Viral Evolution as a Tool to Improve the Tetracycline-regulated Gene Expression System*

Received for publication, December 19, 2003, and in revised form, January 29, 2004
Published, JBC Papers in Press, February 2, 2004, DOI 10.1074/jbc.M313895200

Atze T. Das, Xue Zhou, Monique Vink, Bep Klaver, Koen Verhoef, Giuseppe Marzio, and Ben Berkhout†
From the Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

We present viral evolution as a novel and powerful method to optimize non-viral proteins. We used this approach to optimize the tetracycline (Tc)-regulated gene expression system (Tet system) for its function in mammalian cells. The components of the Tet system were incorporated in the human immunodeficiency virus (HIV)-1 virus such that viral replication is controlled by this regulatory system. Upon long term replication of this HIV-rtTA virus in human T cells, we obtained a virus variant with an enhanced replication potential resulting from an improved rtTA component of the introduced Tet system. We identified a single amino acid exchange, F86Y, which enhances the transcriptional activity and doxycycline (dox) sensitivity of rtTA. We generated a new rtTA variant that is 5-fold more active at high dox levels than the initial rtTA, and 25-fold more sensitive to dox, whereas the background activity in the absence of dox is not increased. This new rtTA variant will be very useful in biological applications that require a more sensitive or active Tet system. Our results demonstrate that the viral evolution strategy can be used to improve the activity of genes by making them an integral and essential part of the virus.

Technology for the regulation of gene expression in mammalian cells and tissues is of primary importance for a wide variety of basic and applied biological research areas, including functional genomics, gene therapy, animal models for human diseases, and biopharmaceutical protein production. All these applications require that production of the protein(s) of interest can be regulated in both a quantitative and temporal way. For this purpose, artificial gene expression systems have been developed that are controlled by effector molecules in a dose-dependent and reversible manner. The most frequently used regulatory circuit is the so-called Tet system, which allows stringent control of gene expression by tetracycline (Tc)† or its derivative doxycycline (dox) (1–3). The Tet system is based on the specific, high affinity binding of the Escherichia coli Tet repressor protein (TetR) to the tet operator (tetO) sequence. Tc and dox induce a conformational change in TetR, which impedes the interaction with tetO. Fusion of the activation domain of the herpes simplex virus VP16 protein to TetR resulted in the transcriptional activator rtTA, which enhances gene expression from promoters placed downstream of tetO elements (Ptet) in eukaryotic cells. The presence of Tc or dox abolishes this gene expression. A rtTA variant with four amino acid substitutions in the TetR moiety exhibits a reverse phenotype (4). This reverse rtTA (rtTA) binds to Ptet, and activates gene expression in the presence of dox but not in its absence. The Tet system is now widely applied to control gene expression in eukaryotes, including mammals, plants, and insects (reviewed in Ref. 1). Since the Tet system originates from a bacterial regulatory system, it seems likely that the components can be optimized for their new transcriptional function in mammalian cells.

A commonly used strategy to improve or alter a specific biological function is directed evolution, which involves genetic diversification followed by selection. This strategy mimics natural evolution, but in a guided and accelerated fashion. In the first step of this approach, cloned DNA sequences, for example encoding a protein, are mutated via random mutagenesis or recombination, resulting in a library of related mutant sequences. In the second step, the mutant sequences with improved or novel function are selected from this library through careful screening in an in vitro or in vivo assay. Directed evolution has been used to change the activity, selectivity, or stability of enzymes (reviewed in Refs. 5 and 6) but also to improve viral vector stability (7), cytokine efficacy (8), and stability of enzymes (reviewed in Refs. 5 and 6) but also to improve viral vector stability (7), cytokine efficacy (8), and antibody fragment binding (9). Recently, Yokobayashi et al. (10) successfully applied directed evolution to genes comprising a simple genetic circuit and demonstrated that a nonfunctional circuit containing improperly matched components could evolve rapidly into a functional one (10, 11). The success of directed evolution depends on both the realization of a sufficiently large genetic diversity and the effectiveness of the selection procedure.

Previous optimizations of the Tet system were based on the introduction of rationally designed mutations, and on directed evolution, in which large scale mutagenesis of the components of the Tet system was followed by functional screening of the mutants in bacterial or yeast assay systems (12–15). However, these approaches are labor intensive, and mutations selected in a bacterial or yeast assay system may not be improvements in higher eukaryotes. We here present viral evolution as a novel and powerful method to optimize non-viral proteins. We used this approach to optimize the Tet system for its function in mammalian cells. The components of the Tet system were...
incorporated into the HIV-1 virus such that virus replication is controlled by this regulatory system. During replication of this virus on human T cells, genetic diversity is continuously generated due to the error-prone reverse transcription process, followed by outgrowth of faster replicating variants. Thus, the generation of genetic diversity and selection of improved variants are combined in this natural evolution approach. In this way, we have selected for HIV variants with improved replication capacity resulting from an improved rtTA-component of the introduced Tet system. The observed rtTA mutation does not only improve virus replication but also the Tet system.

EXPERIMENTAL PROCEDURES

Cells and Viruses—SupT1 T cells were grown at 37 °C and 5% CO2 in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10 units/ml penicillin, 100 units/ml streptomycin. SupT1 cells were transfected with HIV-1 molecular clones by electroporation. Briefly, 5 × 106 cells were washed in RPMI 1640 with 20% FBS and mixed with 1–10 μg of DNA in 250 μl of RPMI 1640 with 20% FBS. Cells were electroporated at 0.4–cm voltages at 250 V and 960 microfarads and subsequently resuspended in RPMI 1640 with 10% FBS. Cells were split 1 to 10 twice a week.

HeLa X1/6 cells (15) are derivatives of the HeLa cervix carcinoma cell line and harbor chromosomally integrated copies of the CMV-tetO promoter/luciferase reporter construct pUC13-3 (16). HeLa X1/6 and C33A cervix carcinoma cells (ATCC HTB31) (17) were grown at 37 °C and 5% CO2 as a monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, minimal essential medium nonessential amino acids, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were transfected by the calcium phosphate method. Cells were grown in 1 ml of culture medium in 2-cm2 wells of a 24-well plate to 60% confluence. 1 μg of DNA in 15 μl of water was mixed with 25 μl of 50 mm HEPES (pH 7.1–250 mM NaCl–1.5 mM Na2HPO4 and 10 μM of 0.6 μM CaCl2, incubated at room temperature for 20 min, and added to the culture medium. The culture medium was refreshed after 16 h.

The incorporation of the Tet system into the HIV-1 genome was described previously (18). The HIVrtTA used in this study is the KYK version, which contains the inactivating Y26A mutation in the Tat gene for 48 h in the absence of dox and lysed in 100 μl of passive lysis buffer (Promega). 10 μl of the lysate was mixed with 10 μl of reducing SDS sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 20% glycerol). Proteins were resolved in an SDS-10% polyacrylamide gel, transferred to Immobilon-P membrane (1 h, 80 V), and subsequently blocked with phosphate-buffered saline containing 5% nonfat dry milk. For immunochromatic detection of rtTA, membranes were subsequently incubated with rabbit serum containing polyclonal anti-TetR antibodies (23). Bound antibodies were visualized with peroxidase-linked anti-rabbit IgG and the ECL+ kit (Amer- sham Biosciences) and analyzed with a Storm 880 Imager (Amer sham Biosciences).

RESULTS

The Tet System as an Essential Component of HIV-1 Replication—We have previously reported the construction of an infectious HIVrtTA virus that is critically dependent on dox for its replication (18). HIV-1 gene expression and replication are naturally controlled by the viral Tat protein, which binds to the 5’ TAR hairpin in the nascent RNA transcript (24) and thereby enhances transcription (reviewed in Ref. 25). In the HIVrtTA variant, this Tat-TAR regulatory mechanism was inactivated by mutation of both Tat and TAR and functionally replaced by the components of the Tet system (Fig. 1A). The gene encoding the rtTA transcriptional activator protein was inserted in place of the 5’-terminal nef gene, and eight copies of the tetO binding sites were introduced in the LTR promoter. This virus does not replicate in the absence of dox. Administration of dox induces transcription of the viral genome and expression of the viral proteins, including rtTA. This rtTA protein subsequently activates transcription, gene expression, and virus replication. Other groups have also tried to implement the Tet system in HIV and SIV (simian immunodeficiency virus), but these attempts failed to produce an efficiently replicating virus (26, 27).
Although our HIV<sub>rtTA</sub> virus can initiate a spreading infection in the presence of dox, replication was relatively poor when compared with the parental HIV<sub>LAI</sub> virus. Instead of trying to improve HIV<sub>rtTA</sub> by additional molecular-biological manipulations, we set out to let nature select for virus variants with improved replication capacity.

**Improved Viral Replication through Evolution-driven Optimization of the rtTA Gene**—We previously reported that a characteristic change in the LTR-tetO promoter occurred in multiple, independent long term virus cultures (21, 22). In a particular culture with greatly accelerated virus replication we also observed amino acid changes in the rtTA protein. Genetic analysis at 114 days of culture indicated that HIV<sub>rtTA</sub> had acquired two amino acid changes in the rtTA protein; the phenylalanine at position 86 was replaced by tyrosine (F86Y) and the alanine at position 209 by threonine (A209T; Fig. 1A). The F86Y mutation is striking because it changes a strictly conserved residue in the TetR domain that is in direct contact with the Tc/dox effector molecule (Fig. 1C). To directly compare the replication capacity of this evolved HIV<sub>rtTA-F86Y</sub>, the original HIV<sub>rtTA</sub> and the wild-type HIV<sub>LAI</sub>, we infected SupT1 T cells with equal amounts of virus (Fig. 1B). Replication potential of the evolved HIV<sub>rtTA</sub> virus was significantly improved when compared with the original virus and approached that of the parental HIV<sub>LAI</sub>. Like the original HIV<sub>rtTA</sub> virus, the new HIV<sub>rtTA</sub> variant does not replicate in the absence of dox.

To demonstrate that the rtTA mutations are responsible for the observed improvement in viral replication, we constructed viruses with the individual F86Y mutation (HIV<sub>rtTA-F86Y</sub>) or the combined F86Y and A209T mutations (HIV<sub>rtTA-F86Y A209T</sub>) and assayed replication of these variants at different dox concentrations (Fig. 2A). The fully wild-type HIV<sub>LAI</sub> isolate of which the replication is not influenced by dox was included as a control (Fig. 2A). The parental HIV<sub>rtTA</sub> did not replicate in the absence of dox and a low level of replication was observed at 100 ng/ml dox (Fig. 2B). Increasing the dox concentration resulted in a gradual increase in replication rate. Replication of HIV<sub>rtTA-F86Y A209T</sub> was also completely dependent on dox, but a high level of replication was already apparent at 100 ng/ml dox (Fig. 2C). At this dox concentration, HIV<sub>rtTA-F86Y</sub> replicates even more efficiently than HIV<sub>rtTA</sub> at 1000 ng/ml dox. The HIV<sub>rtTA-F86Y A209T</sub> showed the same dox dependence and replication potential as HIV<sub>rtTA-F86Y</sub> (Fig. 2D). These results indicate that the F86Y rtTA mutation is sufficient to significantly improve viral replication both at low and high dox concentrations. The superior replication capacity of HIV<sub>rtTA-F86Y</sub> was confirmed in a direct competition experiment with HIV<sub>rtTA</sub>-SupT1 cells were infected with equal amounts of the two viruses in the presence of 1000 ng/ml dox. The F86Y variant dominated the viral population within 1 week of culture (more than 80% of the population-based sequence), and this variant was exclusively detected at later times (results not shown).

We also tested the effect of the rtTA mutations in a virus production assay by transiently transfecting plasmids encoding wt and mutant HIV<sub>rtTA</sub> proviral genomes into C33A cervix carcinoma cells. These cells support viral gene expression and virion production but do not support viral replication because they lack the appropriate receptors. HIV<sub>rtTA</sub> virus production gradually increases with increasing dox concentration and reaches its maximal level at 250 ng/ml dox (Fig. 3). HIV<sub>rtTA-F86Y A209T</sub> virus production is also dox-dependent, but a high production level is already reached at 30 ng/ml dox. Thus, the rtTA mutant significantly improves expression of the proviral genome at low dox concentrations.

**The Evolved rtTA Variant Greatly Improves the Tet System**—To test whether the evolved rtTAs do also improve the Tet gene expression system, we assayed the rtTA variants in a regular Tet system, out of the HIV<sub>rtTA</sub> virus context. Expression plasmids encoding the wt (rtTA<sub>wt</sub>) and mutant rtTA proteins (rtTA<sub>F86Y</sub> and rtTA<sub>F86Y A209T</sub>) were co-transfected into C33A cells with a plasmid encoding the luciferase reporter gene under the control of the viral LTR-23<sub>U</sub> promoter (Fig. 4A).
The luciferase level measured 2 days after transfection reflects the transcriptional activity of the rtTA protein. The wt and mutant rtTAs show no activity in the absence of dox (Fig. 4B). rtTAwt activity is detectable first at 500 ng/ml dox and increases further at 1000 ng/ml. rtTA F86Y and rtTA F86Y A209T activity is detectable at dox levels as low as 50 ng/ml, and this activity gradually increases with higher dox concentrations. Moreover, these mutant rtTAs are ~4-fold more active than the rtTAwt at higher dox levels. Identical activities are observed with rtTA F86Y and rtTA F86Y A209T, confirming that the F86Y mutation is solely responsible for the increased rtTA activity.

This optimization of the rtTA protein by spontaneous virus evolution is not a specific adaptation to the LTR-2 tetO promoter, since similar results were obtained in assays with a standard reporter gene construct in which the luciferase gene is under control of seven tet operators coupled to a minimal CMV promoter (Fig. 4C). We also assayed the rtTA activity in HeLa X1/6 cells that contain a chromosomally integrated copy of this CMV-7tetO reporter construct (15) (Fig. 4D). Also in these cells, both rtTA F86Y and rtTA F86Y A209T show activity at much lower dox concentrations when compared with rtTAwt, and the mutants are more active than the wild type at high dox levels. Thus, the F86Y mutation improves rtTA activity independent of the type of promoter and the episomal or chromosomal status of the promoter.

In their random mutagenesis studies, Urlinger et al. (12) recently identified an S12G mutation that improved rtTA activity. We made expression vectors encoding this mutant (rtTAS12G; previously named rtTA2S-M2 in Ref. 12) and a mutant in which the S12G mutation was combined with the F86Y and A209T mutations (rtTAS12G F86Y A209T). The rtTAs were tested in combination with the LTR-2 tetO reporter construct in C33A cells (Table I). rtTAS12G is indeed more active than rtTAwt, both at low and high dox levels. When compared with rtTA F86Y A209T, rtTAS12G is more active at the lowest dox concentration assayed but less active at high dox levels. Combining the mutations in rtTAS12G F86Y A209T yielded the highest activity at both low and high dox concentrations. To reach an activity that is comparable with the activity of rtTAwt at 1000 ng/ml dox, rtTAS12G F86Y A209T only needs 40 ng/ml dox, which can be translated into a 25-fold increased dox sensitivity (Table I). Importantly, like the other mutants, rtTAS12G F86Y A209T does not show any activity in the absence of dox. The optimal performance of this rtTA is also obvious when considering the fold induction of gene expression by dox. The rtTAS12G F86Y A209T activity is induced up to 47-fold, whereas rtTAwt can only be induced 9-fold in this assay system (Table I). Similar results were obtained when this mutant was tested with the CMV-7tetO reporter construct in C33A cells and in HeLa X1/6 cells.

To exclude the possibility that the enhanced activity observed with the mutant rtTAs resulted from an increased protein level, we determined the intracellular steady state amount of the rtTA proteins. Lysates of HeLa X1/6 cells transfected with rtTA expression plasmids were subjected to SDS-PAGE followed by Western blot analysis with polyclonal anti-TetR antibodies. As shown in Fig. 5, an equal amount of wild-type and mutant rtTA protein was detected. These results indicate that the enhanced activity and dox sensitivity of the mutant rtTAs are intrinsic features of the protein, and do not result from an increased production or stability of the proteins.
We demonstrate that the F86Y mutation significantly improves both the rtTA with which we started (rtTA2 S-S2; Ref. 12) and an S12G-mutated rtTA (rtTA2 S-M2; Ref. 12). The optimal rtTA contains both the F86Y and S12G mutations (rtTAS12G F86Y A209T) and is 5-fold more active at high dox levels than the initial rtTA. Moreover, this new rtTA is 25-fold more sensitive to dox, whereas the background activity observed in the absence of dox is not increased. This new rtTA variant will be very useful in biological applications that require a more sensitive Tet system, for example in gene therapy in tissues where relatively low dox levels can be reached (e.g. the brain). Moreover, the new rtTA improves replication of the 

HIVrtTA virus that we have proposed as a safe live attenuated virus vaccine (18, 21).

There are seven TetR classes (A–E, G, H) with a highly conserved sequence, and the phenylalanine at position 86 is strictly conserved. There is no crystal structure available of the rtTA protein at this moment. However, the structure of TetR, bound either to Tc or tetO DNA, has been solved (28–31). Phe86 is one of the four hydrophilic amino acids (the others are His64, Asn82, and Gln116) that anchor and position the Tc ring A by hydrogen bonding at the far end of a binding tunnel (Fig. 1C). Replacement of this Phe by Tyr, conserving the aromatic nature of this residue, may improve binding of dox, which could be the reason for the increased dox-sensitivity of this rtTA variant. Like the original rtTA, the F86Y-mutated rtTA is hardly activated by Tc (data not shown), which suggests that the mutation specifically improves binding of dox.

In the viral evolution approach, the two steps of directed evolution, i.e. generation of sequence diversity and the selection of improved variants, are combined. The virus automatically generates genetic diversity and performs selection of the most fit virus in this all-in-one system. Selection of fitter HIV-1 variants has previously been observed in in vitro cell culture infections (e.g. adaptation to specific cell lines, repair of virus mutants) and in vivo in HIV-infected individuals (e.g. appearance of drug-resistant variants). We here show that this powerful virus evolution can also be used for the optimization of exogenous genes that are inserted in the viral genome. Our viral evolution approach somehow resembles the compartmentalized self-replication (CSR) strategy, which has been used for directed evolution of Taq DNA polymerase (32). In CSR, genetic diversity is created by error-prone mutagenesis PCR, followed by the expression of mutant polymerases in E. coli and the enrichment of more active variants in a subsequent PCR reaction. Although there are obvious differences, one round of CSR can be compared with one replication cycle of the virus. Major advantages of the viral evolution approach are the continuous cascade of replication cycles and the continuous generation of genetic diversity. Camps et al. (33) recently presented a targeted gene evolution approach in E. coli with an error-prone DNA polymerase I. These bacteria were used to modify an antibiotic resistance gene and to select for improved variants. In this approach, the generation of genetic diversity and the selection of improved genes are coupled, like in our
long term replication of the HIVrtTA virus in the presence of variant with enhanced activity and dox sensitivity through dox. In another HIV rtTA culture in which virus replication likely due to a rearrangement of the tetO promoter elements as served increase in virus replication capacity in this culture was mutation did not improve virus replication. Instead, the ob-
suitable for the optimization of protein function in E. coli

TABLE I

Improved dox sensitivity and activity of new rtTAs

Transfection assays were performed with the LTR-2tetO luciferase reporter construct (mean values for three experiments are shown; ± S.D.).

| Dox (ng/ml) | No | wt | F86Y A209T | S12G | S12G F86Y A209T |
|------------|----|----|-------------|------|-----------------|
| 0          | 12.5 ± 1.6 | 11.0 ± 1.0 | 11.6 ± 1.7 | 11.6 ± 0.2 | 11.4 ± 0.7     |
| 10         | 12.8 ± 1.2 | 11.6 ± 0.7 | 14.4 ± 1.5 | 24.8 ± 1.2 | 23.3 ± 9.4     |
| 50         | 11.9 ± 0.1 | 15.2 ± 1.6 | 62.7 ± 6.8 | 67.1 ± 1.1 | 124.4 ± 51.9   |
| 100        | 11.8 ± 0.2 | 19.9 ± 2.1 | 134.7 ± 3.9 | 106.6 ± 13.6 | 217.3 ± 49.0   |
| 500        | 10.8 ± 0.7 | 65.5 ± 0.9 | 358.5 ± 23.1 | 246.6 ± 30.2 | 418.9 ± 47.9   |
| 1000       | 10.9 ± 0.9 | 100.0 ± 11.4 | 439.5 ± 59.0 | 320.4 ± 38.2 | 529.9 ± 22.8   |

- Fold induction

| Dox (ng/ml) | No | wt | F86Y A209T | S12G | S12G F86Y A209T |
|------------|----|----|-------------|------|-----------------|
| 0          | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0     |
| 10         | 1.0 ± 0.0 | 1.1 ± 0.1 | 1.2 ± 0.1 | 2.1 ± 0.1 | 2.0 ± 0.7     |
| 50         | 1.0 ± 0.1 | 1.4 ± 0.1 | 5.4 ± 0.2 | 5.8 ± 0.0 | 10.8 ± 3.8     |
| 100        | 1.0 ± 0.1 | 1.8 ± 0.4 | 11.7 ± 1.3 | 9.2 ± 1.3 | 19.0 ± 3.1     |
| 500        | 0.9 ± 0.2 | 6.0 ± 0.5 | 31.4 ± 6.0 | 21.2 ± 2.9 | 36.8 ± 1.9     |
| 1000       | 0.9 ± 0.2 | 9.1 ± 0.4 | 38.7 ± 9.9 | 27.6 ± 3.5 | 46.7 ± 3.2     |

- Fold sensitivity toward dox

**FIG. 5. Intracellular level of rtTA variants.** HeLa X1/6 cells were transfected with rtTA expression plasmids. At 2 days after transfection, total cellular extracts were prepared and subjected to SDS-PAGE, followed by Western blot analysis with rabbit serum containing polyclonal anti-TetR antibodies. In lane 1, purified TetR protein (4 ng) was loaded on the SDS-PAGE gel. In lane 2, cells were transfected with the pBlue-script control plasmid. In lanes 3–7, cells were transfected with the plasmid encoding wt or mutant rtTA protein, as indicated on top. The position and size of the rtTA and TetR proteins are indicated (on the right and left, respectively). The asterisk indicates background staining of cellular proteins that are equally present in all cell extracts.

viral evolution approach. Whereas their approach seems to be suitable for the optimization of protein function in E. coli, our approach allows the optimization in mammalian cells.

In this study, we used viral evolution to obtain an rtTA variant with enhanced activity and dox sensitivity through long term replication of the HIV rtTA virus in the presence of dox. In another HIV rtTA culture in which virus replication improved significantly, we observed a threonine to isoleucine mutation at rtTA position 103 (T103I). However, recloning of this rtTA variant into the HIV rtTA virus demonstrated that this mutation did not improve virus replication. Instead, the observed increase in virus replication capacity in this culture was likely due to a rearrangement of the tetO promoter elements as previously described (21, 22). These results underline the genetic flexibility of HIV rtTA, which can be further exploited for the functional modification of the Tet system and to further improve the HIV rtTA virus. For instance, long term replication of HIV rtTA at reduced dox concentrations may further improve the dox sensitivity of rtTA. These studies are currently being performed and already revealed that the F86Y mutation is stably maintained in multiple independent virus cultures. Moreover, adapting HIV rtTA to other, dox-like compounds that do not, or not efficiently, activate the current rtTA protein, such as Te or Minocycline, may result in rtTA variants that do efficiently respond to these compounds. Tet systems with new effector specificity will help in the generation of additional regulatory circuits and allow the independent regulation of multiple (trans)genes with different effector molecules. Moreover, when the virus is adapted to new compounds that lack antibiotic activity, Tet systems can be generated that are no longer responsive to antibiotics frequently used in medical practice. This would have several advantages, especially for applications in animals and humans. First, usage of a non-antibiotic compound to activate rtTA will avoid disturbance of the bowel flora. Second, dox can still be used as an antibiotic for medical purposes and does not unintentionally activate rtTA. Such an rtTA variant would be of particular advantage when the HIV rtTA virus is used as an anti-HIV-1 vaccine, since the risk of unintended reactivation of the virus after vaccination would be reduced.

Acknowledgments—We thank Rogier Sanders for drawing the TetR crystal structure and Christel Kruger, Christian Berens, and Wolfgang Hillen (University of Erlangen, Germany) for their generous gift of polyclonal anti-TetR antibodies and TetR protein.

REFERENCES

1. Gossen, M., and Bujard, H. (2001) in Tetracyclines in Biology, Chemistry and Medicine (Nelson, M., Hillen, W., and Greenwald, R. A., eds) pp. 139–157, Birkhäuser Verlag, Basel
2. Baron, U., and Bujard, H. (2000) Methods Enzymol. 327, 401–421
3. Freundlieb, S., Baron, U., Bonin, A. L., Gossen, M., and Bujard, H. (1997) Methods Enzymol. 283, 159–173
4. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Science 268, 1766–1769
5. Farinas, E. T., Bulte, T., and Arnold, F. H. (2001) Curr. Opin. Biotechnol. 12, 545–551
6. Sutherland, J. D. (2000) Curr. Opin. Chem. Biol. 4, 263–269
7. Powell, S. K., Kalos, M. A., Finkstaff, A., McKee, R., Burinski, I., Pensiero, M., Otto, E., Stemmer, W. P., and Song, N. W. (2000) Nat. Biotechnol. 18, 1279–1282
8. Leong, S. R., Chang, J. C., Ong, R., Dawes, G., Stemmer, W. P., and Punnazonen, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1163–1168
9. Yokobayashi, Y., Weiss, R., and Arnold, F. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16587–16591
10. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
11. Utsinger, S., Pardini, N., Thellung, M., Hassan, M. T., Bujaud, H., and Hillen, W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7963–7968
12. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
13. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
14. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
15. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
16. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
17. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
18. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
19. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
20. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
21. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
22. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
23. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
24. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
25. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
26. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
27. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
28. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
29. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
30. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
31. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
32. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
33. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
34. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
35. Hasty, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16516–16518
Viral Evolution as a Tool to Improve the Tetracycline-regulated Gene Expression System
Atze T. Das, Xue Zhou, Monique Vink, Bep Klaver, Koen Verhoef, Giuseppe Marzio and Ben Berkhout

J. Biol. Chem. 2004, 279:18776-18782.
doi: 10.1074/jbc.M313895200 originally published online February 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313895200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 16 of which can be accessed free at
http://www.jbc.org/content/279/18/18776.full.html#ref-list-1