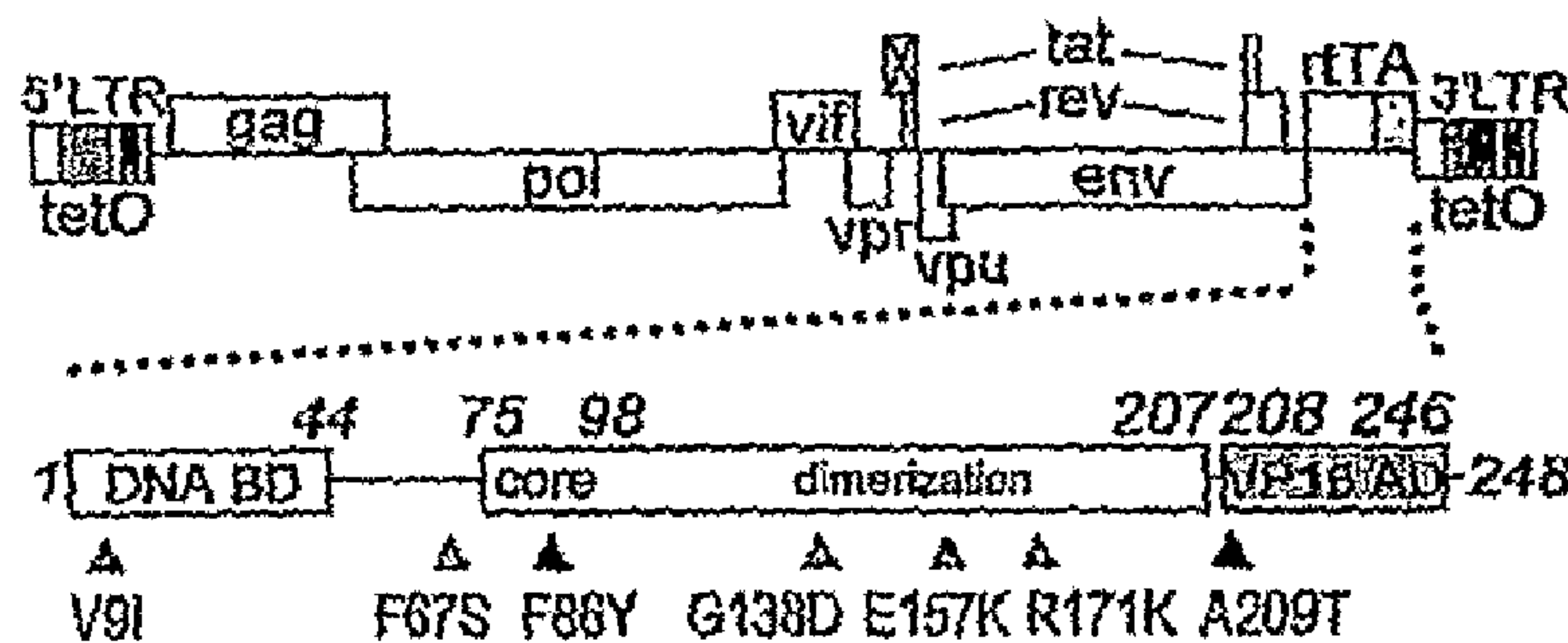




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(54) **Titre : SYSTEMES D'EXPRESSION INDUCTIBLE**
(54) **Title: INDUCIBLE EXPRESSION SYSTEMS**



(57) **Abrégé/Abstract:**

The invention provides improved rtTA and single chain rtTA variants and uses thereof for inducible expression of a nucleic acid of interest. Nucleic acid sequences comprising an improved rtTA and/or sc rtTA sequence according to the invention are also provided, as well as vectors, replicons and cells comprising such nucleic acid sequences.

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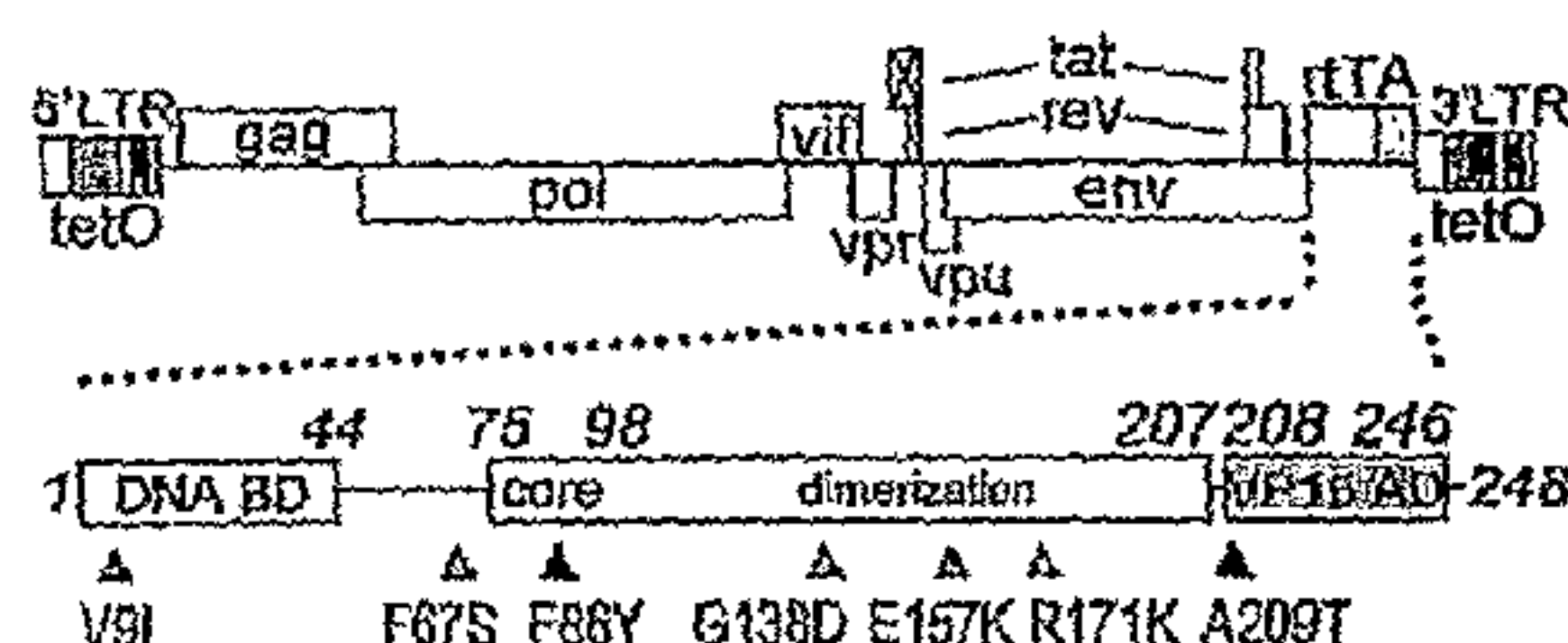
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(54) Title: INDUCIBLE EXPRESSION SYSTEMS



(57) **Abstract:** The invention provides improved rtTA and single chain rtTA variants and uses thereof for inducible expression of a nucleic acid of interest. Nucleic acid sequences comprising an improved rtTA and/or sc rtTA sequence according to the invention are also provided, as well as vectors, replicons and cells comprising such nucleic acid sequences.

Title: Inducible expression systems

The invention relates to molecular biology, in particular to improved expression systems of nucleic acids.

Systems to modulate nucleic acid expression are important for a wide
5 variety of basic and applied biological research areas, including functional
genomics, gene therapy, vaccination, animal models for human diseases and
biopharmaceutical protein production. In these applications expression of a
nucleic acid(s) of interest is preferably controlled in a quantitative and
temporal way. Several artificial gene expression systems that are regulated by
10 non-toxic effector molecules in a dose-dependent and reversible manner are
currently available. The Tet system, in which gene expression is stringently
controlled by tetracycline (Tc) or its derivative doxycycline (dox), is the most
widely used regulatory circuit (Baron et al. 2000; Gossen et al. 2001; Berens et
al. 2003). This system is based on the sequence-specific, high-affinity binding
15 of the *Escherichia coli* Tet repressor protein (TetR) to the tet operator (tetO)
DNA sequence. Tc or dox binds to TetR and triggers a conformational change
that prevents the repressor protein from binding to tetO. Fusion of the VP16
activation domain of herpes simplex virus to TetR resulted in the
transcriptional activator tTA, which induces nucleic acid expression from tetO-
20 containing promoters (P_{tet}) in eukaryotic cells (Gossen et al. 1992). The
presence of Tc or dox abolishes tTA-tetO interaction and switches off gene
expression (Tet-off system). A tTA variant with four amino acid substitutions
in the TetR moiety was identified, which exhibits a reverse phenotype (Gossen
et al. 1995). This reverse tTA (called rtTA) binds to P_{tet} exclusively in the
25 presence of dox, but not in its absence (Tet-on system). Both Tet systems are
now widely applied to control nucleic acid expression in eukaryotes, including
mammals, plants and insects (reviewed in (Gossen et al. 2001)). Because long-
term exposure to effectors is often undesirable, the Tet-on system is preferred

in applications in which nucleic acid expression is to be sustained in a switched-off state for long periods, or when rapid induction of nucleic acid expression is required.

5 Unfortunately, the amino acid substitutions in rtTA that confer the reverse phenotype also affect its binding affinity for effectors. As a consequence, rtTA has lost the ability to be activated by Tc and other Tc-like compounds, and it requires 100-fold more dox for maximal induction than that is needed for tTA inhibition. These characteristics severely limit the *in vivo*
10 use of the Tet-on system. For example, to activate Tet-on controlled transgene expression in the rat brain, the animals have to be fed with high doses of dox that are nearly toxic (Baron et al. 1997). Therefore, the Tet-on system, particularly its effector-sensitivity, has to be improved.

 Previously, the Tet system has been optimized by introduction of
15 rationally designed mutations (Baron et al. 1997; Baron et al. 1999), and by directed evolution in which random mutagenesis of the components of the Tet system was followed by functional screening of the mutants in bacterial or yeast assay systems (Gossen et al. 1995; Urlinger et al. 2000). However, these approaches are labor intensive, and mutations selected in bacterial or yeast
20 assay systems are not necessarily improvements in higher eukaryotes.

 Another disadvantage of current rtTA systems is the risk of reduced dox-dependence after multiple rounds of replication. This problem for instance arises during vaccination applications where replication of at least part of a
25 pathogen is under control of an rtTA system. In such vaccination applications, protection against said pathogen is acquired by controlled, inducible replication of said at least part of a pathogen, preferably during a restrained time span. If however the rtTA system loses its dox-dependence, said at least part of a pathogen will constitutively replicate, resulting in too much
30 pathogenic nucleic acid and/or proteins, involving a safety problem. The same

kind of problem arises during other applications involving various rounds of amplification of rtTA. It is therefore desired to improve the genetic stability of current rtTA systems.

5 It is an object of the present invention to provide rtTA and single chain rtTA variants. Preferably rtTA and single chain rtTA variants are provided with at least one improved property.

 The invention provides a method for inducibly expressing a nucleic
10 acid sequence of interest, the method comprising:
- providing a nucleic acid construct comprising said nucleic acid sequence of interest operably linked to an inducible gene expression system which comprises an rtTA encoding nucleic acid sequence and/or a single chain rtTA encoding nucleic acid sequence, said rtTA encoding nucleic acid sequence
15 and/or single chain rtTA encoding nucleic acid sequence comprising a mutation in a codon at rtTA amino acid position 9, and/or 19, and/or 37, and/or 56, and/or 67, and/or 68, and/or 138, and/or 157, and/or 171, and/or 177, and/or 195;
- introducing said nucleic acid construct to a suitable expression system; and
20 - allowing for inducible expression of said nucleic acid sequence of interest.

 The invention also provides a method for inducibly expressing a nucleic acid sequence of interest, comprising:
- providing a nucleic acid construct comprising said nucleic acid sequence of interest operably linked to an inducible gene expression system which
25 comprises a nucleic acid sequence encoding a reverse tetracycline transactivator (rtTA) and/or a single chain rtTA according to the amino acid sequence of SEQ ID NO:27, said rtTA and/or single chain rtTA encoding nucleic acid sequence comprising a mutation in a codon at rtTA amino acid position 67;
30 - introducing said nucleic acid construct to a suitable expression system; and
- allowing for inducible expression of said nucleic acid sequence of interest.

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The invention also provides a method for inducibly expressing a nucleic acid sequence of interest, comprising:

- providing a nucleic acid construct comprising said nucleic acid sequence of interest operably linked to an inducible gene expression system which
5 comprises a nucleic acid sequence encoding a reverse tetracycline transactivator (rtTA) and/or a single chain rtTA, wherein said rtTA and/or single chain rtTA comprises an amino acid sequence comprising an amino acid substitution at position 67 in the amino acid sequence of SEQ ID NO:27;
- 10 - introducing said nucleic acid construct to a suitable expression system; and
- allowing for inducible expression of said nucleic acid sequence of interest.

According to the present invention, a mutation in at least one of the above
15 mentioned codons of an rtTA nucleic acid or a single chain rtTA (sc rtTA) nucleic acid results in an improved rtTA or sc rtTA activator as compared to currently used Tet-on systems, such as described in (Gossen et al. 1995), (Urlinger et al. 2000) and (Krueger et al. 2003). Using an rtTA or sc rtTA variant of the present invention, an improved rtTA or sc rtTA system is
20 provided which has a higher transcriptional activity, a higher dox-sensitivity, a higher genetic stability and/or a lower level of transcription in the absence of an inducer, as compared to currently used rtTA or sc rtTA systems. The level of transcription in the absence of an inducer is called herein

basal activity. Furthermore, rtTA and sc rtTA systems are provided which are inducible by antibiotics other than doxycycline. Hence, the use of an rtTA and/or sc rtTA of the present invention is preferred for inducibly expressing a nucleic acid sequence of interest.

5 A preferred embodiment provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence further comprises a mutation in a codon at rtTA amino acid position 12, and/or 86, and/or 209. It has been shown that such additional mutations result in improved characteristics of the resulting
10 rtTA and sc rtTA systems.

 A single chain rtTA (sc rtTA) is a monomer comprising the same transregulating properties as the rtTA dimer in kind, not necessarily in amount. Said sc rtTA preferably comprises two TetR moieties and one
15 eukaryotic regulatory domain. Said two TetR moieties are preferably connected to each other by a linker, said linker preferably comprising a sequence encoding an (SG₄)₅ linker which is long and flexible enough to allow intramolecular assembly of the two TetR proteins. Non-limiting examples of single-chain Tet transregulators are described in (Krueger et al. 2003).
20 Methods for generating a single chain tTA and/or a single chain rtTA are described therein on page 3050, last paragraph and page 3051. These methods are non-limiting examples of generating sc rtTA.

 According to the present invention, a mutation in a sc rtTA which
25 corresponds to a mutation according to the present invention in an rtTA dimer transregulator results in an improved sc rtTA. A mutation in a sc rtTA corresponds to a mutation according to the invention in an rtTA dimer when a mutation in a sc rtTA is present in a codon encoding an amino acid residue at a position within said sc rtTA which is comparable to rtTA amino acid position 9,
30 12, 19, 37, 56, 67, 68, 86, 138, 157, 171, 177, 195 and/or 209.

By inducibly expressing a nucleic acid sequence of interest is meant herein that expression of a nucleic acid of interest is at least in part influenced by at least one inducer. Hence, by regulating the amount of inducer that is administered to said expression system, one is capable of regulating the amount of expression of said nucleic acid sequence of interest. Said inducer preferably comprises an exogenous compound, meaning that said compound is not naturally present within said expression system. Preferably, expression of a nucleic acid sequence of interest is dependent on the presence of an inducer. This means that said nucleic acid is expressed in the presence of an inducer, while it is expressed to a significant lesser extent in the absence of said inducer. Preferably said nucleic acid sequence is essentially not expressed in absence of said inducer.

A nucleic acid sequence of interest is operably linked to an inducible nucleic acid expression system when said inducible nucleic acid expression system is capable of expressing said nucleic acid sequence of interest. Preferably said nucleic acid sequence of interest is under control of a tetO-containing promoter. Expression of said nucleic acid of interest is at least in part inhibited in absence of an inducer, since rtTA and/or sc rtTA does not activate tetO-driven expression when an inducer is absent. In the presence of an inducer, rtTA and/or sc rtTA are able to activate tetO-driven expression of said nucleic acid of interest.

An rtTA or sc rtTA nucleic acid sequence according to the invention is defined as an rtTA or sc rtTA nucleic acid sequence derived from an rtTA or sc rtTA sequence (Urlinger et al. 2000; Das et al. 2004; Krueger et al. 2003), which rtTA or sc rtTA sequence has been provided with at least one mutation according to the present invention. Preferably at least one mutation according to the invention is introduced into the rtTA sequence depicted in Figure 19. A method according to the invention is therefore preferably provided wherein

said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises at least one mutation or combination of mutations according to the invention as compared to an rtTA encoding nucleic acid sequence depicted in Figure 19.

5

The invention also provides an isolated, synthetic or recombinant nucleic acid comprising a nucleic acid sequence encoding an rtTA and/or a single chain rtTA according to the amino acid sequence of SEQ ID NO:27, which rtTA and/or single chain rtTA encoding nucleic acid sequence
10 comprises a mutated codon at rtTA amino acid position 67.

The invention also provides an isolated, synthetic or recombinant nucleic acid comprising a nucleic acid sequence encoding an rtTA and/or a single chain rtTA, wherein said rtTA and/or single chain rtTA comprises an amino acid sequence comprising an amino acid substitution at position 67 in
15 the amino acid sequence of SEQ ID NO:27.

An rtTA nucleic acid and/or a sc rtTA nucleic acid is provided with a mutation of the present invention in a variety of ways. It is for instance possible to artificially introduce at least one mutation according to the present invention in an rtTA or sc rtTA nucleic acid, for instance via site
20 directed mutagenesis. Various methods for artificially introducing a specific mutation are known in the art and do not require further explanation here. Once a mutation or a combination of mutations according to the invention is introduced, an rtTA or sc rtTA nucleic acid of the invention is preferably further amplified. Amplified rtTA or sc rtTA comprising a mutation
25 according to the invention is thus also herewith provided. It is clear that it is no longer necessary to artificially introduce a mutation according to the invention once an rtTA or sc rtTA nucleic acid sequence of the invention is available, since a mutation of the invention is retained during amplification.

It is also possible to select an rtTA and/or sc rtTA with at least one
30 mutation according to the invention from a collection of rtTA/sc rtTA nucleic acids. For instance, non-specific mutations are introduced into a collection of rtTA/sc rtTA nucleic acids, and a nucleic acid molecule comprising at least

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one mutation according to the invention is selected (optionally after amplification). In a preferred embodiment rtTA or sc rtTA nucleic acid of the invention is selected from a collection of amplified rtTA or sc rtTA via an evolution and selection method. Since a mutation of the invention provides
5 at least one advantage to an rtTA /sc rtTA nucleic acid, it is possible to select a nucleic acid with a mutation of the invention on the basis of such advantage. For instance, a mutation of the invention resulting in enhanced sensitivity for dox is selected using very small amounts of dox. An inducible gene expression system is

incubated with a very small amount of dox, and sensitive systems are selected. As another example, a mutation of the invention resulting in diminished basal activity is selected by selecting an inducible nucleic acid expression system with very low - if at all - activity in the absence of an inducer.

5 In one embodiment, forced evolution is used in order to generate and select an rtTA and/or sc rtTA nucleic acid with at least one mutation according to the invention. In such method, amplification of rtTA or sc rtTA is performed with a method which involves the introduction of mismatches. This is preferably performed using a genome of a virus comprising RNA, because the
10 error-prone nature of its replication machinery (e.g. the reverse transcriptase (RT) enzyme or RNA polymerase enzyme) allows for the generation of modified nucleic acid sequences. This way, altered rtTA/sc rtTA nucleic acid molecules are produced. If such altered nucleic acid sequence comprises a mutation according to the invention, said nucleic acid will have an advantage over
15 nucleic acid sequences without a mutation of the invention. As a result, nucleic acid molecules comprising at least one mutation according to the invention will outgrow nucleic acid molecules without a mutation of the invention. As a result, an rtTA and/or sc rtTA nucleic acid comprising at least one mutation according to the invention is easily selected.

20 In one preferred embodiment a forced evolution method is used with help of a Human Immunodeficiency Virus-1 (HIV-1) genome, which forced evolution method is described in WO 01/20013 page 21, lines 5-28.

25 As used herein, an rtTA or sc rtTA variant is represented by the term "X[number]Y", wherein X represents the kind of amino acid residue present in a currently used rtTA activator, [number] represents the position of said amino acid residue in said rtTA, and Y represents the amino acid residue that is currently present at said position in said variant. For instance, V9I means a
30 variant which comprises at rtTA amino acid position 9 an isoleucine residue

instead of a valine residue. Variants comprising multiple mutations are represented by multiple X[number]Y indications. Hence, variant V9I F67S R171K F86Y means a variant which comprises at rtTA amino acid position 9 an isoleucine instead of a valine and which comprises at rtTA amino acid position 67 a serine instead of a phenylalanine and which comprises at rtTA amino acid position 171 a lysine instead of an arginine and which comprises at rtTA amino acid position 86 a tyrosine instead of a phenylalanine.

Which mutation according to the invention, or which combination of mutations according to the invention, is used in a specific application is for instance dependent on the kind of advantage(s) that is desired. For instance, for inducible *in vivo* transgene expression in a brain, a sensitive rtTA and/or sc rtTA nucleic acid is particularly desired, since only a small amount of inducer is capable of passing the blood brain barrier. For such application, an rtTA / sc rtTA nucleic acid with a mutation according to the invention at least resulting in improved sensitivity is preferred. In that case, variant V9I F67S G138D F86Y, V9I F67S G138D F86Y A209T, V9I F67S E157K F86Y, V9I F67S E157K F86Y A209T, V9I F67S R171K F86Y, V9I F67S R171K F86Y A209T, E37Q F67S F86Y, E37Q F67S F86Y A209T, V9I C68R G138D F86Y, V9I C68R G138D F86Y A209T, V9I G19M G138D F86Y, V9I G19M G138D F86Y A209T, V9I E37Q G138D F86Y, V9I E37Q G138D F86Y A209T, V9I G19M F67S G138D F86Y, V9I G19M F67S G138D F86Y A209T, V9I S12G F67S G138D F86Y, V9I S12G F67S G138D F86Y A209T, V9I F67S C68R G138D F86Y and/or V9I F67S C68R G138D F86Y A209T is preferred because these variants have more than 100-fold doxycyclin sensitivity as compared to rtTA, with no or low basal activity in the absence of inducer (as indicated in Figure 14B). If some level of basal activity in the absence of inducer is not a problem, a V9I G19M F67S G138D F86Y and/or V9I G19M F67S G138D F86Y A209T rtTA variant is particularly preferred, which is more than 300 times more sensitive for doxycyclin induction as compared to rtTA (indicated in Figure 14).

Furthermore, an rtTA or sc rtTA variant comprising an alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, glutamine, arginine, serine, threonine valine or tyrosine residue at rtTA amino acid position 19 has an improved

5 transcriptional activity as compared to currently known rtTA. Said variants have an increased transcriptional activity at a low doxycycline concentration (between 10 and 100 ng/ml) and/or an increased transcriptional activity at a high doxycycline concentration (between 100 and 1000 ng/ml) as compared to currently known rtTA. One embodiment of the invention therefore provides an

10 isolated, synthetic or recombinant amino acid sequence comprising an rtTA sequence and/or a sc rtTA sequence, which rtTA sequence and/or sc rtTA sequence comprises an alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, glutamine, arginine, serine, threonine valine or tyrosine at rtTA amino acid position 19.

15 An isolated, synthetic or recombinant nucleic acid sequence comprising a sequence encoding an rtTA sequence and/or a sc rtTA sequence, which rtTA sequence and/or sc rtTA sequence comprises an alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, glutamine, arginine, serine, threonine valine or tyrosine at rtTA

20 amino acid position 19 is also herewith provided, as well as a use of said nucleic acid sequence in a method according to the invention for inducible expressing a nucleic acid sequence of interest.

An rtTA or sc rtTA variant comprising a cysteine, methionine, glutamine, arginine or threonine residue at rtTA amino acid position 37 has an

25 improved transcriptional activity as compared to currently known rtTA. Said variants have an increased transcriptional activity at a low doxycycline concentration (between 10 and 100 ng/ml) and/or an increased transcriptional activity at a high doxycycline concentration (between 100 and 1000 ng/ml) as compared to currently known rtTA. One embodiment of the invention therefore

30 provides an isolated, synthetic or recombinant amino acid sequence comprising

an rtTA sequence and/or a sc rtTA sequence, which rtTA sequence and/or
sc rtTA sequence comprises a cysteine, methionine, glutamine, arginine or
threonine residue at rtTA amino acid position 37. An isolated, synthetic or
recombinant nucleic acid sequence comprising a sequence encoding an rtTA
5 sequence and/or a sc rtTA sequence, which rtTA sequence and/or sc rtTA
sequence comprises a cysteine, methionine, glutamine, arginine or threonine
residue at rtTA amino acid position 37 is also herewith provided, as well as a
use of said nucleic acid sequence in a method according to the invention for
inducible expressing a nucleic acid sequence of interest.

10

If an rtTA and/or sc rtTA nucleic acid of the invention is used for
vaccination purposes involving controlled expression of a (pathogen-derived)
nucleic acid sequence of interest, it is important that basal activity is minimal.
Expression of a pathogenic nucleic acid of interest in absence of an inducer is
15 undesired because it would result in continuous presence of said pathogenic
nucleic acid of interest. In that case an organism would be challenged too much
with pathogenic nucleic acid, which could for instance result in disease and/or
tolerance of the immune system for said pathogenic nucleic acid of interest. If
tolerance is induced, protection against a subsequent challenge with said
20 pathogen is diminished. At least in part avoiding basal activity is particularly
important if the replication of a viable pathogen is inducibly controlled by an
rtTA and/or sc rtTA system. Continuous replication of said pathogen involves
the risk of spreading and outgrowth of too many pathogenic organisms,
resulting in disease. For vaccination purposes, an rtTA and/or sc rtTA variant
25 of the invention with a very low basal activity - if any - is therefore preferred.
In such case, variant F67S F86Y, F67S F86Y A209T, G138D F86Y, G138D
F86Y A209T, E157K F86Y, E157K F86Y A209T, R171K F86Y, R171K F86Y
A209T, V9I G138D F86Y, V9I G138D F86Y A209T, V9I E157K F86Y, V9I
E157K F86Y A209T, V9I R171K F86Y, V9I R171K F86Y A209T, F177L F86Y,
30 F177L F86Y A209T, F67S F177L F86Y, F67S F177L F86Y A209T, C195S

F86Y, C195S F86Y A209T, G138S F86Y, G138S F86Y A209T, C68R F86Y, C68R F86Y A209T, F67S G138D F86Y, F67S G138D F86Y A209T, F67S E157K F86Y, F67S E157K F86Y A209T, F67S R171K F86Y, F67S R171K F86Y A209T, V9I F67S R171K F86Y, V9I F67S R171K F86Y A209T, S12G F67S F86Y, S12G F67S F86Y A209T, G19M F67S F86Y and/or G19M F67S F86Y A209T is preferably used. These variants have a very low basal activity of 0.1 percent or less. A V9I F67S R171K F86Y and/or V9I F67S R171K F86Y A209T rtTA variant is most preferably used. These variants have a very low basal activity and are very sensitive since they have a more than 100-fold doxycyclin sensitivity as compared to rtTA (indicated in Figure 14).

The invention furthermore provides rtTA and sc rtTA variants with altered inducer-specificities. rtTA and sc rtTA variants are provided that are inducible by antibiotics other than doxycycline. Hence, although other dox-like compounds such as tetracycline (Tc) and minocycline (Mc) do not effectively activate wild type rtTA, the invention provides variants that have become inducible by at least one of these antibiotics. This provides amongst other things the advantage that tetracycline is suitable as inducer, which is cheaper than doxycycline. Furthermore, rtTA and sc rtTA variants according to the invention that are inducible by antibiotics other than doxycycline are suitable for the development of rtTA and/or sc rtTA variants with an altered specificity which are inducible by at least one antibiotic other than doxycycline but not by doxycycline itself. Figure 15 shows preferred variants according to the invention that are responsive to tetracycline and/or minocycline, except for the wild type rtTA and the F86Y A209T mutant.

The invention therefore provides an isolated, synthetic or recombinant nucleic acid sequence comprising an rtTA encoding nucleic acid sequence and/or a single chain rtTA encoding nucleic acid sequence, which rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a mutation or a combination of mutations as depicted in

Figure 15 except for the F86Y A209T mutation (and, of course, except for the wild type variant). A use of at least one of said nucleic acid sequences for tetracycline-inducible and/or minocycline-inducible expression of a nucleic acid sequence of interest is also herewith provided.

5 Preferably, mutant F67S V9I G138D F86Y A209T, C68R V9I G138D F86Y A209T, G19M V9I G138D F86Y A209T, E37Q V9I G138D F86Y A209T, G19M F67S V9I G138D F86Y A209T, S12G F67S V9I G138D F86Y A209T and/or C68R F67S V9I G138D F86Y A209T is used for tetracycline-inducible expression of a nucleic acid sequence of interest since these mutants are
10 particularly sensitive for tetracycline, meaning that a small amount of tetracycline is sufficient for inducing gene expression. Most preferably, mutant F67S V9I G138D F86Y A209T, C68R V9I G138D F86Y A209T and/or S12G F67S V9I G138D F86Y A209T is used for tetracycline-inducible expression of a nucleic acid sequence of interest since these mutants are very sensitive for
15 tetracycline and show low background activity in the absence of any effector.

In a further preferred embodiment, mutant V9I G138D F86Y A209T, F67S V9I G138D F86Y A209T, F67S V9I E157K F86Y A209T, F67S V9I R171K F86Y A209T, F67S E37Q F86Y A209T, C68R V9I G138D F86Y A209T, G19M V9I G138D F86Y A209T, E37Q V9I G138D F86Y A209T, G19M F67S
20 V9I G138D F86Y A209T, S12G F67S V9I G138D F86Y A209T and/or C68R F67S V9I G138D F86Y A209T is used for minocycline-inducible expression of a nucleic acid sequence of interest since these mutants are particularly sensitive for minocycline, meaning that a small amount of minocycline is sufficient for inducing gene expression. Most preferably, mutant F67S V9I G138D F86Y
25 A209T, F67S E37Q F86Y A209T, C68R V9I G138D F86Y A209T and/or S12G F67S V9I G138D F86Y A209T is used for minocycline-inducible expression of a nucleic acid sequence of interest since these mutants are very sensitive for minocycline and show low background activity in the absence of any effector.

The invention further provides rtTA and sc rtTA variants that are genetically stable. Currently used inducible Tet-on systems are at risk of converting into a system constitutively expressing a nucleic acid sequence of interest. This is preferably avoided, for instance (amongst other things) when
5 replication of a pathogen is controlled by a Tet-on system. Constitutive replication of said pathogen would result in the presence of too many pathogens, involving the risk of disease and/or tolerance.

According to the present invention, the genetic stability of an inducible gene expression system comprising rtTA and/or sc rtTA nucleic acid is
10 improved by altering a codon at rtTA amino acid position 19, 37 and/or 56 (and/or the corresponding codons in sc rtTA) such that loss of inducer dependency is at least in part prevented. Reduction and/or loss of inducer-dependence of currently used rtTA and sc rtTA result from at least one mutation at rtTA amino acid position 19, 37 and/or 56, such as for instance a
15 G19E, E37K, E37A, E37S and/or a P56S mutation. According to the invention, replacement of the glycine residue at rtTA amino acid position 19 by a glutamic acid residue results in at least partial loss of inducer-dependence of rtTA/sc rtTA. Moreover, replacement of the glutamic acid residue at rtTA amino acid position 37 by a lysine, alanine or serine residue results in at least
20 partial loss of inducer-dependence of rtTA/sc rtTA. Moreover, replacement of the proline residue at rtTA amino acid position 56 by serine, tyrosine, cysteine, histidine, asparagine, alanine or glycine results in at least partial loss of inducer-dependence of rtTA/sc rtTA. The invention therefore provides modified rtTA and/or sc rtTA nucleic acids wherein spontaneous mutations that would
25 result in at least partial loss of inducer-dependence are less likely to occur as compared to currently used rtTA/sc rtTA. Such variants are obtained as described in the following paragraph.

In currently used rtTA, a G19E mutation requires only one nucleotide
30 change in codon 19, namely the codon change GGA to GAA. The G-to-A

transition is the most frequent error during reverse transcription of RNA. According to the present invention, reduction and/or loss of inducer-dependency of rtTA and/or sc rtTA is at least in part prevented by using an altered codon at position 19 that is more difficultly converted into a glutamic acid codon. This is preferably performed by using a codon at position 19 that differs in at least two nucleotides from a glutamic acid codon. If such codon is used, an undesired G19E mutation would require a much more difficult two-hit mutation. Hence, when an rtTA and/or sc rtTA nucleic acid is used with a codon at rtTA position 19 which differs in at least two nucleotides from a glutamic acid codon, an undesired G19E mutation is less likely to evolve as compared to currently used Tet-on systems. Reduction and/or loss of inducer-dependence is therefore at least in part prevented. The invention therefore provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon.

In one embodiment an alternative glycine codon at position 19 is used which alternative glycine codon differs in at least two nucleotides from a glutamic acid codon. In this embodiment, the alternative glycine codon GGU or GGC is used (instead of GGA which is present in currently used rtTA). A G19E mutation is much more difficult in this embodiment because it requires a GGU to GAA, a GGU to GAG, a GGC to GAA or a GGC to GAG change. Hence, in all those cases a two-hit mutation would be required. Since this is less likely to occur, an rtTA and/or sc rtTA with an alternative glycine codon according to this embodiment is less likely to lose its inducer-dependence. When an alternative glycine codon according to this embodiment is used, the resulting amino acid residue of the rtTA or sc rtTA activator at rtTA position 19 is the same as the activator encoded by currently used rtTA and sc rtTA nucleic acid. One preferred embodiment therefore provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single

chain rtTA encoding nucleic acid sequence comprises a glycine codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon.

In a more preferred embodiment, an rtTA or sc rtTA nucleic acid is used which comprises an alanine, cysteine, phenylalanine, histidine, isoleucine, leucine, methionine, asparagine, arginine, serine, threonine, valine, tryptophan or tyrosine codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon. A nucleic acid according to this embodiment is not only genetically more stable, but - except for the G19W variant - is also more sensitive for doxycycline. One embodiment therefore provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises an alanine, cysteine, phenylalanine, histidine, isoleucine, leucine, methionine, asparagine, arginine, serine, threonine, valine, tryptophan or tyrosine codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon. Suitable codons at rtTA amino acid position 19 which differ in at least two nucleotides from a glutamic acid codon are codon UUN (with N corresponding to G, A, U, or C (coding for Phenylalanine or Leucine), UCN (Serine), UAY (with Y corresponding to U or C; Tyrosine), UGU (Cysteine), UGC (Cysteine), UGG (Tryptophan), CUN (Leucine), CAY (Histidine), CGN (Arginine), AUN (Isoleucine or Methionine), ACN (Threonine), AAY (Asparagine), AGN (Serine or Arginine), GUY (Valine) and GCY (Alanine).

In another preferred embodiment, a method according to the invention is provided wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a cysteine, phenylalanine, isoleucine, leucine, arginine, serine or threonine codon at rtTA amino acid position 19 which differs in three nucleotides from a glutamic acid codon. Such variant is in particular genetically stable because three mutations

would be required in order to generate a G19E variant. Suitable codons at rtTA amino acid position 19 which differ in at least three nucleotides from a glutamic acid codon are codon UUY (with Y corresponding to U or C; coding for Phenylalanine), UCY (Serine), UGY (Cysteine), CUY (Leucine), CGY (Arginine), AUU (Isoleucine), ACU (Threonine) and AGU (Serine).

An rtTA or sc rtTA nucleic acid comprising a codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine and a serine codon is also provided. If such variant is used, spontaneous E37K, E37A and E37S mutation is less likely to occur as compared to currently used rtTA/sc rtTA because that would require a much more difficult two-hit mutation. As a consequence, loss of inducer dependency is at least in part avoided. The invention therefore provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon. Suitable codons at rtTA amino acid position 37 which differ in at least two nucleotides from an alanine, a lysine or a serine codon are codon CUN (coding for leucine, N stands for U, C, A or G), CAU, CAC (both CAU and CAC coding for histidine), CGA and CGG (both CGA and CGG coding for arginine). A rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprising codon CUN, CAU, CAC, CGA or CGG at rtTA amino acid position 37 is therefore preferably provided.

One preferred embodiment provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon and a codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon. Such variant is particularly

genetically stable, since spontaneous G19E, E37K, E37A and E37S mutations are at least in part prevented.

An rtTA and/or sc rtTA nucleic acid comprising an altered codon at rtTA amino acid position 56 also provides enhanced stability. It has been found that in the absence of doxycycline, rtTA variants are at risk of evolving to variants that have an altered amino acid residue at rtTA amino acid position 56 and that are no longer dependent on doxycycline. The invention therefore provides an isolated, recombinant or synthetic nucleic acid sequence comprising an rtTA and/or sc rtTA encoding nucleic acid sequence which comprises a codon at rtTA amino acid position 56 which differs in at least one nucleotide, preferably a transversion, from a codon that mediates transcriptional activity in the absence of an inducer. Most of said doxycycline-independent rtTA variants contain either a serine, tyrosine, cysteine, histidine, asparagine, alanine or glycine residue at position 56 instead of a proline. In order to at least in part avoid the development of such variant, an rtTA and/or sc rtTA encoding nucleic acid is preferably provided which comprises a CAA or CAG codon encoding glutamine or a AAA or AAG codon encoding lysine at rtTA amino acid position 56.

A transversion is defined herein as a substitution of a purine into a pyrimidine, or a substitution of a pyrimidine into a purine. A transversion is less likely to occur during natural evolution as compared to a substitution of a purine into another purine, or a substitution of a pyrimidine into another pyrimidine. Therefore, an rtTA and/or sc rtTA encoding nucleic acid sequence which comprises a codon at rtTA amino acid position 56 which differs in at least one transversion from a codon encoding a serine, tyrosine, cysteine, histidine, asparagine, alanine or glycine residue is genetically more stable as compared to current rtTA and sc rtTA.

According to the present invention, an rtTA or sc rtTA nucleic acid with at least one mutated codon at rtTA amino acid position 9, 19, 37, 56, 67, 68, 138, 157, 171, 177, and/or 195 comprises at least one improved characteristic as compared to currently available Tet-on systems. Preferably, a method
5 according to the invention is provided wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 9 encoding isoleucine, and/or a codon at rtTA amino acid position 19 encoding alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine,
10 glutamine, arginine, serine, threonine, valine, tryptophan or tyrosine, and/or a codon at rtTA amino acid position 37 encoding threonine, histidine, leucine, arginine, cysteine, methionine or glutamine, and/or a codon at rtTA amino acid position 56 encoding lysine or glutamine, and/or a codon at rtTA amino acid position 67 encoding serine, and/or a codon at rtTA amino acid position 68
15 encoding arginine, and/or a codon at rtTA amino acid position 86 encoding tyrosine, and/or a codon at rtTA amino acid position 138 encoding aspartic acid or serine, and/or a codon at rtTA amino acid position 157 encoding lysine, and/or a codon at rtTA amino acid position 171 encoding lysine, and/or a codon at rtTA amino acid position 177 encoding leucine, and/or a codon at rtTA
20 amino acid position 195 encoding serine, and/or a codon at rtTA amino acid position 209 encoding threonine. Any of these mutations, or any combination of them, is preferred since they particularly improve at least one property of an inducible nucleic acid expression system.

In order to improve rtTA and/or sc rtTA, it is sufficient to introduce one
25 mutation according to the invention into an rtTA and/or sc rtTA encoding nucleic acid sequence. Preferably however at least two mutations according to the invention are introduced, since a combination of at least two mutations according to the invention further improves at least one property of rtTA and/or sc rtTA. In a more preferred embodiment an rtTA nucleic acid and/or a
30 sc rtTA nucleic acid comprises at least three mutations according to the

present invention. Most preferably, an rtTA nucleic acid and/or a sc rtTA nucleic acid comprises at least four mutations according to the present invention.

5 In Figure 14 various preferred variants of rtTA and/or sc rtTA according to the present invention are depicted. These variants are particularly preferred for use in an inducible gene expression system. The invention therefore further provides a method according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence
10 comprises at least one variant as depicted in Figure 14. In one preferred embodiment a nucleic acid sequence of interest is inducibly expressed by rtTA variant V9I G19M F67S G138D F86Y and/or by variant V9I G19M F67S G138D F86Y A209T. These variants are about 385-fold more sensitive for doxycycline as compared to currently used rtTA. Hence, these variants are
15 particularly suitable for applications wherein small amounts of inducer is available and/or desired (for instance during transgene expression in a brain). In another preferred embodiment a nucleic acid sequence of interest is inducibly expressed by rtTA variant V9I F67S R171K F86Y and/or by variant V9I F67S R171K F86Y A209T. These variants are about 100-fold more
20 sensitive for doxycycline as compared to currently used rtTA, while at the same time basal activity is very low (about 0.1 percent). Hence, these variants are particularly suitable for applications wherein sensitivity for doxycycline is desired, while basal activity is undesired (for instance during inducible expression of a pathogen).

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 A mutation according to the present invention is furthermore suitable for improving at least one characteristic of alternative rtTA and/or TetR derived molecules, besides rtTA and sc rtTA. Such alternative rtTA and/or TetR derived molecule for instance comprises an alternative transcriptional
30 activation domain (see for example [Akagi et al. 2001] and [Kamper et al.

2002]), or a transcriptional silencer in which the activation domain has been replaced by a transcriptional repressor domain (tTS, see for example [Deuschle et al. 1995]), or the tTA transcriptional activator, which is active in the absence of an effector and repressed by an effector (Gossen and Bujard, 1992).

5

Any inducible nucleic acid expression system comprising an rtTA and/or sc rtTA nucleic acid sequence according to the present invention and/or an alternative molecule derived thereof is suitable for inducibly expressing a nucleic acid sequence of interest. *In vivo* as well as *ex vivo* applications are possible. In one embodiment a prokaryotic nucleic acid expression system is used. Preferably said nucleic acid of interest is expressed in a eukaryotic expression system, because an rtTA and/or sc rtTA sequence comprising a VP16 activation domain of herpes simplex virus is particularly suitable for regulating nucleic acid expression from tetO-containing promoters in eukaryotic cells. An rtTA and/or sc rtTA nucleic acid sequence according to the present invention, and alternative molecules derived thereof, are suitable for use in a lower eukaryotic expression system. Moreover, an rtTA and/or sc rtTA nucleic acid sequence according to the present invention and alternative molecules derived thereof are suitable for use in a higher eukaryotic expression system. In one embodiment said nucleic acid of interest and/or an alternative molecule derived thereof is expressed in a mammalian cell.

In principle, any nucleic acid sequence of interest is inducibly expressed by a nucleic acid expression system according to the present invention. For instance, suitable applications for an inducible gene expression system according to the present invention are the production of protein pharmaceuticals, *in vivo* expression of therapeutic proteins and production of transgenic animals wherein a (pathogenic) gene of interest is inducibly expressed, to name just a few. In one embodiment at least one viral sequence essential for replication of a virus or replicon is inducibly expressed by a

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nucleic acid expression system of the invention. This is particularly suitable for vaccination purposes in order to provide at least partial protection to a viral pathogen, wherein it is important that said virus or replicon replicates in order to obtain an efficacious immune response, but wherein it is also important that
5 said replication does not go beyond the level required for said immune response. A replicon is defined as a nucleic acid molecule capable of replication in a suitable environment, such as a permissive cell, because it has all the necessary elements for replication in such an environment. We call it a replicon, because it will not always be directly derived from the nucleotide
10 sequences of the original pathogen.

By placing at least one viral sequence essential for replication of a virus or replicon under control of an rtTA and/or sc rtTA nucleic acid sequence of the invention, said virus or replicon replicates in a controlled manner. The amount of replication necessary for eliciting a good immune response without any
15 replication beyond that level is thus regulated by regulating the amount of inducer that is administered to an inducible nucleic acid expression system according to the present invention. In order to prevent leakage, it is preferred to have a combination of essential genes under such control and it is even more preferred to have at least two different repressor/activator combinations in
20 control of at least one, but preferably more than one gene essential for replication. In most (viral) pathogens a number of genes is essential for replication, but most of them also have a sort of "master switch", usually an early gene, usually transactivating other genes. A first candidate to put under direct control of a repressor/activator is of course such a master switch, which
25 then indirectly provides control over other essential genes for replication. Still it is even then preferred to put at least one other essential gene under control of an inducible repressor/activator.

In one embodiment at least part of a HIV genome essential for
30 replication is inducibly expressed under control of an rtTA and/or sc rtTA

nucleic acid sequence of the invention. This is for instance suitable for improved AIDS prophylaxis as compared to currently known methods. In this embodiment a master switch is not required since an HIV genome is under control of a single transcription unit.

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A nucleic acid sequence comprising an rtTA nucleic acid sequence and/or a sc rtTA nucleic acid sequence, said rtTA nucleic acid sequence and/or sc rtTA nucleic acid sequence comprising at least one mutation according to the present invention, finds utility in a wide variety of applications. Said nucleic acid sequence is particularly suitable for use in an inducible nucleic acid sequence expression system. The invention thus provides an isolated, synthetic or recombinant nucleic acid sequence comprising an rtTA encoding nucleic acid sequence and/or a single chain rtTA encoding nucleic acid sequence, which rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a mutated codon at rtTA amino acid position 9, and/or 19, and/or 37, and/or 56, and/or 67, and/or 68, and/or 138, and/or 157, and/or 171, and/or 177, and/or 195. In one embodiment said nucleic acid sequence further comprises a mutated codon at rtTA amino acid position 12, and/or 86, and/or 209. A nucleic acid sequence comprising such combination of mutations is also improved as compared to currently known rtTA.

In a preferred embodiment a nucleic acid sequence of the invention with an improved genetic stability as compared to currently used Tet-on systems is provided. This is particularly desired in applications involving multiple rounds of amplification of a nucleic acid sequence according to the invention, for instance during controlled replication of a viral pathogen or replicon. As explained above, genetic stability is improved by designing an rtTA and/or sc rtTA nucleic acid sequence with a codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon, with a codon at rtTA position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon, and/or with a codon at rtTA position 56 encoding

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lysine or glutamine. Further provided is therefore an isolated, synthetic or recombinant nucleic acid sequence according to the invention wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 19 which differs
5 in at least two nucleotides from a glutamic acid codon, a codon at rtTA position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon, and/or a codon at rtTA position 56 encoding lysine or glutamine. In one embodiment said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a glycine codon at rtTA
10 amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon, so that the resulting amino acid residue of the rtTA or sc rtTA activator at rtTA position 19 is the same as the activator encoded by currently used rtTA and sc rtTA nucleic acid.

Preferably an rtTA or sc rtTA nucleic acid is used which comprises an
15 alanine, cysteine, phenylalanine, histidine, isoleucine, leucine, methionine, asparagine, arginine, serine, threonine, valine, tryptophan or tyrosine codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon. A nucleic acid according to this embodiment is not only genetically more stable as compared to currently used Tet-on systems, but -
20 except for the G19W variant - is also more sensitive for doxycycline. One preferred embodiment provides an isolated, synthetic or recombinant nucleic acid sequence according to the invention, wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a leucine, a histidine or an arginine codon at rtTA amino acid
25 position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon.

A further preferred embodiment provides an isolated, synthetic or recombinant nucleic acid sequence according to the invention, wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic
30 acid sequence comprises a codon at rtTA amino acid position 56 which differs

in at least one nucleotide, preferably a transversion, from a codon that mediates transcriptional activity in the absence of inducer. Said codon at rtTA amino acid position 56 preferably encodes a glutamine or a lysine residue.

5 More preferably an isolated, synthetic or recombinant nucleic acid sequence according to the invention is provided which comprises a codon according to the invention at at least two rtTA amino acid positions which are chosen from the group consisting of positions 19, 37 and 56. Preferably provided is therefore an isolated, synthetic or recombinant nucleic acid
10 sequence according to the invention which comprises a codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon and a codon at rtTA position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon. Further provided is an isolated, synthetic or recombinant nucleic acid sequence according to the invention
15 which comprises a codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon and a codon at rtTA position 56 encoding lysine or glutamine. Further provided is an isolated, synthetic or recombinant nucleic acid sequence according to the invention which comprises a codon at rtTA position 37 which differs in at least two nucleotides from an
20 alanine, a lysine or a serine codon and a codon at rtTA position 56 encoding lysine or glutamine.

 An isolated, synthetic or recombinant nucleic acid sequence according to the invention most preferably comprises a codon according to the invention at
25 rtTA amino acid position 19, 37 and 56. A preferred embodiment therefore provides an isolated, synthetic or recombinant nucleic acid sequence according to the invention which comprises a codon at rtTA amino acid position 19 which differs in at least two nucleotides from a glutamic acid codon, a codon at rtTA position 37 which differs in at least two nucleotides from an alanine, a lysine

or a serine codon, and a codon at rtTA position 56 encoding lysine or glutamine.

As stated hereinbefore, a nucleic acid sequence according to the present invention preferably comprises an rtTA encoding nucleic acid sequence and/or a sc rtTA encoding nucleic acid sequence, wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 9 encoding isoleucine, and/or a codon at rtTA amino acid position 19 encoding alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, glutamine, arginine, serine, threonine, valine, tryptophan or tyrosine, and/or a codon at rtTA amino acid position 37 encoding threonine, histidine, leucine, arginine, cysteine, methionine or glutamine, and/or a codon at rtTA amino acid position 56 encoding lysine or glutamine, and/or a codon at rtTA amino acid position 67 encoding serine, and/or a codon at rtTA amino acid position 68 encoding arginine, and/or a codon at rtTA amino acid position 86 encoding tyrosine, and/or a codon at rtTA amino acid position 138 encoding aspartic acid or serine, and/or a codon at rtTA amino acid position 157 encoding lysine, and/or a codon at rtTA amino acid position 171 encoding lysine, and/or a codon at rtTA amino acid position 177 encoding leucine, and/or a codon at rtTA amino acid position 195 encoding serine, and/or a codon at rtTA amino acid position 209 encoding threonine. These mutations are preferred since each of them particularly improves at least one property of an inducible nucleic acid expression system. Hence, either one of these mutations or any combination thereof is preferably present in a nucleic acid sequence of the invention.

Further provided is an isolated, synthetic or recombinant nucleic acid sequence according to the invention, wherein said rtTA encoding nucleic acid sequence and/or single chain rtTA encoding nucleic acid sequence comprises at least one variant as depicted in Figure 14.

A nucleic acid sequence according to the invention preferably comprises an rtTA encoding nucleic acid sequence and/or a single chain rtTA encoding nucleic acid sequence which comprises at least one mutation as compared to a rtTA or sc rtTA encoding nucleic acid sequence published in (Gossen et al. 1995), (Urlinger et al. 2000) and (Krueger et al. 2003) and/or depicted in Figure 19.

The invention furthermore provides an isolated, synthetic or recombinant amino acid sequence encoded by a nucleic acid sequence according to the invention. Said amino acid sequence preferably comprises an rtTA sequence and/or a single chain rtTA sequence, which rtTA sequence and/or single chain rtTA sequence comprises an isoleucine at position 9, and/or an alanine, cysteine, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, arginine, serine, threonine, valine, aspartic acid, glutamine, tryptophan or tyrosine at position 19, and/or a threonine, histidine, leucine, arginine, cysteine, methionine or glutamine at position 37, and/or a lysine or glutamine at position 56, and/or a serine at position 67, and/or an arginine at position 68, and/or a tyrosine at position 86, and/or an aspartic acid or serine at position 138, and/or a lysine at position 157, and/or a lysine at position 171, and/or a leucine at position 177, and/or a serine at position 195, and/or a threonine at position 209. Each of these mutations particularly confer at least one improved property to an rtTA and/or sc rtTA activator.

As explained above, a nucleic acid sequence of the invention and/or an amino acid sequence encoded by a nucleic acid sequence of the invention is particularly suitable for inducibly expressing a nucleic acid sequence of interest. Use of an isolated, synthetic or recombinant nucleic acid sequence and/or amino acid sequence according to the invention for inducible expression of a nucleic acid sequence of interest is therefore also herewith provided.

Said amino acid sequence preferably comprises at least one of the above mentioned mutations.

Further provided is a vector comprising a nucleic acid sequence according to the invention. Such vector is suitable for a variety of applications. For instance, a vector of the invention comprising a therapeutically beneficial
5 nucleic acid sequence is suitable for therapeutic applications. Administration of such vector to an individual in need thereof results in expression of said therapeutic nucleic acid sequence *in vivo*. Of course, said vector also finds utility in applications involving *in vitro* expression of a nucleic acid sequence of interest. Methods for constructing a vector with a particular nucleic acid
10 sequence are well known in the art. Non-limiting examples of vectors suitable for generating a vector of the invention are retroviral and lentiviral vectors.

An inducible viral replicon, comprising a nucleic acid sequence according to the invention and at least one viral sequence which is essential for
15 replication under direct or indirect control of said nucleic acid sequence is also herewith provided. As explained before, a replicon is defined as a nucleic acid molecule capable of replication in a suitable environment, such as a permissive cell, because it has all the necessary elements for replication in such an environment. We call it a replicon, because it will not always be directly
20 derived from the nucleotide sequences of the original pathogen, for instance in the case of single stranded DNA viruses, RNA viruses, etc. Typically, in order to manipulate nucleic acids, double stranded forms are necessary, typically double stranded DNA forms. Therefore preferred replicons will be double stranded DNA nucleic acids in at least one stage of their life cycle.

25 A replicon is also intended to reflect that the actual pathogen, or its attenuated live vaccine relative, usually comprises more than just nucleic acid. The nucleic acid is typically packaged into a (viral) particle. Therefore a replicon according to the invention preferably also encodes a functional packaging signal, allowing for the nucleic acid in its wild-type-like form (RNA
30 in the case of a retrovirus, etc.) to be packed into a viral particle. In order for

the replicon to be able to replicate in a host, it is preferred that said replicon also carries the structural genes for the proteins of the envelope and/or capsid, be it in wild-type format or in a somewhat different format (reduced or enhanced target binding, etc.). In order to enhance inducer-dependency of a viral replicon according to the invention and/or to at least in part prevent loss of inducer-dependency, an inducible viral replicon according to the invention preferably comprises all viral sequences which are essential for replication under direct or indirect control of said nucleic acid sequence

10 A viral replicon of the invention is preferably derived from any virus comprising a stage wherein at least part of its genome is present in the form of DNA, such that the Tet-on machinery is capable of regulating expression of a nucleic acid of interest. Such viruses for instance comprise DNA viruses and retroviruses. In one embodiment at least part of said viral sequences in said
15 inducible replicon is RNA.

In one preferred embodiment a replicon according to the invention is derived from a human immunodeficiency virus. A replicon according to the invention is now further exemplified by the preferred embodiments relating to a replicon derived from HIV. However, the invention is also applicable to
20 replicons derived from other pathogens.

Typically a replicon of the invention derived from HIV would be an infectious double stranded DNA clone of an HIV strain. Preferably said HIV strain is already an attenuated strain, or is made into an attenuated strain by introducing mutations, such as functional deletions, e.g. those described
25 herein. Any repressor/activator elements that are inducible are in principle applicable in the present invention. In the case of double or more inducible controls, leakage of a single repressor/activator becomes less important, although essentially no leakage is still highly preferred. As a safety valve, it
30 would be advantageous to provide the replicon with a suicide gene that can be

activated when unwanted effects occur such as replication beyond what is necessary for an immune response or rescue by wild type virus, etc. Such a suicide gene is e.g. HSV-tk, which can be induced by adding gancyclovir or a functional equivalent thereof. Upon induction said suicide gene will kill the
5 infected cell, and thereby inhibit further replication and infection of other cells. Thus in yet another preferred embodiment the invention provides a replicon according to the invention which further comprises a suicide gene.

In order to attenuate an HIV replicon and/or the resulting virus it is
10 preferred that the replicon is provided with a functional deletion of the TAR-element. Thus in yet another preferred embodiment the invention provides a replicon according to the invention, which further comprises an inactivated TAR element.

In order to attenuate an HIV replicon according to the invention it is
15 preferred to functionally delete the Tat element. Thus the invention also provides a replicon according to the invention, which further comprises an inactivated Tat element. Preferably both elements mentioned above are functionally deleted. Functional deletion means that at least their function in the replication of the replicon is at least partially inhibited. Essential genes for
20 replication typically should not be completely dysfunctional.

Proteins necessary for removing repression or initiating activation elements which are present upstream of the essential genes to be put under control are preferably encoded by a replicon according to the invention and are preferably inserted in a non-essential gene. Thus the invention also provides in
25 a preferred embodiment a replicon according to the invention wherein at least one functional part of said inducible repressor and/or activator, preferably an rtTA and/or sc rtTA nucleic acid sequence according to the present invention, is inserted into the *nef* gene. The functional part in this case of course refers to any proteinaceous substance capable of activating the element in control of the
30 essential gene. Preferably space is created for the sequence encoding said

proteinaceous substance. Thus the invention also provides a replicon according to the invention in which at least part of the nef gene is deleted to create space for insertion.

To further attenuate a replicon according to the invention further
5 elements of the wild-type virus can be functionally deleted. Thus the invention further provides a replicon according to the invention, in which at least one NF-kB element has been deleted. It is preferred that the motif to be activated is a tetO motif, preferably present in an LTR. Thus the invention also provides a replicon according to the invention, which comprises at least one tetO motif
10 in at least one functional LTR.

It is preferred to have more than one element before an essential gene. Thus the invention also provides a replicon which comprises at least 2, 4, 6, or 8 such elements in at least one functional LTR.

At least one LTR is preferably modified in order to at least in part avoid
15 reversion to wild type virus.

The invention further provides methods using the replicons to produce dependent viruses, meaning viruses needing an inducing agent in order to be able to replicate. Thus the invention provides a method for producing a virus dependent on an inducing agent for replication, comprising providing a
20 permissive cell with a replicon according to the invention, culturing said cell in the presence of said inducing agent and harvesting said dependent virus from said culture. Again such methods are preferably applied to HIV. Thus the invention provides a method in which said dependent virus is a human immunodeficiency virus, preferably an attenuated virus.

25 In one embodiment the inducing agent is doxycyclin or a functional analog thereof. In another embodiment however said inducing agent comprises an antibiotic other than doxycyclin, preferably tetracyclin and/or minocyclin. As stated before, if tetracyclin and/or minocyclin-dependency is desired, a replicon according to the invention is preferred which comprises an rtTA
30 and/or sc rtTA encoding nucleic acid sequence comprising

a mutation or a combination of mutations as depicted in Figure 15, except for the wild type rtTA and the F86Y A209T variant.

The invention also provides the use of an isolated or recombinant nucleic acid comprising a nucleic acid sequence encoding an rtTA and/or a single chain rtTA according to the amino acid sequence of SEQ ID NO:27, which rtTA and/or single chain rtTA encoding nucleic acid sequence comprises a mutation at position 67 or one of the following combination of mutations comprising a mutation at position 67: (a) F67S, F86Y and A209T; (b) F67S, V9I, G138D, F86Y and A209T; (c) F67S, V9I, E157K, F86Y and A209T; (d) F67S, V9I, R171K, F86Y and A209T; (e) F67S, V9I, F86Y and A209T; (f) F67S, S12G, F86Y and A209T; (g) F67S, E37Q, F86Y and A209T; (h) F67S, S12G, V9I, G138D, F86Y and A209T; or (i) F67S, C68R, V9I, G138D, F86Y and A209T, for tetracycline-inducible and/or minocycline-inducible expression of a nucleic acid of interest.

The invention also provides the use of an isolated or recombinant nucleic acid comprising a nucleic acid sequence encoding an rtTA and/or a single chain rtTA for tetracycline-inducible and/or minocycline-inducible expression of a nucleic acid of interest, wherein said rtTA and/or single chain rtTA comprises:

- (i) an amino acid sequence comprising an amino acid substitution at position 67 in the amino acid sequence of SEQ ID NO:27; or
- (ii) an amino acid sequence comprising one of the following combination of substitutions: (a) F67S, F86Y and A209T; (b) F67S, R171K, F86Y and A209T; (c) F67S, V9I, G138D, F86Y and A209T; (d) F67S, V9I, E157K, F86Y and A209T; (e) F67S, V9I, R171K, F86Y and A209T; (f) F67S, V9I, F86Y and A209T; (g) F67S, S12G, F86Y and A209T; (h) F67S, E37Q, F86Y and A209T; (i) F67S, S12G, V9I, G138D, F86Y and A209T; or (j) F67S, C68R, V9I, G138D, F86Y and A209T, in the amino acid sequence of SEQ ID NO:27.

More preferably, a replicon according to the invention comprising mutations F67S V9I G138D F86Y A209T, C68R V9I G138D F86Y A209T, G19M V9I G138D F86Y A209T, E37Q V9I G138D F86Y A209T, G19M F67S

31a

V9I G138D F86Y A209T, S12G F67S V9I G138D F86Y A209T and/or C68R F67S V9I G138D F86Y A209T is used for tetracycline-inducible expression of a nucleic acid sequence of interest since these replicons are particularly sensitive for tetracycline, meaning that a small amount of tetracycline is sufficient for inducing gene expression. Most preferably, a replicon according to the invention comprising mutation F67S V9I G138D F86Y A209T, C68R V9I G138D F86Y A209T and/or S12G F67S V9I G138D F86Y A209T is used for tetracycline-inducible expression of a nucleic acid sequence of interest since these replicons are very sensitive for tetracycline and show low background activity in the absence of effector.

In a further preferred embodiment, a replicon according to the invention comprising mutations V9I G138D F86Y A209T, F67S V9I G138D F86Y A209T, F67S V9I E157K F86Y A209T, F67S V9I R171K F86Y A209T, F67S E37Q F86Y A209T, C68R V9I G138D F86Y A209T, G19M V9I G138D F86Y A209T, E37Q V9I G138D F86Y A209T, G19M F67S V9I G138D F86Y A209T, S12G F67S V9I G138D F86Y A209T and/or C68R F67S V9I G138D F86Y A209T is used for minocycline-inducible expression of a nucleic acid sequence of interest since these replicons are particularly sensitive for minocycline, meaning that a small amount of minocycline is sufficient for inducing gene expression. Most preferably, a replicon according to the invention comprising mutations F67S V9I G138D F86Y A209T, F67S E37Q F86Y A209T, C68R V9I G138D F86Y A209T and/or S12G F67S V9I G138D F86Y A209T is used for minocycline-inducible expression of a nucleic acid sequence of interest since these replicons are very sensitive for minocycline and show low background activity in the absence of effector.

Also part of the present invention are viruses produced by said methods or which can be produced by said methods. Thus the invention also provides a virus dependent on an inducing agent for replication obtainable by a method according to the invention, preferably again a human immunodeficiency virus, again preferably attenuated. Methods for producing a replicon and/or virus according to the present invention are known in the art. For instance, non-limiting examples of methods for producing an inducible viral replicon derived from HIV, comprising an rtTA sequence and TetO elements, and uses thereof, are described in WO01/20013 page 9 line 13 to page 18 line 27.

10

A replicon and/or virus according to the invention is particularly suitable for immunization and vaccination. Administration of a replicon and/or virus according to the invention to an individual allows for controlled replication of said replicon and/or virus within said individual, resulting in an immune response in said individual. The extent of replication of said replicon or virus and, as a result, the extent of elicited immune response is controlled by regulating the presence and/or amount of inducer. In one embodiment an immune response is elicited in an individual in order to provide said individual with at least partial protection against infection by the kind of virus from which said replicon or virus of the invention is derived. In another embodiment an immune response is elicited in a non-human animal in order to produce a binding compound (such as for instance antibodies and/or T cells) and/or a cell capable of producing such binding compound (such as a B cell). Said antibodies, T cells and/or B cells, or a functional part and/or a nucleic acid thereof, are preferably harvested for further use, for instance for the production of monoclonal antibodies.

25

Of course, various alternative methods and applications involving immunization and/or vaccination are known in the art. The use of a replicon

and/or virus according to the invention in such methods and applications is also within the scope of the present invention.

The invention thus provides an immunogenic composition comprising a replicon according to the invention and/or a virus according to the invention.

5 An immunogenic composition comprising a nucleic acid sequence according to the invention is also provided. An immunogenic composition of the invention preferably comprises a suitable adjuvant and/or carrier. Adjuvants and carriers are well known in the art. For instance, an Aluminum Salt Adjuvant and/or a saline solution is used.

10 In one embodiment said immunogenic composition further comprises an amount of inducing agent. This is however not necessary: an inducing agent can be administered at any time. In one preferred embodiment said immunogenic composition comprises a vaccine capable of eliciting full protection against the kind of virus from which said replicon or virus according to the invention is derived. This means that subsequent infection with the kind of virus from which said replicon or virus according to the invention is derived does essentially not result in disease.

An immunogenic composition or a vaccine may comprise a single dosage unit, but it may also comprise at least one inducing agent separately, or it may be made on the spot from a replicon and/or virus that are reconstituted with a liquid excipient such as saline, optionally together with an adjuvant and/or an inducing agent. Viral vaccines are well known in the field. General rules of thumb applicable to known vaccines will also apply to immunogenic compositions and vaccines of the present invention. Doses will be found through the normal dose finding studies performed during (pre)clinical trials, e.g. by simple titration of the amount of doxycycline as inducing agent. An immunogenic composition or vaccine may be sufficient on its own, but it may also be used in addition to other therapeutic and/or prophylactic compounds.

The inducing agent may be needed over a longer period of time and can be provided separately.

Again a preferred immunogenic composition and/or vaccine of the invention is one for at least partial prophylaxis of infection with a human
5 immunodeficiency virus.

The invention also provides a use of said immunogenic composition and/or vaccine in that it provides a method for at least partial prophylaxis and/or treatment of AIDS, comprising administering an immunogenic composition and/or vaccine according to the invention to an individual and
10 allowing for viral replication for a limited time by providing said inducing agent. Booster vaccinations are possible, preferably by simple readdition of the said inducing agent at later times.

The invention also provides a method for the controlled replication of a virus or a viral replicon comprising providing a permissive cell with a replicon
15 or a virus according to the invention, culturing said cell in the presence of said inducing agent and manipulating the amount of inducing agent present.

As explained above, a replicon, virus and/or nucleic acid sequence according to the invention is suitable for eliciting an immune response against
20 a virus of interest. Said immune response is capable of at least in part preventing subsequent infection, replication and/or spreading by said virus of interest. Moreover, an immune response of an individual that is already suffering from an infection by said virus of interest is enhanced by a replicon, virus and/or nucleic acid sequence according to the invention, resulting in a
25 better counteraction of disease.

Replicons, viruses and nucleic acid sequences according to the invention are thus suitable for use as a medicament and/or vaccine. A replicon or virus according to the present invention for use as a medicament and/or vaccine is therefore herewith provided, as well as an isolated or recombinant nucleic acid
30 sequence according to the invention for use as a medicament and/or vaccine. It

is possible to place at least one HIV sequence essential for replication under direct or indirect control of an rtTA and/or sc rtTA nucleic acid of the present invention. This way, controlled replication of HIV has become possible allowing for at least partial prophylaxis and/or treatment of AIDS. A use of an
5 isolated or recombinant replicon, virus and/or nucleic acid sequence according to the invention for the preparation of a medicament or immunogenic composition for at least in part preventing and/or treating AIDS is therefore also herewith provided.

One further embodiment provides an isolated cell comprising a nucleic
10 acid sequence, a replicon and/or a virus according to the invention.

15 The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention.

EXAMPLES

Example 1

- 5 We have previously reported on the construction of an infectious HIV-rtTA virus that is critically dependent on dox for replication (Verhoef et al. 2001; Das et al. 2004b; Marzio et al. 2001). In this virus, the natural transcriptional activator Tat and its TAR binding site were inactivated by mutation and functionally replaced by the components of the Tet-on system (Fig. 1A). The
- 10 gene encoding the transcriptional activator rtTA was inserted in place of the nef gene, and the tetO DNA binding sites were introduced in the viral LTR promoter. This virus does not replicate in the absence of dox. Upon dox administration, rtTA activates transcription from the LTR-tetO promoter, resulting in expression of the viral proteins and viral replication.
- 15 Subsequently, a variant has been provided that has 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) (Das et al. 2004a).
- 20 We started multiple, independent virus cultures of the HIV-rtTA_{F86Y A209T} variant, which contains both the optimized LTR-tetO promoter configuration and the improved rtTA gene. After culturing the virus with dox for up to 200 days, the rtTA gene was sequenced. The F86Y and A209T mutations were stably maintained in all analyzed cultures. Several viruses from independent
- 25 cultures had acquired additional mutations in the rtTA gene. A virus variant should have a replication advantage to become the dominant sequence in a virus population. Mutations in rtTA may improve rtTA function, and thus enhance viral replication. To increase the chance of identifying such beneficial mutations, we focused on the rtTA mutations that were observed in multiple
- 30 cultures (Fig. 1A and Table 1). All these amino acid substitutions are located

in the TetR part of rtTA: V9I is in the $\alpha 1$ helix within the DNA-binding domain, F67S is in the loop following $\alpha 4$, and G138D, E157K, and R171K are within the $\alpha 8$ - $\alpha 9$ region of the regulatory core domain (Fig. 1B). V9I was found both as an individual mutation and in combination with G138D, E157K, or
5 R171K. A combination of F67S and R171K was also observed. There are seven natural variants of TetR (A-E, G, H) and rtTA is based on class B (TetR^B). Interestingly, only TetR^B has a Phe at position 67, whereas most TetRs have a Ser at this position (Table 1). Other amino acid substitutions observed in the evolved rtTAs are never naturally present in TetR variants.

10

Characterization of the evolved rtTA variants.

To test whether the evolved rtTAs exhibit improved transcriptional activity, we assayed rtTA activity in a regular Tet system. Expression plasmids encoding the original (wild-type, this is the rtTA2^S-S2 variant described in
15 Urlinger et al. 2000) and mutant rtTA proteins (V1-V10, Table 1) were constructed and transfected into C33A cells with a plasmid expressing luciferase reporter under the control of the viral LTR-2 Δ tetO promoter. The luciferase level measured two days after transfection reflects rtTA activity (Fig. 2A). Wild-type and all mutant rtTAs show no activity in the absence of
20 dox. Wild-type rtTA activity is detectable first at 500 ng/ml dox and increases further at 1000 ng/ml. rtTA V1 (F86Y A209T) activity is already detectable at 50 ng/ml dox and gradually increases with higher dox concentrations. At 1000 ng/ml dox, the V1 variant is 2.5-fold more active than the wild-type. All mutants did evolve from rtTA V1, and their activity should thus be compared
25 with this variant. rtTA V2 is more active than V1 at the lowest dox concentration tested, but less active at high dox levels. The other rtTAs with a single amino acid substitution (V3-V6) are more active than V1 both at low and high dox levels. The variants V7, V8, and V9 combine the V2 mutation with the V4, V5, and V6 mutation, respectively. These combinations further
30 improve rtTA activity both at low and high dox levels. The V10 variant, which

combines the V3 and V6 mutations, is the most active rtTA of the naturally evolved variants. Therefore, the viral evolution strategy resulted in several novel rtTA variants with enhanced transcriptional activity and dox-sensitivity compared with wild-type rtTA and the V1 variant that was used to start the evolution experiment.

To test whether this rtTA optimization reflects a specific adaptation to the viral LTR-2 Δ tetO promoter, we assayed rtTA activity with a reporter gene construct in which luciferase expression is under the control of a minimal CMV promoter coupled to an array of seven tetO elements (Gossen et al., 1992). All evolved rtTA variants demonstrate improved activity with this promoter construct (Fig. 2B), which mimics the result with the LTR-2 Δ tetO construct (Fig. 2A). Thus, the observed mutations in rtTA are not virus-specific adaptations, but are general improvements of the Tet-on system. We also assayed rtTA activity in HeLa X1/6 cells (Baron et al., 1997) that contain chromosomally integrated copies of the CMV-7tetO luciferase reporter construct (Fig. 2C). In these cells, the evolved rtTAs show a similar pattern of activity as with episomal reporter gene constructs in C33A cells. Thus, these mutations improve rtTA activity independent of the type of promoter and the episomal or chromosomal status of the target gene.

To compare the dox-sensitivity of the rtTA variants in another way, we calculated the dox concentration that each rtTA variant needs to reach an activity similar to that of the wild-type rtTA at 1000 ng/ml dox (Fig. 3). The V10 variant needs only 44 ng/ml dox to reach this activity level, which reflects a 23-fold higher dox-sensitivity than the wild-type rtTA. This makes the V10 variant the most dox-sensitive and most active rtTA (6.6-fold more active than the wild-type, Fig. 3) of the naturally evolved variants.

Combining the beneficial mutations further improves rtTA activity. Analysis of the evolved rtTA variants revealed that the double mutants exhibit a higher activity and dox-sensitivity than the single mutants. For instance, V6 (R171K) is 4.4-fold more sensitive than the wild-type rtTA, and the double

mutant V9 (V9I R171K) is 14.9-fold more dox-sensitive. We therefore constructed additional rtTA variants in which the observed mutations were combined (V11-V18, Table 1), and assayed their activity (Fig. 2D). As shown in Fig. 3, all combination variants demonstrate a higher transcriptional activity and dox-sensitivity than the naturally evolved variants. The triple mutants V14, V15, and V16 are the most active and most dox-sensitive rtTAs. When compared with wild-type rtTA, these triple mutants are 7-fold more active at high dox levels and 100-fold more sensitive to dox. The V15 and V16 variants do not show any basal activity without dox, whereas we frequently observed a low, but distinct basal activity with the V14 variant (less than 0.1% of the induced level).

A more extensive list of novel rtTA variants that carry mutations observed in HIV-rtTA evolution and that demonstrate improved transcriptional activity and dox-sensitivity is shown in figure 14B.

To exclude the possibility that the enhanced activity observed for the mutant rtTAs resulted from an increased protein level, we determined the intracellular steady state level 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. An equal amount of rtTA protein was detected for all naturally evolved and constructed variants (Fig. 4, and data not shown). These results indicate that the enhanced activity and dox-sensitivity are intrinsic properties of the mutant rtTA proteins and do not result from increased expression or protein stability.

Novel rtTA variants can be activated by dox-like compounds.

Dox is the most efficient effector that controls the Tet-on system. Other dox-like compounds, such as tetracycline (Tc) and minocycline (Mc), do not effectively activate the wild-type rtTA and the original HIV-rtTA virus. To test if the novel rtTA variants with improved activity and dox-sensitivity have a broader effector-specificity, we assayed the activity of a subset of these rtTA

variants at different Tc and Mc concentrations (Fig. 5). Whereas the wild-type rtTA and the V1 variant are not activated by Tc and Mc, mutant V3 shows a low level of activity at a high concentration of Tc or Mc (10000 ng/ml). V7 activity is already detectable at 1000 ng/ml Tc or Mc, and this activity
5 increases at higher effector levels. V14, which combines the V3 and V7 mutations, shows the highest activity with Tc and Mc. The activity at 10000 ng/ml Tc is similar to that of the wild-type rtTA at 1000 ng/ml dox. At 1000 ng/ml Mc, V14 is even more active than the wild-type rtTA at 1000 ng/ml dox. Thus, we have generated rtTA variants with a broadened effector-specificity.
10 A more extensive list of novel rtTA variants that are responsive to Tc and/or Mc is shown in figure 15.

rtTA variants improve HIV-rtTA replication.

To test if the rtTA variants with enhanced activity and dox-sensitivity can also
15 improve HIV-rtTA replication, we constructed viral variants with the rtTA genes encoding either mutant V7 or V14, and assayed their replication in SupT1 cells at different dox concentrations (Fig. 6). The original HIV-rtTA_{V1} (HIV-rtTA_{F86Y A209T}) was included as a control. This control HIV-rtTA_{V1} does not replicate in the absence of dox or at low dox levels, efficient replication was
20 observed at 100 ng/ml dox, and the replication rate further increased at 1000 ng/ml dox. The replication of HIV-rtTA_{V7} and HIV-rtTA_{V14} was also completely dependent on dox. The HIV-rtTA_{V7} showed a low level of replication at 1 ng/ml dox and efficient replication at 10 ng/ml. For HIV-rtTA_{V14}, a high level of replication was already apparent at 1 ng/ml dox. These
25 results demonstrate that the variants V7 and V14 significantly improve HIV-rtTA replication at low dox concentrations. Importantly, like the original HIV-rtTA, these viruses do not replicate in the absence of dox. Apparently, the low basal rtTA-activity observed with the V14 variant in the absence of dox is not sufficient to support viral replication.

We also assayed replication of these new HIV-rtTA variants in the presence of Tc and Mc (Fig. 7). Whereas the control HIV-rtTA_{v1} did not replicate in the presence of 500 ng/ml Tc or Mc, both HIV-rtTA_{v7} and HIV-rtTA_{v14} show efficient replication with these effectors. These results confirm that the rtTA variants V7 and V14 can be effectively activated by Tc and Mc.

Conclusion

Amino acid substitutions in rtTA at position 9, 19, 37, 67, 68, 86, 138, 157, 171, 177, 195 and/or 209, which are observed during evolution of the HIV-rtTA virus, enhance the transcriptional activity and/or inducer-sensitivity of rtTA. Moreover, these mutations broaden the inducer-specificity of rtTA. The most optimal rtTA variants (V15 and V16) are 7-fold more active at high dox levels and 100-fold more sensitive to dox than the original rtTA. Importantly, these rtTA variants do not show any basal activity in the absence of dox. High activity and dox-sensitivity of these novel rtTAs significantly improve the performance of the Tet-on system.

Materials and Methods

Cell cultures. The human T-lymphocyte cell line SupT1 (Smith et al. 1984) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 U/ml). HeLa X1/6 (Baron et al. 1997) is a HeLa-derived cervix carcinoma cell line, containing chromosomally integrated copies of the CMV-7tetO promoter/luciferase reporter construct pUHC13-3 (Gossen et al. 1992). HeLa X1/6 and C33A cervix carcinoma cells (ATCC HTB31) (Auersperg, 1964) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, minimal essential medium nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 U/ml). All cell cultures were kept at 37°C and 5% CO₂.

Virus replication. Construction of the HIV-rtTA molecular clone was described previously (Verhoef et al. 2001; Das et al. 2004b). The HIV-rtTA variant used in this study contains the 2 Δ tetO configuration (Marzio et al. 2001; Marzio et al. 2002) in both the 5' and the 3' LTR. SupT1 cells (5×10^6) were transfected with 5 μ g of the HIV-rtTA molecular clone by electroporation (250 V and 975 μ F). Viral replication was induced with doxycycline (dox, D-9891, Sigma, St. Louis, MO, USA), tetracycline (Tc, Sigma T-3383) or minocycline (Mc, Sigma M-9511). The CA-p24 level in the cell-free culture supernatant was determined by antigen capture enzyme-linked immunosorbent assay (ELISA) (Back et al. 1996).

For the selection of evolved viruses, SupT1 cells were transfected with the HIV-rtTA_{F86Y A209T} molecular clone (Das et al. 2004a), and cultured in the presence of 1 μ g/ml dox for up to 200 days. The virus containing culture supernatant was passaged onto fresh SupT1 cells at the peak of infection, as determined by the massive appearance of syncytia. At regular intervals, cell and supernatant samples were taken from the culture and stored at -80°C for subsequent analysis.

Proviral DNA analysis and cloning of evolved sequences. Total cellular DNA from infected cells was isolated as described previously (Das et al. 1997). The proviral rtTA genes were PCR amplified with the sense primer tTA1 (5'-ACAGCCATAGCAGTAGCTGAG-3'; SEQ ID NO:1) and the antisense primer tTA-rev2 (5'-GATCAAGGATATCTTGTCTTCGT-3'; SEQ ID NO:2), and sequenced with the bigdyeTM terminator cycle sequencing kit (Applied Biosystems, Foster city, CA, USA). The PCR products were digested with *Xba*I and *Sma*I and used to replace the corresponding fragment in pCMV-rtTA, in which the expression of wild-type rtTA (rtTA2^S-S2, (Urlinger et al. 2000)) is controlled by the human cytomegalovirus (CMV) immediate-early promoter. Mutant rtTA genes were cloned from pCMV-rtTA into the shuttle vector pBlue3'LTRext-deltaU3-rtTA_{F86Y A209T}-2 Δ tetO (Das et al. 2004a) using the *Xcm*I and *Nde*I sites and

subsequently cloned back into the HIV-rtTA molecular clone as *Bam*HI-*Bgl*II fragments. To introduce the F67S and G138D mutations into evolved rtTA variants, mutagenesis PCR (Mikaelian et al. 1992) was performed with the corresponding pCMV-rtTA plasmid and the mutagenic primer (primer M) tTA-F67S (5'-CATACCCACTCCTGCCCCCTGGAAGGCGA-3' (SEQ ID NO:3), mismatching nucleotide underlined) or tTA-G138D (5'-GTCCGCCGTGGACCACTTTACACTGGGCT-3'; SEQ ID NO:4) and the general primers 5'-TGGAGACGCCATCCACGCT-3' (primer 1; SEQ ID NO:5), 5'-TGAAATCGAGTTTCTCCAGGCCACATATGA-3' (primer 2; SEQ ID NO:6), and 5'-TCACTGCATTCTAGTTGTGGT-3' (primer 3; SEQ ID NO:7). Briefly, PCR reactions were performed with primer M plus primer 3, and with primer 1 plus primer 2. The PCR products were purified, mixed, and PCR amplified with primers 1 and 3 (see reference (Mikaelian et al. 1992) for details). The resulting mutated rtTA genes were cloned as *Eco*RI-*Bam*HI fragments into pCMV-rtTA. All constructs were verified by sequence analysis.

rtTA activity assay. Two firefly luciferase reporter constructs with different promoter configurations were used. pLTR-2ΔtetO-luc contains the LTR-2ΔtetO promoter derived from the HIV-rtTA molecular clone (Marzio et al. 2001; Marzio et al. 2002). pCMV-7tetO-luc, previously named pUHC13-3 (Gossen et al. 1992), contains seven tetO elements located upstream of a minimal CMV promoter. The plasmid pRL-CMV (Promega, Madison, WI, USA), in which the expression of *Renilla* luciferase is controlled by the CMV promoter, was used as an internal control to allow correction for differences in transfection efficiency.

C33A and HeLa X1/6 cells were grown in 2-cm² wells to 60% confluency and transfected by the calcium phosphate precipitation method (Das et al. 1999). C33A cells were transfected with 0.4 ng pCMV-rtTA (wild-type or mutant), 20 ng pLTR-2ΔtetO-luc or pCMV-7tetO-luc, 0.5 ng pRL-CMV, and 980 ng pBluescript as carrier DNA. HeLa X1/6 cells were transfected with 8 ng

pCMV-rtTA, 2.5 ng pRL-CMV, and 990 ng pBluescript. The amount of the DNA constructs was optimized for each cell type to keep rtTA-mediated transactivation within the linear range and to avoid squelching of transcription factors. Cells were cultured for 48 hours in the presence of
5 different concentrations of dox, Tc or Mc, and lysed in Passive Lysis Buffer (Promega). Firefly and *Renilla* luciferase activities were determined with the dual-luciferase reporter assay (Promega). The activity of the rtTA variants was calculated as the ratio of the firefly and *Renilla* luciferase activities, and corrected for between-session variation.

10

Western blot analysis. HeLa X1/6 cells were transfected at 90% confluency with 1 µg of wild-type or mutant pCMV-rtTA and 2 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 2-cm² wells. Cells were cultured for 48 hours and lysed in 100 µl of Passive Lysis Buffer. 10 µl of the lysate was
15 subjected to SDS-polyacrylamide gel separation, and transferred to Immobilon-P membrane (Millipore, Billerica, MA, USA). For immunochemical detection of rtTA variants, membranes were incubated with rabbit serum containing polyclonal anti-TetR antibodies (Krueger et al. 2003). Bound antibodies were visualized with peroxidase-linked anti-rabbit IgG and the ECL+ kit
20 (Amersham Biosciences, Freiburg, Germany) and analyzed with a Storm 860 Imager (Amersham Biosciences).

25

Example 2

HIV-1 vaccines based on a live-attenuated virus have shown promise in the SIV-macaque model, but are generally considered unsafe for use in humans.

30 The major safety concern is that chronic low-level replication of the attenuated

virus may eventually lead to selection of fitter and more pathogenic virus variants. Ideally, one would like to restrict replication of a vaccine virus to the time window that is needed to elicit a protective immune response. We previously presented a novel vaccine approach that uses a conditional-live
5 HIV-1 virus. In this HIV-rtTA virus, the viral transcriptional activator Tat and its TAR binding site were inactivated by mutation and functionally replaced by the components of the Tet-on system. This system, in which gene expression is stringently controlled by the non-toxic effector doxycycline (dox), is widely applied to regulate gene expression in eukaryotes. The rtTA gene encoding the
10 transcriptional activator was inserted in place of the nef gene, and the tet-operator (tetO) DNA binding sites were placed in the viral LTR promoter. This HIV-rtTA virus does not replicate in the absence of dox. Binding of dox to rtTA triggers a conformational change that allows the protein to bind tetO DNA, resulting in transcriptional activation and subsequent viral replication. Upon
15 vaccination with this virus, replication can be temporarily activated and controlled to the extent needed for induction of the immune system by transient dox administration.

The potential use of this dox-dependent HIV-rtTA virus as a vaccine
20 raises new safety questions concerning the genetic stability of the introduced Tet-on system. There are several hypothetical evolutionary routes toward a constitutively replicating virus. First, the virus may restore the function of the Tat-TAR system, despite the multiple inactivating mutations that were introduced in both elements to avoid simple reversion to the wild-type
25 sequence. In this scenario, the dox-controlled rtTA-tetO system will become superfluous, and may be inactivated over time by mutation or deletion. Second, the viral LTR promoter could become a constitutive transcription element, for instance by acquisition of a binding site for a constitutively expressed cellular transcription factor. Replication of such a virus is not dependent on a virally
30 encoded transactivator, neither Tat nor rtTA. Third, the introduced rtTA-tetO

axis may lose dox-dependence, thereby creating an uncontrolled Tet system. This scenario would for instance occur through acquired mutations in the rtTA protein that shift its conformation into the DNA-binding mode, even in the absence of dox.

5 To address these safety issues, we followed the evolution of HIV-rtTA in multiple, independent virus cultures. We observed loss of dox-control in several cultures, which in all cases resulted from a typical amino acid substitution either at position 19 or 37 in the rtTA protein. We developed novel
10 rtTA variants with alternative amino acids at these positions, and demonstrated that the corresponding HIV-rtTA variants did not lose dox-control in long-term cultures. Thus, we improved the genetic stability of the Tet-on system and the HIV-rtTA vaccine candidate by blocking unwanted evolutionary routes.

15 Materials and Methods

Virus cultures. The HIV-rtTA infectious molecular clone is a derivative of the HIV-1 LAI proviral plasmid (Peden et al. 1991) and was described previously (Das et al. 2004b; Verhoef et al. 2001). HIV-rtTA used in this study is the KYK version, which contains the inactivating Y26A mutation in the Tat gene and
20 five nucleotide substitutions in the TAR hairpin motif. This virus contains the rtTA2^S-S2 gene (Urlinger et al. 2000) in place of the nef gene and eight tetO sequences in the LTR promoter region. The HIV-rtTA 2ΔtetO clone is identical to HIV-rtTA, but with the optimized 2ΔtetO promoter configuration (Marzio et al. 2001; Marzio et al. 2002). HIV-rtTA_{F86Y A209T} contains the LTR-2ΔtetO
25 promoter and the recently described rtTA_{F86Y A209T} gene (Das et al. 2004a).

SupT1 T cells were grown at 37°C and 5% CO₂ in RPMI1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. SupT1 cells were transfected with HIV-rtTA molecular clones by electroporation. Briefly, 5 x 10⁶ cells were washed in RPMI1640 with
30 20% FBS and mixed with 5 μg of DNA in 250 μl RPMI1640 with 20% FBS.

Cells were electroporated in 0.4-cm cuvettes at 250 V and 975 μ F and subsequently resuspended in RPMI1640 with 10% FBS. The CA-p24 level in the cell-free culture supernatant was determined by antigen capture enzyme-linked immunosorbent assay (ELISA) (Back et al. 1996).

5 The 24-well evolution experiment was started with transfection of 40 μ g of the HIV-rtTA proviral plasmid into 2×10^7 SupT1 cells. Cells were split into 24 independent cultures and maintained in the presence of 1 μ g/ml dox (Sigma D-9891) for up to 200 days. The virus containing culture supernatant was passaged onto fresh SupT1 cells at the peak of infection, as determined by the
10 massive appearance of syncytia. At regular intervals, supernatant samples were taken from the culture and tested in parallel infections with and without dox. Cell samples were stored at -80°C for subsequent analysis.

Proviral DNA analysis and cloning of evolved sequences.

HIV-rtTA infected cells were pelleted by centrifugation and washed with
15 phosphate-buffered saline. DNA was solubilized by resuspending the cells in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-0.5% Tween 20™, followed by incubation with 200 μ g/ml of proteinase K at 56°C for 60 min and 95°C for 10 min. Proviral DNA sequences were PCR amplified from total cellular DNA. The first exon of the Tat gene was amplified with the primers KV1
20 (5'-CCATCGATACCGTCGACATAGCAGAATAGG-3'; SEQ ID NO:8) and 3'TAT (5'-CGGGAATTCTTACTGCTTTGATAGAGAAAC-3'; SEQ ID NO:9). The LTR-tetO region was amplified with the primers tTA-tetO1 (5'-CTCCCCGGGTAAGTAAGGAT-3'; SEQ ID NO:10) and C(N1) (5'-GGTCTGAGGGATCTCTAGTTACCAGAGTC-3'; SEQ ID NO:11). The rtTA
25 gene was amplified with the primers tTA1 (5'-ACAGCCATAGCAGTAGCTGAG-3'; SEQ ID NO:1) and tTA-rev2 (5'-GATCAAGGATATCTTGTCTTCGT-3'; SEQ ID NO:2). All PCR fragments were sequenced with the bigdye™ terminator cycle sequencing kit (Applied Biosystems). For the cloning of the G19E or E37K mutated rtTA sequences
30 into the HIV-rtTA provirus, rtTA PCR fragments were digested with XcmI and

*Sma*I and cloned into the corresponding sites of the shuttle vector pBlue3'LTRext-deltaU3-rtTA-2ΔtetO (16). The *Bam*HI-*Bgl*II fragment of the shuttle vector was used to replace the corresponding sequence in HIV-rtTA 2ΔtetO.

5

Construction of novel HIV-rtTA variants and rtTA expression plasmids.

HIV-rtTA variants with an alternative G codon (GGU instead of GGA) at rtTA position 19 and with a wild-type (E) or alternative amino acid (D, F, L, N, Q, R, S) at position 37 were constructed by oligonucleotide directed mutagenesis.

The oligonucleotide G19

(5'-ATAACCATGTCTAGACTGGACAAGAGCAAAGTCATAAACTCTGCTCTG
GAATTACTCAATGGTGTCGGTATCGAAGGCCTGACGACAAGGAAACTCGC
T-3', mutated nucleotide underlined; SEQ ID NO:12) was annealed to the
oligonucleotide rev-37

(5'-AGCAGGGCCCGCTTGTCTTCACGTGCCAGTACAGGGTAGGCTGXXXA
ACTCCCAGCTTTTGAGCGAGTTTCCTTGTCGTCAGGCCTTCGA-3', with
XXX corresponding to amino acid 37; this triplet is CTC for E, ATC for D, GAA
for F, AAG for L, ATT for N, CTG for Q, GCG for R, and AGA for S; SEQ ID
NO:13), both strands were completed with Klenow DNA polymerase in the
presence of dNTPs, digested with *Xcm*I and *Apa*I, and ligated into the
similarly digested shuttle vector pBlue3'LTRext-deltaU3-rtTA_{F86Y A209T}-2ΔtetO
(Das et al. 2004a). The *Bam*HI-*Bgl*II fragment of the shuttle vector was used to
replace the corresponding sequence in HIV-rtTA 2ΔtetO.

The plasmid pCMV-rtTA contains the rtTA^{2S}-S2 gene in the expression vector pUHD141-1/X (Urlinger et al. 2000). To generate rtTA variants with different amino acids at position 19 or 37, PCR was performed on pCMV-rtTA with the sense primer random-rtTA-19

(5'-TTCACCATGTCTAGACTGGACAAGAGCAAAGTCATAAACTCTGCTCTG
GAATTACTCAATNNKGTCGGTATCGAAGGCCTGACGA-3', mutated

30

nucleotide underlined with K corresponding to T or G, and N corresponding to T, C, A or G; SEQ ID NO:14) plus the antisense primer CMV2 (5'-TCACTGCATTCTAGTTGTGGT-3'; SEQ ID NO:15) or with the sense primer CMV1 (5'-TGGAGACGCCATCCACGCT-3'; SEQ ID NO:16) plus the
 5 antisense primer random-rtTA-37 (5'-AGCAGGGCCCGCTTGTTCCTTCACGTGCCAGTACAGGGTAGGCTGMNN AACTCCCAGCTTTTGAGCGA -3', mutated nucleotide underlined with M corresponding to A or C, and N corresponding to T, C, A or G; SEQ ID NO:17), respectively. The mutated rtTA sequences were cloned as *XbaI*-*ApaI* fragments
 10 into pCMV-rtTA_{F86Y A209T} (Das et al. 2004a). All constructs were verified by sequence analysis. To combine the G19F (UUU codon) and E37L (CUU codon) mutations, the E37L-containing *StuI*-*BamHI* fragment of pCMV-rtTA_{E37L} was used to replace the corresponding sequence in pCMV-rtTA_{G19F}, resulting in pCMV-rtTA_{G19F E37L}. The rtTA_{G19F E37L} sequence was cloned into the shuttle
 15 vector pBlue3'LTRext-deltaU3-rtTA_{F86Y A209T}-2ΔtetO (Das et al. 2004a) using the *XcmI* and *NdeI* sites and subsequently cloned into the HIV-rtTA 2ΔtetO molecular clone as a *BamHI*-*BglII* fragment.

rtTA activity assay.

20 HeLa X1/6 cells (Baron et al. 1997) are derived from the HeLa cervix carcinoma cell line and harbor chromosomally integrated copies of the CMV-7tetO firefly luciferase reporter construct pUHC13-3 (Gossen et al. 1992). Cells 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
 25 amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin.

HeLa X1/6 cells were grown in 2-cm² wells to 60% confluency and transfected by the calcium phosphate precipitation method. 1 µg of DNA mixture in 15 µl water was mixed with 25 µl of 50 mM HEPES (pH 7.1)-250 mM NaCl-1.5 mM Na₂HPO₄ and 10 µl of 0.6 M CaCl₂, incubated at room
 30 temperature for 20 min and added to the culture medium. The DNA mixture

consisted of 8 ng pCMV-rtTA, 2.5 ng pRL-CMV, and 990 ng pBluescript as carrier DNA. The plasmid pRL-CMV (Promega), in which the expression of *Renilla* luciferase is controlled by the CMV promoter, was used as an internal control to allow correction for differences in transfection efficiency. Cells were
5 cultured after transfection for 48 hours at different dox concentrations and then lysed in Passive Lysis Buffer (Promega). Firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase Reporter Assay (Promega). The expression of firefly and *Renilla* luciferase was within the linear range and no squelching effects were observed. The activity of the rtTA
10 variants was calculated as the ratio of the firefly and *Renilla* luciferase activities, and corrected for between-session variation (Retrovirology, submitted).

Results

15 **Appearance of HIV-rtTA variants with reduced dox-dependence.** We have previously reported on the construction of a conditional-live HIV-1 variant (Das et al. 2004b; Verhoef et al. 2001), in which the natural Tat-TAR elements that control viral gene expression and replication were inactivated by mutation and functionally replaced by the rtTA-tetO elements of the Tet-on
20 system for inducible gene expression (Fig. 8A). This HIV-rtTA virus does not replicate constitutively, but exclusively in the presence of dox. We recently reported that long-term replication of this virus resulted in rearrangement of the tetO elements and amino acid substitution in the rtTA protein that significantly improved viral replication without a loss of dox-control. We
25 anticipated that the HIV-rtTA virus could also evolve in different directions (see introduction), and therefore focused this study on the appearance of virus variants that no longer relied on dox for replication. We started multiple long-term virus cultures and followed the development of dox-independence. The evolution approach and the flow chart of the subsequent analyses are
30 illustrated in Fig. 8B. The HIV-rtTA virus was passaged extensively in the

presence of dox in 24 independent cultures. At several time points, supernatant samples were taken from the culture and tested in a parallel infection without dox to determine the dox-dependence of the evolved virus. The results for all 24 cultures are summarized in Fig. 8C (black squares). We
5 observed a significant reduction in the number of dox-dependent viruses within 50 days of culturing, and only three cultures remained dox-dependent after 125 days.

The replication curves of the original HIV-rtTA virus and two representative dox-independent virus cultures are shown in Fig 9. Virus
10 sample C5 did replicate without dox, but can still be activated by dox to some extent, whereas virus sample C6 replicated with similar efficiency with and without dox. Total cellular DNA with integrated provirus was isolated from eight dox-independent HIV-rtTA cultures. We analyzed the sequence of both the “old” Tat-TAR motifs and the “new” rtTA-tetO motifs as they were present
15 in the virus population. In all cultures, the Tat and TAR sequences contained the original mutations, indicating that the Tat-TAR axis of transcriptional activation had not been repaired. In contrast, we observed in all cultures the characteristic rearrangement of tetO elements that had previously been shown to improve dox-dependent HIV-rtTA replication (Marzio et al. 2001; Marzio et
20 al. 2002). Moreover, viruses from all dox-independent cultures had acquired either a G19E or an E37K mutation in the rtTA gene (Fig. 8D). Two of the cultures (B6 and C6) contained additional amino acid substitutions. The repeated selection of G19E or E37K in multiple cultures, combined with their absence in the three remaining cultures (data not shown), strongly suggests
25 their linkage to the acquired dox-independent phenotype.

Amino acid substitutions in rtTA confer the loss of dox-control.

To demonstrate that these rtTA mutations are responsible for the observed viral replication without dox, we constructed HIV-rtTA molecular clones with
30 the G19E or E37K mutation in the rtTA gene and assayed their replication at

different dox concentrations (Fig. 10). HIV-rtTA with wild-type rtTA did not replicate without dox and showed a graded increase in viral replication with increasing dox concentrations. HIV-rtTA_{G19E} replicated efficiently both with and without dox. HIV-rtTA_{E37K} also replicated without dox, but replication is
5 more efficient with dox. These results demonstrate that the G19E or E37K mutation is sufficient to reduce the dox-dependence of the HIV-rtTA virus.

The results described above were obtained with the original HIV-rtTA virus, which replicates relatively poor. We also tested the genetic stability of two improved HIV-rtTA variants in a similar 24-well long-term culture assay.
10 HIV-rtTA 2ΔtetO is identical to HIV-rtTA, but with the improved LTR-2ΔtetO promoter (Marzio et al. 2001; Marzio et al. 2002), and HIV-rtTA_{F86Y A209T} contains in addition the improved rtTA_{F86Y A209T} gene (Das et al. 2004a). With both viruses we again observed the appearance of variants that replicated without dox, albeit at a significantly slower rate compared with the original
15 HIV-rtTA (Fig. 8C). Whereas the original HIV-rtTA lost dox-control in 50% of the cultures within 50 days, 50% of the HIV-rtTA 2ΔtetO cultures lost dox-dependence in approximately 75 days, and more than 50% of the HIV-rtTA_{F86Y A209T} cultures were still fully dox-dependent after 120 days. Apparently, these new HIV-rtTA variants do not only have an improved replication capacity, but
20 also a lower tendency to lose dox-control. Sequence analysis of two dox-independent HIV-rtTA_{F86Y A209T} cultures revealed the G19E mutation in both cases.

HIV-rtTA variants with alternative codons at rtTA positions 19 and 37.

25 In the evolution experiments, we observed very specific amino acid substitutions that reduced dox-dependence at only two rtTA positions (G19E and E37K). The introduction of alternative rtTA codons may make such specific amino acid substitutions more difficult or even prevent these unwanted evolutionary routes. For instance, the G19E mutation involves a
30 GGA to GAA codon change, and the G-to-A transition is the most frequent

error during HIV-1 reverse transcription. Introduction of an alternative Gly codon (GGU or GGC) would require a much more difficult two-hit mutation, including one transversion, to create a Glu codon (GAA or GAG). Such a difference in the mutational frequency strongly influences the course of HIV-1 evolution.

A similar strategy is not possible for E37K because all possible Glu codons (GAA and GAG) require only a single G-to-A mutation to turn into a Lys codon (AAA or AAG). As an alternative blocking strategy, we could replace the E37-codon with a non-Glu codon that would be more difficult to transform into a Lys codon. However, such an amino acid substitution should ideally not affect the activity or dox-dependence of the rtTA protein. We first examined natural variation at this position in the Tet repressor (TetR). The rtTA protein is based on the *E. coli* class B TetR (TetR^B), but there are six additional TetR classes (A, C-E, G, H). TetR from class D, E and H also have the Glu at position 37, but TetR from class A, C and G have a Gln instead. Evolution of a Gln codon (CAA or CAG) to a Lys codon (AAA or AAG) would require only a single C-to-A mutation, but this transversion is less frequently observed in HIV-1 evolution. We therefore constructed an HIV-rtTA variant with a Gln codon (CAG) at position 37 (E37Q). In addition, we constructed variants with either an Asp (GAU; E37D), Asn (AAU; E37N), Ser (UCU; E37S), Arg (CGC; E37R), Phe (UUC; E37F) or a Leu codon (CUU; E37L). The E37D substitution leaves the acidic nature of the residue intact. The E37N and E37S mutations, like the natural variant E37Q, result in polar, uncharged residues. The E37F and E37L mutations result in hydrophobic residues. The E37R substitution creates a basic residue that is similar to the E37K mutation selected through viral evolution. When allowed by the degeneracy of the genetic codon, we chose the codon that requires most mutations to convert into a Lys codon. For example, a CGC rather than an AGA codon was chosen for the E37R variant. Moreover, all new HIV-rtTA variants contain the alternative Gly codon (GGU) at position 19.

We tested replication of these novel HIV-rtTA variants in SupT1 cells with and without dox (Fig. 11). As expected, the virus with the silent codon change at position 19 (E37) replicated dox-dependently. The E37L, E37N, E37F, E37Q and E37R variants also showed efficient and dox-dependent replication. The E37D variant did not replicate with or without dox. Interestingly, the E37S variant replicated efficiently both with and without dox, which is a phenotype similar to that of the E37K variant. This initial survey demonstrates that the HIV-rtTA phenotype is difficult to predict from the chemical nature of the residue, e.g. E37R is similar to E37K, but does not reduce dox-dependence. To construct a more stable dox-dependent virus, it therefore seems necessary to know the impact of all possible amino acid substitutions at position 37.

Testing all possible position 37 variants of rtTA.

We constructed rtTA expression plasmids with all possible amino acids at position 37. The activity of these variants was assayed by transfection into HeLa X1/6 cells (Baron et al. 1997) that contain stably integrated copies of the CMV-7tetO luciferase reporter construct (Gossen et al. 1992). Transfected cells were cultured for two days in the presence of 0-1000 ng/ml dox. We subsequently determined the intracellular luciferase level, which reflects rtTA activity. As shown in Fig. 12A, the activity of these 20 rtTA variants varies considerably. Most variants show no or very low activity in the absence of dox, and their activity increases with increasing dox levels.

Comparison of the rtTA activity data (Fig. 12A) with the replication capacity of the selected set of HIV-rtTA variants (Fig. 11) allows us to determine the level of rtTA activity that is required for viral replication. The 37F, 37L, 37N, 37Q and 37R variants show no or very low activity at zero dox (less or equal to 0.06% of the wild-type rtTA activity at 1000 ng/ml dox), and viruses carrying these rtTA variants do not replicate without dox. The low activity (0.09%) of the 37D variant at 1000 ng/ml dox is not sufficient for viral

replication either. The 37K and 37S variants show 0.19% and 1% activity without dox, respectively. This level of activity is apparently sufficient to drive a low level of viral replication. The threshold of rtTA activity that is sufficient for HIV-rtTA replication was therefore set at 0.1%. This would mean that not only HIV-rtTA_{E37K} and HIV-rtTA_{E37S}, but also HIV-rtTA_{E37A} will replicate in the absence of dox. The codons corresponding to these amino acids are therefore dark grey (but not black) in the codon table (Fig. 12C), and evolution toward these codons should be prevented. All other variants, except for the inactive 37D mutant, show a phenotype similar to wild-type rtTA, i.e. activity below 0.1% at zero dox and much higher than 0.1% at 1000 ng/ml dox. HIV-rtTA viruses with these variants are thus expected to replicate in a dox-dependent manner. These amino acids are light grey in the codon table, and evolution toward them would not result in a loss of dox-dependence. The D and stop codons are marked in black, as the corresponding viruses will not be replication competent.

In the codon table, every change in row or column represents a single nucleotide substitution. This colored codon table (Fig. 12C) thus facilitates the identification of position 37 codons that preserve dox-dependence (light grey) and that require multiple nucleotide mutations to convert into a codon that allows replication in the absence of dox (dark grey). The Leu codons CUN meet these safety requirements.

Testing all possible position 19 variants of rtTA.

Like the E37K mutation, the G19E mutation causes viral replication in the absence of dox. To reveal whether other amino acid substitutions at this position would similarly result in a loss of dox-dependence, we constructed rtTA expression plasmids with all possible amino acids at position 19. The activity of these rtTA variants was analyzed as described above for the position 37 variants. As shown in Fig. 12B, most variants show no or very low activity (less than 0.1%) without dox, and their activity increases with increasing dox

levels. In contrast, the 19P variant is inactive, and the 19E variant shows 3% activity without dox. This relatively high basal activity of 19E is in agreement with the efficient replication of the corresponding HIV-rtTA virus without dox. There are multiple codons possible at position 19 that preserve dox-

5 dependence (colored light grey in Fig. 12D) and that require multiple nucleotide mutations to convert into a codon that allows replication in the absence of dox (colored dark grey). For example, the Phe codon UUU meets these safety requirements very well, since it requires three transversions to convert into a Glu codon.

10

rtTA with safety-lock mutations prevents the loss of dox-control.

We constructed an rtTA variant that combines the two safety-lock mutations: Phe (UUU) at position 19 (G19F) and Leu (CUU) at position 37 (E37L). This rtTA variant shows very low basal activity (less than 0.1%) and its activity

15 gradually increases with increasing dox levels (figure 13A). Although rtTA_{G19F E37L} is less active than wild-type rtTA at low dox concentrations, it is highly active at high dox levels. Accordingly, HIV-rtTA_{G19F E37L} does not replicate in the absence of dox or at low dox levels, but does replicate efficiently at high dox levels (Fig. 13C). We tested the genetic stability of this virus in 24 long-term

20 cultures with dox (Fig. 8C). The HIV-rtTA_{G19F E37L} virus never lost dox-control up to 200 days of culturing. This result demonstrates the increased genetic stability, and thus improved safety, of the novel HIV-rtTA variant.

rtTA variants with an alternative amino acid at position 19 or 37

25 **demonstrate an improved transcriptional activity and dox-sensitivity.**

Most rtTA variants with an alternative amino acid at position 19 (alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, glutamine, arginine, serine, threonine, valine or tyrosine) and some of the rtTA variants with an alternative amino acid at

30 position 37 (cysteine, methionine, glutamine, arginine or threonine) show an

increased transcriptional activity at a low dox concentration and/or an increased transcriptional activity at a high dox concentration when compared with the original (wild-type) rtTA (Fig. 12 A and B). These results demonstrate that these amino acid substitutions at position 19 and 37 enhance the activity
5 and/or dox-sensitivity of rtTA.

Conclusions

When currently known rtTA is incorporated in a replicating system (e.g. in a replicon), rtTA is at risk of losing dox-control due to mutations at rtTA amino
10 acid position 19 and/or 37 acquired during evolution of the system. Such undesired evolution is prevented by the introduction of alternative codons at these amino acid positions. Preferred alternative codons require multiple nucleotide substitutions to convert into a codon encoding an amino acid that would mediate loss of dox-control of rtTA. As an example we demonstrate that
15 a phenylalanine codon (UUU) at rtTA amino acid position 19 and a leucine codon (CUU) at position 37 improve the genetic stability of rtTA and prevent at least in part the loss of dox-control. Our results demonstrate that other amino acid codons at position 19 (encoding alanine, cysteine, phenylalanine, histidine, isoleucine, leucine, methionine, asparagine, arginine, serine,
20 threonine, valine, tryptophane or tyrosine) and position 37 (encoding histidine, leucine or arginine) similarly improve the genetic stability of rtTA.

The introduction of alternative amino acids at rtTA amino acid position 19 and/or 37 improve the transcriptional activity and/or inducer-sensitivity of
25 rtTA. Specifically, introduction of an alanine, cysteine, aspartic acid, phenylalanine, histidine, isoleucine, lysine, leucine, methionine, asparagine, glutamine, arginine, serine, threonine, valine or tyrosine at rtTA amino acid position 19, and/or the introduction of a cysteine, methionine, glutamine, arginine or threonine at rtTA amino acid position 37 results in an increased
30 transcriptional activity and/or dox-sensitivity of rtTA.

5 Example 3: Improved sc rtTA variants

Single-chain Tet transregulators have recently been developed, in which two TetR domains are connected by a peptide linker and one VP16 activation domain or KRAB repressor domain is positioned at the C-terminal end
10 (Krueger et al. 2003). These transregulators fold intramolecularly and do not dimerize with each other. Unfortunately, the single-chain version of rtTA (sc rtTA) exhibits reduced activity when compared with the regular rtTA, and this low activity may thwart its use in applications that require an active Tet-on system.

15 We have incorporated the rtTA gene and the tetO elements into the HIV-1 genome to control virus replication. During culturing of this dox-dependent virus, spontaneous viral evolution selected for improved virus variants, in which the introduced Tet-on system was optimized. We have identified several amino acid substitutions in the rtTA gene that greatly
20 enhance the transcriptional activity and dox-sensitivity of the transactivator. To test whether these mutations similarly improve other TetR-based transactivators, we introduced them into sc rtTA. All mutations enhanced sc rtTA activity. The most active sc rtTA variant is ~30-fold more active than the original sc rtTA, and is almost as active as the regular rtTA.

25

Materials and Methods

Construction of sc rtTA variants. The plasmids pCMV-rtTA and pCMV-scrTA contain the rtTA2^S-S2 and sc rtTA2-S2 genes, respectively, cloned in the expression vector pUHD141-1/X (Krueger et al. 2003; Urlinger et al. 2000).
30 The sc rtTA gene contains two TetR domains and a single activation domain.

To introduce mutations into the N-terminal TetR domain, the *EcoRI*-*BfuAI* fragment of pCMV-scrTA was replaced with the corresponding fragment of the appropriate pCMV-rtTA plasmid. Mutations were introduced into the C-terminal TetR domain of sc rtTA by mutagenesis PCR (Mikaelian et al. 1992) on pCMV-scrTA with the mutagenic primers (primer M) scrTA-V9I (5'-GGCTCTAGATCTCGTTTAGATAAAAAGTAAAATCATTAACAGCGCA-3'; SEQ ID NO:18), scrTA-F67S (5'-AGGCACCATACTCACTCTTGCCCTTTA-3'; SEQ ID NO:19), scrTA-F86Y (5'-AACGCTAAAAGTTATAGATGTGCT-3'; SEQ ID NO:20), or scrTA-G138D (5'-CAGCGCTGTGGACCACTTTACTTTA-3'; SEQ ID NO:21) and the primers 5'-TAATCATATGTGGCCTGGAGAA-3' (primer 1; SEQ ID NO:22), 5'-AGGCGTATTGATCAATTCAAGGCCGAATAAG-3' (primer 2; SEQ ID NO:23), and 5'-TCACTGCATTCTAGTTGTGGT-3' (primer 3; SEQ ID NO:24) as described above for the tTA mutations. The final PCR products were digested with *BglII* and *SmaI* and used to replace the corresponding fragment of pCMV-scrTA. All constructs were verified by sequence analysis.

Cell culture and rtTA activity assay. The activity of rtTA and sc rtTA was assayed in HeLa X1/6 cells (Baron et al. 1997), which are HeLa-derived cells containing chromosomally integrated copies of the CMV-7tetO luciferase reporter construct pUHC13-3 (Gossen et al. 1992). Cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, minimal essential medium nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were grown in 2-cm² wells to 60% confluency and transfected with the pCMV-rtTA or pCMV-scrTA expression plasmids and the plasmid pRL-CMV (Promega) by the calcium phosphate precipitation method. pRL-CMV expresses *Renilla* luciferase from the CMV promoter and was used as an internal control to allow correction for differences in transfection efficiency. 1 µg of DNA mixture in 15 µl water was mixed with 25 µl of 50 mM HEPES (pH 7.1)-250 mM NaCl-1.5 mM Na₂HPO₄ and 10 µl of 0.6 M CaCl₂, incubated at room temperature for 20 min, and added to the culture medium. The DNA mixture consisted of 20 ng pCMV-

scrtTA or pCMV-rtTA, 2 ng pRL-CMV, and 978 ng pBluescript for sc rtTA or rtTA activity assay. Cells were cultured after transfection for 48 hours at different dox (D-9891, Sigma) concentrations and then lysed in Passive Lysis Buffer (Promega). Firefly and *Renilla* luciferase activities were determined
5 with the Dual-Luciferase Reporter Assay (Promega). The expression of firefly and *Renilla* luciferase was within the linear range and no squelching effects were observed. The activity of the transactivators was calculated as the ratio of the firefly and *Renilla* luciferase activities, and corrected for between-session variation.

10

Results

Mutations observed in rtTA improve sc rtTA activity.

In sc rtTA, two TetR domains are connected head to tail by a peptide linker,
15 and a single activation domain is fused to the C-terminal TetR domain. The mutations that did improve rtTA activity are all positioned within the TetR domain of the protein (Fig. 16). To test whether these beneficial mutations of rtTA can also improve the activity and dox-sensitivity of sc rtTA, we introduced them into either one or both of the TetR domains of sc rtTA.
20 Activity of these variants was analyzed in HeLa X1/6 cells and compared with the activity of rtTA and the original (wild-type) sc rtTA (Fig. 18). Both rtTA and wild-type sc rtTA show no background activity without dox and their activity increases gradually with increasing dox levels. However, the induced activity of sc rtTA is much lower than that of rtTA at all tested dox
25 concentrations. For example, sc rtTA is about 40-fold less active than rtTA at 1000 ng/ml dox (Fig. 18A). Introduction of the F86Y mutation in the N-terminal TetR domain increased sc rtTA activity ~10-fold at all dox levels, but did not affect background activity. The additional introduction of the V9I mutation into the F86Y variant also improved sc rtTA activity (albeit
30 marginally), whereas the addition of the F67S, G138D, or V9I plus G138D

mutations further improved sc rtTA activity ~2-fold at all dox levels. The background activity of these variants was not increased.

Similar results were obtained upon introduction of the mutations into the C-terminal TetR domain of sc rtTA (Fig. 18B). However, none of these
5 variants are as active as their counterparts with mutations in the N-terminal TetR domain. The F86Y mutation increased sc rtTA activity ~3-fold, and the addition of the F67S, G138D, or V9I plus G138D mutations further increased activity ~2-fold. These results demonstrate that the activity of sc rtTA is improved by mutations in either TetR domain. Mutations introduced into the
10 N-terminal TetR domain have a larger effect on sc rtTA activity than the same mutations in the C-terminal domain.

Introduction of the mutations in both TetR domains resulted in the most active sc rtTA variants (Fig. 18C). At high dox levels (500-1000 ng/ml), all these variants demonstrate a higher transcriptional activity than the
15 corresponding variants with mutations in only one of the two TetR domains (Fig. 18A and 18B). For instance, the sc rtTA with the F86Y mutation in both TetR domains is ~13-fold more active than wild-type sc rtTA at 1000 ng/ml dox, whereas the same mutation in the N-terminal or in the C-terminal TetR domain increased sc rtTA activity ~10-fold and ~3-fold, respectively. The
20 variants carrying the F67S, G138D, or V9I plus G138D mutations in addition to the F86Y mutation in both TetR domains are not only more active at high dox levels, but also more active at low dox levels (10-100 ng/ml). In fact, these variants demonstrate a transcriptional activity and dox-sensitivity similar to rtTA.

25

Discussion

We have identified amino acid substitutions in rtTA that greatly improve the transcriptional activity and dox-sensitivity of the transactivator. In this
30 Example, we tested whether these mutations similarly affect sc rtTA. Our

results demonstrate that all mutations did significantly enhance sc rtTA activity. Both the transactivators rtTA and sc rtTA are activated by doxycycline. Our results demonstrate that the sc rtTA activity is significantly improved by introduction of at least one mutation that enhances rtTA activity.

5 The most active sc rtTA variant in this study was obtained by introducing beneficial mutations in both TetR domains. However, sc rtTA is also improved by at least one mutation in only one of the TetR domains. The sc rtTA variants with beneficial mutations in the N-terminal TetR domain appear to be more active than the variants with the same mutations in the C-terminal TetR

10 domain.

The sc rtTA variant with the F67S and F86Y mutations in both TetR domains is ~30-fold more active than the original sc rtTA at high dox levels, and does not show any background activity in the absence of dox. This novel sc rtTA is almost as active and dox-sensitive as rtTA, and is therefore suitable for

15 replacing the regular rtTA in applications where multiple TetR-based regulatory systems are used in the same cell or organism.

Conclusion

The transcriptional activity and inducer-sensitivity of single chain rtTA

20 activity is significantly improved by the introduction of amino acid substitutions that were found by us to improve the transcriptional activity and inducer-sensitivity of rtTA. We thus for instance generated sc rtTA variants with an up to ~30-fold increased transcriptional activity and an increased dox-sensitivity by the introduction of a F86Y, a V9I, a F67S and/or a G138D amino

25 acid substitution into the original sc rtTA.

Example 4: Development of novel rtTA variants with improved genetic stability; Introduction of alternative amino acids at rtTA position 19, 37 and 56.

5 We have demonstrated that long-term replication of HIV-rtTA resulted in virus variants that no longer depend on dox for replication. This reduced dox-dependence was associated with an amino acid substitution in the rtTA protein either at position 19 (glycine to glutamic acid; G19E) or at position 37 (glutamic acid to lysine; E37K). We developed an HIV-rtTA variant with
10 safety-lock mutations (G19F and E37L) in the rtTA gene to block these undesired evolutionary routes. This novel variant showed improved genetic stability and did not lose dox-control in long-term cultures with dox (see Example 2).

As a vaccine, replication of HIV-rtTA would be temporally switched on
15 to induce anti-viral immune responses. Subsequent dox-withdrawal impose alternative evolutionary pressure on the virus than long-term culturing with dox. Specifically, there is a risk of rtTA variants with a tTA-like phenotype, which are active without dox and inhibited by dox, appearing in dox-washout experiments, whereas such variants are counter selected in the presence of
20 dox. We therefore followed evolution of HIV-rtTA in multiple, independent virus cultures that were transiently activated by dox. The virus did indeed lose dox-control in a significant number of cultures after dox-withdrawal. We identified a typical amino acid substitution at position 56 in the rtTA protein, which was found to be responsible for the dox-independent replication. This
25 mutation had never been observed in long-term cultures with dox. We developed a novel rtTA variant that blocks this undesired evolutionary route and thus improves the genetic stability and safety of HIV-rtTA.

Materials and Methods

Virus cultures. The HIV-rtTA infectious molecular clone is a derivative of the HIV-1 LAI proviral plasmid (Peden et al, 1991) and was described previously (Das et al, 2004b; Verhoef et al, 2001). HIV-rtTA used in this study contains
5 the inactivating Y26A mutation in the Tat gene, five nucleotide substitutions in the TAR hairpin motif, the rtTA_{F86Y A209T} gene (Das et al, 2004a) in place of the nef gene, and the LTR-2ΔtetO promoter configuration (Marzio et al, 2001; Marzio et al, 2002).

SupT1 T cells were cultured at 37°C and 5% CO₂ in RPMI1640 medium
10 containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. SupT1 cells were transfected with HIV-rtTA molecular clones by electroporation. Briefly, 5 x 10⁶ cells were washed in RPMI1640 with 20% FBS and mixed with 5 μg of DNA in 250 μl RPMI1640 with 20% FBS. Cells were electroporated in 0.4-cm cuvettes at 250 V and 975 μF and
15 subsequently resuspended in RPMI1640 with 10% FBS. The CA-p24 level in the cell-free culture supernatant was determined by antigen capture enzyme-linked immunosorbent assay (ELISA) (Back et al, 1996).

The evolution experiment was started with transfection of 15 μg HIV-rtTA proviral plasmid into 1.5 x 10⁷ SupT1 cells. Cells were split into 12
20 independent cultures and dox (Sigma D-9891) was added to initiate viral replication. Three days after transfection, dox was removed from the cultures by washing the cells twice with medium, each followed by a 30 min incubation at 37°C and 5% CO₂ to allow release of dox from cells. Cells were subsequently resuspended in medium and cultured without dox. If virus replication was
25 apparent as indicated by the formation of syncytia, the virus containing culture supernatant was passaged onto fresh SupT1 cells. Infected cell samples were used to analyze the proviral rtTA sequence.

Proviral DNA analysis of evolved sequences. HIV-rtTA infected cells were
30 pelleted by centrifugation and washed with phosphate-buffered saline. Total

cellular DNA was solubilized by resuspending the cells in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-0.5% Tween 20™, followed by incubation with 200 µg/ml of proteinase K at 56°C for 60 min and 95°C for 10 min. The proviral rtTA genes were PCR amplified with primers tTA1 (5'-ACAGCCATAGCAGTAGCTGAG-3';
 5 SEQ ID NO:1) and tTA-rev2 (5'-GATCAAGGATATCTTGTCTTCGT-3'; SEQ ID NO:2), and sequenced with the bigdye™ terminator cycle sequencing kit (Applied Biosystems).

Construction of novel rtTA expression plasmids and HIV-rtTA

variants. The plasmid pCMV-rtTA contains the rtTA2^S-S2 gene in the
 10 expression vector pUHD141-1/X (Urlinger et al, 2000). To introduce the P56S mutation, the proviral PCR product with this mutation was digested with *Xba*I and *Sma*I and used to replace the corresponding fragment in pCMV-rtTA. To generate rtTA variants with the G19F and E37L mutations and different amino acids at position 56, mutagenesis PCR was performed on pCMV-
 15 rtTA_{G19F E37L} (example 2) with the sense primer random-rtTA-56 (5'-AAGCGGGCCCTGCTCGATGCCCTGNNKATCGAGATGCTGGACAGGC-3', with K corresponding to G or T, and N corresponding to G, A, T or C; SEQ ID NO:25) plus the antisense primer CMV2 (5'-TCACTGCATTCTAGTTGTGGT-3'; SEQ ID NO:15). Mutant rtTA sequences were cloned as *Apa*I-*Bam*HI
 20 fragments into pCMV-rtTA_{G19F E37L}. Novel rtTA sequences were cloned into the shuttle vector pBlue3'LTRext-deltaU3-rtTA_{F86Y A209T}-2ΔtetO (Das et al, 2004a) using the *Xcm*I and *Nde*I sites and subsequently cloned into the HIV-rtTA molecular clone as *Bam*HI-*Bgl*II fragments. All constructs were verified by sequence analysis.

25 **rtTA activity assay.** pLTR-2ΔtetO-luc expresses firefly luciferase from the LTR-2ΔtetO promoter derived from the HIV-rtTA molecular clone (Marzio et al, 2001; Marzio et al, 2002). pCMV-7tetO-luc, previously named pUHC13-3 (Gossen & Bujard, 1992), contains seven tetO elements located upstream of a minimal CMV promoter and the firefly luciferase gene. The plasmid pRL-CMV
 30 (Promega), in which the expression of *Renilla* luciferase is controlled by the

CMV promoter, was used as an internal control to allow correction for differences in transfection efficiency. HeLa X1/6 cells are derived from the HeLa cervix carcinoma cell line and harbor chromosomally integrated copies of the CMV-7tetO firefly luciferase reporter construct (Baron et al, 1997). HeLa
5 X1/6 and C33A cervix carcinoma cells (ATCC HTB31) (Auersperg, 1964) were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, minimal essential medium nonessential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin.

C33A and HeLa X1/6 cells were grown in 2-cm² wells to 60% confluency
10 and transfected by the calcium phosphate precipitation method. 1 µg of DNA mixture in 15 µl water was mixed with 25 µl of 50 mM HEPES (pH 7.1)-250 mM NaCl-1.5 mM Na₂HPO₄ and 10 µl of 0.6 M CaCl₂, incubated at room temperature for 20 min and added to the culture medium. The DNA mixture consisted of 0.4 ng pCMV-rtTA, 20 ng pLTR-2ΔtetO-luc or pCMV-7tetO-luc, 0.5
15 ng pRL-CMV, and 980 ng pBluescript as carrier DNA for C33A cells, or 8 ng pCMV-rtTA, 2.5 ng pRL-CMV, and 990 ng pBluescript for HeLa X1/6 cells. Transfected cells were cultured for 20 hours at different dox concentrations, washed with DMEM, and subsequently cultured for 24 hours with fresh medium containing dox (the same concentrations as before the wash step).
20 Cells were then lysed in Passive Lysis Buffer (Promega), and firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase Reporter Assay (Promega) using a GloMax microplate luminometer (Promega). The expression of firefly and *Renilla* luciferase was within the linear range and no squelching effects were observed. The activity of the rtTA variants was
25 calculated as the ratio of the firefly and *Renilla* luciferase activities, and corrected for between-session variation.

Results

Evolution of HIV-rtTA after transient dox administration. To test the
30 genetic stability of HIV-rtTA upon removal of the effector dox, we started 12

independent virus cultures in SupT1 T cells with dox (Fig. 20B). Viral replication resulted in the production of CA-p24 and the appearance of syncytia in all cultures. At day 3, we washed the cultures to remove dox, which resulted in silencing of viral replication as was obvious from the decrease in CA-p24 levels and the disappearance of syncytia in all cultures. However, CA-p24 levels started to increase again at day 10-20, and continued culturing resulted in high CA-p24 levels and formation of large syncytia. At the peak of infection, the virus was passaged onto fresh SupT1 cells and cultured without dox. All viruses were able to initiate a spreading infection, indicating that they had lost dox-control. Total cellular DNA with integrated proviruses was isolated from the cultures and the rtTA gene was PCR-amplified and sequenced. In all cultures, the virus had acquired a point mutation (CCA to UCA) in the rtTA gene that resulted in a Proline to Serine substitution at position 56 (P56S).

Similar results were obtained with HIV-rtTA_{V9I G138D}, an improved HIV-rtTA variant with two rtTA mutations (V9I and G138D) (example 1). The evolved viruses started to replicate without dox in 10 of the 12 cultures (Fig. 20C). Nine virus cultures had acquired the P56S mutation, whereas one culture had obtained the previously described G19E mutation (example 2). In the two remaining cultures, CA-p24 levels stably decreased after dox removal and no viral replication was observed upon prolonged culturing. At day 64, these cultures were split and continued with and without dox. While the cultures without dox remained negative for CA-p24, spreading infections were apparent in the cultures with dox (Fig. 20C). Thus, the virus in these two cultures remained dox-dependent and can be readily reactivated.

P56S mutation causes a tTA-like phenotype. The repeated selection of the P56S mutation in multiple, independent cultures strongly suggests its linkage to the observed loss of dox-control. To demonstrate that this amino acid substitution is indeed responsible for an altered rtTA phenotype, we cloned the

P56S-mutated rtTA gene into the expression plasmid pCMV-rtTA and assayed its activity in a regular Tet-on system. The rtTA expression plasmid was transfected into C33A cells together with a reporter plasmid in which luciferase expression is controlled by the viral LTR-2 Δ tetO promoter (Marzio et al, 2001; Marzio et al, 2002). Transfected cells were cultured for two days at different dox concentrations. We subsequently determined the intracellular luciferase level, which reflects rtTA activity (Fig. 21A). Wild-type rtTA shows no activity without dox or with a low dox level (10 ng/ml), and its activity gradually increases at higher dox concentrations. In contrast, the P56S variant exhibits a very high activity without dox, and its activity is inhibited, instead of activated, by increasing dox concentrations. This phenotype is similar to that of the transcriptional activator tTA, which differs from rtTA by four amino acids, including an Alanine instead of Proline at position 56 (Urlinger et al, 2000). The high activity of the P56S variant in the absence of dox explains its appearance in the dox-washout experiments, whereas its low activity with dox explains why we never observed this mutation in long-term cultures of HIV-rtTA in the presence of dox.

We also analyzed rtTA activity in C33A cells transfected with a luciferase reporter under the control of a minimal CMV promoter coupled to an array of seven tetO elements (Gossen & Bujard, 1992), and in HeLa X1/6 cells that contain stably integrated copies of this CMV-7tetO luciferase construct (Baron et al, 1997). In both assays, we observed similar results as with the viral LTR-2 Δ tetO promoter construct (Fig. 21B and 21C), demonstrating that the tTA-like phenotype of rtTA_{P56S} is not dependent on the type of promoter, nor on the episomal or chromosomal state of the reporter gene.

HIV-rtTA_{G19F E37L} can lose dox-control by a P56S mutation. We have previously constructed an HIV-rtTA variant with the safety-lock mutations G19F and E37L that prevent the virus from losing dox-control during long-term culturing with dox (example 2). We now tested the stability of HIV-

rtTA_{G19F E37L} in the dox-washout experiment. This virus did lose dox-control in only one of the 12 cultures, and all other cultures did not show any replication in the absence of dox (Fig. 20D). Sequence analysis revealed that the escape variant had acquired the P56S mutation. This result demonstrates that

5 although HIV-rtTA_{G19F E37L} showed a lower tendency to lose dox-control than the original virus without safety-lock mutations (Fig. 20B), the escape route at position 56 is preferably blocked in order to further improve the genetic stability of the virus.

10 **Safety-lock mutation at position 56.** The P56S mutation is caused by a single nucleotide substitution (CCA to UCA). Such single nucleotide transitions (pyrimidine-pyrimidine or purine-purine substitutions) occur at a much higher frequency than single nucleotide transversions (pyrimidine-purine substitutions) or multiple nucleotide changes during HIV-1 reverse

15 transcription (Berkhout et al, 2001; Berkhout & de Ronde, 2004). This mutational bias strongly influences the course of virus evolution (Keulen et al, 1996; Keulen et al, 1997). Accordingly, the undesired evolutionary route at position 56 is blocked by introducing alternative amino acid codons that require multiple nucleotide changes for HIV-rtTA to lose dox-control. In fact,

20 we have successfully blocked the escape routes at positions 19 and 37 by such safety-lock mutations, which demonstrate the effectiveness of this strategy (example 2). To block all three observed escape routes of HIV-rtTA at the same time, the position 56 safety-lock mutation is ideally combined with the positions 19 and 37 mutations. To identify suitable amino acid substitutions,

25 we made rtTA expression plasmids with all possible amino acids at position 56 in combination with the G19F and E37L mutations, and assayed their activity in HeLa X1/6 cells.

The activity of these 20 rtTA variants varies considerably (Fig. 22A). Like the S variant, the A, C and H variants exhibit a tTA-like phenotype, since

30 their activity is relatively high in the absence of dox and drops with increasing

dox levels. Except for the F and M variants that are completely inactive, the other variants exhibit an rtTA phenotype, since their activity increases with a rising dox level. However, the basal and induced activities of these variants (at 0 and 1000 ng/ml dox, respectively) differ significantly. Because the L variant shows an rtTA phenotype with a very low basal activity, we introduced this variant into HIV-rtTA and tested viral replication in SupT1 T cells. This virus did not replicate without dox, but also not with dox (data not shown), indicating that the induced activity of the L variant (~0.3% of the wild-type rtTA activity at 1000 ng/ml dox) is not sufficient for HIV-rtTA replication. This is in agreement with our observation that the wild-type rtTA does not support viral replication at 10 ng/ml dox (~0.4% rtTA activity; wt in Fig. 22A) and rtTA_{G19F E37L} does not support replication at 100 ng/ml dox (~0.4% rtTA activity; P variant in Fig. 22A). All these results indicate that the E, F, L and M variants with both their basal and induced activities lower than 0.4% will not support viral replication. We therefore colored the codons corresponding to these amino acids and the stop codons in black (Fig. 22B). The basal activity of the A, C, G, H, N, S, and Y variants is higher than 0.4%. Since the corresponding HIV-rtTA viruses are at risk of replicating without dox, their codons are dark grey (but not totally black). The other variants that show a low basal activity (<0.4%) and a high induced activity (>0.4%) result in dox-dependent viruses, and their codons are light grey.

In the codon table, every change in row or column represents a single nucleotide substitution. Apparently, the only position 56 codon that preserves dox-dependence (light grey) and requires more than a single nucleotide mutation to be converted to a codon that allows replication without dox (dark grey) is the AUA codon encoding an Isoleucine. However, the activity of the I variant at 1000 ng/ml dox is only 1% of the wild-type level (Fig. 22A), which may result in a poorly replicating virus. The K and Q variants, which show a dox-dependent activity similar to the P variant (rtTA_{G19F E37L}), require at least one nucleotide transversion to be converted to a dox-independent variant. It

has been shown that transversions occur less frequently than transitions during HIV-1 reverse transcription (Berkhout et al, 2001; Berkhout & de Ronde, 2004). For instance, we did frequently observe a P56S mutation (caused by a CCA to UCA transition), but never a P56A mutation (would require a CCA to GCA transversion) in the dox-washout experiment, although both mutations would cause a similarly high activity in the absence of dox (Fig. 22A). Therefore, introduction of an AAG (K) or CAG (Q) codon at rtTA position 56 blocks the appearance of dox-independent virus variants upon dox-withdrawal.

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Blocking loss of dox-control by triple safety-lock rtTA variant. We constructed HIV-rtTA molecular clones carrying triple safety-lock mutations G19F, E37L and P56K or P56Q, and tested their replication in SupT1 T cells with and without dox (Fig. 23). Both viruses replicated in a dox-dependent manner. However, whereas replication of HIV-rtTA_{G19F E37L P56K} was as efficient as the double safety-lock variant HIV-rtTA_{G19F E37L}, HIV-rtTA_{G19F E37L P56Q} replicated less efficiently. We therefore focused our studies on the HIV-rtTA_{G19F E37L P56K} variant and tested the genetic stability of this virus in long-term cultures with dox and in dox-washout experiments. We started 24 long-term cultures with dox and tested virus replication in the presence and absence of dox at several time points (as described previously in example 2). All virus cultures stayed fully dox-dependent during the 100 days of culture, and sequence analysis revealed that the safety-lock mutations were stably maintained in all cultures (data not shown). To test the genetic stability of HIV-rtTA_{G19F E37L P56K} after transient dox administration, we started 24 virus cultures with dox (Fig. 24). Virus replication resulted in the production of detectable amounts of CA-p24 and the appearance of syncytia in all cultures. Upon dox-withdrawal at day 3, the CA-p24 level dropped and syncytia disappeared, and no sign of viral replication could be detected in any of the 24 cultures in the following months. At day 60, all cultures were split and

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continued with and without dox. While there was no viral replication in the cultures without dox, administration of dox did result in spreading infections, which indicate that the virus in all cultures stayed dox-dependent, and the undetectable CA-p24 levels in dox minus cultures were not due to loss of proviral genomes or total silencing of the viral promoter. Thus, replication of HIV-rtTA_{G19F E37L P56K} stays dox-dependent in both long-term cultures with dox and transiently activated cultures, demonstrating that the triple safety-lock mutations at rtTA positions 19, 37 and 56 completely block the loss of dox.

10 Conclusions

Our virus evolution experiments demonstrate that HIV-rtTA is at risk of escaping from dox-control by an amino acid substitution in rtTA at position 19, 37 or 56. To generate a safe HIV-rtTA virus, all three evolutionary routes are preferably blocked. We have previously blocked the position 19 and 37 routes by safety-lock mutations (e.g. G19F and E37L) that require multiple nucleotide changes to lose dox-control (example 2). We here demonstrate that the position 56 escape route is efficiently blocked by the introduction of an alternative amino acid at position 56 (e.g. P56K or P56Q) that requires at least one nucleotide transversion to convert rtTA into a dox-independent variant.

Table 1: Naturally evolved and constructed rtTA variants

rtTA	Mutations	Times in culture	Natural variation in TetR							
			A	B	C	D	E	G	H	
wild-type ^a	-	-				-				
V1	F86Y A209T ^b	-	F	F	F	F	F	F	F	
V2	V9I	2	V	V	V	V	V	V	V	
V3	F67S	2	S	F	S	S	S	S	V	
V4	G138D	7	S	G	S	S	S	S	A	
V5	E157K	2	E	E	E	D	E	E	E	
V6	R171K	1	R	R	Q	R	Q	H	T	
V7	V9I G138D	1				-				
V8	V9I E157K	1				-				
V9	V9I R171K	1				-				
V10	F67S R171K	1				-				
V11	V9I F67S	-				-				
V12	F67S G138D	-				-				
V13	F67S E157K	-				-				
V14	V9I F67S G138D	-				-				
V15	V9I F67S E157K	-				-				
V16	V9I F67S R171K	-				-				
V17	V9I G138D E157K	-				-				
V18	V9I G138D R171K	-				-				

^a The wild-type rtTA was previously described as rtTA2^S-S2 (Urlinger et al. 2000).

^b All variants (V1-V18) contain the F86Y (in the TetR domain) and A209T (in the VP16 activation domain) mutations.

Brief description of the drawings

Figure 1. Mutation of the rtTA gene through viral evolution. (A) In the HIV-rtTA virus, the Tat-TAR axis of transcription regulation has been inactivated by mutation of both Tat and TAR (crossed boxes). Transcription and replication of the virus were made dox-dependent by introduction of tetO elements in the LTR promoter region and replacing the nef gene by the rtTA gene. This 248-amino acid protein is a fusion of the *E. coli* Tet repressor (TetR) and the VP16 activation domain (AD) of the herpes simplex virus. The TetR part can be subdivided in a DNA binding domain (BD) (α -helices 1-3) and a regulatory core domain (α -helices 5-10) with a dimerization surface (α -helices 7-10). The F86Y (dark grey triangle) and A209T (black triangle) mutations were present in the starting virus and maintained in all long-term cultures. Light grey triangles indicate additional amino acid exchanges in rtTA that were observed in multiple, independent cultures of HIV-rtTA_{F86Y A209T}. (B) The crystal structure of the TetR homodimer (one monomer in dark grey, the other in light grey) complexed with Tc (light grey) and Mg²⁺ (grey ball) (Hinrichs et al, 1994; Kisker et al, 1995). Residue 86 is shown in dark grey. Additional mutated amino acids (positions 9, 67, 138, and 171) are shown in light grey. Residue 157 is not shown, because the segment 156 to 164 is flexible and not determined in the TetR crystal structure. A close up of the Tc-binding region is shown at the right. There are seven classes of TetR proteins (A-E, G, H) with a highly conserved sequence. The high resolution crystal structure that is shown is based on class D (TetR^D). rtTA is based on class B (TetR^B), which shares 63% sequence identity with TetR^D. The crystal structure of TetR^B at medium resolution revealed an identical polypeptide fold (Hinrichs et al, 1994). Therefore, we can assume that the interactions of TetR with Tc and Mg²⁺ will be nearly identical in both classes. Figures are drawn using the 2TCT coordinates from the Protein Data Bank and the MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt et al, 1997) programs.

Figure 2. Novel rtTA variants show increased activity and dox-sensitivity in different Tet systems. The transcriptional activity of rtTA variants was measured in C33A cells transfected with a plasmid carrying the firefly luciferase reporter gene under the control of the viral LTR-2 Δ tetO promoter (LTR-2 Δ tetO; A) or under the control of a minimal CMV-derived promoter coupled to seven tetO elements (CMV-7tetO; B). Furthermore, rtTA activity was measured in HeLa X1/6 cells (Baron et al, 1997) that contain a chromosomally integrated copy of the CMV-7tetO reporter construct (CMV-7tetO-integrated; C, D). Variants V1 to V10 were compared in all three Tet systems (panels A-C) and variants V11 to V18 in the cells with the integrated reporter (panel D). Cells were transfected with the indicated rtTA expression plasmid or pBluescript as a negative control, and a plasmid constitutively expressing *Renilla* luciferase to correct for differences in transfection efficiency. Cells were cultured in the presence of different dox concentrations (0-1000 ng/ml). The ratio of the firefly and *Renilla* luciferase activities measured 2 days after transfection reflects the rtTA activity. All values were related to the wild-type (wt) rtTA activity at 1000 ng/ml dox, which was arbitrarily set at 100%. In (C and D), average values of three transfections are shown with error bars indicating the standard deviation.

Figure 3. Transcriptional activity and dox-sensitivity of the naturally evolved and constructed rtTA variants. Transfection assays were performed in HeLa X1/6 cells, see Fig. 2 for details. Transcriptional activity observed at 1000 ng/ml dox is shown as average value of three transfections with error bars indicating the standard deviation. The wild-type rtTA activity was set at 100%. Dox-sensitivity is compared with the wild-type rtTA of which the sensitivity is arbitrarily set at 1. For each rtTA variant, the dox concentration (ng/ml) that results in an activity comparable to that of the wild-

type rtTA activity at 1000 ng/ml dox is indicated between brackets. (nd, not determined)

Figure 4. Mutations do not affect the intracellular rtTA protein level.

5 HeLa X1/6 cells were transfected with the indicated rtTA expression plasmid (lanes 3 to 6) or pBluescript as a negative control (lane 2). Total cellular extracts were prepared at 2 days after transfection and analyzed on Western blot that was stained with polyclonal anti-TetR rabbit serum (Krueger et al, 2003). Detection of purified TetR protein (2ng) is shown in lane 1. The position
10 and molecular weight (in kDa) of the rtTA and TetR proteins are indicated.

Figure 5. Novel rtTA variants can be activated by dox-like compounds.

The rtTA activity was measured in HeLa X1/6 cells, see Fig. 2 for details. Cells were cultured in the presence of different concentrations of Tc or Mc (0-10000
15 ng/ml). The wild-type (wt) rtTA activity at 1000 ng/ml dox (not shown) was set at 100%. Average values of three transfections are plotted with error bars indicating the standard deviation.

Figure 6. rtTA variants improve HIV-rtTA replication. The rtTA

20 variants V7 and V14 were cloned into the HIV-rtTA proviral genome. SupT1 cells were transfected with 5 µg of the molecular clones and cultured in the presence of different dox concentrations (0-1000 ng/ml). Virus replication was monitored by CA-p24 ELISA on culture supernatant samples.

25 **Figure 7. HIV-rtTA replication induced by dox-like compounds.** SupT1

cells were transfected with 5 µg of the HIV-rtTA clones and cultured in the presence of 500 ng/ml Tc or Mc. Virus replication was monitored by CA-p24 ELISA on culture supernatant samples.

Figure 8. Evolution of HIV-rtTA can result in loss of dox-control. (A) Schematic of the HIV-rtTA genome. The inactivated Tat-TAR elements (crossed boxes) and the introduced rtTA-tetO elements are indicated. rtTA is a fusion protein of the *E. coli* Tet repressor (TetR) and the VP16 activation domain (AD) of herpes simplex virus. TetR contains a DNA-binding domain (DNA BD) (residues 1-44) and a regulatory core domain (residues 75-207) with a dimerization surface. (B) Flow-chart of the 24-well evolution experiment. Further details are provided in the text. (C) Gradual loss of dox-control in HIV-rtTA, HIV-rtTA 2ΔtetO (carrying the improved 2ΔtetO promoter configuration (Marzio et al. 2001; Marzio et al. 2002) and HIV-rtTA_{F86Y A209T} (carrying the LTR-2ΔtetO promoter and the improved rtTA_{F86Y A209T} gene (Das et al. 2004a). The HIV-rtTA_{G19F E37L} variant developed in this study does not escape from dox-control. Plotted is the number of dox-dependent cultures as a function of the culture time. Each experiment was started with 24 independent cultures. (D) Amino acid substitutions observed in HIV-rtTA cultures that lost dox-control. In all cases, the G19E substitution resulted from a GGA to GAA codon mutation and the E37K substitution from a GAG to AAG mutation.

Figure 9. Replication of evolved HIV-rtTA variants. Replication of the original HIV-rtTA virus, the virus from culture C6 or from culture C5 (both harvested after 50 days of culturing) was compared by infecting SupT1 T cells with equal amounts of virus (5 ng/ml CA-p24) in the absence or presence of dox (1 μg/ml). Sequence analysis revealed that the C6 virus carried the G19E and E156K mutations in the rtTA gene, and the C5 virus carried the E37K mutation (Fig. 1D).

Figure 10. Amino acid substitutions at rtTA position 19 or 37 confer the loss of dox-control. The G19E and E37K mutated rtTA sequences were cloned into the HIV-rtTA 2ΔtetO proviral genome (Marzio et al, 2001; Marzio et al, 2002). SupT1 cells were transfected with 2.5 μg of the molecular clones

and cultured in the presence of 0-1000 ng/ml dox. Virus replication was monitored by CA-p24 ELISA on culture supernatant samples.

Figure 11. Replication of HIV-rtTA variants with alternative amino acids at position 37. SupT1 cells were transfected with HIV-rtTA 2 Δ tetO proviral plasmids (2.5 μ g) carrying the wild-type (E) or an alternative amino acid (K, D, L, N, F, Q, R, S) at rtTA position 37, and cultured with or without 1 μ g/ml dox. All viruses, except for the E37K mutant, have the alternative G codon (GGU instead of GGA) at rtTA position 19, which does not affect viral replication (data not shown), and the F86Y and A209T mutations (Das et al. 2004a).

Figure 12. Transcriptional activity of rtTA variants with alternative amino acids at position 19 or 37. (A and B) rtTA activity was measured in HeLa X1/6 cells (Baron et al. 1997) that contain stably integrated copies of the CMV-7tetO firefly luciferase reporter construct (Gossen et al. 1992). Cells were transfected with the indicated rtTA expression plasmid (all rtTA variants contain the F86Y and A209T mutations that improve rtTA activity (Das et al. 2004a) or pBluescript as a negative control (-), and a plasmid constitutively expressing *Renilla* luciferase to correct for differences in transfection efficiency. Cells were cultured in the presence of different dox concentrations (0-1000 ng/ml). The ratio of the firefly and *Renilla* luciferase activities measured two days after transfection reflects rtTA activity. All values were related to the wild-type (37E in A, and 19G in B) rtTA activity at 1000 ng/ml dox, which was arbitrarily set at 100%. Average values of two transfections are plotted with the error bar indicating the standard deviation. (C and D) Codon tables of rtTA variants with all possible amino acids at position 19 or 37. The dox-dependent phenotype is marked in light grey, variants active in the absence of dox in dark grey, and inactive variants in black. See the text for details.

Figure 13. Activity of the novel rtTA variant with safety-lock mutations. (A) The activity of wild-type and safety-lock rtTA (G19F E37L) was measured in HeLa X1/6 cells, see Fig. 5 for details. Cells were cultured in the presence of different dox concentrations (0-1000 ng/ml). All values were related to the wild-type rtTA activity at 1000 ng/ml dox, which was arbitrarily set at 100%. Average values of two transfections are plotted with the error bar indicating the standard deviation. (B and C) Replication of HIV-rtTA_{F86Y A209T} and HIV-rtTA_{G19F E37L} (which also carries the F86Y and A209T mutations (Das et al. 2004a). SupT1 cells were transfected with 5 µg of the molecular clones and cultured with or without 1 µg/ml dox. Virus replication was monitored by CA-p24 ELISA on culture supernatant samples.

Figure 14 A+B. Transcriptional activity and dox-sensitivity of wild type, naturally evolved and constructed rtTA variants. Transfection assays were performed in HeLa X1/6 cells, see Fig. 2 for details. Transcriptional activity observed at 1000 ng/ml dox is shown as average value of three transfections with error bars indicating the standard deviation. The wild-type rtTA activity was set at 100%. Dox-sensitivity is compared with the wild-type rtTA of which the sensitivity is arbitrarily set at 1. For each rtTA variant, the dox concentration (ng/ml) that results in an activity comparable to that of the wild-type rtTA activity at 1000 ng/ml dox is indicated between brackets (Part of these results is also shown in figure 3).

Figure 14C. rtTA variants according to the present invention. Each column row depicts suitable rtTA variants.

Figure 15. Novel rtTA variants can be activated by dox-like compounds. The rtTA activity was measured in HeLa X1/6 cells, see Fig. 2 for details. Cells were cultured in the presence of different concentrations of Tc

or Mc (0-10000 ng/ml). The wild-type (wt) rtTA activity at 1000 ng/ml dox (not shown) was set at 100%.

Figure 16. TetR-based transactivators. (A and B) In the homodimeric
5 rtTA, each monomer contains an N-terminal *E. coli*-derived TetR domain and
a C-terminal herpes simplex virus VP16-derived activation domain. The V9I,
F67S, F86Y and G138D mutations that enhance rtTA activity are all located in
the TetR domain. sc rtTA is a single-chain version of rtTA. It contains two
10 TetR domains connected head to tail by a peptide linker and a single activation
domain at the C-terminal end.

**Figure 17. Mutations that enhance rtTA activity do not improve tTA
activity.** The transcriptional activity of tTA variants was measured in HeLa
X1/6 cells (Baron et al. 1997) containing chromosomally integrated copies of
15 the CMV-7tetO luciferase reporter construct. Cells were transfected with the
indicated tTA expression plasmids or pBluescript (-) as a negative control and
a plasmid constitutively expressing *Renilla* luciferase to correct for differences
in transfection efficiency. Cells were cultured in the presence of different dox
concentrations (0-20 ng/ml). The ratio of the firefly and *Renilla* luciferase
20 activities measured two days after transfection reflects the tTA activity. All
values were related to the original (wild-type) tTA activity in the absence of
dox, which was arbitrarily set at 100%. Average values of two transfections are
shown with the error bar indicating the standard deviation.

25 **Figure 18. Mutations observed in rtTA can improve sc rtTA activity.**
The transcriptional activity of rtTA and sc rtTA was measured in HeLa X1/6
cells, see Fig. 17 for details. Cells were cultured in the presence of different dox
concentrations (0-1000 ng/ml). All values were related to the original (wild-
type) sc rtTA activity at 1000 ng/ml dox, which was arbitrarily set at 100%.

Average values of two transfections are plotted with the error bar indicating the standard deviation.

Figure 19. Nucleotide and amino acid sequence of rtTA. Shown is the
 5 nucleotide sequence (upper line; SEQ ID NO:26) and amino acid sequence
 (lower line; SEQ ID NO:27) of the rtTA2^S-S2 variant (Urlinger et al, 2000).

Figure 20. Evolution of HIV-rtTA after transient dox administration.
 (A) Schematic of the HIV-rtTA genome. The inactivated Tat-TAR elements
 10 (crossed boxes) and the introduced rtTA-tetO elements are indicated. rtTA is a
 fusion protein of the *E. coli* Tet repressor (TetR) and the VP16 activation
 domain (AD) of herpes simplex virus. TetR contains a DNA-binding domain
 (DNA BD) (amino acids 1-44) and a regulatory core domain (amino acids 75-
 207) with a dimerization surface. (B-D) Loss of dox-control in cultures of HIV-
 15 rtTA after transient activation. SupT1 cells were transfected with HIV-rtTA
 and cultured at 100 ng/ml dox (B), HIV-rtTA_{V9I G138D} at 10 ng/ml dox (C), or
 HIV-rtTA_{G19F E37L} at 1000 ng/ml dox (D). Each experiment was started with 12
 independent cultures (different symbols represent different cultures). At day 3,
 dox was washed out and the cultures were continued with dox-free medium.
 20 The cultures in which the virus did not lose dox-control were split in two parts
 at day 64 (C) or day 66 (D) and dox was added to one of the samples. Virus
 production was monitored by CA-p24 ELISA on culture supernatant samples.

Figure 21. The P56S mutation causes a tTA-like phenotype. The activity
 25 of wild-type and P56S-mutated rtTA was measured in C33A cells transfected
 with a reporter plasmid carrying the firefly luciferase gene under the control of
 the viral LTR-2ΔtetO promoter (LTR-2ΔtetO; A) or under the control of a
 minimal CMV promoter coupled to an array of seven tetO elements (CMV-
 7tetO; B). Furthermore, rtTA activity was measured in HeLa X1/6 cells (Baron
 30 et al, 1997) that contain chromosomally integrated copies of the CMV-7tetO

luciferase construct (CMV-7tetO-integrated; C). Cells were transfected with the indicated rtTA expression plasmid (both rtTA variants carry the F86Y and A209T mutations (Das et al, 2004a) or pBluescript as a negative control (-), and a plasmid constitutively expressing *Renilla* luciferase to correct for
 5 differences in transfection efficiency. Cells were cultured with different dox concentrations (0-1000 ng/ml). The ratio of the firefly and *Renilla* luciferase activities measured two days after transfection reflects the rtTA activity. All values were related to the wild-type rtTA activity at 1000 ng/ml dox, which was arbitrarily set at 100%.

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Figure 22. Activity of rtTA_{G19F E37L} variants with all possible amino acids at position 56. (A) The activity of rtTA was measured in HeLa X1/6 cells, see Fig. 21 for details. All variants carry the G19F, E37L, F86Y and A209T mutations in combination with different amino acids at position 56. The
 15 wild-type rtTA (wt) carrying only the F86Y and A209T mutations was included as a control, of which the activity at 1000 ng/ml dox was arbitrarily set at 100%. Average values of two transfections are shown with the error bar indicating the standard deviation. (B) Codon table of rtTA_{G19F E37L} variants with all possible amino acids at position 56. The corresponding codons of
 20 inactive variants are marked in black, of dox-dependent variants in light grey, and of variants that are active without dox in dark grey. See the text for details.

Figure 23. Replication of HIV-rtTA_{G19F E37L} variants with different amino acids at position 56. SupT1 cells were transfected with 5 µg of HIV-rtTA molecular clones encoding different rtTA alleles, and cultured with or
 25 without 1 µg/ml dox. All rtTA variants contain the F86Y and A209T mutations. Virus replication was monitored by CA-p24 ELISA on culture supernatant samples.

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Figure 24. Blocking the loss of dox-control by triple safety-lock mutations. SupT1 cells were transfected with HIV-rtTA containing triple safety-lock mutations (HIV-rtTA_{G19F E37L P56K}) at 1000 ng/ml dox and split into 24 independent cultures (different symbols represent different cultures). At
5 day 3, dox was washed out and the cultures were continued with dox-free medium. At day 60, all cultures were split in two parts and dox (1000 ng/ml) was added to one of the samples. Virus production was monitored by CA-p24 ELISA on culture supernatant samples.

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Claims

1. A method for inducibly expressing a nucleic acid sequence of interest, comprising:
 - providing a nucleic acid construct comprising said nucleic acid sequence of interest operably linked to an inducible gene expression system which comprises a nucleic acid sequence encoding a reverse tetracycline transactivator (rtTA) and/or a single chain rtTA, wherein said rtTA and/or single chain rtTA comprises an amino acid sequence comprising an amino acid substitution at position 67 in the amino acid sequence of SEQ ID NO:27;
 - introducing said nucleic acid construct to a suitable expression system; and
 - allowing for inducible expression of said nucleic acid sequence of interest.
2. The method according to claim 1, wherein said amino acid sequence further comprises an amino acid substitution at one or more of rtTA amino acid positions 9, 12, 37, 68, 86, 138, 157, 171, 177, 195 and 209 in the amino acid sequence of SEQ ID NO:27.
3. The method according to claim 2, wherein said rtTA and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon.
4. The method according to claim 3, wherein said rtTA and/or single chain rtTA encoding nucleic acid sequence comprises a histidine, a leucine or an arginine codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon.
5. The method according to any one of claims 2-4, wherein said rtTA and/or single chain rtTA encoding nucleic acid sequence comprises (i) a codon at rtTA amino acid position 9 encoding isoleucine, (ii) a codon at rtTA amino acid position 37 encoding cysteine, methionine, glutamine, threonine, histidine, leucine or arginine, (iii) a codon at rtTA amino acid position 67 encoding serine, (iv) a codon at rtTA amino acid position 68 encoding arginine, (v) a codon at rtTA amino acid position 86 encoding tyrosine, (vi) a codon at rtTA amino acid position 138 encoding aspartic acid or serine, (vii) a codon at rtTA amino acid position 157 encoding lysine, (viii) a codon at rtTA amino acid position 171 encoding lysine, (ix) a codon at rtTA amino acid position 177 encoding leucine, (x) a

codon at rtTA amino acid position 195 encoding serine, (xi) a codon at rtTA amino acid position 209 encoding threonine, or (xii) any combination of (i) to (xi).

6. The method according to claim 5, wherein (i) said codon at rtTA amino acid position 138 encodes aspartic acid, (ii) said codon at rtTA amino acid position 37 encodes glutamine, or (iii) a combination of (i) and (ii).

7. The method according to any one of claims 1-6, wherein said nucleic acid of interest is expressed in a higher eukaryotic expression system.

8. The method according to claim 7, wherein said nucleic acid of interest is expressed in a mammalian cell.

9. The method according to any one of claims 1-8, wherein said nucleic acid of interest comprises a viral sequence essential for replication.

10. The method according to any one of claims 1-9, wherein said nucleic acid of interest comprises at least part of a HIV genome essential for replication.

11. An isolated, synthetic or recombinant nucleic acid comprising a nucleic acid sequence encoding an rtTA and/or a single chain rtTA, wherein said rtTA and/or single chain rtTA comprises an amino acid sequence comprising an amino acid substitution at position 67 in the amino acid sequence of SEQ ID NO:27.

12. The isolated, synthetic or recombinant nucleic acid according to claim 11, wherein said amino acid sequence further comprises an amino acid substitution at one or more of rtTA amino acid positions 9, 12, 37, 68, 86, 138, 157, 171, 177, 195 and 209 in the amino acid sequence of SEQ ID NO:27.

13. The isolated, synthetic or recombinant nucleic acid according to claim 12, wherein said rtTA and/or single chain rtTA encoding nucleic acid sequence comprises a codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon.

14. The isolated, synthetic or recombinant nucleic acid according to claim 13, wherein said rtTA and/or single chain rtTA encoding nucleic acid sequence comprises a histidine, a leucine or an arginine codon at rtTA amino acid position 37 which differs in at least two nucleotides from an alanine, a lysine or a serine codon.

15. The isolated, synthetic or recombinant nucleic acid according to claim 12, wherein said rtTA and/or single chain rtTA encoding nucleic acid sequence comprises (i) a codon at rtTA amino acid position 9 encoding isoleucine, (ii) a codon at rtTA amino acid position 37 encoding cysteine, methionine, glutamine, threonine, histidine, leucine or arginine, (iii) a codon at rtTA amino acid position 67 encoding serine, (iv) a codon at rtTA amino acid position 68 encoding arginine, (v) a codon at rtTA amino acid position 86 encoding tyrosine, (vi) a codon at rtTA amino acid position 138 encoding aspartic acid or serine, (vii) a codon at rtTA amino acid position 157 encoding lysine, (viii) a codon at rtTA amino acid position 171 encoding lysine, (ix) a codon at rtTA amino acid position 177 encoding leucine, (x) a codon at rtTA amino acid position 195 encoding serine, (xi) a codon at rtTA amino acid position 209 encoding threonine, or (xii) any combination of (i) to (xi).

16. The isolated, synthetic or recombinant nucleic acid sequence according to claim 15, wherein (i) said codon at rtTA amino acid position 138 encodes aspartic acid, (ii) said codon at rtTA amino acid position 37 encodes glutamine, or (iii) a combination of (i) and (ii).

17. An isolated, synthetic or recombinant polypeptide encoded by the nucleic acid according to any one of claims 11-16.

18. Use of the isolated, synthetic or recombinant nucleic acid according to any one of claims 11-16 for inducible expression of a nucleic acid sequence of interest.

19. Use of a polypeptide encoded by the nucleic acid sequence according to any one of claims 11-16 or the polypeptide according to claim 17 for inducible expression of a nucleic acid sequence of interest.

20. Use of an isolated or recombinant nucleic acid comprising a nucleic acid sequence encoding an rtTA and/or a single chain rtTA for tetracycline-inducible and/or minocycline-inducible expression of a nucleic acid of interest, wherein said rtTA and/or single chain rtTA comprises:

(i) an amino acid sequence comprising an amino acid substitution at position 67 in the amino acid sequence of SEQ ID NO:27; or

(ii) an amino acid sequence comprising one of the following combination of substitutions: (a) F67S, F86Y and A209T; (b) F67S, R171K, F86Y and A209T; (c) F67S, V9I, G138D, F86Y and A209T; (d) F67S, V9I, E157K, F86Y and A209T; (e) F67S, V9I, R171K, F86Y and A209T; (f) F67S, V9I, F86Y and A209T; (g) F67S, S12G, F86Y and A209T; (h) F67S, E37Q, F86Y and A209T; (i) F67S, S12G, V9I, G138D, F86Y and A209T; or (j) F67S, C68R, V9I, G138D, F86Y and A209T, in the amino acid sequence of SEQ ID NO:27.

21. The use of claim 20, wherein said amino acid sequence comprises the following combination of substitutions: F67S, R171K, F86Y and A209T.

22. A vector comprising the nucleic acid sequence defined in any one of claims 11-16.

23. An isolated cell comprising the nucleic acid according to any one of claims 11-16.

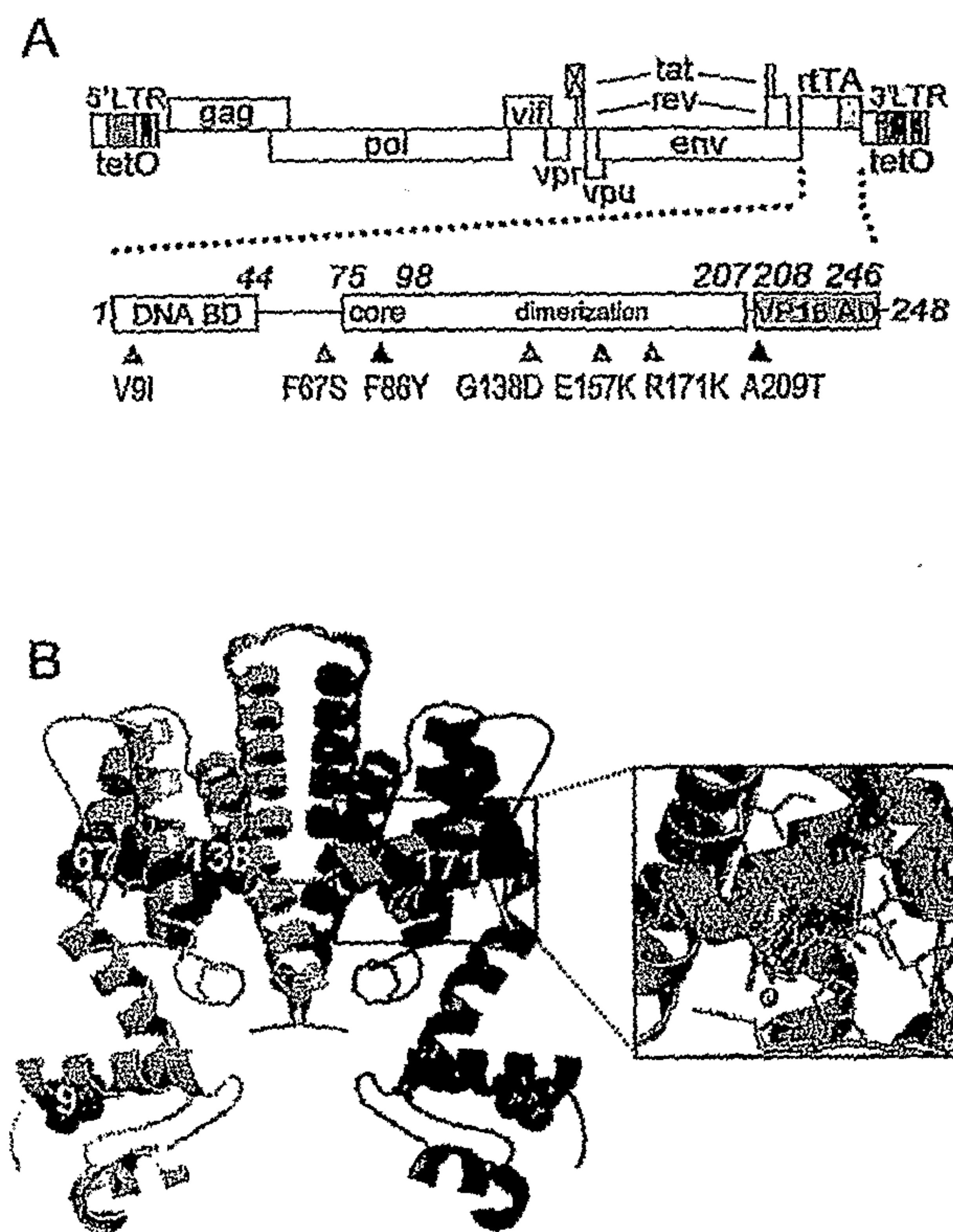


Figure 1

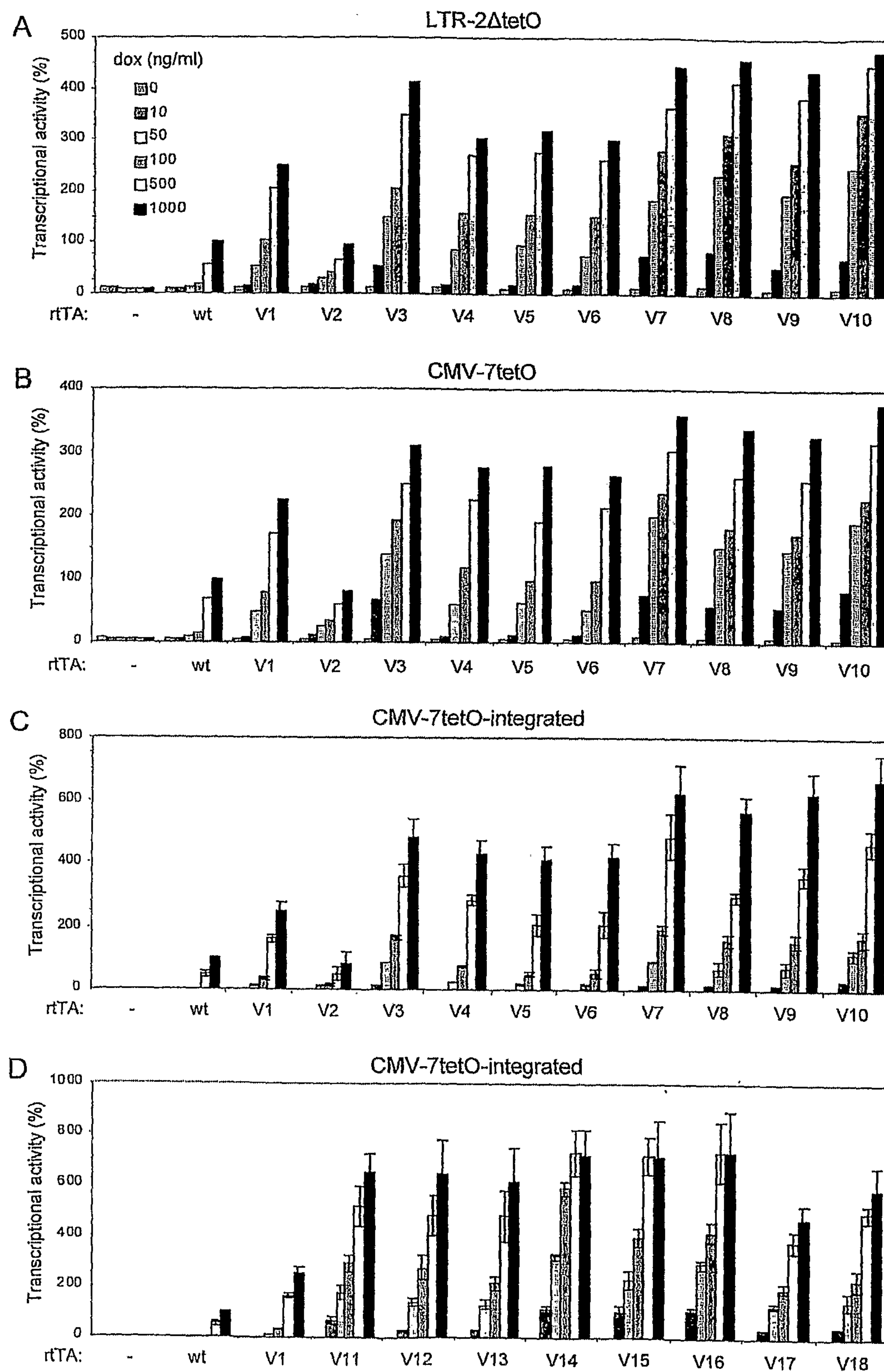


Figure 2

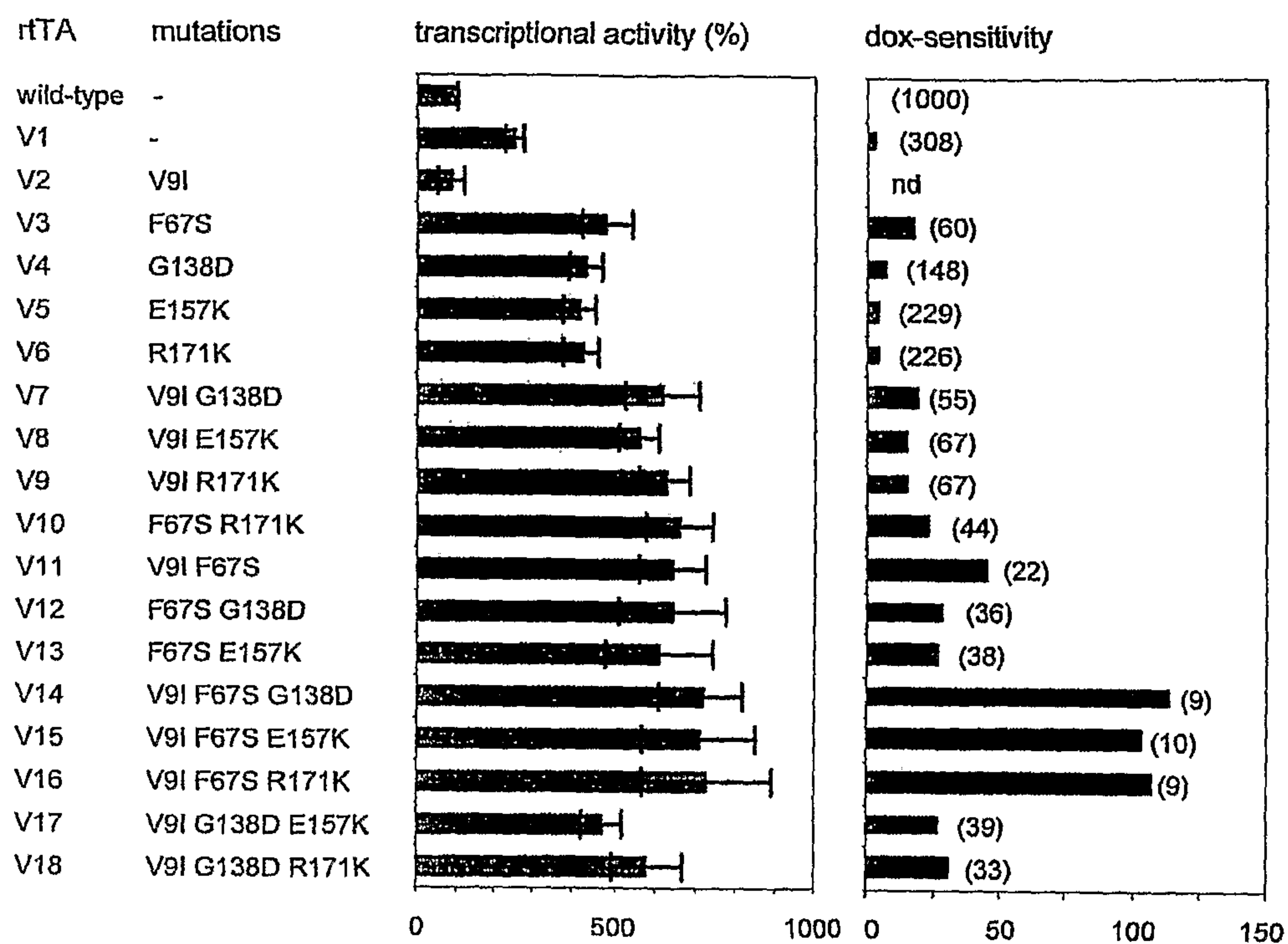


Figure 3

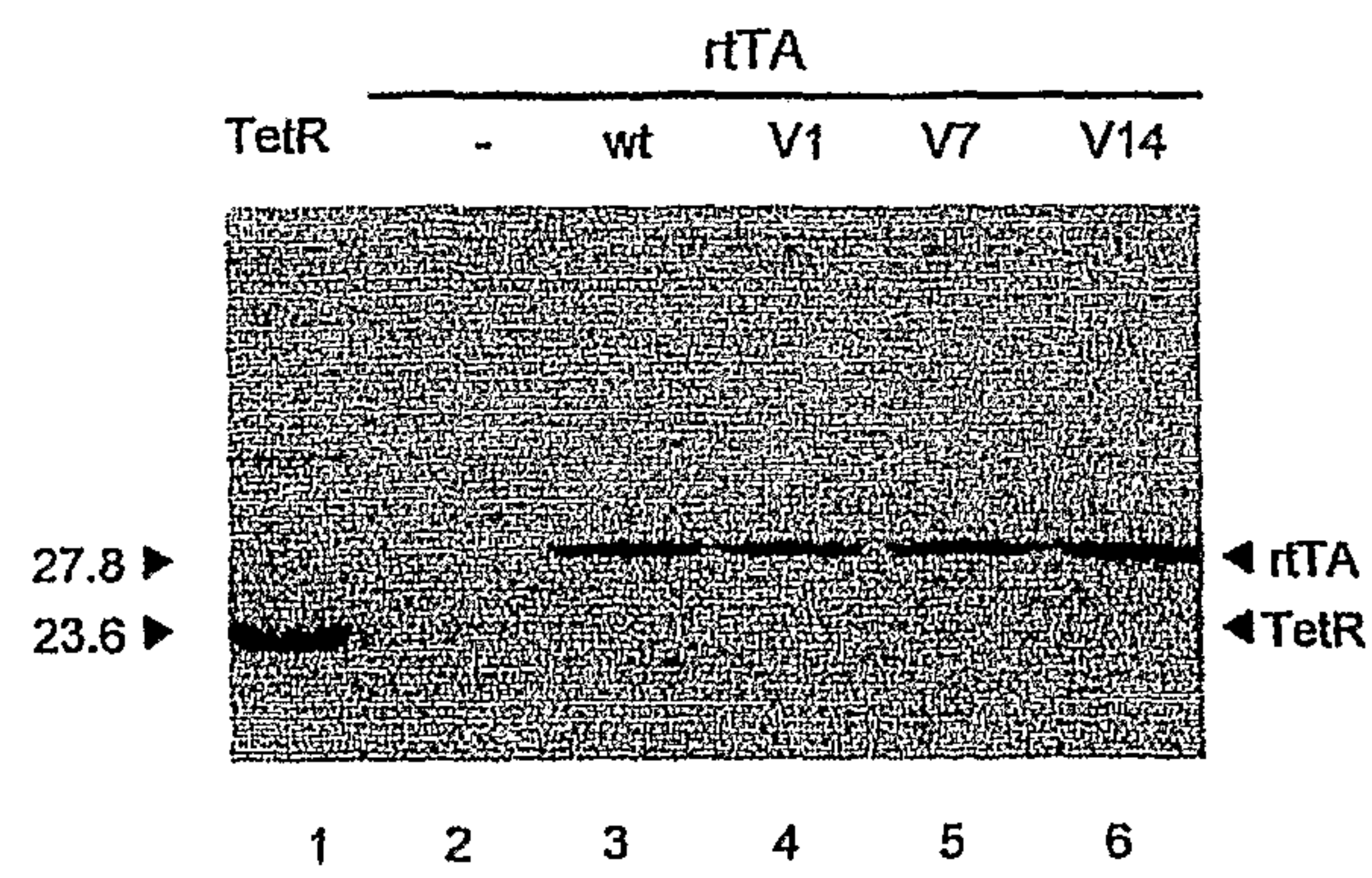


Figure 4

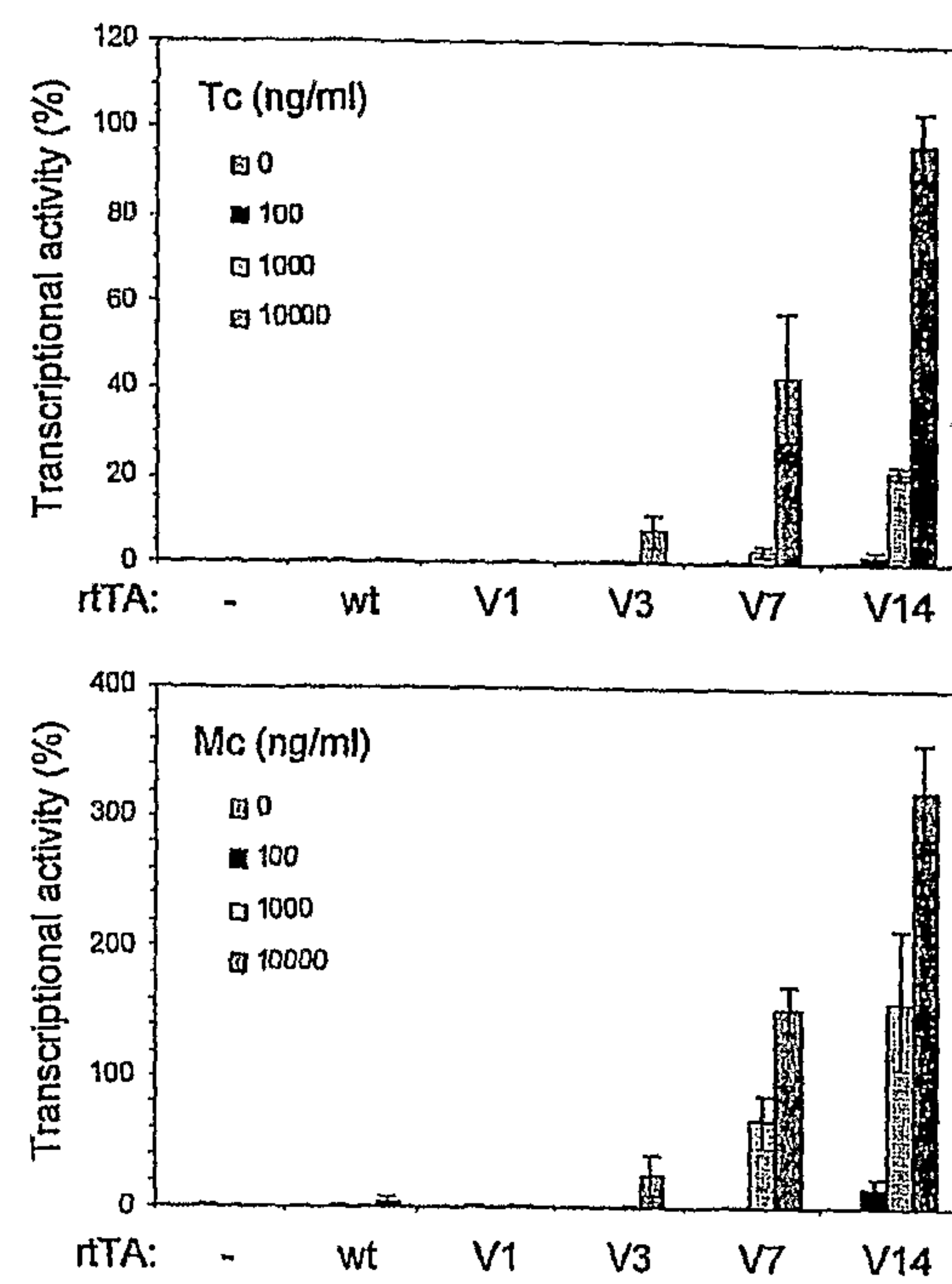


Figure 5

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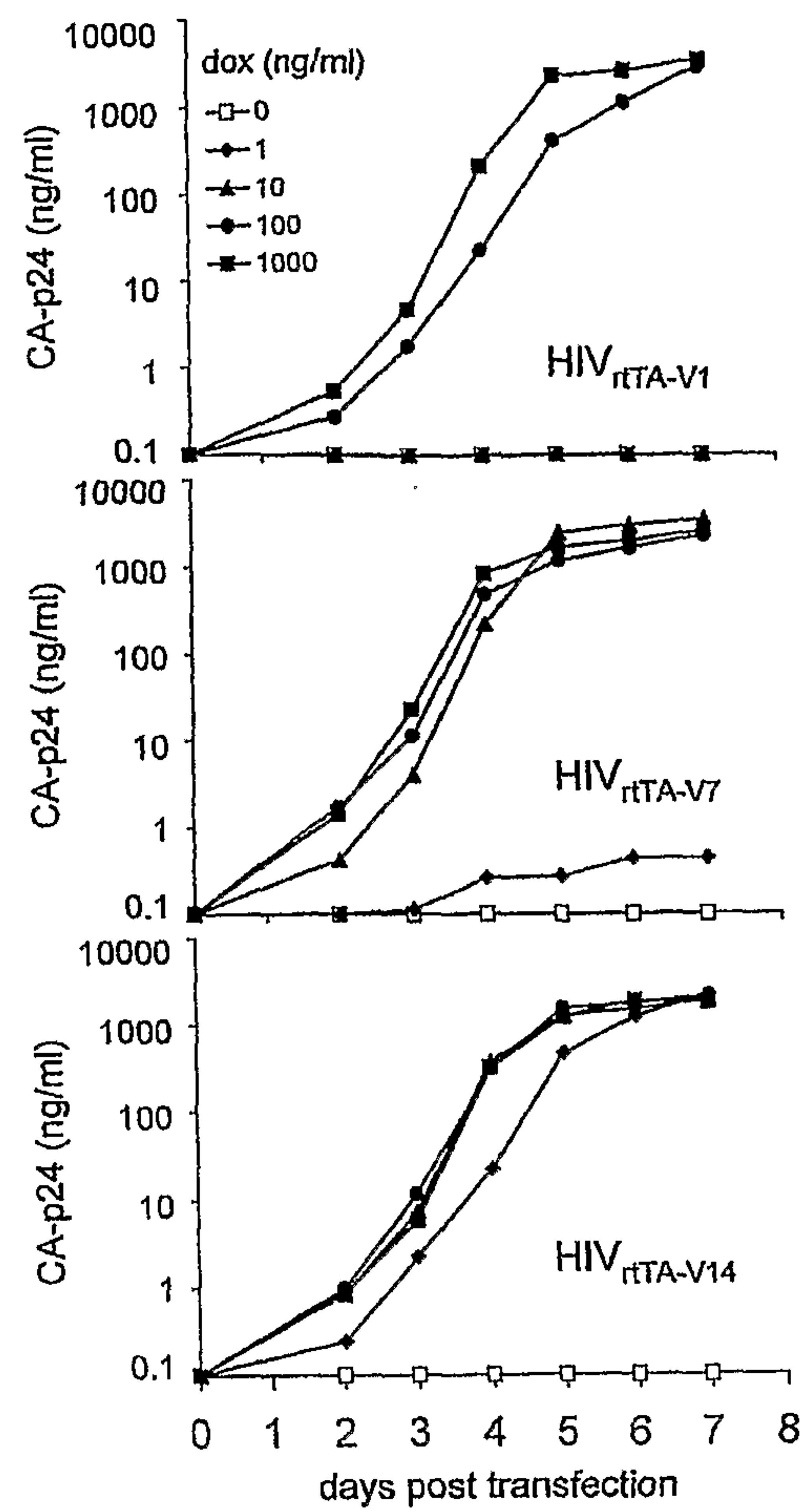


Figure 6

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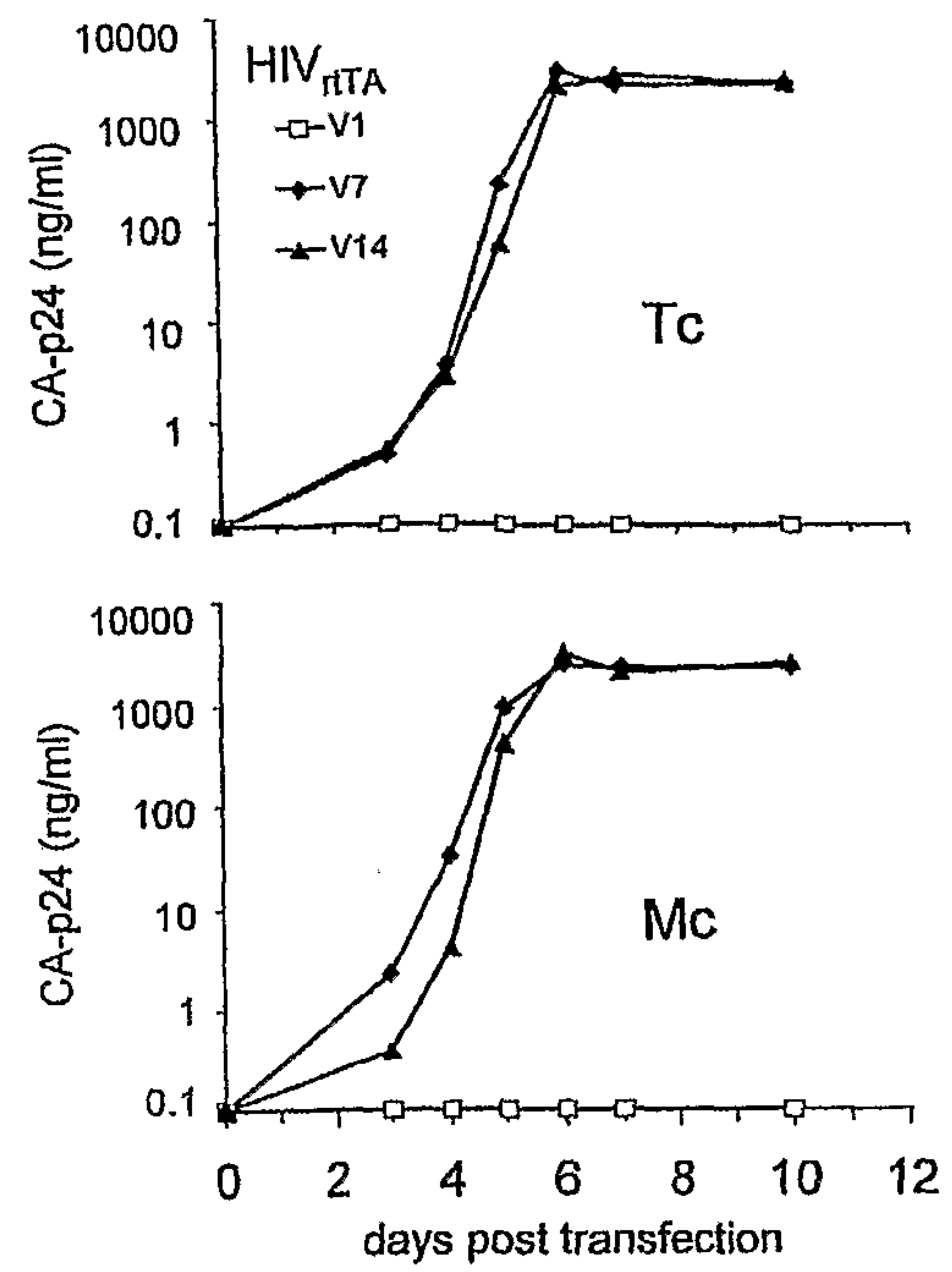


Figure 7

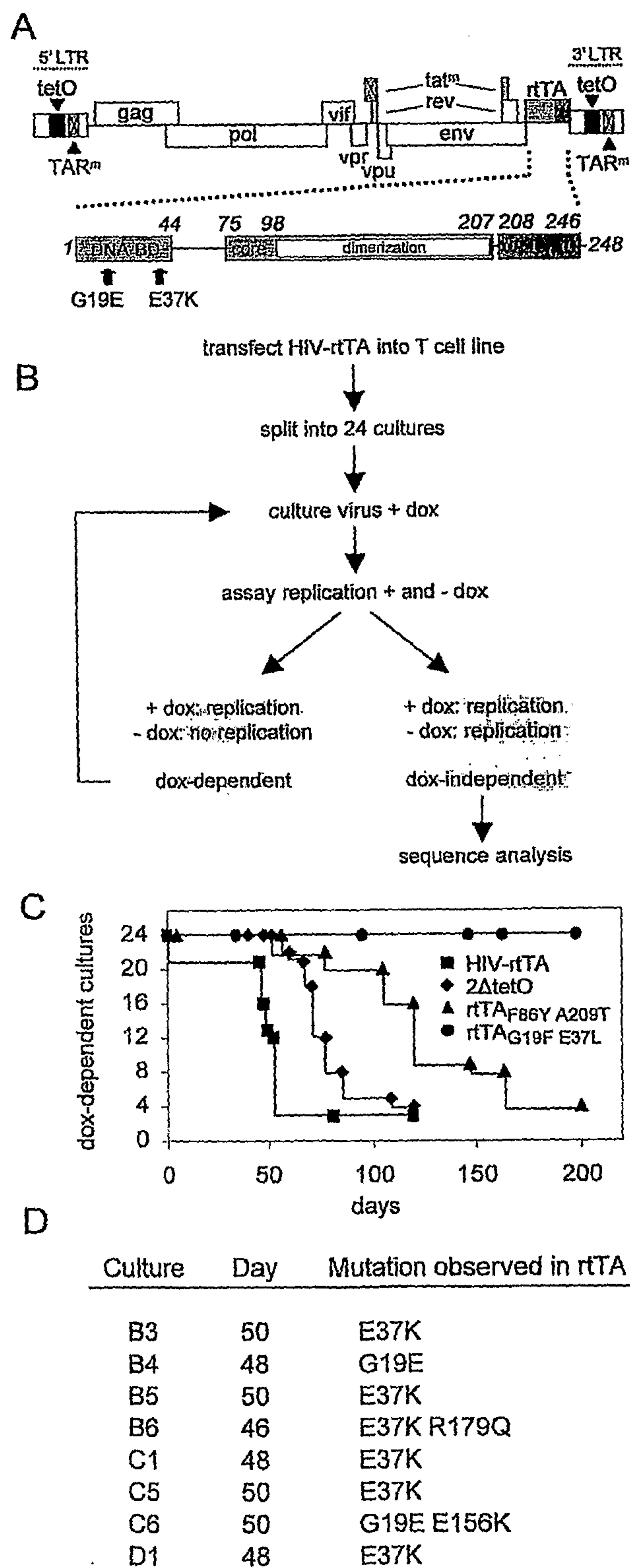


Figure 8

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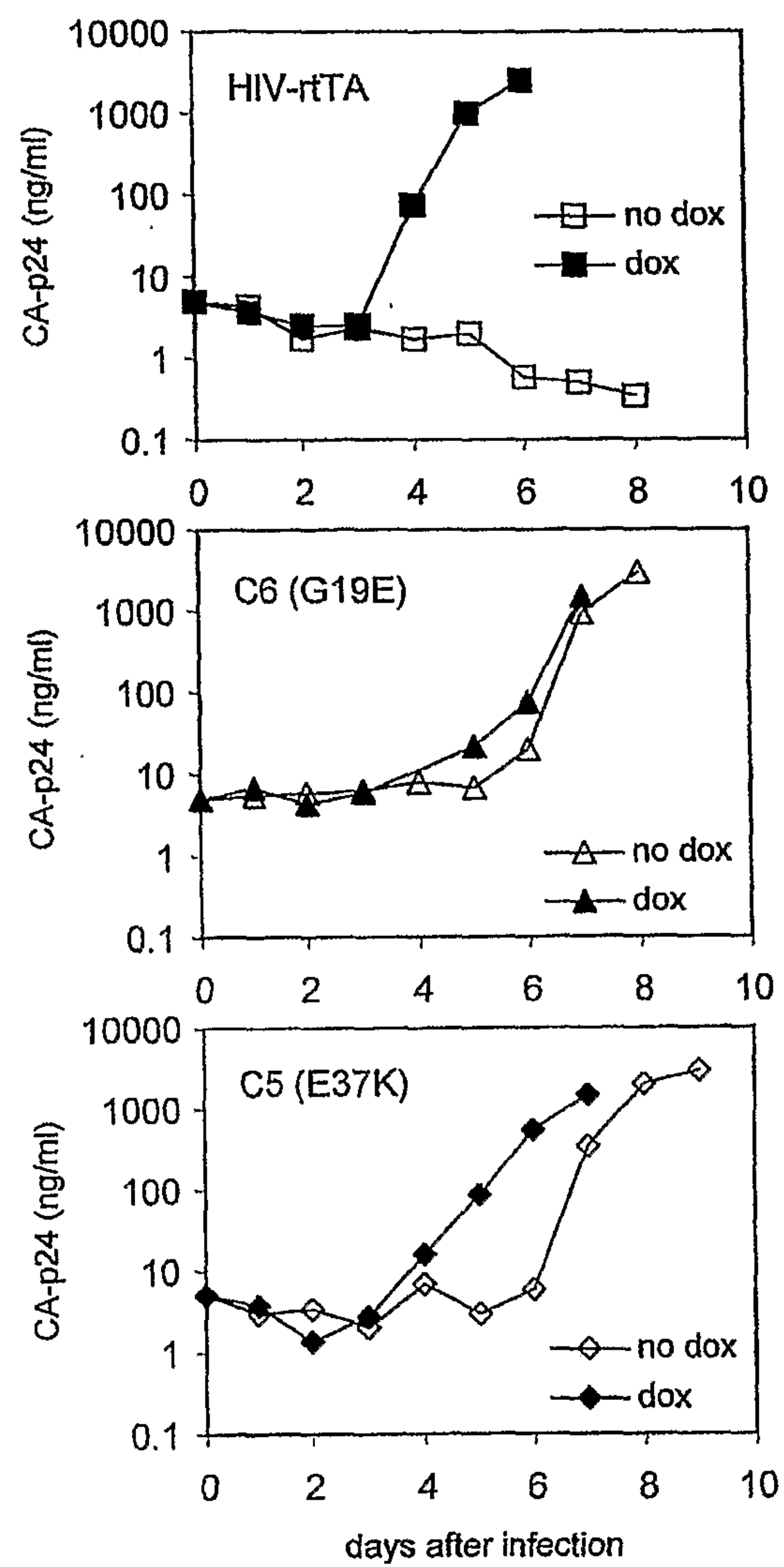


Figure 9

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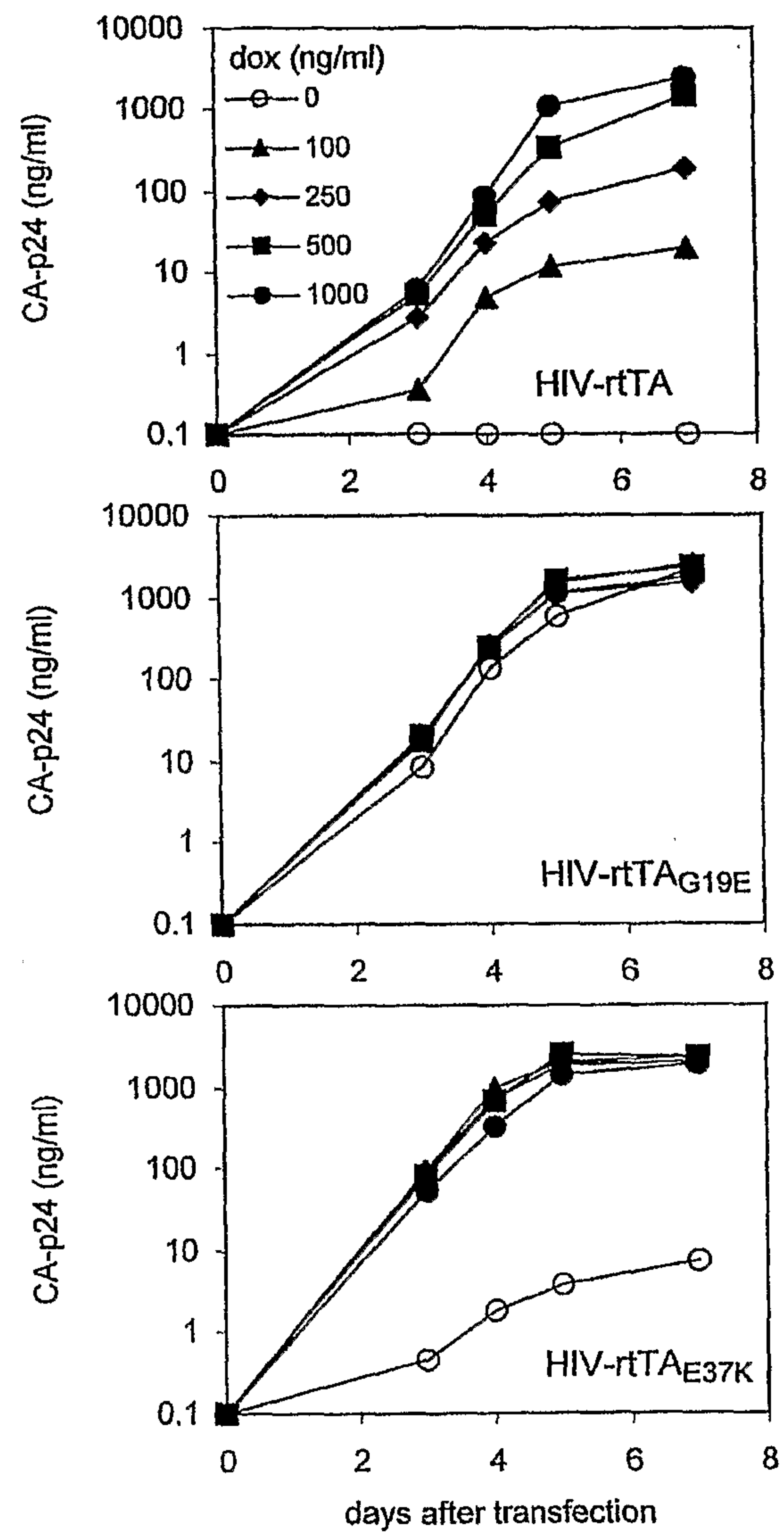


Figure 10

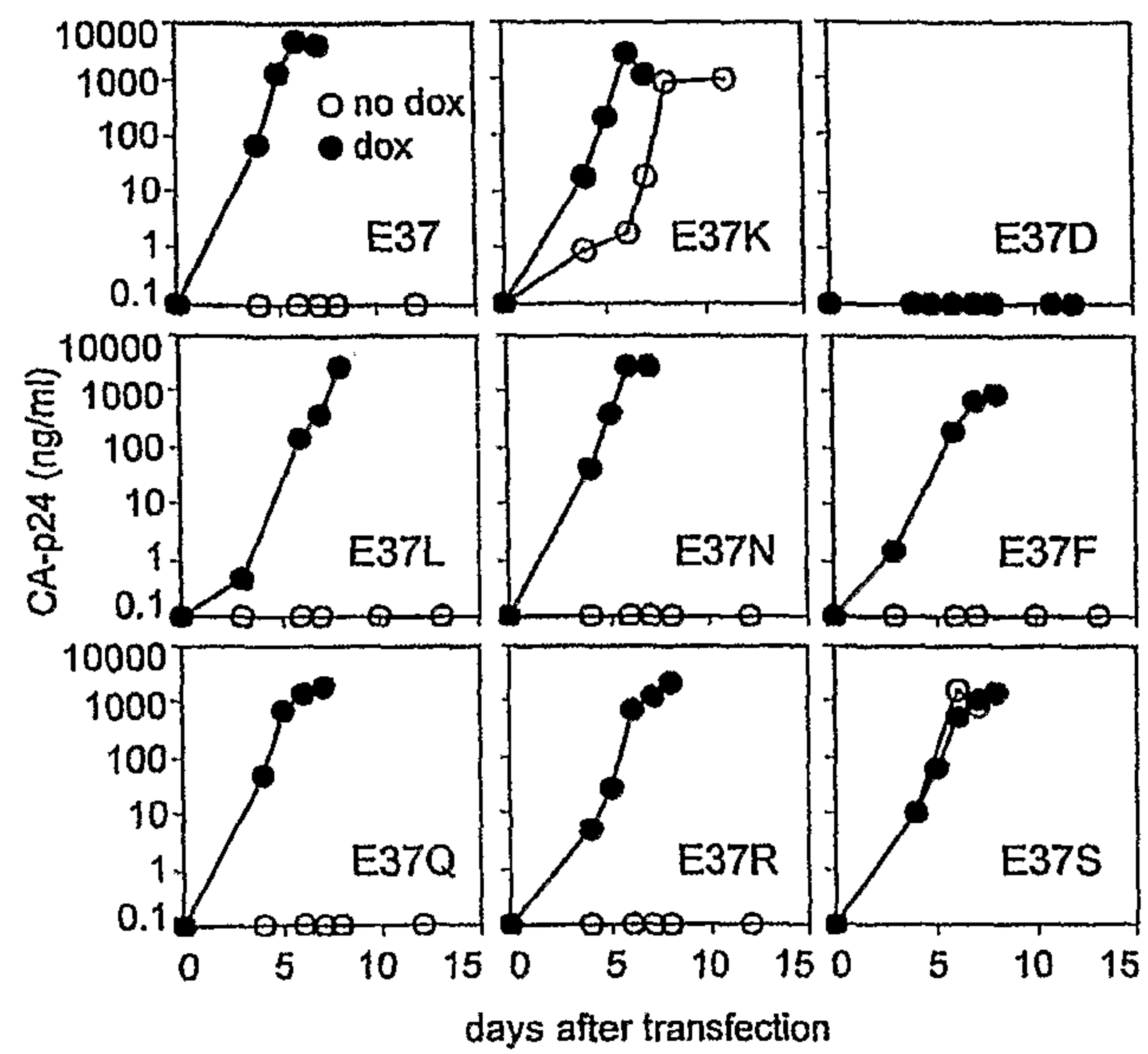


Figure 11

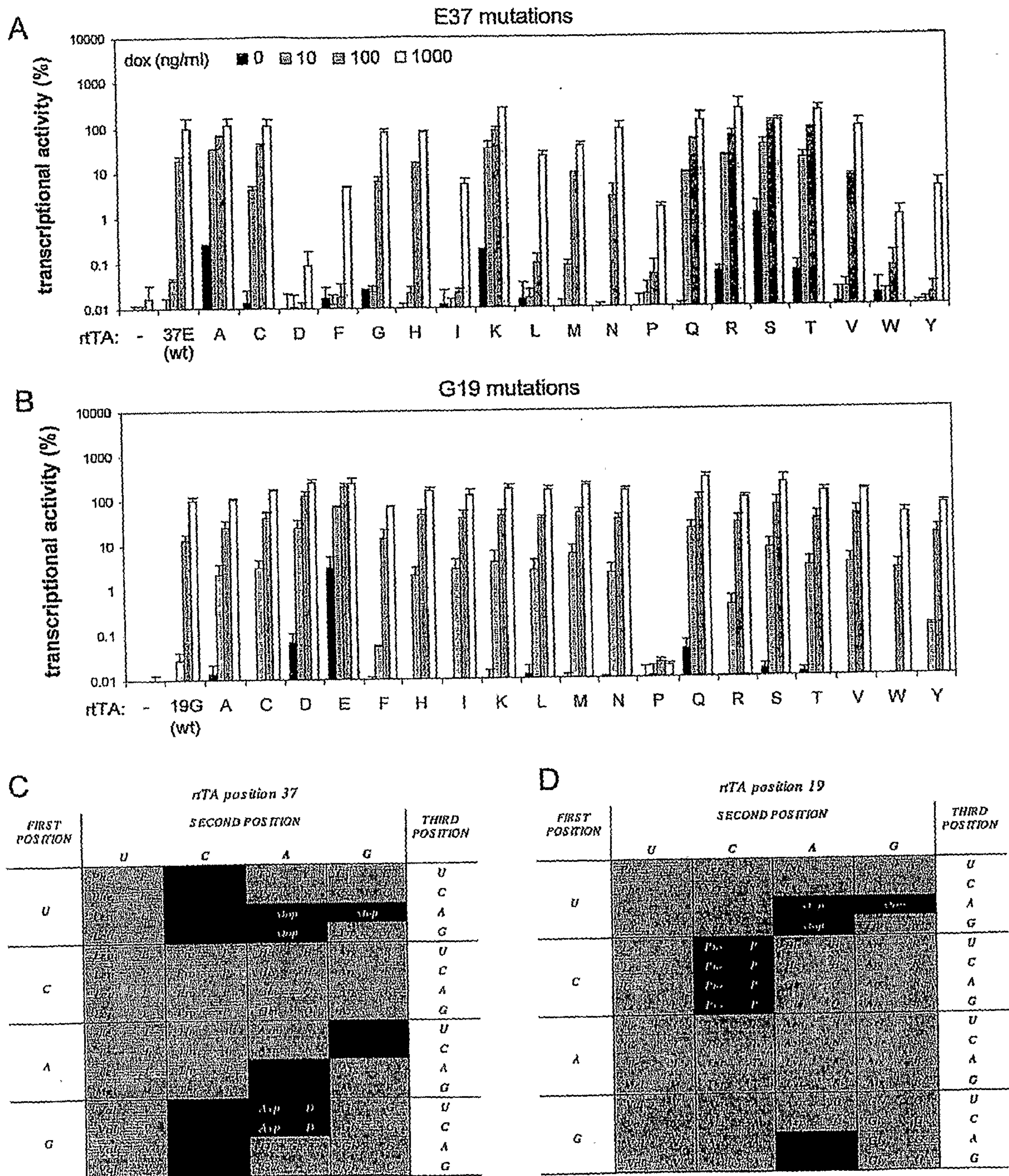


Figure 12

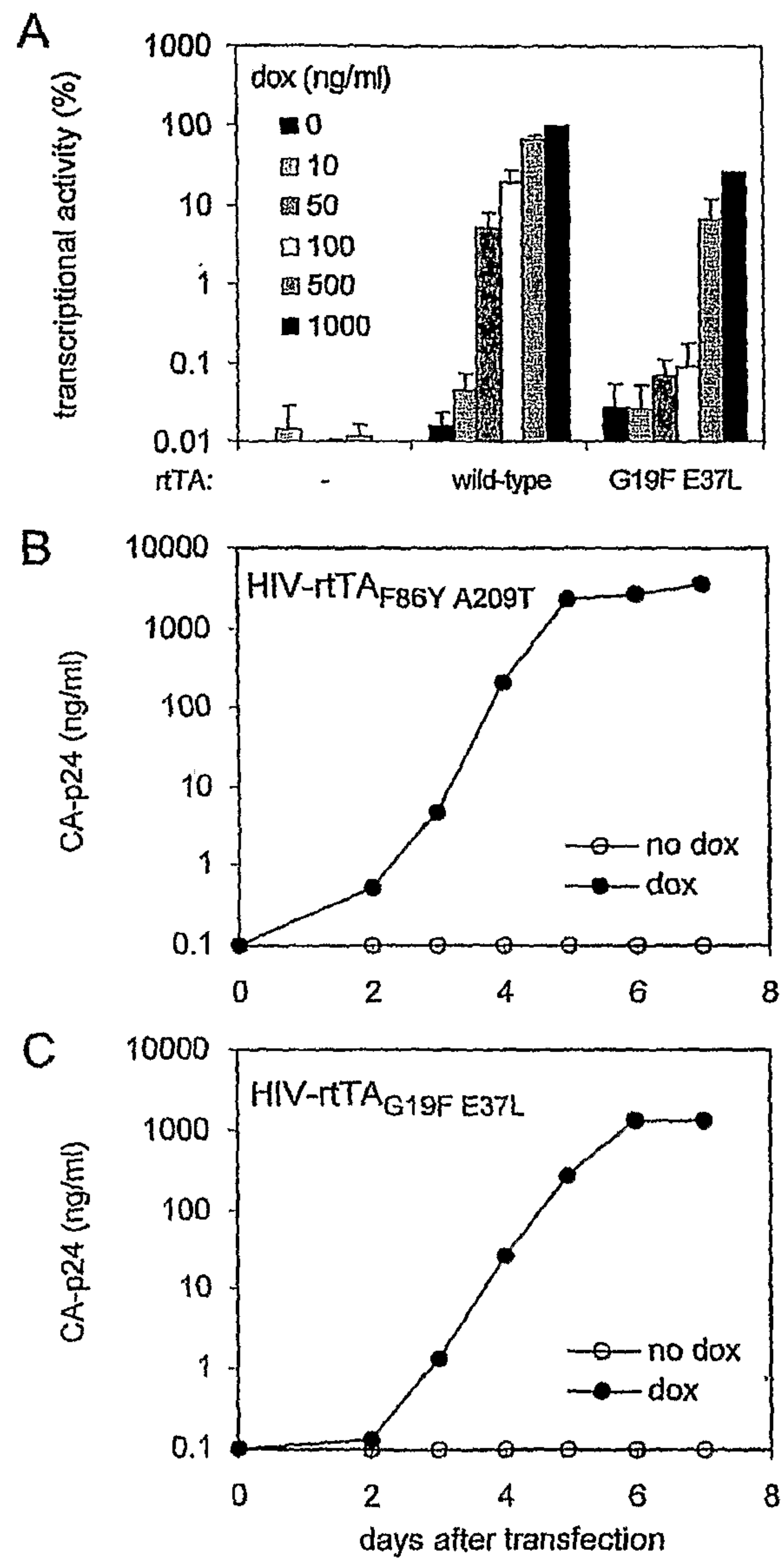


Figure 13

A

rtTA		basal activity	max activity	fold sensitivity	dox(ng/ml)
wt		0	100	1	1000
F86Y		0.1	253	3.2	314.3
F86Y A209T		0	250	3.2	308.2

B

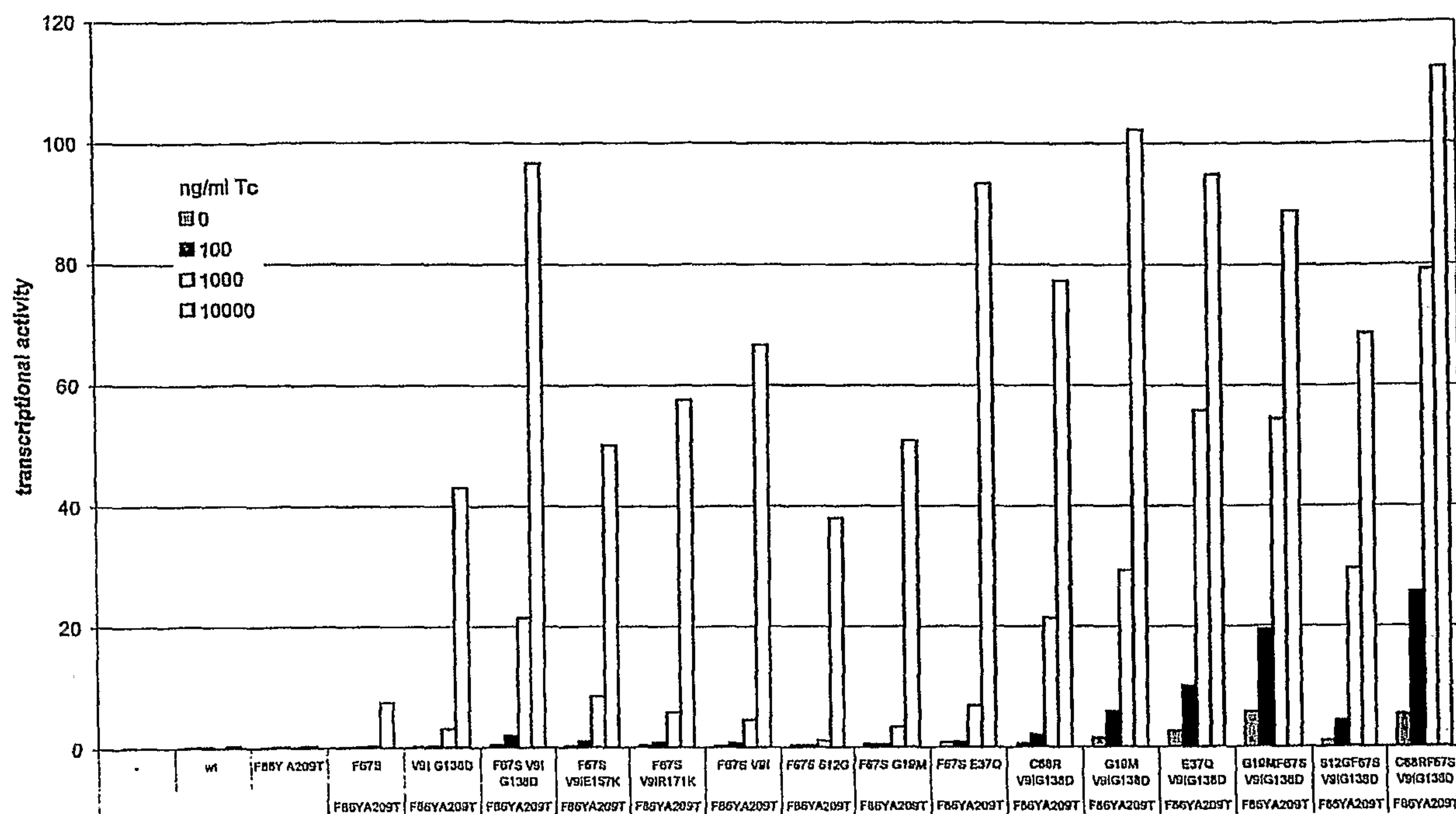
F67S	+ F86Y A209T	0	482	16.7	59.8
G138D	+ F86Y A209T	0	429	6.8	147.5
E157K	+ F86Y A209T	0	414	4.4	228.5
R171K	+ F86Y A209T	0	421	4.4	225.9
V9I G138D	+ F86Y A209T	0	621	18.2	54.9
V9I E157K	+ F86Y A209T	0	560	14.9	67.3
V9I R171K	+ F86Y A209T	0	624	14.9	67.3
F177L	+ F86Y A209T	0	358	4	249
F67S F177L	+ F86Y A209T	0	485	17.5	57.2
C195S	+ F86Y A209T	0	242	3.2	314.6
G138S	+ F86Y A209T	0	39	4.9	206.1
C68R	+ F86Y A209T	0	379	5.2	193
V9I F67S	+ F86Y A209T	0.3	646	44.6	22.4
F67S G138D	+ F86Y A209T	0	644	28	35.7
F67S E157K	+ F86Y A209T	0	615	26.1	38.4
F67S R171K	+ F86Y A209T	0	663	22.7	44
V9I F67S G138D	+ F86Y A209T	0.7	715	113.9	8.8
V9I F67S E157K	+ F86Y A209T	0.3	711	102.8	9.7
V9I F67S R171K	+ F86Y A209T	0.1	730	107	9.3
V9I G138D E157K	+ F86Y A209T	0.5	471	26	38.5
V9I G138D R171K	+ F86Y A209T	0.2	582	30	33.4
S12G F67S	+ F86Y A209T	0	641	45.5	22
G19M F67S	+ F86Y A209T	0	758	56.2	17.8
E37Q F67S	+ F86Y A209T	0.3	647	112.4	8.9
C68R G138D	+ F86Y A209T	0.3	626	23.6	42.3
G19M G138D	+ F86Y A209T	0.4	744	34.2	29.2
E37Q G138D	+ F86Y A209T	0.7	635	34.2	29.2
V9I C68R G138D	+ F86Y A209T	0.5	525	106.4	9.4
V9I G19M G138D	+ F86Y A209T	1.1	751	128.2	7.8
V9I E37Q G138D	+ F86Y A209T	2.7	689	135.1	7.4

Figure 14 A and B

F67S	F67S F86Y	F67S A209T
G138D	G138D F86Y	G138D A209T
E157K	E157K F86Y	E157K A209T
R171K	R171K F86Y	R171K A209T
V9I G138D	V9I G138D F86Y	V9I G138D A209T
V9I E157K	V9I E157K F86Y	V9I E157K A209T
V9I R171K	V9I R171K F86Y	V9I R171K A209T
F177L	F177L F86Y	F177L A209T
F67S F177L	F67S F177L F86Y	F67S F177L A209T
C195S	C195S F86Y	C195S A209T
G138S	G138S F86Y	G138S A209T
C68R	C68R F86Y	C68R A209T
V9I F67S	V9I F67S F86Y	V9I F67S A209T
F67S G138D	F67S G138D F86Y	F67S G138D A209T
F67S E157K	F67S E157K F86Y	F67S E157K A209T
F67S R171K	F67S R171K F86Y	F67S R171K A209T
V9I F67S G138D	V9I F67S G138D F86Y	V9I F67S G138D A209T
V9I F67S E157K	V9I F67S E157K F86Y	V9I F67S E157K A209T
V9I F67S R171K	V9I F67S R171K F86Y	V9I F67S R171K A209T
V9I G138D E157K	V9I G138D E157K F86Y	V9I G138D E157K A209T
V9I G138D R171K	V9I G138D R171K F86Y	V9I G138D R171K A209T
S12G F67S	S12G F67S F86Y	S12G F67S A209T
G19M F67S	G19M F67S F86Y	G19M F67S A209T
E37Q F67S	E37Q F67S F86Y	E37Q F67S A209T
C68R G138D	C68R G138D F86Y	C68R G138D A209T
G19M G138D	G19M G138D F86Y	G19M G138D A209T
E37Q G138D	E37Q G138D F86Y	E37Q G138D A209T
V9I C68R G138D	V9I C68R G138D F86Y	V9I C68R G138D A209T
V9I G19M G138D	V9I G19M G138D F86Y	V9I G19M G138D A209T
V9I E37Q G138D	V9I E37Q G138D F86Y	V9I E37Q G138D A209T
V9I G19M F67S G138D	V9I G19M F67S G138D F86Y	V9I G19M F67S G138D A209T
V9I S12G F67S G138D	V9I S12G F67S G138D F86Y	V9I S12G F67S G138D A209T
V9I F67S C68R G138D	V9I F67S C68R G138D F86Y	V9I F67S C68R G138D A209T

Figure 14 C

Mutations improve Tc response



Mutations improve Mc response

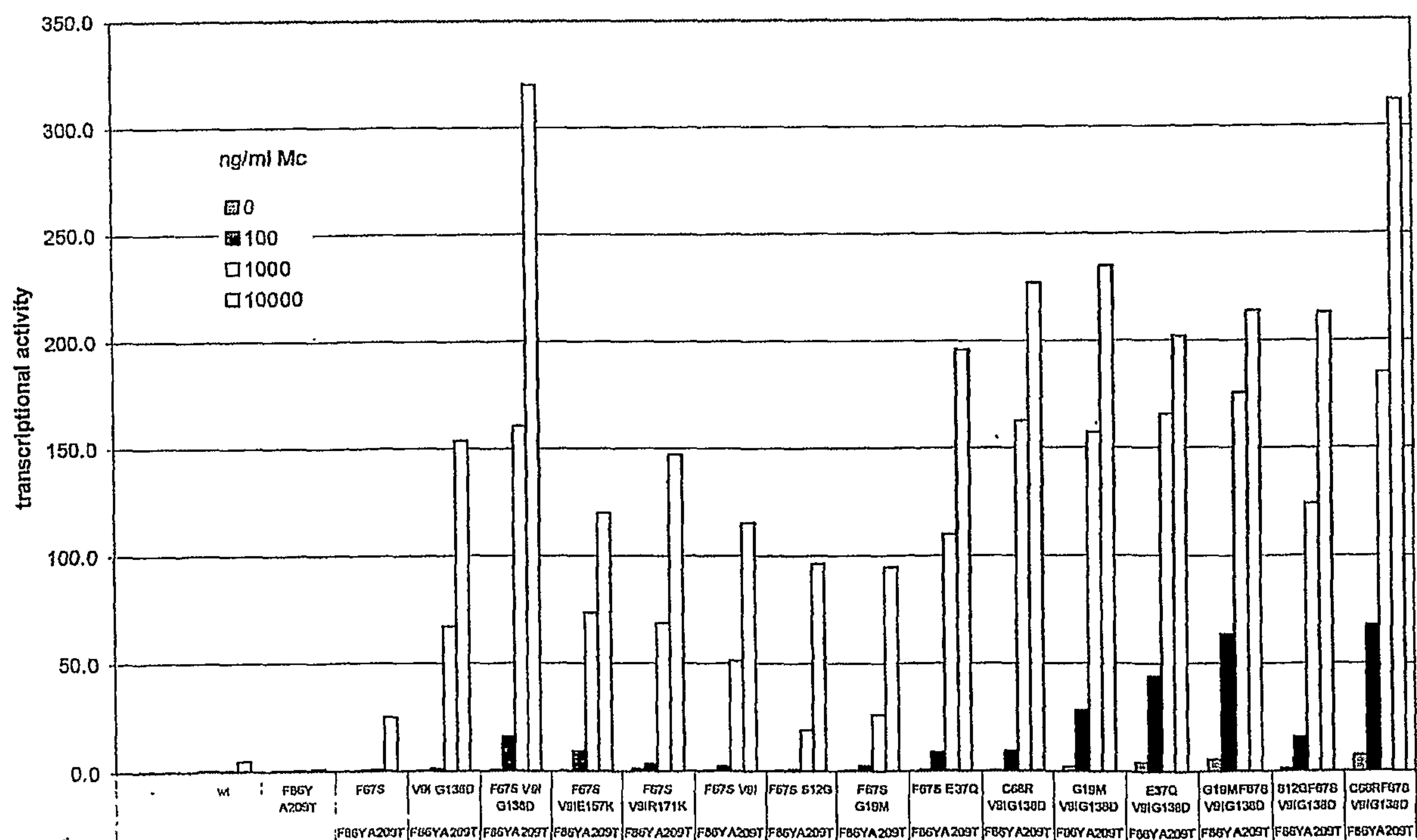
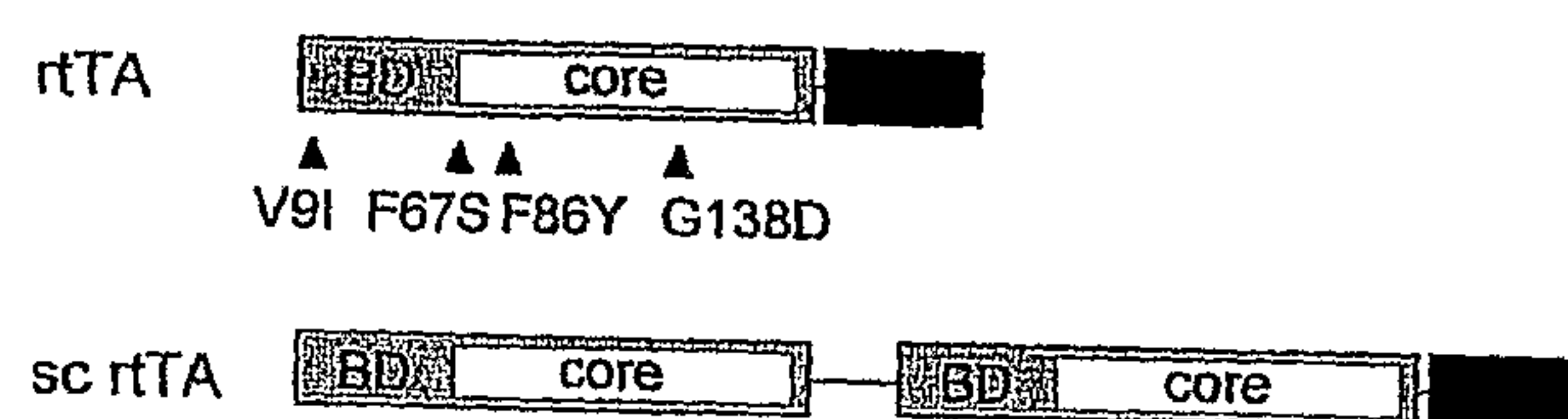


Figure 15

A



B

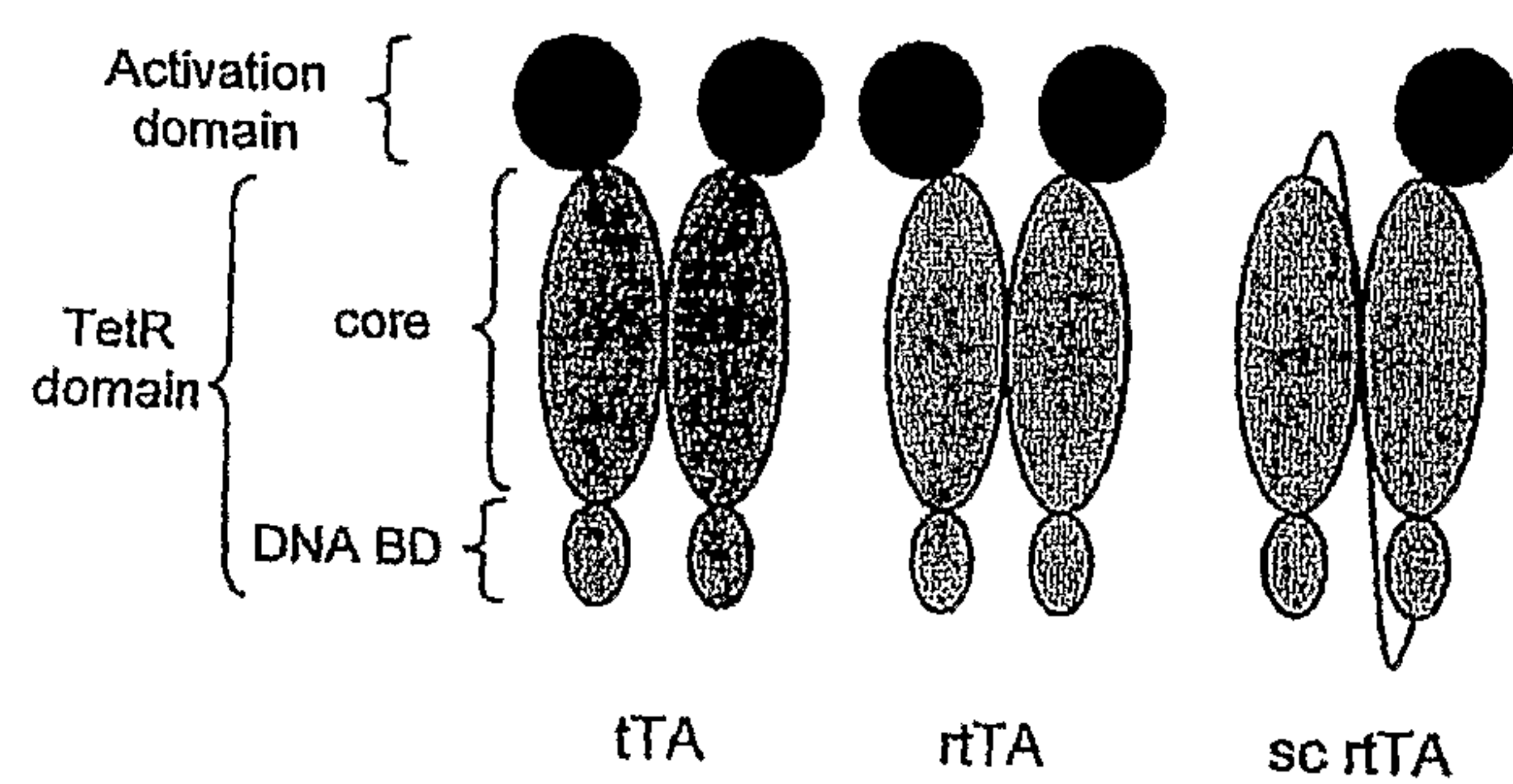


Figure 16

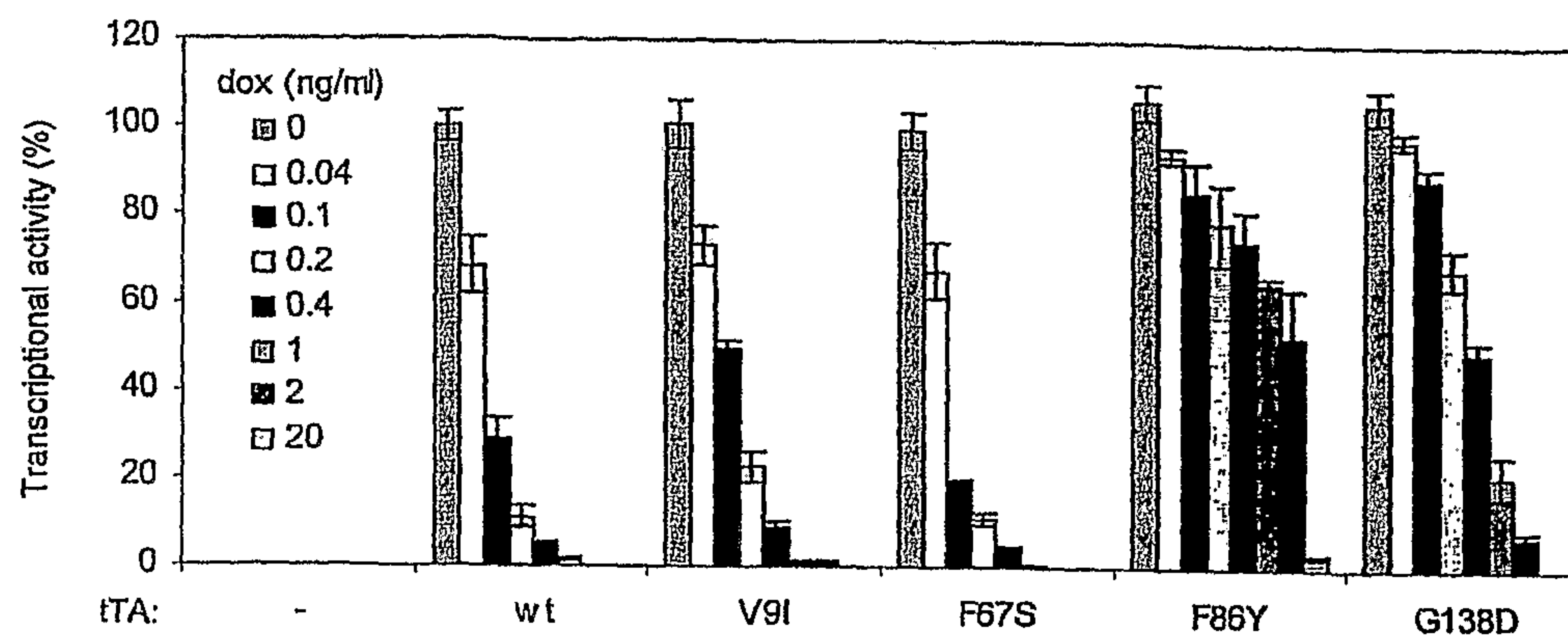


Figure 17

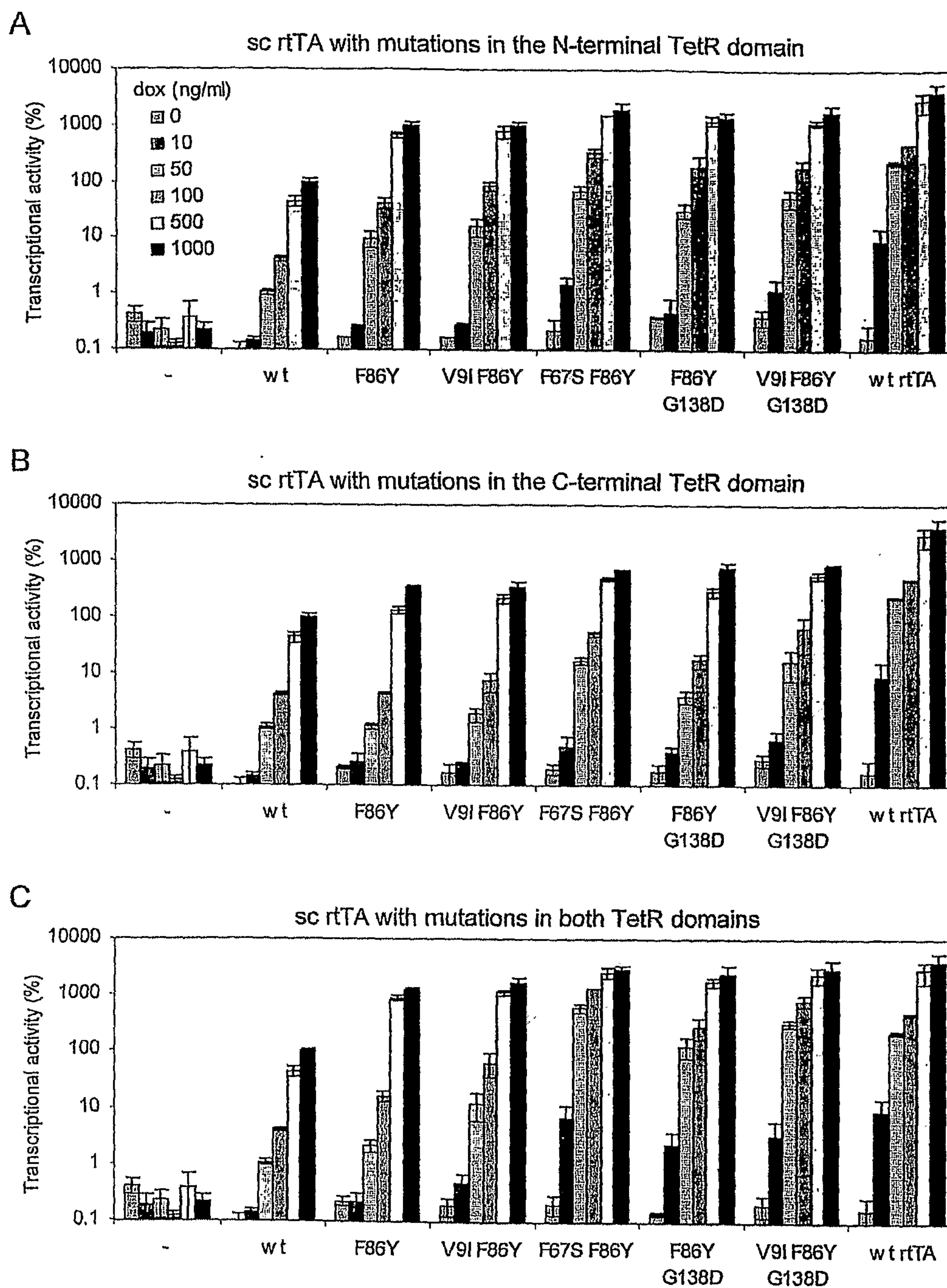


Figure 18

Figure 19: Nucleotide and amino acid sequence of rtTA

1	ATGTCTAGACTGGACAAGAGCAAAGTCATAAACTCTGCTCTGGAATTACTCAATGGAGTC
1	M S R L D K S K V I N S A L E L L N G V
61	GGTATCGAAGGCCTGACGACAAGGAACTCGCTCAAAAGCTGGGAGTTGAGCAGCCTACC
21	G I E G L T T R K L A Q K L G V E Q P T
121	CTGTACTGGCACGTGAAGAACAAGCGGGCCCTGCTCGATGCCCTGCCAATCGAGATGCTG
41	L Y W H V K N K R A L L D A L P I E M L
181	GACAGGCATCATACCCACTTCTGCCCCCTGGAAGGCGAGTCATGGCAAGACTTTCTGCGG
61	D R H H T H F C P L E G E S W Q D F L R
241	AACAACGCCAAGTCATTCCGCTGTGCTCTCCTCTCACATCGCGACGGGGCTAAAGTGCAT
81	N N A K S F R C A L L S H R D G A K V H
301	CTCGGCACCCGCCCAACAGAGAAACAGTACGAAACCCTGGAAAATCAGCTCGCGTTCCTG
101	L G T R P T E K Q Y E T L E N Q L A F L
361	TGTCAGCAAGGCTTCTCCCTGGAGAACGCACTGTACGCTCTGTCCGCCGTGGGCCACTTT
121	C Q Q G F S L E N A L Y A L S A V G H F
421	ACACTGGGCTGCGTATTGGAGGAACAGGAGCATCAAGTAGCAAAAGAGGAAAGAGAGACA
141	T L G C V L E E Q E H Q V A K E E R E T
481	CCTACCACCGATTCTATGCCCCCACTTCTGAGACAAGCAATTGAGCTGTTGACCGGCAG
161	P T T D S M P P L L R Q A I E L F D R Q
541	GGAGCCGAACCTGCCTTCCTTTTCGGCCTGGAACATAATCATATGTGGCCTGGAGAAACAG
181	G A E P A F L F G L E L I I C G L E K Q
601	CTAAAGTGCGAAAGCGGCGGGCCGGCCGACGCCCTTGACGATTTTGACTTAGACATGCTC
201	L K C E S G G P A D A L D D F D L D M L
661	CCAGCCGATGCCCTTGACGACTTTGACCTTGATATGCTGCCTGCTGACGCTCTTGACGAT
221	P A D A L D D F D L D M L P A D A L D D
721	TTTGACCTTGACATGCTCCCCGGGTAA
241	F D L D M L P G *

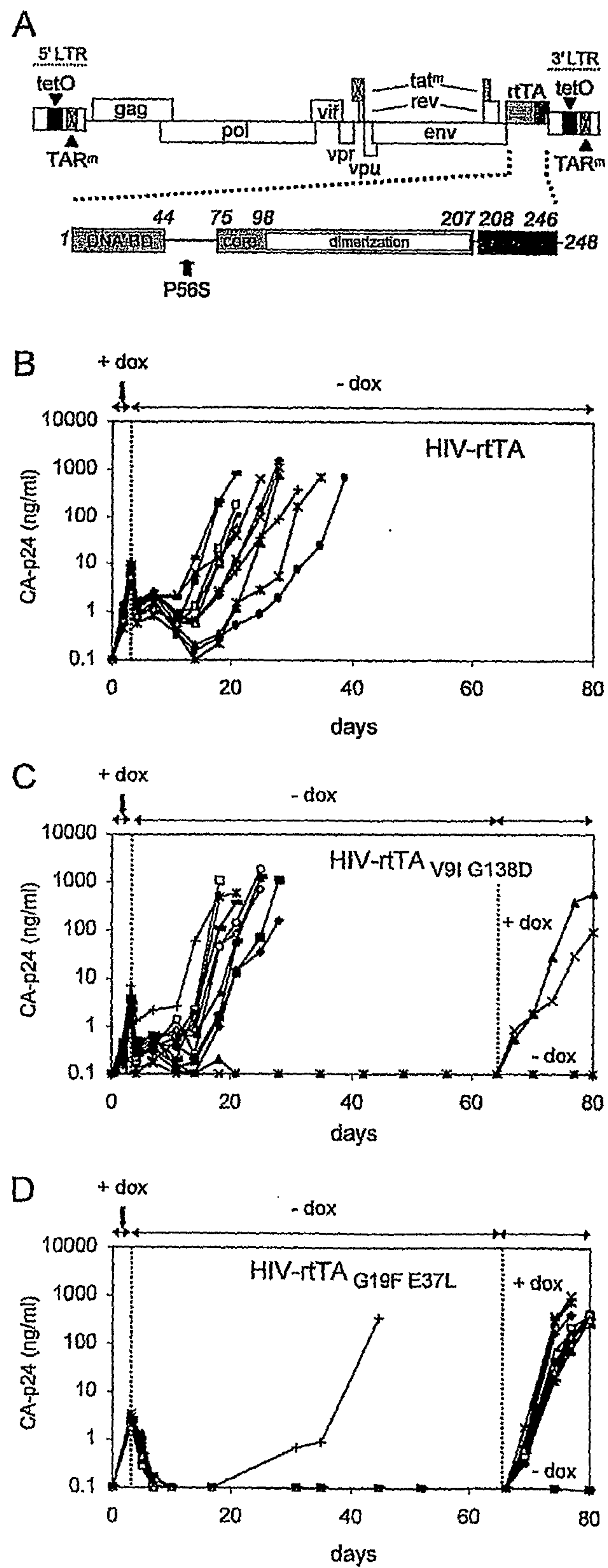


Figure 20

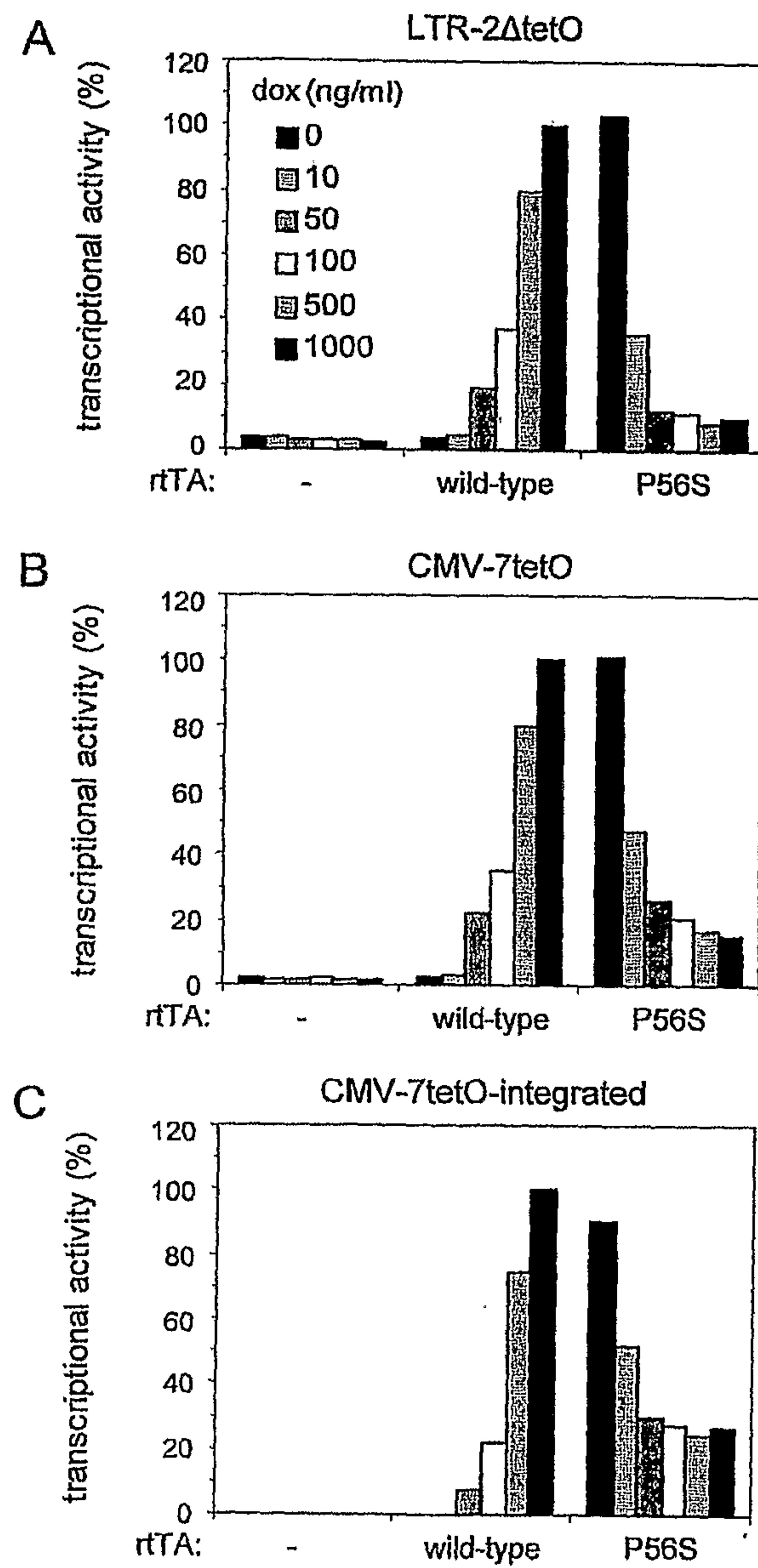


Figure 21

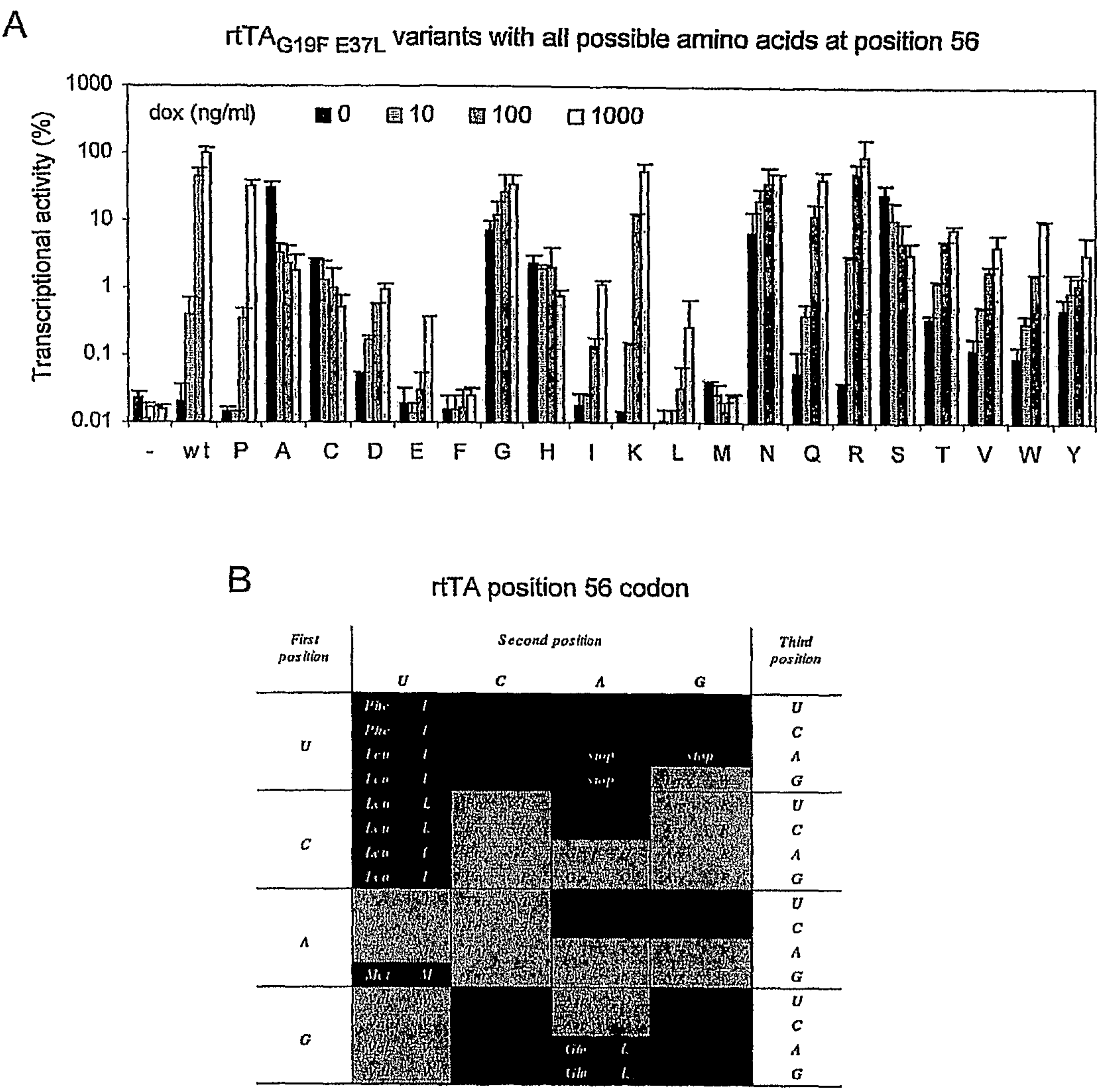


Figure 22

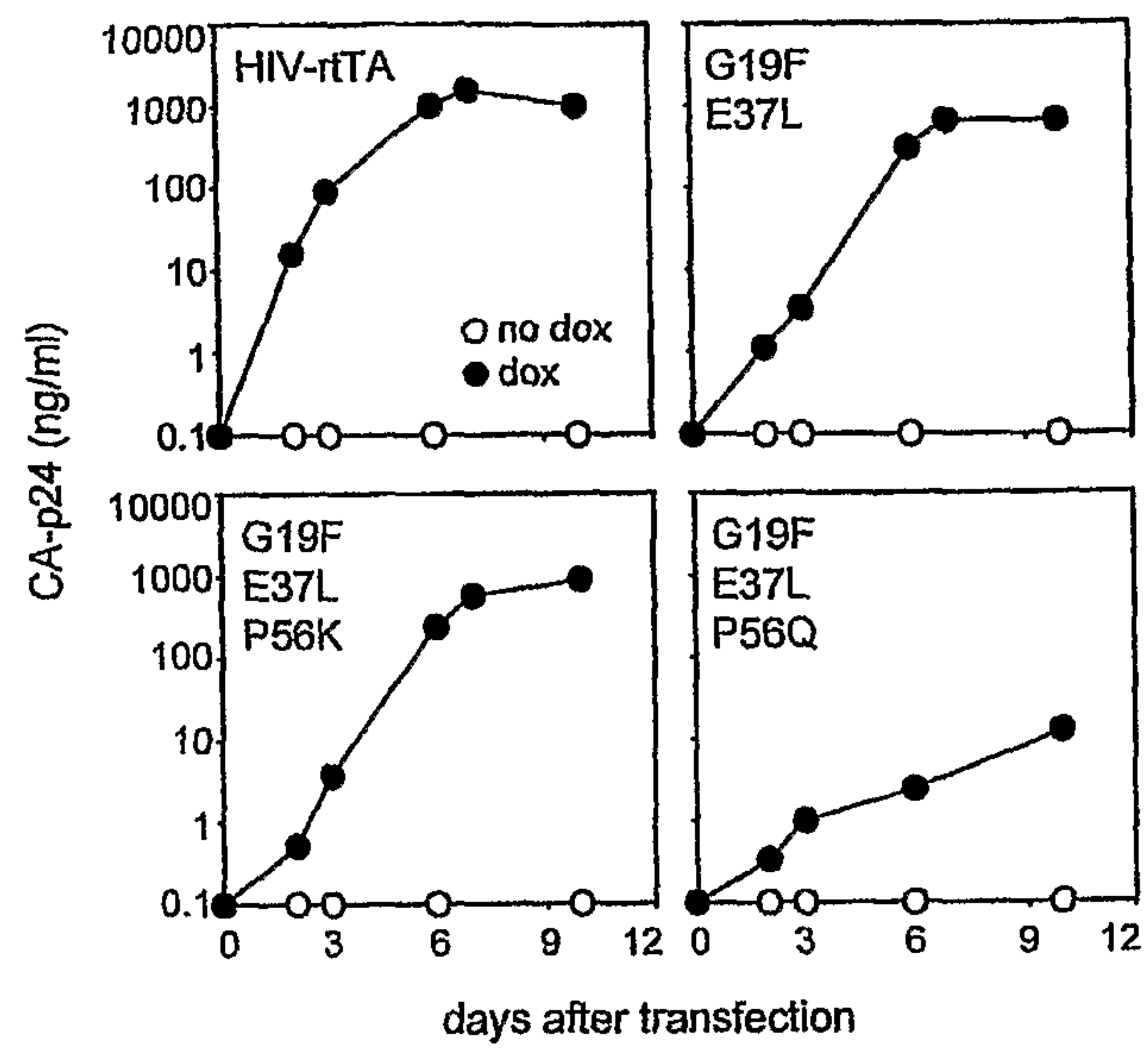


Figure 23

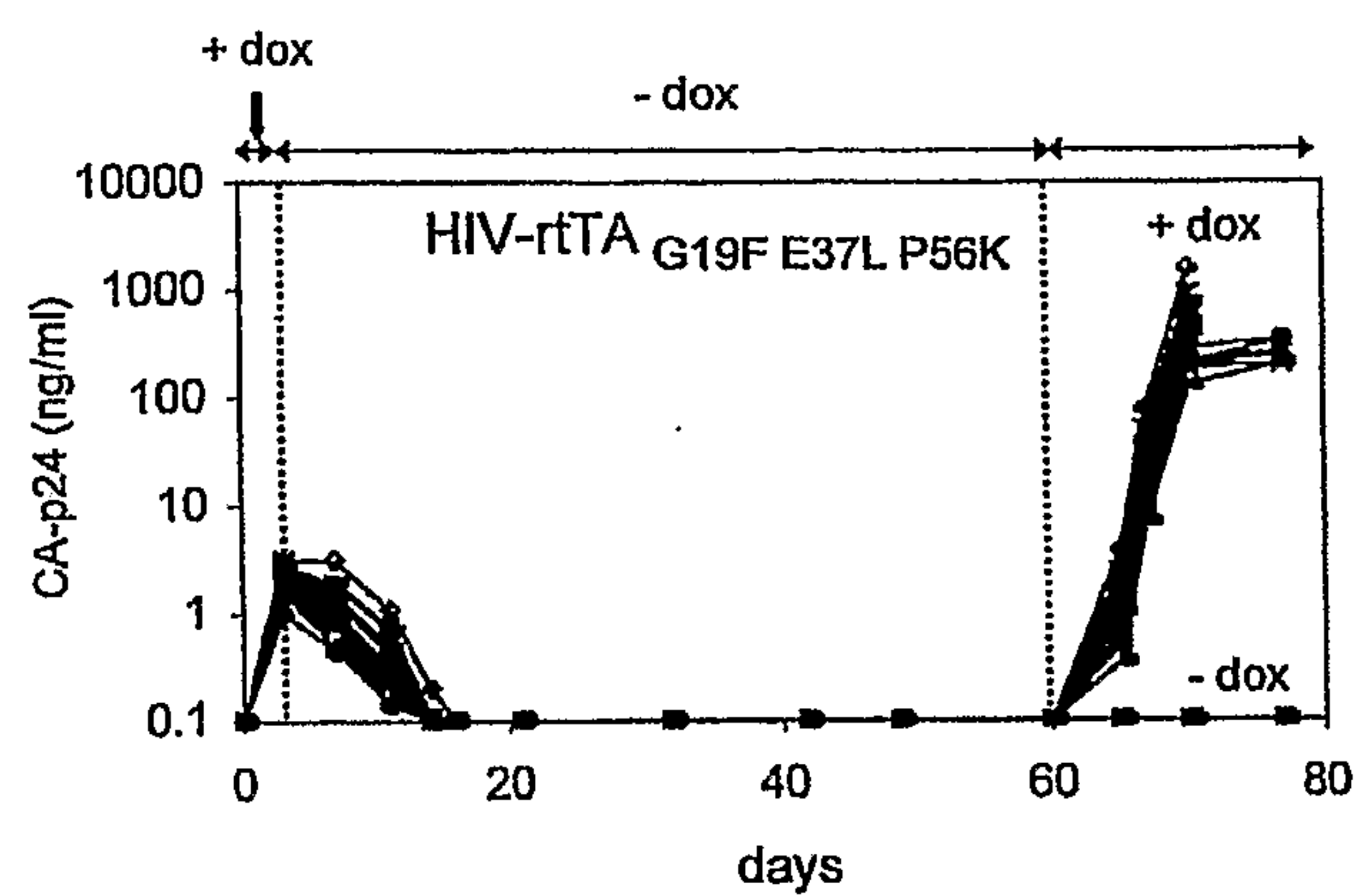


Figure 24

