 2.5 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM dithiothreitol, and 40 mM β-glycerophosphate, supplemented with 1 mM phenylmethylsulfonyl fluoride, aprotinin (20 μg/ml), and leupeptin]. Hemagglutinin (HA)-tagged Erk2, MEK1, and PKB were immunoprecipitated from the centrifuged lysates with mouse monoclonal antibodies to HA (anti-HA, 12CA5 from Babco) for 1 to 2 hours at 4°C and immobilized on Gamma-bind Sepharose (Pharmacia). Beads were washed twice with phosphate-buffered saline containing 1% NP-40 and 2 mM Na_3VO_4, followed by washes with 100 mM tris (pH 7.5) with 0.5 M LiCl and MAPK reaction buffer [12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM Na_3VO_4]. Myelin basic protein was the substrate for Erk2 and MEK1; for PKB assays, crosstide peptide was used [R. Meier, D. R. Alessi, P. Cron, M. Andjelkovic, B. A. Hemmings, *J. Biol. Chem.* **272**, 30491 (1997)].

16. We thank K.-L. Guan for MEK, R. Marais for Raf, T. Franke for PKB expression plasmids, S. Gutkind for plasmids described in (*11*), B. Hemmings and R. Meier for help with the PKB assay and crosstide peptide, and A. Schneider for criticism. Supported by Swiss National Science Foundation grant 3100-50506.97 (to M.P.W.).

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Controlling Gene Expression in Living Cells Through Small Molecule–RNA Interactions

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Short RNA aptamers that specifically bind to a wide variety of ligands in vitro can be isolated from randomized pools of RNA. Here it is shown that small molecule aptamers also bound their ligand in vivo, enabling development of a method for controlling gene expression in living cells. Insertion of a small molecule aptamer into the 5′ untranslated region of a messenger RNA allowed its translation to be repressible by ligand addition in vitro as well as in mammalian cells. The ability of small molecules to control expression of specific genes could facilitate studies in many areas of biology and medicine.

In vitro genetic selections (*1*) have been used to isolate nucleic acid sequences (aptamers) that bind small molecules with high affinity and specificity (*2*). The ability to control gene expression by using cell-permeable small molecules offers several advantages, and small molecule manipulation of gene expression at the levels of transcription (*3*) and signal transduction (*4*) has been reported.

We selected RNA aptamers that specifically bound to the related aminoglycoside antibiotics kanamycin A and tobramycin (Fig. 1A) (*5*). We analyzed the ability of these aptamers to function in vivo by expressing them in *Escherichia coli* and testing for a drug-resistant phenotype (*6*). In the absence of drug, bacterial strains expressing no aptamer (bl-RSETA), the kanamycin aptamer (bl-kan1), or the tobramycin aptamer (bl-tob1) grew similarly (Fig. 1B). In the presence of 10 μM kanamycin A, bl-kan1 grew to saturation, whereas growth of bl-RSETA and bl-tob1 was negligible (Fig. 1C). In the presence of 10 μM tobramycin, bl-tob1 grew to saturation, and bl-kan1 grew to a subsaturating concentration (Fig. 1C) (*7*). Increasing the number of aptamers in the expression vector from one to three enhanced growth in the presence of antibiotic (Fig. 1D). Thus, a specific drug-resistant phenotype was conferred by expression of an aminoglycoside aptamer (*8*), which demonstrates a small molecule–aptamer interaction in vivo.

We next asked whether small molecule aptamers could be used to regulate gene expression. Eukaryotic translation initiation typically involves 5′-to-3′ scanning from the 5′-m^7G cap to the start codon (*9*). Binding of a protein

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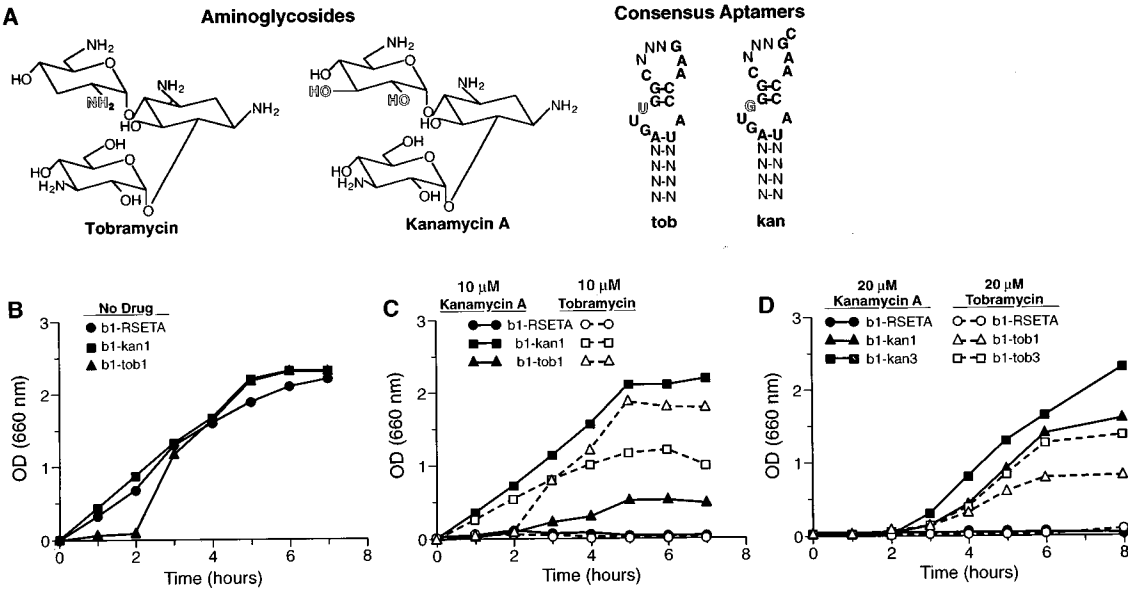

Fig. 1. Selective interaction between aminoglycosides and aminoglycoside aptamers in vivo. (**A**) Structures of aminoglycoside antibiotics and their aptamers. Consensus aptamers were identified after 10 to 12 rounds of selection. (**B** to **D**) Growth curves. Overnight cultures of *E. coli* BL-21 transformed with plasmids expressing RSETA, tob1, tob3, kan1, or kan3 were diluted 1:100 into medium containing the indicated concentration of aminoglycoside antibiotic. Optical density (660 nm) was measured at fixed intervals over 8 hours of growth at 37°C. (B) Growth in the absence of drug. (C) Growth in the presence of 10 μM kanamycin A or tobramycin. (D) Growth in the presence of 20 μM kanamycin A or tobramycin.

between the cap and start codon can repress translation, presumably by blocking either scanning or the ribosome-mRNA interaction (*10*). We thus asked whether the presence of a small molecule–aptamer complex within the 5′ untranslated region (UTR) would repress translation.

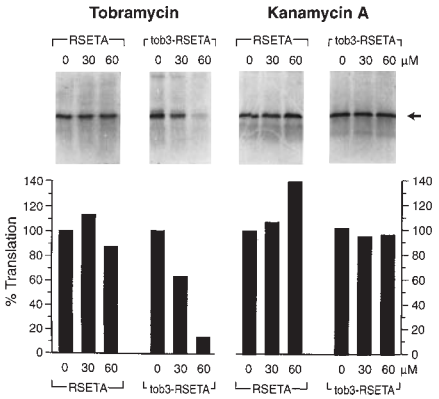

Fig. 2. An aminoglycoside aptamer translation switch functions in vitro. RNA transcripts containing zero (RSETA) or three copies of the tob aptamer cloned into the 5′ UTR of RSETA (tob3-RSETA) were translated in a wheat germ extract in the presence of [^{35}S]methionine and 0, 30, or 60 μM tobramycin or kanamycin A. Protein products were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (arrows) and quantitated by densitometry. For each transcript, translation in the absence of drug was set at 100%.

We constructed an mRNA that contained three copies of the tob aptamer inserted in the 5′ UTR of RSETA (tob3-RSETA). In vitro translation (*11*) of the control RSETA mRNA was unaffected by all concentrations of tobramycin or kanamycin tested, whereas addition of tobramycin inhibited in vitro translation of the tob3-RSETA mRNA in a dose-dependent fashion (Fig. 2) (*12*). In vitro translation of the tob3-RSETA mRNA was not inhibited by comparable concentrations of kanamycin A, which is not recognized by the tob aptamer.

We next attempted to reconfigure the system for regulating gene expression in vivo. Because aminoglycosides are relatively impermeable to the plasma membrane, can be cytotoxic, and have a general inhibitory effect on translation at high concentrations (*13*), we used a cell-permeable small molecule as the translation regulator.

Hoechst dye 33258 (H33258) and the closely related drug H33342 (Fig. 3A) are relatively nontoxic and cell-permeable (*14*). We isolated RNA aptamers that bound specifically to H33258 (*15*), two of which—H10 and H19—are shown (Fig. 3B). Both H10 and H19 bound to an H33258 affinity column and required a relatively high concentration (25 mM) of free H33258 for elution (Fig. 3C) (*16*).

To demonstrate that the H33258 aptamer could be used to regulate translation, we inserted one copy of H10 and H19 in tandem into the 5′ UTR of RSETA. Addition of H33258 inhibited in vitro translation of H2-RSETA but not the control RSETA in a dose-dependent fashion (Fig. 3D).

To test whether this small molecule–aptamer interaction could be used to control gene expression in vivo, we inserted one copy of H10 and H19 into the 5′ UTR of a mammalian β-galactosidase expression plasmid, SVβGal (Promega), generating the construct SVH2βgal. Chinese hamster ovary (CHO) cells were cotransfected with SVH2βGal or, as a control, the parental vector SVβGal and a luciferase reporter gene to provide an internal control (*17*). After transfection, cells were grown for 24 hours in the presence of 0, 5, or 10 μM H33342 and analyzed for β-galactosidase and luciferase activities (*18*).

In the absence of drug, two H33258 aptamers in the 5′ UTR had no effect on gene expres-

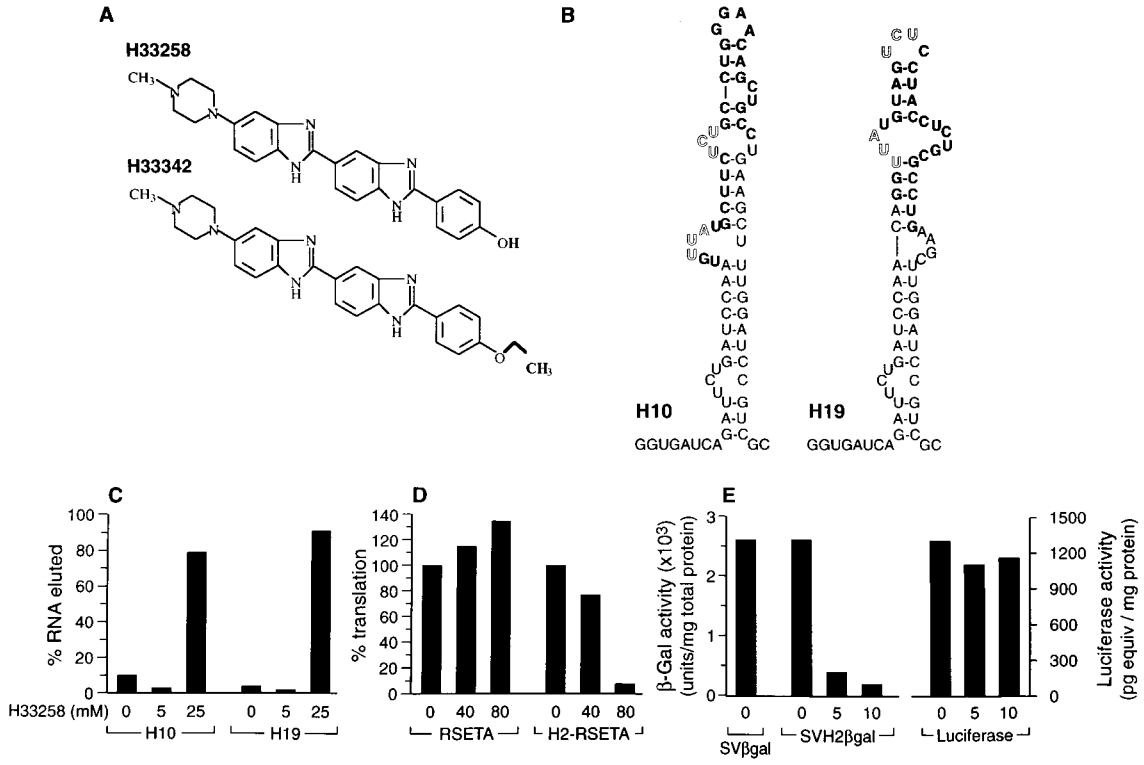

Fig. 3. Controlling translation in vitro and in vivo with cell-permeable small molecules. (**A**) Structure of Hoechst dyes H33258 and H33342. (**B**) Sequences and predicted secondary structures of two H33258 aptamers, H10 and H19, based on the computer modeling program Mulfold. A Hoechst dye aptamer consensus sequence (UUAN$_{4-5}$UCU) was identified after 10 rounds of selection. The fixed-primer binding regions are shown in normal print, selected bases are boldface, and the consensus sequence is indicated with outline print. (**C**) Interaction of H10 and H19 aptamers with H33258. [^{32}P]UTP-labeled aptamer (200,000 cpm) was loaded onto a 0.25-ml H33258 Sepharose column. Each column was then washed sequentially with 6 ml of binding buffer, 1 ml of binding buffer containing 5 mM H33258, and 1 ml of binding buffer containing 25 mM H33258. Percentages of total bound RNA eluted in each step are indicated. (**D**) In vitro translation. RNA transcripts containing zero (RSETA) or two copies of an H33258 aptamer cloned into the Bsa I site of pRSETA (H2-RSETA) were translated in a wheat germ extract in the presence of [^{35}S]methionine and 0, 40, or 80 μM H33258. Protein products were analyzed by SDS-PAGE and quantitated by densitometry. For each transcript, translation in the absence of drug was set at 100%. (**E**) In vivo expression. H33258 aptamers H10 and H19 were cloned in tandem into the 5′ UTR (Sfi I–Avr II sites) of a β-galactosidase reporter gene (SVβgal; Promega) to generate SVH2βgal. CHO cells were cotransfected with 1 μg of SVβgal or SVH2βgal and 1 μg of a luciferase expression vector (pGL3). Cells were grown in the presence of 0, 5, or 10 mM H33342. Twenty-four hours after transfection, cell extracts were prepared and β-galactosidase and luciferase activities were determined.

sion (compare SVβgal and SVH2βgal) (Fig. 3E), consistent with the in vitro translation data of Fig. 3D. Expression of the luciferase reporter was also not inhibited by H33342 (*19*). However, H33342 reduced β-galactosidase activity from SVH2βGal by greater than 90% in a dose-dependent fashion (Fig. 3E).

We have described how a small molecule and its RNA aptamer can be used to design a translation switch for controlling gene expression in living cells. The results also establish the possibility of using small molecules to regulate expression of endogenous genes.

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5. The initial 70-nucleotide RNA pool, containing 31 random nucleotides, was constructed as described [R. Singh, J. Valcarcel, M. R. Green, *ibid.* **268**, 1173 (1995)]. Tobramycin and kanamycin A were covalently linked to cyanogen bromide–activated Sepharose 4B. The RNA pool was dissolved in selection buffer [50 mM tris-HCl (pH 8.3), 250 mM KCl, 2 mM $MgCl_2$), loaded onto a preselection column (0.25 ml of glycine-Sepharose), eluted with two column volumes of selection buffer, and immediately loaded onto a 0.5-ml aminoglycoside-Sepharose column. Columns were washed with 10 column volumes of selection buffer (rounds 1 to 5), 10 column volumes of buffer with 5 mM competitor aminoglycoside (rounds 6 to 9), or 10 column volumes of buffer with 10 mM competitor (rounds 10 to 14). The competitor aminoglycoside for tobramycin aptamer selection was kanamycin A and vice versa. Bound RNA was eluted with the cognate aminoglycoside (5 mM) and amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) using flanking primers. The PCR products were transcribed into RNA with T7 RNA polymerase and purified by polyacrylamide gel electrophoresis. Pools were subcloned into the plasmid pBluescript (Stratagene) and sequenced after rounds 10, 12, and 14.
6. One or three copies of the kanamycin A (kan) or the tobramycin (tob) aptamer were cloned into the Nde I site (one copy) or Nde I and Bsa I sites (three copies) of T7 RNA polymerase-driven expression vector pRSETA (Invitrogen) and transformed into a bacterial strain containing an isopropyl β-D-thiogalactopyranoside (IPTG)–inducible T7 RNA polymerase. Bacterial strains were grown in liquid culture overnight, induced with 0.1 mM IPTG for 1 hour, and then diluted into medium containing antibiotic and 0.1 mM IPTG.
7. The partial resistance of bl-kan1 to tobramycin is consistent with the fact that the kan aptamer binds both drugs, whereas the tob aptamer is more selective and binds only tobramycin.
8. None of the strains exhibited increased resistance to the unrelated antibiotics tetracycline and gentamicin.
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11. In vitro transcription reaction mixtures contained 5 μg of pRSETA (or RSET derivative); 0.5 mM $m^7G(5')ppp(5')G$; 0.5 mM adenosine triphosphate, cytidine triphosphate, and uridine triphosphate (UTP); 0.05 mM guanosine triphosphate; 10 mM dithiothreitol; and 40 units of T7 RNA polymerase in 50 μl of a solution of 40 mM tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl. After incubation for 1 hour at 37°C, RNA was purified by extraction with phenol and chloroform, precipitated with ethanol, and resuspended in 30 μl of H_2O. Translation reactions were carried out in 10 μl containing 5 μl of wheat germ extract, 0.8 μl of 1 mM amino acid mixture (without methionine), 2 μl of RNA transcript as described above, [^{35}S]methionine (0.5 μl; 1200 Ci/mmol), and 0 to 80 μM drug. Reaction mixtures were incubated at 25°C for 15 min and terminated by addition of 2× sample loading buffer. Translation products were separated by electrophoresis on an 18% polyacrylamide gel, visualized by autoradiography, and quantitated by densitometry.
12. Translation repression was more efficient with multiple aptamers than with a single aptamer. Repression was also more efficient when the aptamers were positioned near the 5′ end of the mRNA.
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15. Isolation of H33258 aptamers was carried out by covalently linking H33258 to epoxy-activated Sepharose 6B. The ligand solution was mixed at 37°C for 16 hours. The resin was then washed and excess active groups were blocked with 1 M ethanolamine (pH 10). Preselection columns were prepared with ethanolamine alone. H33258 selection buffer contained 50 mM tris-HCl (pH 7.3), 200 mM KCl, 2 mM $MgCl_2$. In selection rounds 1 to 6, columns were washed with 20 column volumes of selection buffer and eluted with 2 column volumes of 10 mM H33258. In selection rounds 7 to 10, columns were washed with 20 column volumes of buffer and 20 column volumes of 10 mM benzimidazolepropionic acid (in selection buffer) before elution.
16. H10 and H19 bound H33258 and the closely related H33342 comparably.
17. CHO cells (80% confluent) were pretreated with 0, 5, or 10 mM H33342 and then cotransfected (Lipofectamine, Gibco-BRL) with 1 μg of pSVβgal or pH2βgal and 1 μg of the luciferase reporter gene pGL3 (Promega). Two hours after transfection H33342 was reapplied to the cells. Twenty-four hours after transfection cells were harvested and cell extracts were prepared. Cell extracts were normalized for total protein (Bradford assay). β-Galactosidase and luciferase activities in the extracts were determined relative to standard curves generated with the purified β-galactosidase and luciferase enzymes (Promega).
18. In these experiments, H33342 was used instead of H33258 because it is about 10 times more cell-permeable (*14*).
19. The parental expression vector SVβGal was also not inhibited by 5 or 10 μM H33342.

20. We are grateful to R. Singh for advice and for providing the randomized RNA pool and to M. Zapp for helpful discussions. This work was supported by an NIH grant to M.R.G. M.R.G. is an investigator of the Howard Hughes Medical Institute.

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Transition from Moderate to Excessive Drug Intake: Change in Hedonic Set Point

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Differential access to cocaine self-administration produced two patterns of drug intake in rats. With 1 hour of access per session, drug intake remained low and stable. In contrast, with 6 hours of access, drug intake gradually escalated over days. After escalation, drug consumption was characterized by an increased early drug loading and an upward shift in the cocaine dose-response function, suggesting an increase in hedonic set point. After 1 month of abstinence, escalation of cocaine intake was reinstated to a higher level than before. These findings may provide an animal model for studying the development of excessive drug intake and the basis of addiction.

A critical problem in drug addiction research is to understand the differences between controlled and uncontrolled drug use, the latter being an essential feature of drug addiction (*1, 2*). These two patterns of drug use may be observed simultaneously in different individuals or they may represent successive stages in the same individuals. The transition from drug use to addiction often involves a gradual process of escalated drug intake, whereby an individual's consumption becomes exaggerated with chronic exposure to a drug (*2*). Because escalation of drug use defines a common feature of drug addiction, the study of the factors that govern its development may help to explain the transition from drug use to drug addiction.

In animal models of drug self-administration, availability plays a role in determining the pattern of drug intake, as suggested by different studies with different drug access conditions (*3*). Numerous studies have restricted drug access to a few hours per day and produced a regular and stable pattern of consumption. In contrast, others have shown that, with continuous access to the drug, different patterns of drug intake develop, including the binge-like patterns of psychomotor stimulant use observed in both animals and humans (*3, 4*). Unknown,

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