



Office de la Propriété

Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2625981 A1 2007/08/16

(21) **2 625 981**

(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2006/10/11
(87) Date publication PCT/PCT Publication Date: 2007/08/16
(85) Entrée phase nationale/National Entry: 2008/04/14
(86) N° demande PCT/PCT Application No.: ES 2006/070152
(87) N° publication PCT/PCT Publication No.: 2007/090911
(30) Priorité/Priority: 2005/10/14 (ESP200502511)

(51) Cl.Int./Int.Cl. *C12N 9/12*(2006.01),
A61K 38/17(2006.01), *A61P 43/00*(2006.01)

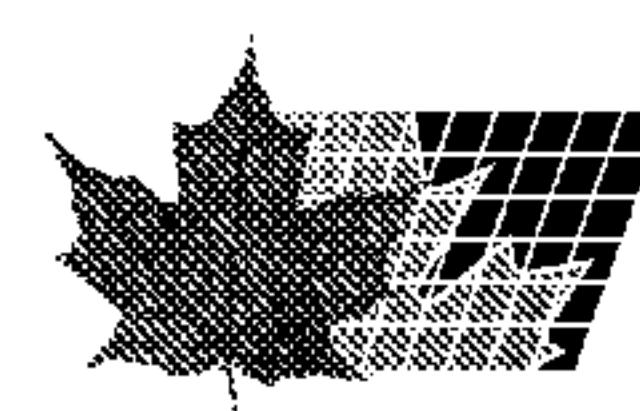
(71) **Demandeurs/Applicants:**
CONSEJO SUPERIOR DE INVESTIGACIONES
CIENTIFICAS, ES;
UNIVERSIDAD AUTONOMA DE MADRID -
FUNDACION GENERAL, ES;
UNIVERSIDAD POLITECNICA DE VALENCIA, ES

(72) **Inventeurs/Inventors:**
PERONA ABELLON, ROSARIO, ES;
MACHADO PINILLA, ROSARIO, ES;
SASTRE GARZON, LEANDRO, ES;
SANCHEZ PEREZ, ISABEL, ES; ...

(54) Titre : SEQUENCE DE NUCLEOTIDES ET DE PEPTIDES GSE 24.2 DE LA DYSKERINE INDUCTEURS DE
L'ACTIVITE TELOMERASE, PROCEDE D'OBTENTION, COMPOSITIONS THERAPEUTIQUES ET LEURS
APPLICATIONS
(54) Title: SEQUENCE OF NUCLEOTIDES AND PEPTIDES GSE 24.2 OF DYSKERIN, WHICH CAN INDUCE
TELOMERASE ACTIVITY, METHOD FOR OBTAINING SAME, THERAPEUTIC COMPOSITIONS AND
APPLICATIONS THEREOF

(57) **Abrégé/Abstract:**

The invention relates to a compound which induces or activates telomerase activity, based on the nucleotide sequence of the GSE 24.2 fragment of dyskerin or the protein or peptide sequence encoded by said nucleotide sequence. The invention also relates to vectors containing said sequence and cells transformed with same and pharmaceutical compositions containing all of the aforementioned elements. Said compositions can be used in the treatment of diseases from the following group: ageing or acceleration of ageing, neurodegenerative diseases and congenital dyskeratosis.



(72) Inventeurs(suite)/Inventors(continued): MURGUIA IBANEZ, JOSE RAMON, ES

(74) Agent: BERESKIN & PARR

ABSTRACT

This invention discloses a compound that induces or activates telomerase activity based on the nucleotide sequence of the GSE 24.2 fragment of dyskerin or the protein or peptide sequence encoded by said nucleotide sequence. Also a part of the invention are vectors that comprise said sequence and cells transformed thereby, and pharmaceutical compositions that contain all these elements. These compositions may be used in the treatment of diseases from the following group: ageing or acceleration of ageing, neurodegenerative diseases and dyskeratosis congenita.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND
PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 38

NOTE : Pour les tomes additionnels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE
VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 38

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

SEQUENCE OF GSE 24.2 NUCLEOTIDES AND PEPTIDES OF DYSKERIN THAT INDUCE TELOMERASE ACTIVITY, METHOD OF OBTAINMENT, THERAPEUTIC COMPOSITIONS AND THE APPLICATIONS THEREOF

FIELD OF THE INVENTION

5 Biotechnological sector with applications related to human health and, more specifically, biological compounds – nucleotide sequences, peptides and transformed human cells – with therapeutic applications for human beings.

STATE OF THE ART

Dyskerin (Figure 1) is a 58-kD nucleolar protein that is associated with 10 the H/ACA box in the SnoRNAs, present in the small ribonucleoprotein particles in charge of pseudouridinising the ribosomal RNA. On the other hand, it is also a component of the telomerase complex, which is responsible for maintaining telomeric repetitions at the chromosome ends. The X-chromosome-linked form 15 of dyskeratosis congenita (Marrone et al., 2003; Besseler et al., 2004) is a congenital syndrome that causes bone marrow failure and is associated with greater susceptibility to cancer. This form of dyskeratosis is caused by a specific mutation of the DKC1 gene, which encodes dyskerin. There is another 20 autosomal dominant dyskeratosis congenita; in this case, the disease is associated with mutations in the RNA component of telomerase (hTR) (Heiss et al., 1998). In the fibroblasts and lymphoblasts of patients with dyskeratosis congenita there is less telomerase activity and the telomeres are shorter than those of cells not affected by the disease (Siriniavin et al., 1975; Trowbridge et al., 1977). In the cells of patients with the X-chromosome-linked form of 25 dyskeratosis congenita, telomerase defects have been overcome by expressing hTERT ectopically (Mitchell et al., 1999). In dyskeratosis congenita caused by mutations in hTR, the only way to recover telomerase activity is by re-expressing hTR (Fu et al., 2003).

Telomeres are composed of 500-2,000 repetitions of the TTAGGG 30 conserved sequence at the chromosome 3' end and their shortening through successive divisions becomes a limitation for the cell's proliferation capacity. Telomeres are susceptible to suffering DNA damage caused by exogenous agents, including cisplatin. It has been described that cisplatin is capable of

inhibiting telomerase activity in different cell lines (Ishibashi et al., 1998; Burger et al., 1997). There are several hypotheses as to how this inhibition may occur. One possibility is the formation of G-Pt-G adducts, typical of cisplatin, in the repeated sequence of TTAGGG telomeres. Alternatively, interactions of 5 cisplatin with sulphhydryl groups essential for the reverse transcriptase catalytic subunit (hTERT) and it could even be due to the reduction in hTERT expression (Burger et al., 1997).

DESCRIPTION

BRIEF DESCRIPTION

10 One object of this invention is a compound that induces or activates telomerase activity, hereinafter activator compound of this invention, based on the nucleotide sequence of the GSE 24.2 fragment of dyskerin or the protein or peptide sequence encoded by said nucleotide sequence which is capable of recovering telomerase activity in the interior of the cells of a mammal, preferably 15 human.

One particular object of the invention is a nucleotide sequence, hereinafter GSE 24.2 gene sequence of this invention, which allows for the expression of a protein or peptide that induces telomerase activity in the interior of the cells of a mammal, preferably human, and which consists of one or 20 several GSE 24.2 nucleotide sequences belonging to the following group:

- a) a nucleotide sequence composed of a human GSE 24.2 nucleotide sequence (SEQ ID NO1),
- b) a nucleotide sequence analogous to the sequence of a),
- c) a fragment of any of sequences a) and b), and
- 25 d) a nucleotide sequence, genetic construction, that comprises any sequence belonging to a), b) and c).

As used in this invention, the term "nucleotide sequence" refers to a DNA, cDNA or mRNA sequence.

A particular embodiment of this invention is the nucleotide sequence of 30 the GSE 24.2 sequence of a) composed of SEQ ID NO1.

Another particular embodiment of this invention is the nucleotide sequence of the GSE 24.2 sequence of b) composed of SEQ ID NO11 or SEQ

ID NO13, which encode the Trub I and Trub II peptide domains, respectively (Example 1.7).

Another particular object of this invention is a GSE 24.2 genetic construction which comprises the GSE 24.2 nucleotide sequence.

5 Another particular object of this invention is a GSE 24.2 expression vector which comprises a GSE 24.2 nucleotide sequence or a GSE 24.2 genetic construction, described in this invention, and which allows for the expression of a protein or peptide capable of recovering telomerase activity in the interior of the cells of mammals, preferably humans. One example of this particular 10 embodiment is the pLNCX 24.2 expression vector of the invention (see examples).

In addition, another particular object of the invention is a protein or peptide, hereinafter GSE 24.2 protein of this invention, which exhibits activity aimed at recovering telomerase in the interior of the cells of a mammal, 15 preferably human, and which comprises one or several amino acid sequences belonging to the following group:

- a) an amino acid sequence composed of a human GSE 24.2 amino acid sequence (SEQ ID NO2),
- b) an amino acid sequence analogous to the sequence of a),
- 20 c) a fragment of any of the sequences of a) and b), and
- d) an amino acid sequence that comprises any sequence belonging to a), b) and c).

Another particular embodiment of this invention is a protein whose amino acid sequence of a) is composed of SEQ ID NO2.

25 Another particular embodiment of this invention is a protein whose amino acid sequence of c), fragment of which is composed of SEQ ID NO12 or SEQ ID NO14.

On the other hand, another, additional object of this invention are 30 genetically modified cells, either eukaryotic – preferably human – or prokaryotic, hereinafter GSE 24.2 cells of the invention, which comprise the GSE 24.2 nucleotide sequence, construction and expression vector of the invention and wherein the GSE 24.2 peptide or protein of the invention may be adequately

expressed.

Therefore, another object of the invention is the use of the GSE 24.2 activator compound of this invention in the preparation of a drug or pharmaceutical composition for the treatment of a disease caused by an 5 alteration of, preferably a reduction in, telomerase activity, belonging, for illustrative purposes and without this limiting the scope of the invention, to the following group: ageing or acceleration of ageing, neurodegenerative diseases and dyskeratosis congenita.

Another object of this invention is a pharmaceutical composition or drug 10 for the treatment of diseases, disorders or pathologies that develop with alterations of telomerase activity, preferably a reduction in activity, hereinafter pharmaceutical composition of this invention, which comprises a therapeutically effective quantity of a compound or agent capable of recovering telomerase activity jointly with, optionally, one or more pharmaceutically acceptable 15 adjuvants and/or vehicles, and which is capable of stimulating the generation and maintenance of telomerase activity.

Another particular object of this invention is a pharmaceutical composition of the invention wherein the compound or agent capable of recovering telomerase activity belongs to the following group: GSE 24.2 20 sequence, genetic construction or vector that allow for the expression of a protein or peptide capable of recovering telomerase activity in the interior of the cells of a mammal, preferably human.

A particular embodiment of the invention is a pharmaceutical composition of the invention wherein the compound or agent capable of recovering 25 telomerase activity is one or several GSE 24.2 sequences belonging to the following group:

- a) a nucleotide sequence composed of a human GSE 24.2 nucleotide sequence (SEQ ID NO1),
- b) a nucleotide sequence analogous to the sequence of a),
- 30 c) a fragment of any of the sequences of a) and b), and
- d) a nucleotide sequence, genetic construction, that comprises any sequence belonging to a), b) and c).

Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the nucleotide sequence of a) is the GSE 24.2 nucleotide sequence (SEQ ID NO1).

Another particular embodiment of this invention is the pharmaceutical 5 composition of the invention wherein the nucleotide sequence of c) is the SEQ ID NO11 or SEQ ID NO 13 nucleotide sequence.

Another particular embodiment of this invention is a pharmaceutical composition of the invention wherein the nucleotide sequence is a vector, preferably the pLNCX 24.2 vector.

10 Another particular object of this invention is a pharmaceutical composition of the invention wherein the compound or agent capable of recovering telomerase activity is a protein or a peptide encoded by the GSE 24.2 sequence, genetic construction or vector of the invention.

A particular embodiment of the invention is a pharmaceutical composition 15 of the invention wherein the GSE 24.2 protein or peptide belongs to the following group:

- a) an amino acid sequence composed of a human GSE 24.2 amino acid sequence (SEQ ID NO2),
- b) an amino acid sequence analogous to the sequence of a),
- 20 c) a fragment of any of the sequences of a) and b), and
- d) an amino acid sequence that comprises any sequence belonging to a),
b) and c).

Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the amino acid sequence of a) is the SEQ 25 ID NO2 sequence.

Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the amino acid sequence of c) is the SEQ ID NO12 or SEQ ID NO14 sequence.

Another particular object of this invention is a pharmaceutical 30 composition of the invention wherein the telomerase-activating compound or agent is a cell, preferably human, transformed by the GSE 24.2 sequence, construction or vector.

Another object of the invention is the use of the pharmaceutical composition of the invention, hereinafter use of the pharmaceutical composition of the invention, in a method of treatment or prophylaxis for a mammal, preferably a human being, affected by a disease, disorder or pathology that

5 develops with alterations of telomerase activity, consisting of the administration of said therapeutic composition in a suitable dose which allows for the recovery of telomerase activity in the interior of its cells.

Another particular object of this invention is the use of the pharmaceutical composition of the invention in a treatment method for a disease or disorder that

10 develops with alterations of telomerase activity and which affects human beings, belonging, for illustrative purposes and without this limiting the scope of the invention, to the following group: ageing or acceleration of ageing, neurodegenerative diseases, dyskeratosis congenita, Cri du chat, ataxia telangiectasia, Nijmegen Breakage Syndrome, Bloom Syndrome, Werner

15 Syndrome, Fanconi's anaemia, ulcerous colitis, vascular ageing, atherosclerosis and cancer.

Another particular embodiment of this invention is the use of the pharmaceutical composition of the invention in a treatment method for a neurodegenerative disease belonging to the following group: Alzheimer's

20 disease, Parkinson's disease, cerebellar ataxia and spinal cord degeneration.

Another particular embodiment of this invention is the use of the pharmaceutical composition of the invention in a treatment method for the X-chromosome-linked form of dyskeratosis congenita

Another particular embodiment of this invention is the use of the pharmaceutical composition of the invention in a treatment method for

25 autosomal dominant dyskeratosis congenita.

DETAILED DESCRIPTION

This invention addresses the problem of providing new therapeutic tools for the treatment of diseases that develop with alterations of telomerase activity

30 and, more specifically, dyskeratosis congenita.

This invention is based on the fact that the inventors have proven that the expression of a cDNA fragment of dyskerin, fragment GSE 24.2 (SEQ ID NO1),

which expresses an internal sequence of dyskerin (SEQ ID NO2), compensates for the defects in telomerase activity in the cells of patients with dyskeratosis congenita and in VA13 cells (Example 3). More specifically, when the cells of patients with dyskeratosis congenita and VA13 cells are transfected with the GSE 24.2 sequence, in addition to recovering telomerase activity, there is an increase in hTERT and hTR levels. In fact, it was observed that the expression of the GSE 24.2 peptide increased the baseline activity of the telomerase promoter and, following treatment with cisplatin, the 24.2 cells also had greater activity. Curiously, Collins et al. had previously described that, in the cells of these same patients, telomerase activity was only recovered by overexpressing the hTERT gene, and not by expressing dyskerin, a protein which is mutated in these patients (Mitchell et al., 1999).

It has been described in the literature that mutations in dyskerin could affect the accumulation of the telomerase RNA (Mochizuki et al., 2004); therefore, the effects caused by the GSE 24.2 peptide could be due to an increase in hTERT levels and a greater stabilisation of hTR, since the increase in hTERT levels could stabilise the formation of the telomerase complex, thereby preventing the degradation of hTR. In this regard, in this invention it has been observed that, in cells transfected with the GSE 24.2 peptide, the hTERT protein promoter, but not hTR, is constitutively activated in a manner dependent on c-myc expression, more specifically through its binding to the NHEIII region, located in the purine-rich P1 region of the promoter (Pu27, see Example 2), thus allowing for a change in the secondary conformation of DNA, in such a way that transcription factors may access the DNA. Any change in the sequence of this region that modifies the secondary structure thereof alters the activity of the GSE 24.2 peptide.

Similarly, a sequence equivalent to the human GSE 24.2 peptide (CBF5 of yeast, *S. cerevisiae* CBF5) exhibits a similar activity in the activation of hTERT (data not shown), indicating a high degree of functional conservation in the activity of this domain of dyskerin, thus defining and illustrating the multiple possibilities of elements or sequences homologous to GSE 24.2 that may be used in this invention.

Moreover, this GSE 24.2 peptide provides human cell lines with a survival capacity to cisplatin (Example 1). The 24.2 cell line increases viability in the face of the inhibitor of telomerase 1. This inhibitor has an action mechanism that is similar to cisplatin, forming G-quadruplexes in the telomeres and 5 reducing telomerase activity (Sun et al., 1997). Sequences have also been described which form G-quadruplexes in an hTERT intron; therefore, it is possible that this inhibitor also reduces hTERT levels, thereby also reducing telomerase activity (Lemateleur et al., 2004).

Bearing in mind that both cisplatin and the inhibitor of telomerase 10 stabilise the formation of G-quadruplexes (Redon et al., 2001), which could in turn block telomerase activity, the GSE 24.2 peptide could reduce the efficacy of these inhibitors by preventing or reducing the formation of these G-quadruplexes, or perhaps it is capable of increasing hTERT levels, thus increasing telomerase activity, through another mechanism.

15 Gene suppression elements (GSEs) are biologically active cDNA fragments that encode peptides or RNA anti-sense inhibitors and act in a dominant fashion on gene expression in mammal cells. GSE 24.2 is a 165-pb fragment that comprises nucleotides 268 to 433, and corresponds to a sequence formed by two highly conserved domains in different species, called 20 TRUB (Figure 2b, see SEQ ID NO12 and 14, respectively). These domains seem to have an important function in the pseudouridinisation of snoRNAs (Zucchini et al., 2003; Pan et al., 2003) and, what is more surprising, nucleotide sequences prepared with such domains, separately cloned, increased the baseline activity of the telomerase promoter like the initially described, entire 25 sequence of 55 amino acids (Example 1.7, Figure 8c). It is interesting to note that this activity, which induces resistance to cisplatin and compensates for defects in telomerase activity, is only located in the region of dyskerin found in the GSE 24.2 sequence, since the complete protein (see SEQ ID 4) or the aminoterminal fragment do not have any activity whatsoever. Finally, we must 30 note that these results open new therapeutic prospects for human disorders or diseases caused by alterations of the telomerase complex (shortening of telomeres and diseases with cell ageing or stem cells), such that the GSE 24.2

element or derivatives thereof may be used as drugs to recover telomerase activity. Moreover, there are different syndromes other than DC, which include a shortening of telomeres amongst the cellular phenotypes, and also bone marrow failures, immunosuppression and predisposition to cancer. The 5 telomerase gene is not affected by mutations in any of them, but telomere replacement and telomerase activity are.

Therefore, one object of this invention is a compound that induces or activates telomerase activity, hereinafter activator compound of this invention, based on the nucleotide sequence of the GSE 24.2 fragment of dyskerin, or the 10 protein or peptide sequence encoded by said nucleotide sequence, which is capable of recovering telomerase activity in the interior of the cells of a mammal, preferably human.

As used in this invention, the term "compound that induces or activates telomerase" refers to a molecule that increases the intensity or prolongs the 15 duration of the biological activity thereof. This definition includes, moreover, those compounds or molecules which allow for the expression of a nucleotide sequence that encodes for a GSE 24.2 protein. An activator compound may be composed of a peptide, a protein or a nucleotide sequence.

Thus, a particular object of the invention is a nucleotide sequence, 20 hereinafter GSE 24.2 gene sequence of this invention, which allows for the expression of a protein or peptide that induces the recovery of telomerase activity in the interior of the cells of a mammal, preferably human, and which is composed of one or several sequences of GSE 24.2 nucleotides belonging to the following group:

25 a) a nucleotide sequence composed of a human GSE 24.2 nucleotide sequence (SEQ ID NO1),
b) a nucleotide sequence analogous to the sequence of a),
c) a fragment of any of the sequences of a) and b), and
d) a nucleotide sequence, genetic construction, that comprises any 30 sequence belonging to a), b) and c).

In the sense used in this description, the term "analogous" is intended to include any nucleotide sequence that may be isolated or constructed on the

basis of the sequence shown in this specification; for example, by introducing conservative or non-conservative nucleotide substitutions, including the insertion of one or more nucleotides, the addition of one or more nucleotides at any of the ends of the molecule or the deletion of one or more nucleotides at 5 any end or in the interior of the sequence, which allows for the encoding of a peptide or protein capable of mimicking the activity of the GSE 24.2 sequence (SEQ ID NO2) or fragments thereof (SEQ ID NO12 and SEQ ID NO14).

The dyskerin enzyme belongs to a family of pseudouridine synthase present in several organisms (see Figure 3B, Mitchel et al., 1999). On the basis 10 of the information described in this invention and of different organisms found in nature, a person skilled in the art may isolate or construct a nucleotide sequence analogous to those described in this invention.

In general, an analogous nucleotide sequence is substantially homologous to the nucleotide sequence described above. In the sense used in 15 this description, the expression "substantially homologous" means that the nucleotide sequences in question have a degree of identity of, at least, 30%, preferably of, at least, 85%, or more preferably of, at least, 95%.

As used in this invention, the term "nucleotide sequence" refers to a 20 DNA, cDNA or mRNA sequence.

A particular embodiment of this invention is the nucleotide sequence of the GSE 24.2 sequence of a) composed of SEQ ID NO1.

Another particular embodiment of this invention is the nucleotide sequence of the GSE 24.2 sequence of b) composed of SEQ ID NO11 or SEQ 25 ID NO13, which encode the Trub I and Trub II peptide domains, respectively (Example 1.7).

The GSE 24.2 nucleotide sequence identified as d) corresponds to a GSE 24.2 gene construction. This GSE 24.2 gene construction of the invention may also comprise, if necessary and in order to allow for better isolation, detection or cytoplasm secretion of the expressed peptide, a nucleotide 30 sequence that encodes a peptide susceptible to being used for purposes of isolation, detection or secretion of said peptide. Therefore, another particular object of this invention is a GSE 24.2 genetic construction that comprises, in

addition to the GSE 24.2 nucleotide sequence, any other nucleotide sequence that encodes a peptide or peptide sequence which allows for the isolation, the detection or the cell cytoplasm secretion of the expressed peptide; for example, for illustrative purposes and without this limiting the scope of the invention, a 5 polyhistidine sequence (6xHis), a peptide sequence that is recognisable by a monoclonal antibody (for example, for the identification thereof, or any other that may be used to purify the resulting fusion protein by immunoaffinity chromatography: tag peptides such as c-myc, HA, E-tag) ("Using antibodies: a laboratory manual". Ed. Harlow and David Lane (1999). Cold Spring Harbor 10 Laboratory Press. New York. Chapter: Tagging proteins. Pp. 347-377).

The GSE 24.2 nucleotide sequence and the GSE 24.2 genetic construction described above may be obtained by a person skilled in the art using techniques that are widely known in the state of the art (Sambrook et al. "Molecular cloning, a Laboratory Manual". 2nd ed., Cold Spring Harbor 15 Laboratory Press, N.Y., 1989. vols. 1-3). Said nucleotide sequences may be integrated in a gene expression vector that allows for the regulation of the expression thereof under suitable conditions in the interior of cells.

Therefore, another particular object of this invention is a GSE 24.2 expression vector that comprises a GSE 24.2 nucleotide sequence or a GSE 20 24.2 genetic construction, described in this invention, which allows for the expression of a protein or peptide capable of recovering telomerase activity in the interior of cells of mammals, preferably humans. One example of a particular embodiment is the pLNCX 24.2 expression vector of the invention (see examples 1 and 2).

25 In general, an expression vector comprises, in addition to the GSE 24.2 nucleotide sequence or the 24.2 genetic construction described in the invention, a promoter that directs the transcription thereof (for example, *pT7*, *plac*, *ptrc*, *ptac*, *pBAD*, *ret*, etc.), whereto it is operatively bound, and other necessary or appropriate sequences which control and regulate said transcription and, if 30 applicable, the translation of the product of interest; for example, transcription initiation and termination signals (*tit2*, etc.), polyadenylation signal, replication origin, ribosome-binding sequences (RBS), transcriptional-regulator-encoding

sequences (enhancers), transcriptional silencers, repressors, etc. Examples of appropriate expression vectors may be selected, on the basis of the conditions and needs of each specific case, amongst expression plasmids, viral vectors (DNA or RNA), cosmids, artificial chromosomes, etc., which may contain, in addition, markers that may be used to select the cells transfected or transformed with the gene or genes of interest. The selection of the vector will depend on the host cell and the type of use intended. Therefore, in accordance with a particular embodiment of this invention, said vector is a plasmid or a viral vector. Said vector may be obtained by conventional methods known by those skilled in the art; similarly, different widely-known methods may be used for the transformation of microorganisms and eukaryotic cells – chemical transformation, electroporation, microinjection, etc. –, which are described in various manuals [Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.].

In addition, another particular object of the invention is a protein or peptide, hereinafter GSE 24.2 protein of this invention, that exhibits activity aimed at recovering telomerase in the interior of the cells of a mammal, preferably human, and which comprises one or several amino acid sequences belonging to the following group:

- a) an amino acid sequence composed of a human GSE 24.2 amino acid sequence (SEQ ID NO2),
- b) an amino acid sequence analogous to the sequence of a),
- c) a fragment of any of the sequences of a) and b), and
- 25 d) an amino acid sequence that comprises any sequence belonging to a), b) and c).

In the sense used in this description, the term "analogous" is intended to include any amino acid sequence that may be isolated or constructed on the basis of the sequence shown in this specification; for example, by the introduction of conservative or non-conservative amino acid substitutions, including the insertion of one or more amino acids, the addition of one or more amino acids at any of the ends of the molecule or the deletion of one or more

amino acids at any end or in the interior of the sequence, and which mimics the telomerase recovery activity of SEQ ID NO2.

The dyskerin enzyme belongs to a family of pseudouridine synthase present in several organisms (see Figure 3B, Mitchel et al., 1999). On the basis 5 of the information described in this invention and of different organisms present in nature, a person skilled in the art may isolate or construct an amino acid sequence analogous to those described in this invention.

In general, an analogous amino acid sequence is substantially homologous to the amino acid sequence discussed above. In the sense used in 10 this description, the expression "substantially homologous" means that the amino acid sequences in question have a degree of identity of, at least, 30%, preferably of, at least, 85%, or more preferably of, at least, 95%.

Another particular embodiment of this invention is a protein whose amino acid sequence of a) is composed of SEQ ID NO2.

15 Another particular embodiment of this invention is a protein whose amino acid sequence of c), fragment of which is composed of SEQ ID NO12 or SEQ ID NO14.

On the other hand, another additional object of this invention are 20 genetically modified cells, either eukaryotic – preferably human – or prokaryotic, hereinafter GSE 24.2 cells of the invention, which comprise the GSE 24.2 nucleotide sequence, construction and expression vector of the invention and wherein the GSE 24.2 peptide or protein of the invention may be adequately expressed. These cells may be transformed, infected or transfected by means 25 of said nucleotide sequences using genetic engineering techniques known by those skilled in the art [Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory] and are a part of this invention. These cells may be useful for the production of peptides capable of recovering telomerase activity, which may form the basis for a pharmaceutical composition, for the recombinant amplification of said 30 nucleotide sequences, or may be useful *per se* as cells for gene therapy, etc. A particular embodiment would be a human cell transformed by these GSE 24.2 nucleotide sequences, from different cell strains, which may be used as cells for

human tissue regeneration.

Gene expression systems may or may not allow for the integration of new genetic material into the host cell's genome. Thus, the GSE 24.2 nucleotide sequence, gene construction or expression vector may be used as a 5 drug to protect host cells, preferably human cells affected by an alteration of telomerase activity, in a gene therapy method of treatment and prophylaxis for a human being affected by a disease that develops with alterations of telomerase activity. Similarly, the GSE 24.2 cells of the invention may be used as a drug for the regeneration or implantation of tissues or cells in human beings. The 10 biopharmaceutical tools and gene therapy procedures are sufficiently known by those skilled in the art such that, with the information described in this invention, they may be developed relatively easily. Moreover, the proteins or peptides and the cells themselves may become biodrugs.

Therefore, another object of the invention is the use of the GSE 24.2 15 activator compound of this invention in the preparation of a drug or pharmaceutical composition for the treatment of a disease caused by an alteration of, preferably a reduction in, telomerase activity, belonging, for illustrative purposes and without this limiting the scope of the invention, to the following group: ageing or acceleration of ageing, neurodegenerative diseases, 20 dyskeratosis congenita, Cri du chat (CdC, OMIM 123450), ataxia telangiectasia (AT, OMIM 208900), Nijmegen Breakage Syndrome (NBS, OMIM 251260), Bloom Syndrome (BS, OMIM 10900), Werner Syndrome (WS, OMIM 277900), Fanconi's anaemia (FA OMIM 227650), ulcerous colitis, vascular ageing, atherosclerosis and cancer.

25 Studies performed in recent years have highlighted the potential relationship between defects in the maintenance of telomerase activity and other syndromes with bone marrow failures. The main cause of death for patients with dyskeratosis congenita is a bone marrow failure caused by opportunistic infections or haemorrhages (in 70% of the cases). Other causes of 30 death are pulmonary disease and cancer.

Cri du chat disease (CdC, OMIM 123450) is a congenital hereditary syndrome associated with deletions of the short arm of chromosome 5 and

appears with a frequency of 1:20,000 and 1:50,000; the frequency in patients affected by profound mental retardation (IQ less than 20) reaches 1%.

Ataxia telangiectasia (AT, OMIM 208900) is an autosomal recessive syndrome caused by mutations in the ATM gene. The problems appear

5 between the second and fifth years of life and consist of progressive neuronal degeneration (cerebral ataxia), ocular telangiectasia, immunodeficiency, hypogonadism, genomic instability, premature ageing, mild diabetes mellitus, small height and predisposition to cancer (lymphomas and leukemias).

Nijmegen Breakage Syndrome (NBS, OMIM 251260) is an autosomal recessive disease caused by mutations or losses of the Nibrin gene. The syndrome is characterised by microcephalia, destructive facial appearance, retarded growth, progressive mental retardation and strong predisposition to lymphomas and respiratory tract infections.

Bloom Syndrome (BS, OMIM 210900) and Werner Syndrome (WS, OMIM 277900). BS is an autosomal recessive syndrome induced by mutations in the recQ gene, a protein with helicase activity. WS is also an autosomal recessive syndrome caused by mutations in helicase recQL2. Both diseases are characterised by accelerated ageing and the symptoms include atherosclerosis, osteoporosis, diabetes mellitus, binocular cataracts and 20 predisposition to some types of tumours, particularly sarcomas (WS) and leukemias (BS).

Fanconi's anaemia (FA, OMIM 227650) is an autosomal recessive disease characterised by a great number of developmental defects, bone marrow failures and a one-thousand-fold increase in the incidence of myeloid leukemias and a strong predisposition to develop solid tumours. The frequency 25 of development of the disease amongst mutation carriers is 1:100.

Ulcerous colitis is a disease which affects one in 100 Spaniards. It is believed to have an autoimmune origin wherein genetic and environmental factors intervene. Ulcerous colitis affects the small intestine and is characterised 30 by chronic inflammation of the colonic sheet, with consequent ulceration, disruption of the mucous barrier and atrophy of the colon mucous membrane. The risk of cancer increases with the duration of the disease and occurs in

numerous organs, such as the colon and lymphomas.

On the other hand, the consequences of cellular senescence include atherosclerosis. On the one hand, telomeric dysfunction is present in blood vessels with atherosclerotic plaques. The reactivation of telomerase in vascular 5 progenitor cells increases these cells' division capacity and vasculogenesis. A loss of these progenitor cells contributes to an important vascular dysfunction; therefore, an anti-senescence therapy would provide a new approach to palliate the vascular effects of ageing and atherosclerosis.

Finally, in relation to other potential applications of the GSE 24.4 element 10 of the invention, we must note that they may be very broad once we know the mechanism, or one of the mechanisms, whereby this element carries out its biological activity, and which is described for the first time in this invention: by binding to the NHEIII region. In this area, there are few examples of this polypurine sequence in other promoters, but we can highlight the fact that this 15 polypurine region is located in the promoter regions of the CCR5 and PDGFA genes, amongst others. On the other hand, few transcription factors that interact with this NHE domain are known, although we must highlight the nm23H1 metastasis-suppressing peptide (Yoshiro O et al., 2001; Grand et al., 2004). In this regard, the GSE 24.2 element of the invention could be used as a 20 suppressing factor for tumour growth and metastasis propagation, an application that is a part of this invention.

As used in this invention, the term "neurodegenerative disease" refers to a disease belonging, amongst others, for illustrative purposes, to the following group: Alzheimer's disease, Parkinson's disease, cerebellar ataxia and spinal 25 cord degeneration.

Another object of this invention is a pharmaceutical composition or drug for the treatment of diseases, disorders or pathologies that develop with alterations of telomerase activity, preferably a reduction in activity, hereinafter pharmaceutical composition of this invention, that comprises a therapeutically 30 effective quantity of a compound or agent capable of recovering telomerase activity jointly with, optionally, one or more pharmaceutically acceptable adjuvants and/or vehicles, which is capable of stimulating the generation and

maintenance of telomerase activity.

The pharmaceutically acceptable adjuvants and vehicles that may be used in said compositions are the adjuvants and vehicles known by those skilled in the art which are habitually used in the preparation of therapeutic compositions.

In the sense used in this description, the expression "therapeutically effective quantity" refers to the quantity of the agent or compound capable of recovering telomerase activity calculated to produce the desired effect and, in general, it will be determined, amongst other causes, by the compounds' characteristics, including the patient's age and condition, the severity of the alteration or disorder, and the route and frequency of administration.

In another particular embodiment, said therapeutic composition is prepared in solid form or aqueous suspension, in a pharmaceutically acceptable diluent. The therapeutic composition provided by this invention may be administered by any appropriate administration route; to this end, said composition will be formulated in the suitable pharmaceutical form for the selected administration route. In a particular embodiment, administration of the therapeutic composition provided by this invention is performed by parenteral route, by oral route, by intraperitoneal route, subcutaneous route, etc. A review of the different pharmaceutical forms for drug administration and the necessary excipients to obtain them may be found, for example, in "Tratado de Farmacia Galénica", C. Faulí i Trillo, 1993, Luzán 5, S.A. Ediciones, Madrid.

Another particular object of this invention is a pharmaceutical composition of the invention wherein the compound or agent capable of recovering telomerase activity belongs to the following group: GSE 24.2 sequence, genetic construction or vector which allow for the expression of a protein or peptide capable of recovering telomerase activity in the interior of the cells of a mammal, preferably human.

A particular embodiment of the invention is a pharmaceutical composition of the invention wherein the compound or agent capable of recovering telomerase activity is one or several GSE 24.2 sequences belonging to the following group:

- a) a nucleotide sequence composed of a human GSE 24.2 nucleotide sequence (SEQ ID NO1),
- b) a nucleotide sequence analogous to the sequence of a),
- c) a fragment of any of the sequences of a) and b), and
- 5 d) a nucleotide sequence, genetic construction, that comprises any sequence belonging to a), b) and c).

Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the nucleotide sequence of a) is the GSE 24.2 nucleotide sequence (SEQ ID NO1).

10 Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the nucleotide sequence of c) is the SEQ ID NO11 or SEQ ID NO 13 nucleotide sequence.

15 Another particular embodiment of this invention is a pharmaceutical composition of the invention wherein the nucleotide sequence is a vector, preferably the pLNCX 24.2 vector.

Another particular object of this invention is a pharmaceutical composition of the invention wherein the compound or agent capable of recovering telomerase activity is a protein or a peptide encoded by the GSE 24.2 sequence, genetic construction or vector of the invention.

20 A particular embodiment of the invention is a pharmaceutical composition of the invention wherein the GSE 24.2 protein or peptide belongs to the following group:

- a) an amino acid sequence composed of a human GSE 24.2 amino acid sequence (SEQ ID NO2),
- 25 b) an amino acid sequence analogous to the sequence of a),
- c) a fragment of any of the sequences of a) and b), and
- d) an amino acid sequence that comprises any sequence belonging to a), b) and c).

Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the amino acid sequence of a) is the SEQ 30 ID NO2 sequence.

Another particular embodiment of this invention is the pharmaceutical

composition of the invention wherein the amino acid sequence of c) is the SEQ ID NO12 or SEQ ID NO14 sequence.

Another particular object of this invention is a pharmaceutical composition of the invention wherein the compound or agent that activates 5 telomerase is a cell, preferably human, that is transformed by the GSE 24.2 sequence, construction or vector.

Another object of the invention is the use of the pharmaceutical composition of the invention, hereinafter use of the pharmaceutical composition of the invention, in a method of treatment or prophylaxis for a mammal, 10 preferably a human being, affected by a disease, disorder or pathology that develops with alterations of telomerase activity, consisting of the administration of said therapeutic composition in a suitable dose that allows for the recovery of telomerase activity in the interior of its cells.

The pharmaceutical composition of this invention may be used in a 15 treatment method in isolation or jointly with other pharmaceutical compounds.

Another particular object of this invention is the use of the pharmaceutical composition of the invention in a treatment method for a disease or disorder that develops with alterations of telomerase activity and which affects human beings, belonging, for illustrative purposes and without this limiting the scope of 20 the invention, to the following group: ageing or acceleration of ageing, neurodegenerative diseases, dyskeratosis congenita, Cri du chat (CdC, OMIM 123450), ataxia telangiectasia (AT, OMIM 208900), Nijmegen Breakage Syndrome (NBS, OMIM 251260), Bloom Syndrome (BS, OMIM 210900), Werner Syndrome (WS, OMIM 277900), Fanconi's anaemia (FA OMIM 25 227650), ulcerous colitis, vascular ageing, atherosclerosis and cancer.

Another particular embodiment of this invention is the use of the pharmaceutical composition of the invention in a treatment method for a neurodegenerative disease belonging to the following group: Alzheimer's disease, Parkinson's disease, cerebellar ataxia and spinal cord degeneration.

30 Another particular embodiment of this invention is the use of the pharmaceutical composition of the invention in a treatment method for the X-chromosome-linked form of dyskeratosis congenita.

Another particular embodiment of this invention is the use of the pharmaceutical composition of the invention in a treatment method for autosomal dominant dyskeratosis congenita.

Bibliographical references

5 Bednarek A, Shilkaitis A, Green A, Lubet R., Kelloff G, Christov K and Aldaz M. 1999. Suppression of cell proliferation and telomerase activity in 4-(hydroxyphenyl)retinamide-treated mammary tumors. *Carcinogenesis.* 20(5): 879-Bessler M, Wilson DB, Mason PJ. 2004. Dyskeratosis congenita and telomerase. *Curr Opin Pediatr.* Feb;16(1):23-8. Review.

10 Burger AM, Double JA and Newell DR. 1997. Inhibition of Telomerase Activity by Cisplatin in Human Testicular Cancer Cells. *Eur J Cancer.* 33(4): 638-44.

15 Cesare AJ and Griffith JD. 2004. Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. *Mol. Cell. Biol.* 24(22): 9948-9957.

Cheng H, Wu Z, Zheng J, Lu G, Yan J, Liu M, Huang D, Lin J. 2003. Inhibition on telomerase activity and cytotoxic effects by cisplatin in cultured human choroidal melanoma cells. *Yan Ke Xue Bao.* 19(1): 54-9.

20 Fu D, Collins K. 2003 Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. *Mol Cell.* 11(5): 1361-72.

Grand CL, Powell TJ, Naglee RB, Bearss BJ, Tye D, Gleason-Guzman M, Hurley LH. Mutations in the G-quadruplex silencer element and their relationship to c-MYC overexpression, NM23 repression, and therapeutic rescue. *Proc Natl Acad Sci U S A.* 2004, 101(16): 6140–6145.

25 Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ, Poustka A, Dokal I. 1998. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet.* 19(1): 32-8.

Ishibashi T, Lippard SJ. 1998 Telomere loss in cells treated with 30 cisplatin. *Proc Natl Acad Sci U S A.* 14; 95(8): 4219-23.

Jun Hyun KIM, Joo Hee KIM, Gun Eui Lee, Sang Woong KIM and In Kwon CHUNG. 2003. Identification of a quinoxaline derivative that is a potent

telomerase inhibitor leading to cellular senescence of human cancer cells. *Biochem J.* 15; 373(Pt 2): 523-9.

5 Lemarteleur T, Gomez D, Paterski R, Mandine E, Mailliet P, Riou JF. 2004. Stabilization of the c-myc gene promoter quadruplex by specific ligands' inhibitors of telomerase. *Biochem Biophys Res Commun.* 22; 323(3): 802-8.

Marrone A, Mason PJ. 2003. Dyskeratosis congenita. *Cell Mol Life Sci* 60(3): 507-17. Review.

10 Mese H, Ueyama Y, Suzuki A, Nakayama S, Sasaki A, Hamakawa H, Matsumura T. 2001. Inhibition of telomerase activity as a measure of tumor cell killing by cisplatin in squamous cell carcinoma cell line. *Chemotherapy.* 47(2): 136-42.

Mitchell JR, Wood E, Collins K. 1999. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature.* Dec 2; 402(6761): 551-5.

15 Mochizuki Y, He J, Kulkarni S, Bessler M, Mason PJ. 2004. Mouse dyskerin mutations affect accumulation of telomerase RNA and small nucleolar RNA, telomerase activity, and ribosomal RNA processing. *Proc Natl Acad Sci U S A.* 20; 101(29): 10756-61.

20 Oh S, Song YH, Kim UJ, Yim J, Kim TK. 1999. In vivo and in vitro analyses of Myc for differential promoter activities of the human telomerase (hTERT) gene in normal and tumor cells. *Biochem Biophys Res Commun.* 263(2):361-5.

25 Pan H, Agarwalla S, Moustakas DT, Finer-Moore J, Stroud RM. 2003. Structure of tRNA pseudouridine synthase TruB and its RNA complex: RNA recognition through a combination of rigid docking and induced fit. *Proc Natl Acad Sci USA* 28;100(22):12648-53.

Poole JC, Andrews LG, Tollefsbol TO. 2001. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene.* 16;269(1-2): 1-12. Review.

30 Redon S, Bombard S, Elizondo-Rojas MA, Chottard JC. 2001. Platination of the (T2G4)4 telomeric sequence: a structural and cross-linking study. *Biochemistry.* 24; 40(29): 8463-70.

Roninson IB, Gudkov AV, Holzmayer TA, Kirschling DJ, Kazarov AR, Zelnick CR, Mazo IA, Axenovich S, Thimmapaya R. 1995. Genetic suppressor elements: new tools for molecular oncology. *Cancer Res.* 15; 55(18): 4023-8.

Sanchez-Perez I, Murguia JR, Perona R. 1998. Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene* 29; 16(4): 533-40.

Siddiqui-Jain A, Grand CL, Bearss DJ, Hurley LH. 2002. Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc Natl Acad Sci U S A.* 3;99(18):11593-8.

10 Sirinavin C and Trowbridge A. 1975. Dyskeratosis congenita: clinical features and genetic aspects. *J. Med. Genet.* 12, 339-354.

Sun D, Thompson B, Cathers BE, Salazar M, Kerwin SM, Trent JO, Jenkins TC, Neidle S, Hurley LH. 1997. Inhibition of human telomerase by a G-quadruplex-interactive compound. *J Med Chem.* 4; 40(14):2113-6.

15 Trowbridge AA, Sirinavin C and Linman JW. 1977. Dyskeratosis congenita: hematologic evaluation of a sibship and review of the literature. *Am. J. Hematol.* 3, 143-152.

Wright WE, Shay JW, Piatyszek MA. 1995. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and 20 sensitivity. *Nucleic Acids Res* 25; 23(18): 3794-5.

Yoshiro O, Tanaka M, Yoshii S, Nakaya NK, Sugimura H. Tumor metastasis suppressor nm23H1 regulates Rac1 G interaction with Tiam1. *Proc Natl Acad Sci USA* 2001, 98 (8): 4385-4390.

Zhang RG, Zhang RP, Wang XW, Xie H. 2002. Effects of cisplatin on 25 telomerase activity and telomere length in BEL-7404 human hepatoma cells. *Cell Res* 12(1): 55-62.

Zucchini C, Strippoli P, Biolchi A, Solmi R, Lenzi L, D'Addabbo P, Carinci P, Valvassori L. 2003. The human TruB family of pseudouridine synthase genes, including the Dyskeratosis Congenita 1 gene and the novel member 30 TRUB1. *Int J Mol Med.* 11(6): 697-704.

DESCRIPTION OF THE FIGURES

Figure 1.- Schematic structure of the telomerase complex. The

hTERT, dyskerin, p23, hsp90 and TEP1 proteins, jointly with the hTR RNA, constitute the ribonucleoprotein complex of telomerase.

Figure 2.- Cell viability and activation of the JNK and p38 death pathways in 293T cell lines: pLNCX and 24.2, treated with cisplatin. A) Cell

5 **viability.** After seeding the 293T cells: expressing the empty vector (pLNCX) and GSE 24.2 (24.2) in 24-well plates, they were incubated with concentrations between 0-100 µg/ml of cisplatin. The cell viability was measured by means of the crystal violet technique after 72 hours of exposure to the drug. The data represent the mean of two experiments performed in quadruplicate. B)

10 **Activation kinetics.** Treatment of both cell lines at the cisplatin concentrations indicated in the figure for 6 hours. Subsequently, the activation of JNK and p38 was studied using antibodies that specifically recognise the active forms. As a load control, the levels of JNK-1 were detected.

15 **Figure 3.- Diagram of the DSK sequences contained in the constructions used and sequence of genes homologous to DSK. A)** Diagram of the cDNA of dyskerin that represents the region whereto GSE 24.2, which covers nucleotides 268 to 433, corresponds; the location of the DSK 5' construction is also shown. B) Comparative sequence in GSE 24.2 amino acids with pseudouridine synthase sequences from other organisms. The TRUB I and

20 TRUB II conserved domains are shown.

25 **Figure 4.- Viability of the 293T cell lines: pLNCX, 24.2, DSK5' and DSK treated with cisplatin.** The cell lines described above (pLNCX and 24.2), the DSK 5' cell line, which expresses a fragment of dyskerin described in the preceding figure, and a fourth one that overexpresses the complete cDNA of dyskerin (DSK) have been used. The viability was determined as shown in figure 1 A. The data represent the mean of two experiments performed in quadruplicate.

30 **Figure 5.- Telomerase activity of the 293T cell lines: pLNCX and 24.2 following treatment with cisplatin.** The cells were seeded in 60-mm plates, pre-treated with 0.5 µg/ml of cisplatin for 3 days and subsequently treated with cisplatin at a dose of 3 µg/ml (unpretreated cells). The telomerase activity was measured by means of the Intergen TRAPeze assay. The experiment was

performed 3 times, with similar results.

Figure 6.- Cell viability and telomerase activity of the 293T cell lines: pLNCX and 24.2 treated with the Inhibitor of telomerase I. A) *Cell viability.* After seeding the 293T cells: pLNCX and 24.2 in 24-well plates, they were 5 incubated with concentrations between 0-20 μ M of the Inhibitor of Telomerase I. The cell viability was measured by means of the crystal violet technique after 72 hours of exposure to the drug. The data represent the mean of two experiments performed in quadruplicate. B) *Telomerase activity at different doses of the Inhibitor of Telomerase I.* Telomerase activity of the 293T cells: 24.2 and 10 pLNCX in the presence of different concentrations of the inhibitor of telomerase I (5-7 μ M) for 3 days. The cells were seeded in 60-mm plates and subsequently treated at the specified concentrations and times. The telomerase activity was measured by means of the Intergen TRAPeze assay. C) *Telomerase activity at different times of treatment with the Inhibitor of Telomerase I.* Telomerase 15 activity in the presence of TII (5 μ M) at 1 and 5 days. The cells were seeded as in B. Subsequently, they were treated with 5 μ M of the Inhibitor at the specified times.

Figure 7.- Expression levels of different genes related to telomerase in 293T cells: pLNCX and 24.2 following treatment with cisplatin. The cells 20 were seeded in 60-mm plates, treated with 3 μ g/ml of cDDP following pre-treatment with 0.5 μ g/ml of cisplatin for 3 days and, subsequently, collected at the times specified in the figure (3 and 7 days). Following the extraction of RNA, RT-PCR was performed with oligos specific for hTERT, hTR and dyskerin in order to study the expression levels of the messenger RNAs. As a starting RNA 25 control, oligos for the amplification of GAPDH were used.

Figure 8.- Activity of the hTERT promoter in the 293T cell lines: pLNCX and 24.2 and in 293T cells transitorily transfected with the pLNCX-24.2 construction and the pLNCX empty plasmid. A) Both the pLNCX and the 24.2 cells were transitorily transfected with 0.25 μ g of the hTERT-luc reporter vector. The cells were seeded in 60-mm plates. After 24 hours of 30 transfection, the cells were lysed and the luciferase activity of 10 μ g of total protein was quantified. As a transfection control, the cells were co-transfected

with a reporter vector, CMV-Renilla. B) The 293T cells were transfected with the pLNCX vector and the 24.2 plasmid (5 µg), and both were co-transfected with 0.25 µg of the hTERT-luc reporter vector. After seeding the cells as in A, they were transfected with pLNCX and 24.2, and co-transfected with the hTERT-luc reporter vector; subsequently, they were treated as in A. As a transfection control, the CMV-Renilla reporter vector was used. The data correspond to the ratio of relative units of luciferase with respect to non-transfected cells. Each point represents, with ±, the standard deviation obtained from 3 independent experiments. C) The 293T cells were transfected with the different GSE 24.4, 10 DSK, TRUB I, TRUB II constructions and the pLNCX empty plasmid (5 µg), and co-transfected with 0.25 µg of the hTERT-luc reporter vector. The cells were seeded in 60-mm plates; after 24 hours of transfection, the cells were lysed and the luciferase activity of 10 µg of total protein was quantified. As a transfection control, the cells were co-transfected with a CMV-Renilla reporter vector. The 15 data correspond to the ratio of relative units of luciferase with respect to non-transfected cells. Each point represents, with ±, the standard deviation obtained from 3 independent experiments.

Figure 9.- Activity of the hTERT promoter following treatment with cisplatin. A) Both the pLNCX cell line and the 24.2 cell line were transitorily transfected with 0.25 µg of the hTERTluc reporter vector. After 24 hours of transfection, they were treated with 3 µg/ml of cisplatin for 8 hours. The cells were seeded in 60-mm plates; after 24 hours of transfection and following treatment with cDDP, the cells were lysed and the luciferase activity of 10 µg of total protein was quantified. As a transfection control, the cells were co- 20 transfected with a CMV-Renilla reporter vector. B) 293T cells which, after being transitorily transfected with pLNCX and 24.2, were co-transfected with 0.25 µg of the hTERT-luc reporter vector and treated as in A. After seeding the cells as in A, they were transfected with pLNCX and 24.2, and co-transfected with the hTERT-luc reporter vector; subsequently, they were treated as in A. As a 25 transfection control, the CMV-Renilla reporter vector was used. The data correspond to the ratio of relative units of luciferase with respect to non-transfected cells. Each point represents, with ±, the standard deviation obtained 30 from 3 independent experiments.

from 3 independent experiments.

Figure 10.- Telomerase activity and expression levels of hTERT and hTR in the cells of patients with dyskeratosis congenita and in VA13 cells transitorily transfected with the 24.2 plasmid or the pLNCX empty vector.

5 A) Telomerase activity measured by means of the TRAP assay in the cells of patients with dyskeratosis congenita (DC-1, DC-2 and DC-3) and in the carrier mother (DC-C) following electroporation of 45 µg of the pLNCX empty plasmid (-) or the pLNCX 24.2 vector (+) by 15 million cells. B) RT-PCR of the RNA from cells of one of the patients with dyskeratosis congenita, DC3 transfected
10 with the empty vector and DC3 transfected with the 24.2 plasmid, using oligos specific for hTERT and hTR. As a starting RNA control, oligos for the amplification of GAPDH were used. (C) Telomerase activity in VA13 cells. The cells were transitorily transfected with 16 µg of the control pLNCX empty vector, expressing DKC or the GSE 24.2 peptide per million cells. 24 hours later, the
15 telomerase activity was analysed as described above, using dilutions of the protein extracts from each transfection. (D) The expression levels of hTERT and hTR in VA13 cells transfected with 16 µg of the different constructions per million cells. RNA levels were detected as described above. GAPDH was used as a control. In A, B, C, and D, the experiments were repeated three times with
20 similar results. (E) Activity of the hTERT promoter in VA13 cells. The cells were co-transfected with the specified constructions (10 µg per million cells) and with the hTERT-luc reporter (1 µg per million cells). After 24 h of transfection, the luciferase activity was determined as described above. CMV-Renilla (0.1 µg/ml per million cells) was used as a control for the transfection efficiency. The data
25 represent the mean of two experiments performed in quadruplicate.

Figure 11.- The GSE 24.2 peptide increases the activity of the hTERT promoter regulated by c-MYC. (A) The 293T cells were co-transfected with different constructions (10 µg/DNA per million cells) and the hTERT-luc reporter (1 µg per million cells). (B) The specified cell lines were co-transfected
30 with the hTERT-luc reporter and different quantities of the Mad/myc expression vector. (C) The specified cell lines were co-transfected with the HIV-luc reporter and with different quantities of the Mad/myc expression vector. After 24 h of

transfection, the cells were stimulated with 50 ng/ml of TNF- α for 6 hours and the luciferase activity was analysed as described above. CMV-Renilla (0.1 μ g/ml per million cells) was used as a control for the transfection efficiency. The data represent the mean of two experiments performed in quadruplicate.

5 **Figure 12.- The promoter activity of c-MYC is induced by the GSE 24.2 peptide.** (A) Schematic representation of the c-MYC promoter, indicating the different constructions used in the experiments. (B) 293T cells were co-transfected with the specified plasmids (10 μ g/DNA per million cells) and with the px3.2 c-MYC-luc reporter (1 μ g per million cells). (C) The specified cell lines 10 were transfected with the px3.2 c-MYC-luc reporter (1 μ g per million cells). (D) The specified cell lines were transfected with the different constructions of the c-MYC-luc reporter (1 μ g per million cells). After 24 hours of transfection, the luciferase activity was analysed as specified above. CMV-Renilla (0.1 μ g/ml per million cells) was used as a control for the transfection efficiency. The data 15 represent the mean of two experiments performed in quadruplicate.

10 **Figure 13.- The activity of the c-MYC promoter induced by the GSE 24.2 peptide is dependent on the NHE III element.** (A) Schematic representation of the mutations generated in the NHE III element. (B) The 293T cells were co-transfected with different constructions (10 μ g/DNA per million 20 cells) and with the different mutants of the px3.2 reporter plasmid (1 μ g per million cells). (C) The cell lines specified in the figure were transfected with the different mutants of the px3.2 reporter (1 μ g per million cells). After 24 h of transfection, the luciferase activity was analysed as specified above. CMV-Renilla (0.1 μ g/ml per million cells) was used as a control for the transfection 25 efficiency. The data represent the mean of two experiments performed in quadruplicate.

EMBODIMENT EXAMPLES

Example 1.- Identification and biological activity of the GSE 24.2 sequence

30 1.1.- Identification of the GSE sequence called 24.2

Resistance to chemotherapy is one of the greatest limitations in the treatment of cancer. In order to study the mechanisms of resistance to cisplatin,

cDNA library sequences that conferred resistance to cisplatin were isolated by tracking the gene suppression elements. This methodology, which has been previously described (Roninson et al., 1995), consists of the expression of cDNA constructions from a human placenta library, normalised in order to 5 equate the abundance in gene expression. Close to 100 different clones that conferred resistance to cisplatin were isolated. After amplifying the cDNA inserts, they were subcloned in the pLNCX plasmid and transfected again in order to ensure that they conferred resistance. One of these GSEs was a 165- 10 pb fragment called 24.2 (see SEQ ID NO1) which corresponded to an internal sequence of human dyskerin.

1.2.- Viability of the 293T cell lines: pLNCX and 24.2 with respect to cisplatin and activation of the JNK and p38 mediating cell death pathways

The pLNCX24-2 plasmid containing GSE of the same name was transfected in a stable manner in the 293T cells and, as a control, the pLNCX 15 empty vector was transfected. After verifying, by means of PCR, that the 24.2 cell line contained this insert (data not shown), the response to cisplatin was studied, creating a viability curve with this drug. Figure 2a shows the viability curve for the two cell lines in response to different doses of cisplatin after 72 hours. There we can observe that the cells that express GSE 24.2 in a stable 20 manner exhibit greater viability in response to cisplatin as compared to those that express the empty vector, specially at low doses, close to the GSE selection dose. JNK and p38 are MAPKs that are activated in response to genotoxic agents (Sánchez-Perez et al., 1998). The activation kinetics of these 25 two proteins in response to cisplatin is related to the capacity to induce cell death; for this reason, the activation of these kinases in the two cell lines was studied (Figure 2b) and it was observed that a greater dose of cisplatin is required to activate both kinases in cells that express GSE 24.2 in a stable manner, thus suggesting that the expression of GSE 24.2 attenuates the cell damage signal that activates both kinases.

30 **1.3.- Viability of the 293T cell lines: pLNCX, DSK (Gene Bank NM 001363.2), DSK-5' and 24.2 in response to cisplatin**

In order to study whether the overexpression of dyskerin could reproduce

the increase in cell viability in response to cisplatin induced by GSE 24.2, a cell line was generated which overexpressed, in a stable manner, the complete cDNA of dyskerin (pLNCX DSK). Also transfected was a construction that contained a fragment of dyskerin described in Figure 3 a), which includes the 5 GSE 24.2 sequence, in order to study whether the effect is exclusive of the region contained in 24.2 or a larger region of dyskerin (pLNCX DSK-5'). Figure 4 shows the viability curve for the four cell lines in response to different doses of cisplatin. There we observe that the cells that express the 24.2 construction (SEQ ID NO1) in a stable manner exhibit greater viability in response to 10 cisplatin as compared to those that express the empty vector, complete dyskerin (DSK) or the 5' fragment of dyskerin (SEQ ID NO3). Therefore, we may say that the increased viability in response to cisplatin is restricted to the 15 cDNA sequence contained in the GSE 24.2 fragment.

1.4.- Telomerase activity of the pLNCX and 24.2 cell lines treated with cisplatin

As discussed above, cisplatin inhibits telomerase activity through a mechanism that is yet to be defined. Since GSE 24.2 corresponds to an internal sequence of dyskerin and the latter is a part of the ribonucleoprotein complex of telomerase, the effect of cisplatin on telomerase activity was studied in the cell 20 line that expressed GSE 24.2; also studied was whether this effect varied with respect to the cell line that expresses the empty vector.

To this end, an assay of telomerase activity was performed using the TRAP method with the pLNCX and pLNCX 24.2 cell lines (Figure 5), pre-treating them for 3 days with 0.5 µg/ml of cisplatin; subsequently, they were 25 treated with 3 µg/ml of cisplatin for 3 and 7 days. The 3-day treatment was performed because it had been described that a specific inhibitor of telomerase needed to act during this time for efficient inhibition of the enzyme (Kim et al., 2003; Bednarek et al., 1999; Gowan et al., 2002). The 7-day treatment in the 293T pLNCX cells caused inhibition of telomerase activity, whereas no inhibition 30 was observed for the 24.2 cell line during this time (Figure 4). It can be concluded that the expression of GSE 24.2 confers resistance to the inhibition of telomerase activity caused by cisplatin and this inhibition is probably

responsible for the greater survival capacity of these cells in response to the drug.

1.5.- Effect of the Inhibitor of Telomerase I in pLNCX and 24.2 cells

The Inhibitor of Telomerase I is a compound that forms G-quadruplexes in the telomeres, thereby inhibiting telomerase activity (Thompson et al., 1997). We wished to study the effect of GSE 24.2 in response to the inhibitor of telomerase I. To this end, a viability curve was performed wherein the pLNCX and 24.2 cell lines were treated with increasing concentrations of the inhibitor of telomerase I (0-20 μ M) for 72 hours. Figure 6a) shows that the 24.2 cells are more resistant to the inhibitor of telomerase I than parental cells. In order to verify whether this protection was accompanied by changes in the sensitivity of telomerase activity, said activity was studied in the pLNCX and pLNCX 24.2 cells treated with different doses of the Inhibitor of Telomerase I for 3 days (Figure 6b). We can observe that inhibition of telomerase activity in the pLNCX cell line is greater than in the pLNCX 24.2 cells. The telomerase activity was also studied by treating the pLNCX and pLNCX 24.2 cells with one dose (5 μ M) and at several times (0-5 days. Figure 6c). In this case, it was also proven that the pLNCX 24.2 cells need greater exposure to the inhibitor in order for telomerase activity to be reduced. Therefore, the effects observed for telomerase activity and cell survival in both cell lines are common for cisplatin and the inhibitor of telomerase I, thus suggesting that the GSE 24.2 fragment could have a significant role in the maintenance of the telomeres' structure, at least in part, since it is capable of preventing the inhibition caused by the inhibitor of telomerase I.

25 1.6.- Effects of cisplatin on the expression levels of hTR, hTERT and dyskerin in the pLNCX and 24.2 cell lines

One of the explanations for the lower sensitivity of pLNCX 24.2 cells to cisplatin could be a change in the levels of some of the components of the telomerase complex. Therefore, the expression levels of different genes involved in the telomerase complex and the effect that cisplatin had on their expression were studied. To this end, the pLNCX and pLNCX 24.2 cells were pre-treated with 0.5 μ g/ml and treated with 3 μ g/ml of cisplatin for 3 and 7 days.

Using oligos specific for hTR, hTERT and dyskerin, the expression levels of these genes and the effect of cisplatin on said expression were studied. It was proven that cisplatin has no effect on the expression of dyskerin and hTR (Figure 7); on the contrary, it was observed that, following 3 days of treatment, 5 there is a reduction in the expression of hTERT in the pLNCX cells; on the other hand, there is no reduction in expression in the pLNCX 24.2 cells until 7 days of treatment. Therefore, the pLNCX 24.2 cells need greater exposure to cisplatin in order to exhibit inhibition of the expression of hTERT. This change in the sensitivity to cisplatin could explain, at least partially, the difference in sensitivity 10 in the telomerase activity assays.

1.7.- Effect of the expression of GSE 24.2 and Trub I and Trub II fragments, in both a stable and a transitory manner, on the activity of the hTERT promoter

Since changes were observed in the expression levels of hTERT in those 15 cells that expressed the GSE 24.2 peptide in response to cisplatin, we decided to study whether the expression of the GSE 24.2 sequence and sequences of fragments thereof – containing the Trub I (SEQ ID NO11) and Trub II (SEQ ID NO 13) domains – had any effect on the activity of the hTERT promoter. The pseudouridine synthase TRUB domain of dyskerin comprises two structural 20 subdomains: the Trub I and II motifs (Zucchini et al., 2003). These two domains are critical to maintain the global protein structure of dyskerin. Moreover, motif II contains at least one residue (asp125) that is essential for enzymatic activity. To this end, the hTERT-luc reporter vector containing a 3,402-pb sequence (Song et al., 1999) of the human hTERT promoter was transfected in the cell lines: 25 pLNCX 24.2 and pLNCX. Figure 8a) shows that, in the pLNCX 24.2 cell line, the activity of the hTERT promoter is greater than in the cells that express the empty vector (pLNCX). The same experiment was performed by transitorily expressing GSE 24.2 in the 293T cells (Figure 8b) and it was observed that the transitory expression of GSE 24.2 also increases the activity of the hTERT 30 promoter as compared to the cells transfected with the empty vector. On the other hand, this experiment was performed with the two fragments of GSE 24.2 that contain each of the Trub I (SEQ ID NO11) and Trub II (SEQ ID NO13)

domains, respectively, in order to identify the smallest sequence with an activity similar to that of the GSE 24.2 fragment (Figure 8c). The results show that the sequence contained in GSE 24.2, the sequences with TruB domains, act by activating the expression of the hTERT promoter.

5 In addition, the sequence of the CBF5 gene of yeast, *S. cerevisiae* CBF5, a sequence equivalent to that of GSE 24.2 (Figure 3b), was subcloned in the pLNCX vector, and it was observed that the expression of said construction increased the activity of hTERT, but not that of the hTR promoter (data not shown), indicating a high degree of functional conservation in the activity of this
10 domain of dyskerin.

1.8.- Effect of the expression of GSE 24.2 on the hTERT promoter in cells treated with cisplatin.

After observing that the expression of GSE 24.2 delayed the inhibition of the expression of hTERT induced by cisplatin, it was studied whether the
15 treatment with cisplatin had any effect on the promoter of said gene and whether the expression of GSE 24.2 influenced the latter. To this end, the pLNCX and pLNCX 24.2 cells were transfected with the hTERT-luc reporter vector. After 24 hours of transfection, they were treated with 3 µg/ml of cisplatin for 8 hours and the telomerase activity was assayed (Figure 9a). It can be
20 observed that, in the pLNCX 24.2 cells, the induction of the activity of the promoter is 4 times greater than in the pLNCX cells following treatment with cisplatin. The same experiment was performed by transitorily expressing GSE 24.2 and comparing them to the 293T cells transfected with the pLNCX empty vector. In this case, the activity of the cells that expressed GSE 24.2 was
25 greater than the baseline activity following treatment with cisplatin.

Example 2.- The NHEIII fragment of the promoter of the c-MYC gene is the target of the GSE 24.2 peptide.

The hTERT promoter contains two E-box regions (CACGTG), binding sites to myc/max heterodimers (Oh et al., 1999; Wu et al., 1999), as well as five
30 sp1 regions. In order to study whether activation of the hTERT promoter by the GSE 24.2 peptide takes place through this E-box, a hybrid molecule containing this binding domain to c-myc DNA and the mad transactivation domain was

transfected, which was capable of inhibiting binding to this E-box, inhibiting the dependent transcription of c-myc. The control cells and cells that express the dyskerin protein (DKC) and the GSE 24.2 peptide were transfected with incremental quantities of said hybrid molecule. The expression of the mad/myc fusion protein inhibited the baseline activity of the hTERT promoter in a dose-dependent manner, in both the control group and the DKC cells. Furthermore, the expression of the myc/mad fusion protein was capable of blocking the GSE-24.2-peptide-mediated transcription in the GSE 24.2 cells, thus suggesting that the activation of the hTERT transcription induced by GSE 24.2 is dependent on c-myc (Figure 11b). This inhibition was specific, since the transfection of the myc/mad construction jointly with an NF κ B-dependent promoter (HIVLuc) did not affect the transcription following stimulation with TNF α in any of the cell lines (Figure 11c).

The transcription of the c-myc gene is controlled by two different promoters and it seems to be repressed by a domain hypersensitive to DNase located between the P0 and P1 promoters (NHEIII). Thus, four different constructions were used that contained 3.2 kb of the transcription initiation point of c-myc bound to the luciferase gene (Figure 12a). This plasmid (px3.2myc) was transfected in the control cells and in the GSE 24.2 cells, and a three-fold increase in activity was observed in the last group of cells (Figure 12b). A similar result was obtained when cells were transfected in 293T cells with the pLNCX vector or with the plasmid that expresses the GSE 24.2 peptide jointly with the px3.2myc reporter (Figure 12c).

Both results indicated that this internal fragment of dyskerin (GSE 24.2) was capable of activating the transcription of the c-myc gene. This transcription factor, c-myc, should therefore activate the transcription of hTERT. Thus, three mutants deleted in the c-myc promoter were used to define the necessary region for the GSE 24.2 peptide to perform its inducing activity. Of all the mutants used, only those contained in the distal region of promoters P1 and P0, which include the NHEIII domain, were capable of maintaining the transcription in the GSE 24.2 cells, but not in the control cells (Figure 12d), thus indicating that the proximal region of promoter P2 is not necessary for the activity of the

GSE 24.2 peptide.

Since the NHEIII region has been described as an inhibitor of the transcription of c-myc, several mutants of the NHEIII region were constructed by Guanine modification of the purine-rich region. The 27 Guanines present in the 5 NHEIII region are involved in maintaining the secondary structure of the G-quadruplexes of the purine-rich region (Pu27). The following were mutated: G12A, located on the second guanine of the second quartet; G17A, on the second triplet of Guanines; and, finally, two consecutive Guanines (G26A/G27A) at the end of the purine-rich region (Figure 13a). These promoter 10 constructions were transfected in cells that express, either the empty vector, or the vectors containing the DKC protein or the GSE 24.2 peptide. The results indicate that only the WT px3,2 myc promoter was capable of being activated when transfected with GSE 24.2. The three mutants in the Pu27 region were activated by means of GSE 24.2, albeit at very low levels (Figure 13b).

15 Interestingly, the transfection of the double mutant of G in the control cells or those that express DKC was capable of showing increased activity in the promoter, indicating that this region is important to maintain a conformation that represses the promoter. Similar results were obtained in transitory transfection assays in 293T cells when the different mutants were transfected 20 with the empty vector, with the DKC protein or the GSE 24.2 peptide (Figure 13c). Overall, these results suggest that the GSE 24.2 peptide is capable of modifying the secondary structure of the Pu27 region towards an active conformation, thus allowing for transcription of the c-myc gene.

Example 3.- Expression of GSE 24.2 in the cells of patients with the 25 X-chromosome-linked form of dyskeratosis congenita and in VA13 cells.

Since in the X-chromosome-linked form of dyskeratosis congenita there are defects in telomerase activity, with low levels of telomerase RNA, the effect of the expression of this GSE 24.2 was tested in the cells of patients with dyskeratosis congenita. To this end, cells of patients with dyskeratosis 30 congenita (DC-1, DC-2, DC-3) and commercially available carrier mothers cells (DC-C) were transfected with the GSE 24.2 vector or with the empty vector. These cells are mortal and normally age in cultivation. 24 hours after the

transfection, the telomerase activity was measured and an increase in telomerase activity was observed in both the carrier mother cells and the cells of patients with dyskeratosis congenita when the GSE 24.2 fragment is expressed (Figure 10a). Curiously, an increase in telomerase activity was not 5 observed with the expression of complete dyskerin (data not shown). On the other hand, the levels of hTR are low in the DC cells that express a mutated form of the dyskerin gene (Mochizuki et al., 2004). It was investigated whether this increase in activity was the consequence of an increase in the expression 10 of any of the components of the telomerase complex, hTERT and hTR. To this end, an RT-PCR assay was performed 24 hours after the transfection, using oligos specific for hTERT and hTR (Figure 10b), and in both cases an increase 15 in the expression levels was observed following expression of GSE 24.2.

Moreover, the expression of the GSE 24.2 peptide of the invention in the VA13 telomerase-deficient cell line (Cesare and Griffith, 2004) was capable of 20 recovering telomerase activity (Figure 10c), and the expression of the hTERT and hTR RNAs (Figure 10d). In this cell line, the expression of motif I of the GSE 24.2 peptide (with a lower efficiency) and the fragment of the CBF5 gene of yeast additionally activated the hTERT promoter (Figure 10e). Overall, the results indicate that the expression of the GSE 24.2 peptide was capable of 25 recovering telomerase activity in both cell lines.

MATERIALS AND METHODS

Constructions and cell lines. The cell lines of patients with X-chromosome-linked dyskeratosis congenita were obtained from the Coriell Cell Repository and kept in RPMI 20% FBS. The VA13 line was obtained from Dr. M 25 Serrano. The DKC family was clinically described (Sirinavin et al., 1975; Trowbridge et al., 1977) and the affected individuals exhibit substitution of a T66A amino acid (data not shown) (DC-1, DC-2, DC-3). The carrier mother cell line (DC-C) expresses a messenger RNA without mutation (data not shown). These cells were grown in RPMI (Gibco) supplemented with 20% fetal bovine 30 serum (Gibco) and 2 mM of Glutamine.

The DSK construction contains the complete cDNA of human dyskerin and the DSK 5' construction (SEQ ID NO5) contains the first 500 nucleotides of

human dyskerin. Both constructions, like GSE 24.2 (SEQ ID NO1), were cloned in the Clal site of the pLNCX plasmid (BD Biosciences Clontech).

The cells derived from patients with DC and their respective controls were transitorily transfected by means of electroporation using 3 µg of the pLNCX or pLNCX 24.2 construction per million cells.

The 293T cell line was obtained from the American Type Culture Collection and the cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 2 mM of Glutamine. This cell line was transfected in a stable manner using the calcium chloride method, 10 µg of plasmid per million cells. These cells were co-transfected with pBABEpur, 1 µg of vector per million cells. 24 hours after the transfection, the cells were treated with puromycin in order to select stable clones. It was confirmed that the cells expressed the GSE 24.2 fragment in a stable manner by means of PCR of the genomic DNA (data not shown).

The hTERT-luc construction was cloned in the pGL3 basic plasmid (Promega) and has been provided by Tae Kook Kim (Kim et al., 1999).

Drugs. Both cisplatin and the Inhibitor of Telomerase I were obtained from Calbiochem and used at the doses specified in each figure.

Telomerase activity assay based on the TRAP method (Wright et al., 1995). The telomerase activity was measured using the TRAPeze telomerase detection kit (Intergen) in accordance with the instruction manual. The protein concentration of each extract was quantified by means of the Bradford method using the BIORAD reagent and after performing a PCR in accordance with the manual instructions; polyacrylamide gel electrophoresis was performed with the reaction products under non-denaturing conditions and, subsequently, it was stained with Ethidium bromide for 30 minutes.

Total protein extraction. The cells were lysed after being washed with PBS in order to eliminate residue from the medium. The lysis buffer was prepared in accordance with standard protocols and the protein inhibitors were added: ABSF, orthovanadate, Leupeptin, pepstatin A, aprotinin and DTT (Sigma). Subsequently, the extracts were centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was recovered. The total protein content

was determined by means of the Bradford method using the BIORAD reagent.

Western Blot and antibodies. 20 µg of protein were separated in SDS-polyacrylamide gels; subsequently, they were transferred to Immobilon-P membranes (Millipore) by humid transfer. The membranes were blocked in a 5% BSA solution or in 5% skimmed milk in TBS (20 mM Tris-HCL, pH 7.5, 150 mM NaCl) 0.1% Tween-20 (Sigma). The membranes were incubated with the corresponding antibodies. The secondary antibodies used were anti-mouse/rabbit (Biorad), directly conjugated with peroxidase. The detection was performed by the ECL method (Pharmacia), as specified in the instruction manual.

The antibodies used for the assays were: anti-pJNK (V7391, Promega), anti-JNK1 (C-17, Santa Cruz Biotechnologies), anti p-P38 (C20, Santa Cruz Technologies).

Expression of genes by RT-PCR. Total RNA extraction from the cells was performed using the Trizol reagent (Life Technologies) following the manufacturer's instructions. In each reaction, 2 µg of total RNA were transcribed to cDNA using the M-Mlv reverse transcriptase (Promega).

The following oligos were used:

Oligo A: 5'-CGGAAGAGTGTCTGGAGCAA-3' (SEQ ID NO5) and

20 Oligo B: 5'- GGATGAAGCGGAGTCGGA -3' (SEQ ID NO6) for hTERT;

Oligo C: 5'- TCTAACCCCTAACTGAGAAGGGCGTAG -3' (SEQ ID NO7) and

Oligo D: 5'- GTTGCTCTAGAATGAACGGTGGAAAG -3' (SEQ ID NO8) for hTR;

Oligo E: 5'- ATGGCGGATGCGGAAGTAATT- 3' (SEQ ID NO9) and

Oligo F: 5'- CCCCTTCAATAGCATTGTGC - 3' (SEQ ID NO10) for dyskerin.

25 The PCR conditions for the amplification of hTERT were the following: 94°C, 45 s; 60°C, 45 s; 72°C, 90 s for 31 cycles. The PCR conditions for hTR were: 94°C, 45 seconds; 55°C, 45 seconds; 72°C, 90 seconds for 28 cycles. The conditions for dyskerin were: 94°C, 40 seconds; 60°C, 60 seconds; 72°C, 120 seconds for 28 cycles (Zhang et al., 2002).

30 **Luciferase assay.** The transcriptional regulation of hTERT was measured by means of the Luciferase reporter gene, preceded by a 3,402-pb sequence of the hTERT promoter. After 24 hours of transfection, the cells were

lysed with the commercial Reporter Lysis Buffer (Promega). The cell lysates were centrifuged and the expression of luciferase was quantified with 10 µg of protein from the supernatant, using a Berthold luminometer. A construction of the CMV promoter, followed by the renilla gene, was used as a transfection 5 control. The luciferase activity is expressed per microgram of protein and is normalised using the luminescence of the renilla in the same extract.

Growth curves. The cell viability was studied by means of the crystal violet technique. The cells were seeded in 24-well plates and treated with different concentrations of the corresponding drug, specified in the figures. After 10 72 hours of incubation, the cells were fixated with 1% glutaraldehyde for 15 minutes and, after being washed with PBS, they were stained with 0.1% of the crystal violet colouring agent. The colouring agent associated with the cells was removed with a 10% acetic acid solution. The number of cells was determined 15 by estimating the absorbance at 595 nm. The figures show the % viability with respect to the cells not subject to treatment and represent the mean of 2 experiments performed in quadruplicate with the corresponding deviations.

Directed mutagenesis. The specific mutations of guanine in the NHEIII region of the c-myc gene promoter, px3.2, were performed using the Quickchange X-L directed mutagenesis kit (Stratagene) in accordance with the 20 manufacturer's instructions.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND
PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 38

NOTE : Pour les tomes additionnels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE
VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 38

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS

1. Compound that induces or activates telomerase activity, characterised in that it is a GSE 24.2 nucleotide sequence of dyskerin or that it is the protein or peptide sequence encoded by said nucleotide sequence.
5
2. Nucleotide sequence, according to claim 1, characterised in that it allows for the expression of a protein or peptide that induces the recovery of telomerase activity in the interior of the cells of a mammal, preferably human, 10 and that it is composed of one or several nucleotide sequences belonging to the following group:
 - a) a nucleotide sequence composed of a human GSE 24.2 nucleotide sequence (SEQ ID NO1),
 - b) a nucleotide sequence analogous to the sequence of a),
 - 15 c) a fragment of any of the sequences of a) and b), and
 - d) a nucleotide sequence, genetic construction, that comprises any sequence belonging to a), b) and c).
3. Nucleotide sequence, according to claim 2, characterised in that it is a 20 DNA, cDNA or mRNA sequence.
4. Nucleotide sequence, according to claim 2, characterised in that the GSE 24.2 sequence of a) is composed of SEQ ID NO1.
- 25 5. Nucleotide sequence, according to claim 2, characterised in that the GSE 24.2 sequence of b) is composed of SEQ ID NO11 or SEQ ID NO13.
6. Genetic construction, characterised in that it comprises one or several nucleotide sequences, according to claims 3 to 5.
30
7. Expression vector, characterised in that it comprises a nucleotide sequence, according to claims 3 to 5, or a genetic construction, according to

claim 6.

8. Expression vector, according to claim 7, characterised in that the expression vector is the pLNCX 24.2 plasmid.

5

9. Protein or peptide, characterised in that it induces telomerase activity in the interior of the cells of a mammal, preferably human, and that it comprises one or several amino acid sequences belonging to the following group:

- 10 a) an amino acid sequence composed of a human GSE 24.2 amino acid sequence (SEQ ID NO2),
- b) an amino acid sequence analogous to the sequence of a),
- c) a fragment of any of the sequences of a) and b), and
- d) an amino acid sequence that comprises any sequence belonging to a), b) and c).

15

10. Protein, according to claim 9, characterised in that the amino acid sequence of a) is composed of SEQ ID NO2.

11. Protein, according to claim 9, characterised in that the amino acid 20 sequence of c) is SEQ ID NO12 or SEQ ID NO14.

12. Genetically modified eukaryotic – preferably human – or prokaryotic 25 cells, characterised in that they comprise the nucleotide sequence, according to claims 2 to 5, the construction, according to claim 6, or the expression vector, according to claims 7 and 8, wherein the peptide or protein, according to claims 9 to 11, may be adequately expressed.

13. Cell, according to claim 12, characterised in that it is a human cell.

30 14. Use of the inducer compound, the nucleotide sequence, the construction, the vector, the protein or the cell, according to claims 1 to 13, in the preparation of a drug or pharmaceutical composition for the treatment of a disease caused

by an alteration of, preferably a reduction in, telomerase activity.

15. Use, according to claim 14, characterised in that the disease belongs to the following group: ageing or acceleration of ageing, neurodegenerative

5 diseases and dyskeratosis congenita.

16. Pharmaceutical composition or drug for the treatment of diseases, disorders or pathologies that develop with alterations of telomerase activity,

preferably a reduction in activity, characterised in that it comprises a compound

10 or agent capable of recovering telomerase activity, according to claim 1.

17. Pharmaceutical composition, according to claim 16, characterised in that the compound or agent that induces telomerase activity belongs to the following

group: nucleotide sequence, genetic construction, expression vector, protein or

15 cell, according to claims 2 to 13.

18. Pharmaceutical composition, according to claim 17, characterised in that

the compound is one or several sequences belonging to the following group:

a) a nucleotide sequence composed of a human GSE 24.2 nucleotide

20 sequence (SEQ ID NO1),

b) a nucleotide sequence analogous to the sequence of a),

c) a fragment of any of the sequences of a) and b), and

d) a nucleotide sequence that comprises any sequence belonging to a),

b) and c).

25

19. Pharmaceutical composition, according to claim 18, characterised in that

the nucleotide sequence of a) is the SEQ ID NO1 nucleotide sequence.

20. Pharmaceutical composition, according to claim 18, characterised in that

30 the nucleotide sequence of c) is the SEQ ID NO11 or SEQ ID NO 13 nucleotide sequence.

21. Pharmaceutical composition, according to claim 17, characterised in that the compound is a genetic construction, according to claim 6.
22. Pharmaceutical composition, according to claim 17, characterised in that 5 the compound is an expression vector, according to claims 7 and 8.
23. Pharmaceutical composition, according to claim 22, characterised in that the vector is the pLNCX 24.2 plasmid.

10 24. Pharmaceutical composition, according to claim 17, characterised in that the compound is a protein, according to claims 9 to 11.

25. Pharmaceutical composition, according to claim 24, characterised in that the protein belongs to the following group:

- 15 a) an amino acid sequence composed of a human GSE 24.2 amino acid sequence (SEQ ID NO2),
- b) an amino acid sequence analogous to the sequence of a),
- c) a fragment of any of the sequences of a) and b), and
- d) an amino acid sequence that comprises any sequence belonging to a),

20 b) and c).

26. Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the amino acid sequence of a) is the SEQ ID NO2 sequence.

25

27. Another particular embodiment of this invention is the pharmaceutical composition of the invention wherein the amino acid sequence of c) is the SEQ ID NO12 and SEQ ID NO14 sequence.

30 28. Pharmaceutical composition, according to claim 17, characterised in that the compound is a cell, preferably human, transformed by the sequence, construction or vector, according to claims 2 to 8.

29. Use of the pharmaceutical composition, according to claims 16 to 25, in a method of treatment or prophylaxis for a mammal, preferably a human being, affected by a disease, disorder or pathology that develops with alterations of, 5 preferably a reduction in, telomerase activity, consisting of the administration of said therapeutic composition in a suitable dose that makes it possible to recover telomerase activity in the interior of its cells.

30. Use of the pharmaceutical composition, according to claim 29, 10 characterised in that the disease that develops with alterations of telomerase activity and which affects human beings belongs to the following group: ageing or acceleration of ageing, neurodegenerative diseases, dyskeratosis congenita, Cri du chat, ataxia telangiectasia, Nijmegen Breakage Syndrome, Bloom Syndrome, Werner Syndrome, Fanconi's anaemia, ulcerous colitis, vascular 15 ageing, atherosclerosis and cancer.

31. Use of the pharmaceutical composition, according to claim 30, characterised in that the neurodegenerative disease belongs to the following group: Alzheimer's disease, Parkinson's disease, cerebellar ataxia and 20 spinal cord degeneration.

32. Use of the pharmaceutical composition, according to claim 30, characterised in that the disease is cancer.

25 33. Use of the pharmaceutical composition, according to claim 30, characterised in that the dyskeratosis congenita is the X-chromosome-linked form of dyskeratosis congenita or autosomal dominant dyskeratosis congenita.

Application number / numéro de demande: ES06/70152

^{8abc}

Figures: 1, 2b, 5, 6bc, 7, 9a-d, 11, 12bcd

Pages: _____

Unscannable item(s)
received with this application

To inquire if you can order a copy of the unscannable items, please visit the
CIPO WebSite at [HTTP://CIPO.GC.CA](http://CIPO.GC.CA)

Item(s) ne pouvant être balayés

Documents reçus avec cette demande ne pouvant être balayés.

Pour vous renseigner si vous pouvez commander une copie des items ne
pouvant être balayés, veuillez visiter le site web de l'OPIC au [HTTP://CIPO.GC.CA](http://CIPO.GC.CA)

A)

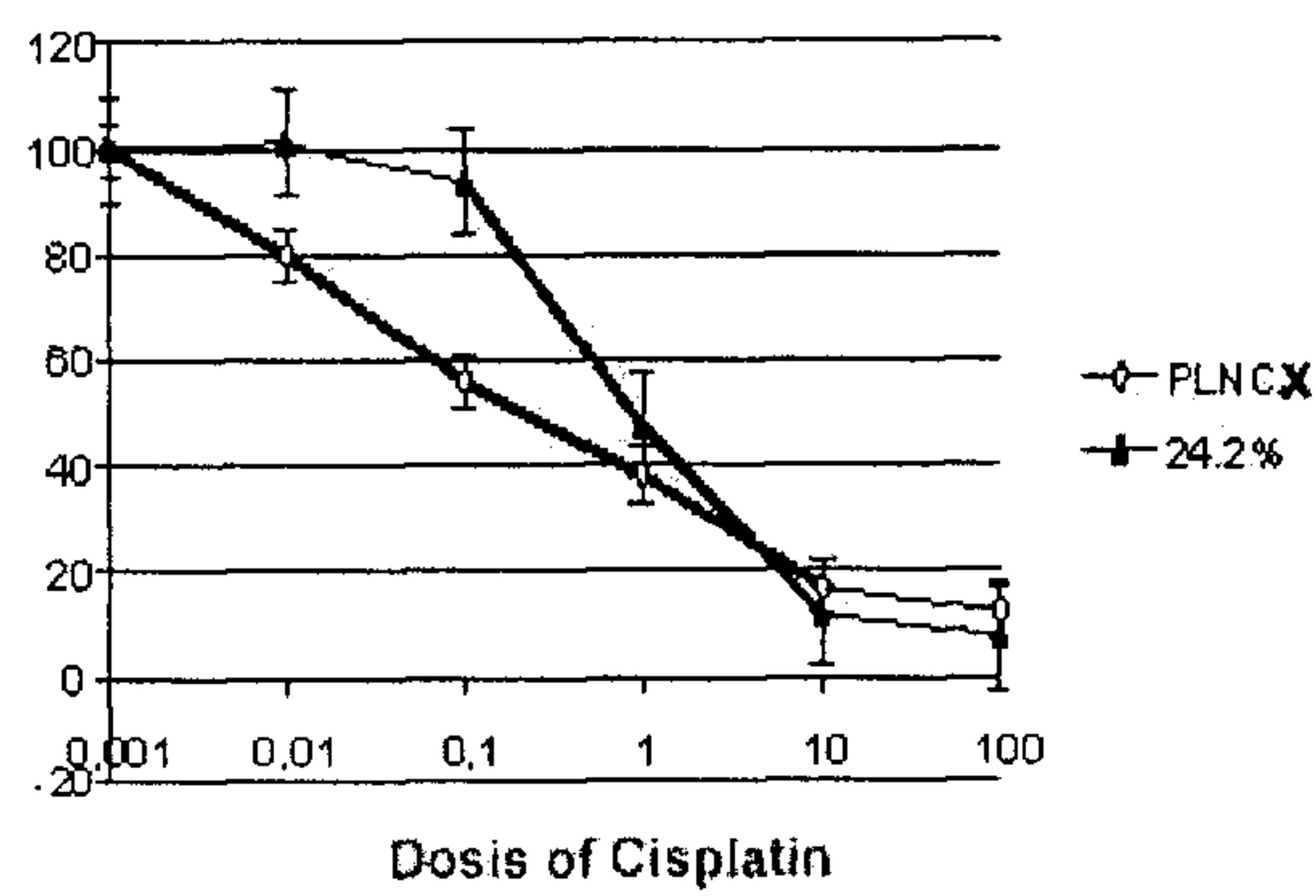


Fig 2

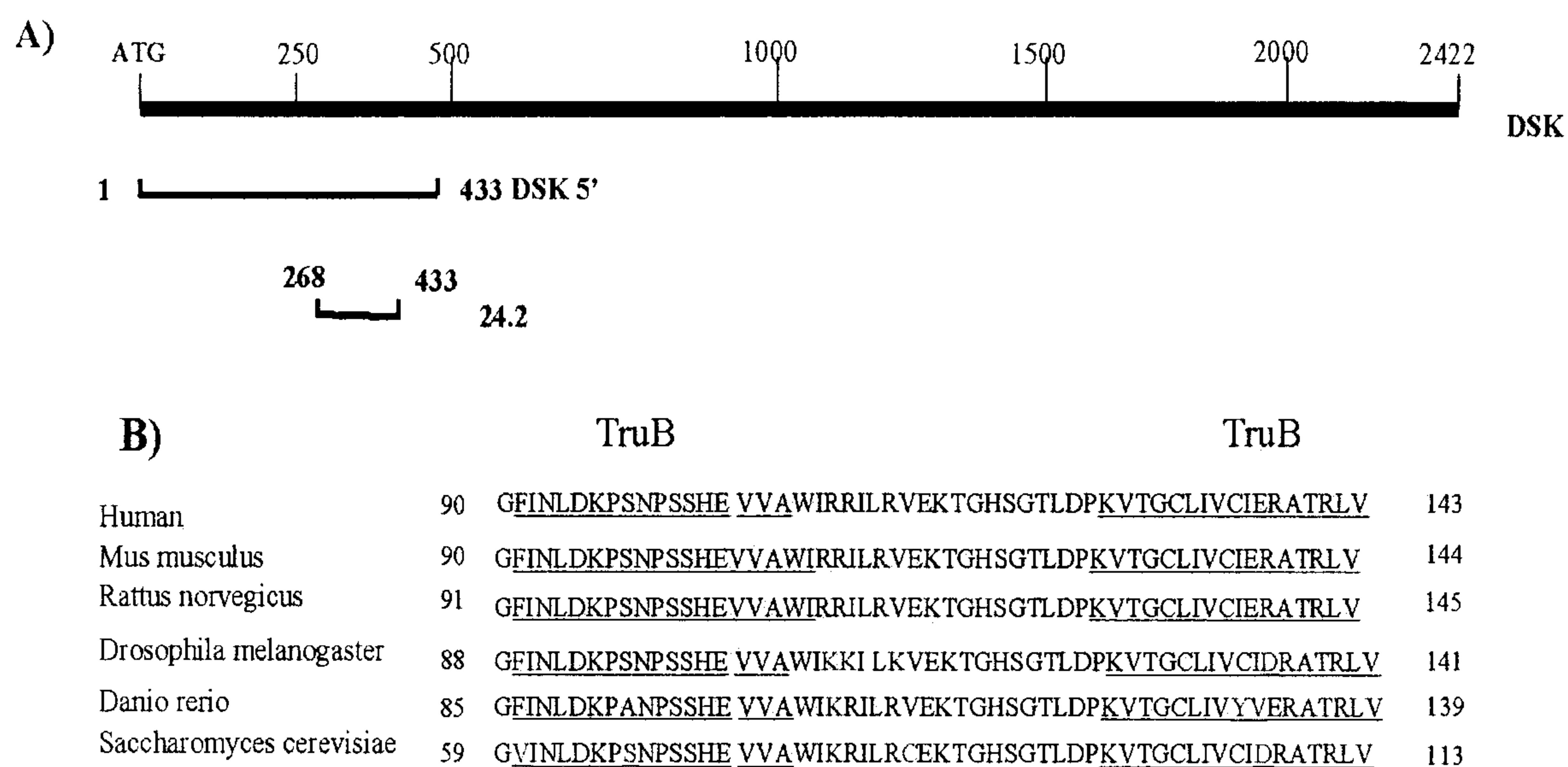


Fig 3

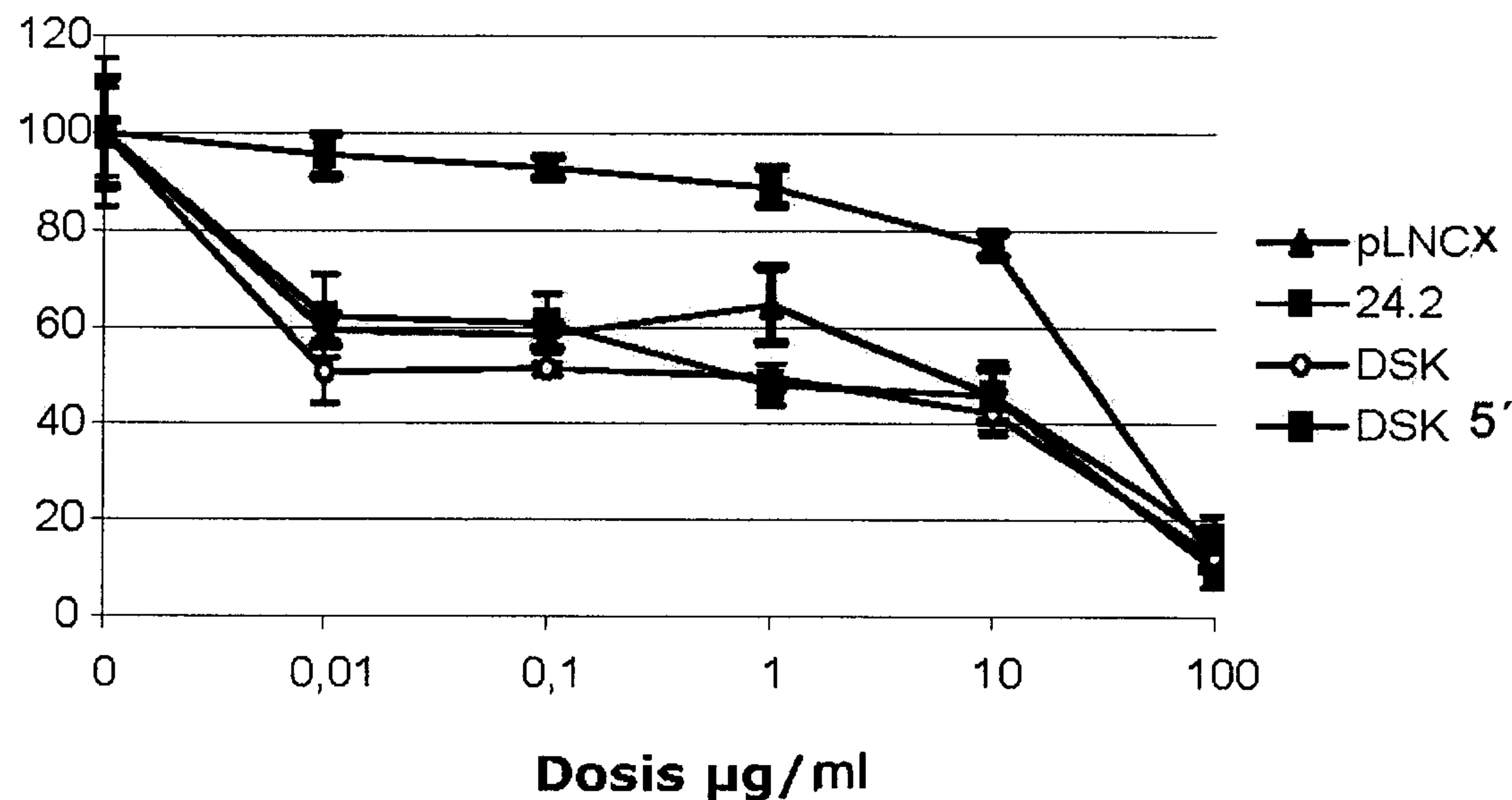


Fig 4

A)

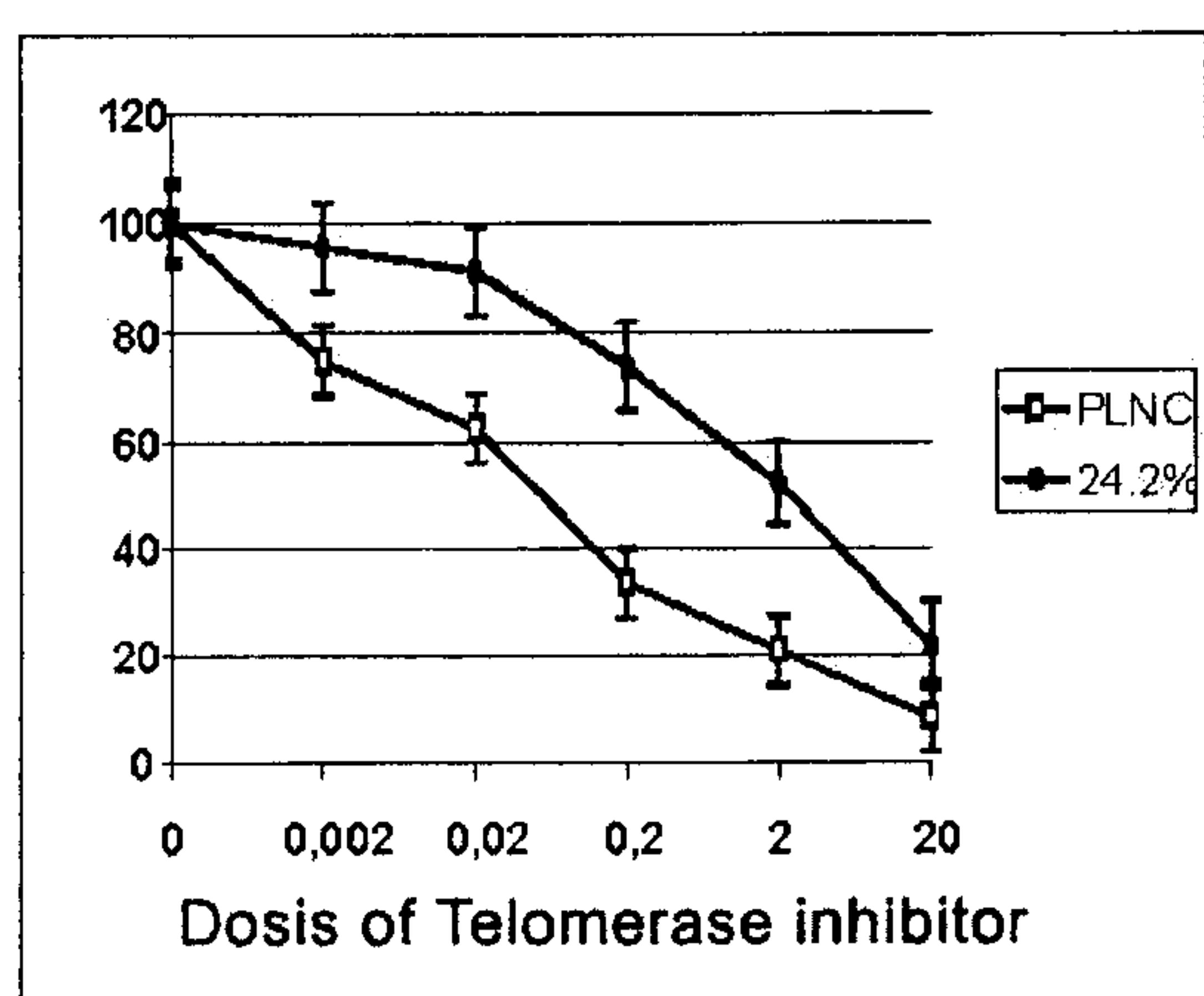


Fig 6

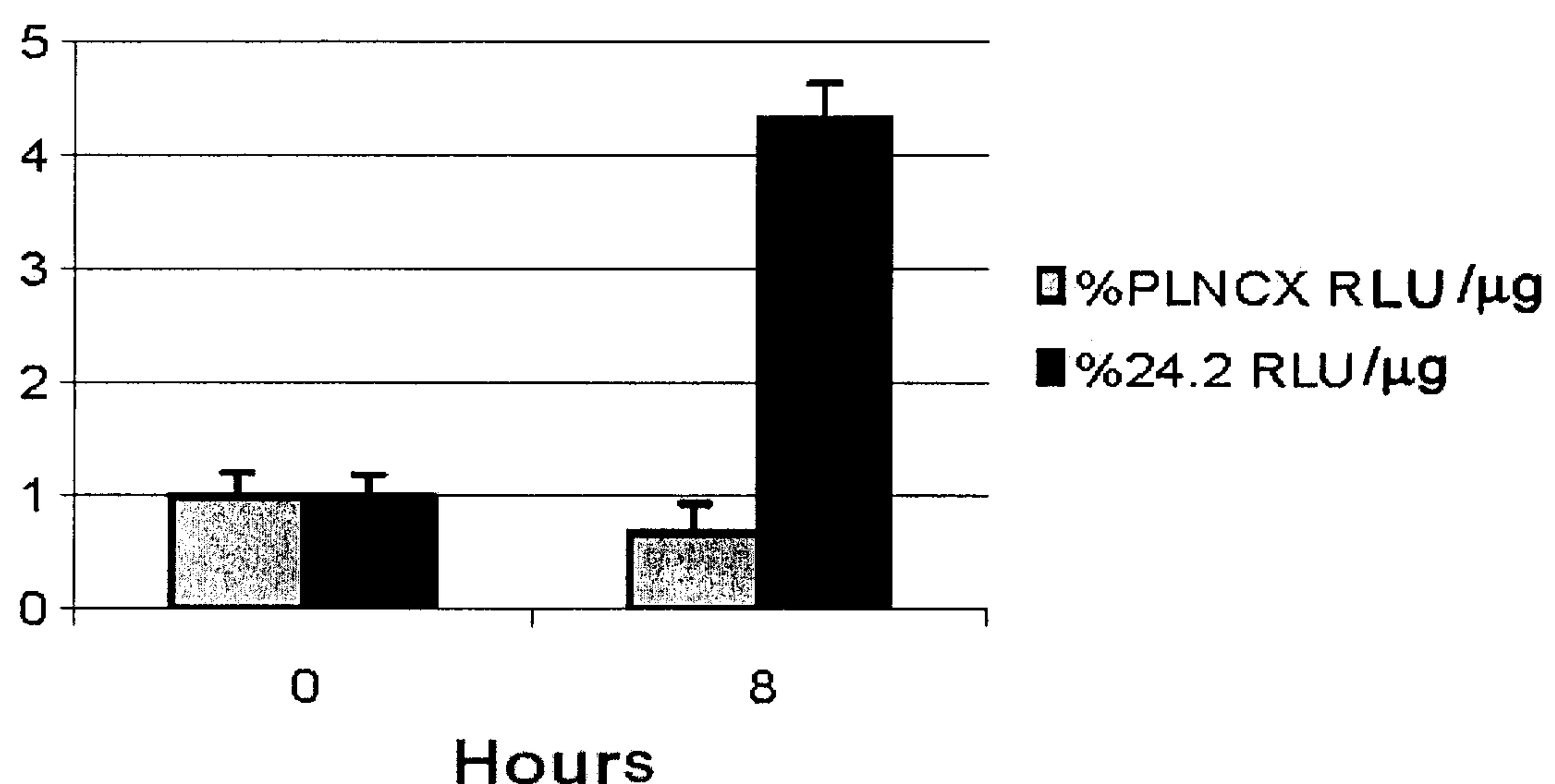
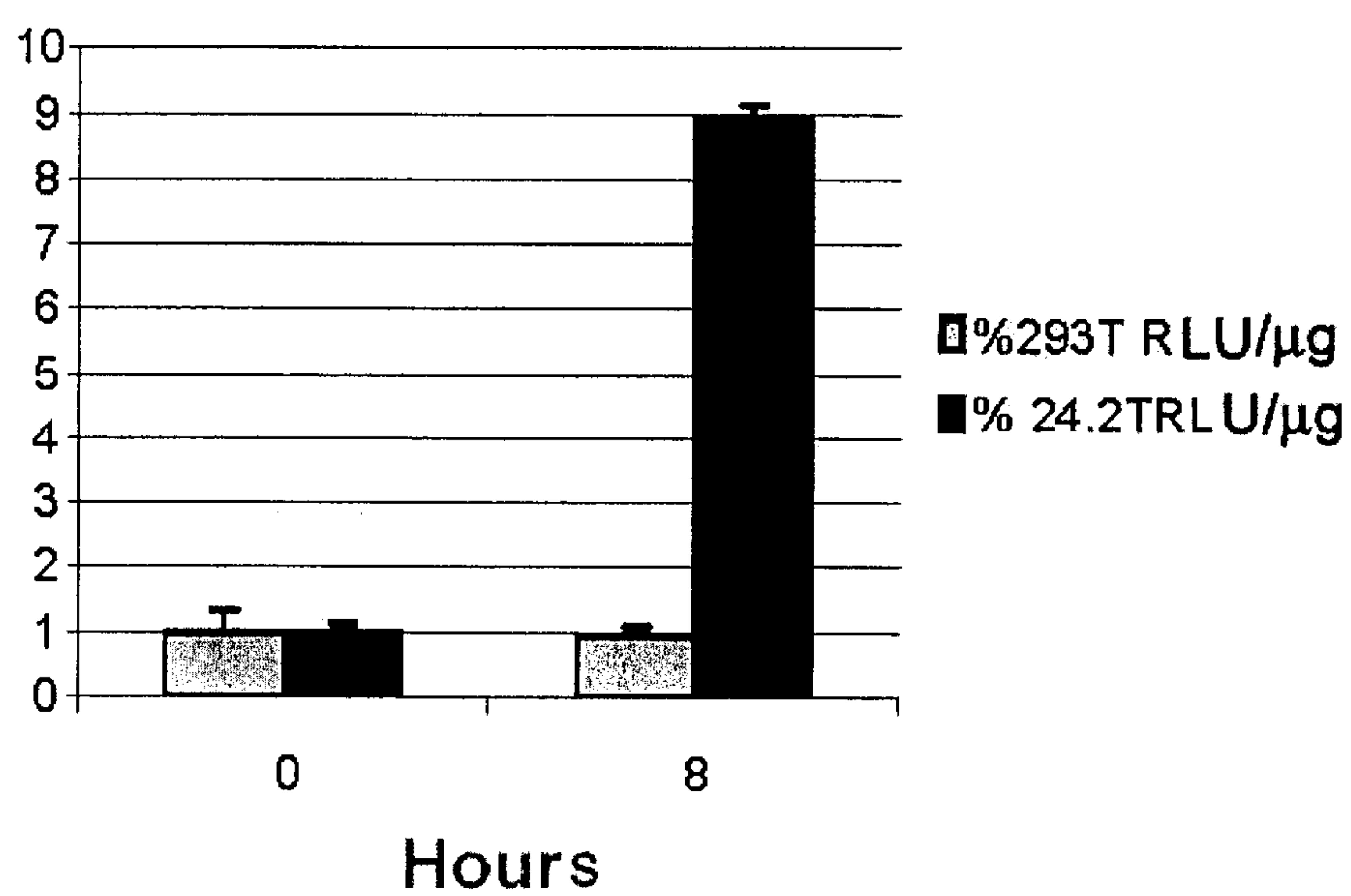
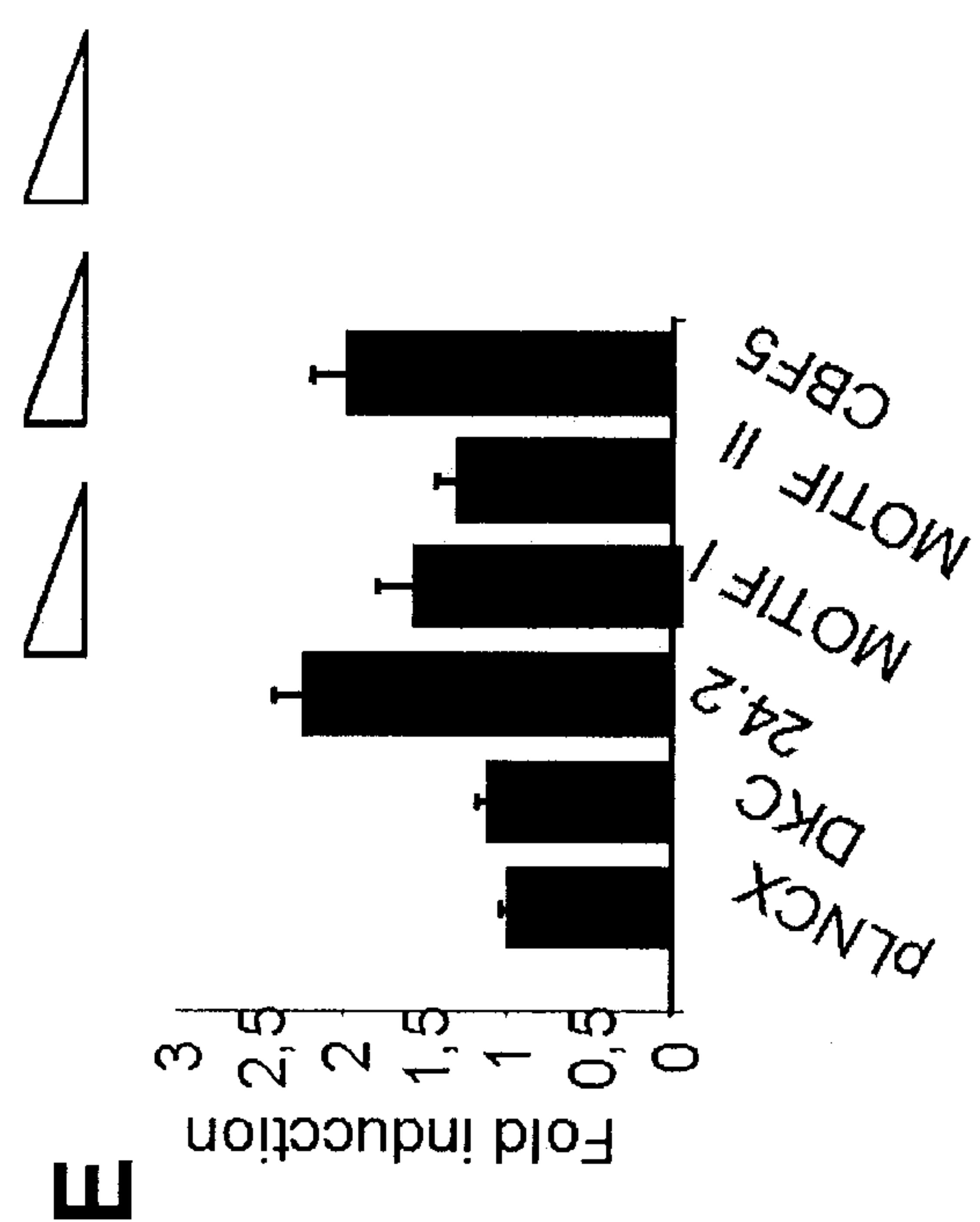
A)**B)**

Fig 9



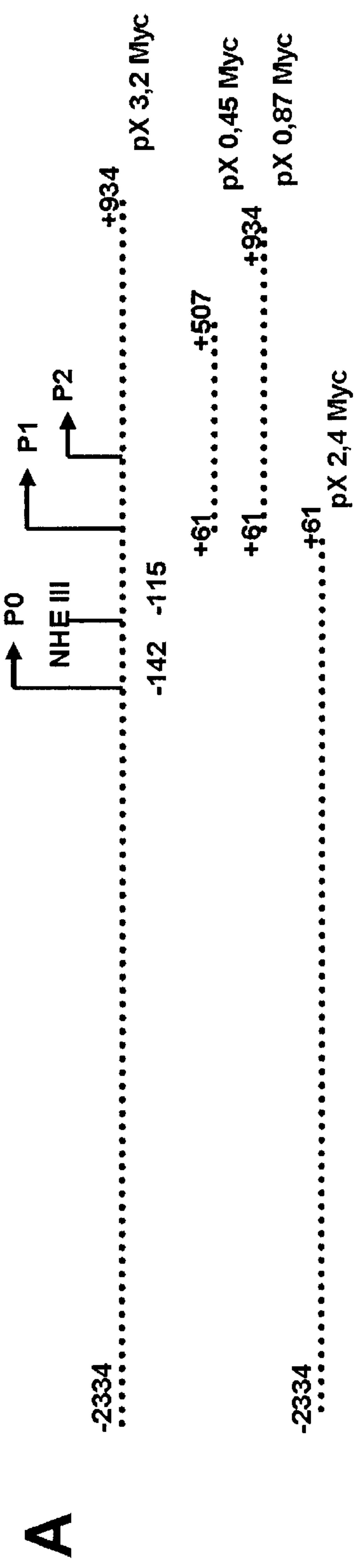


Fig 12

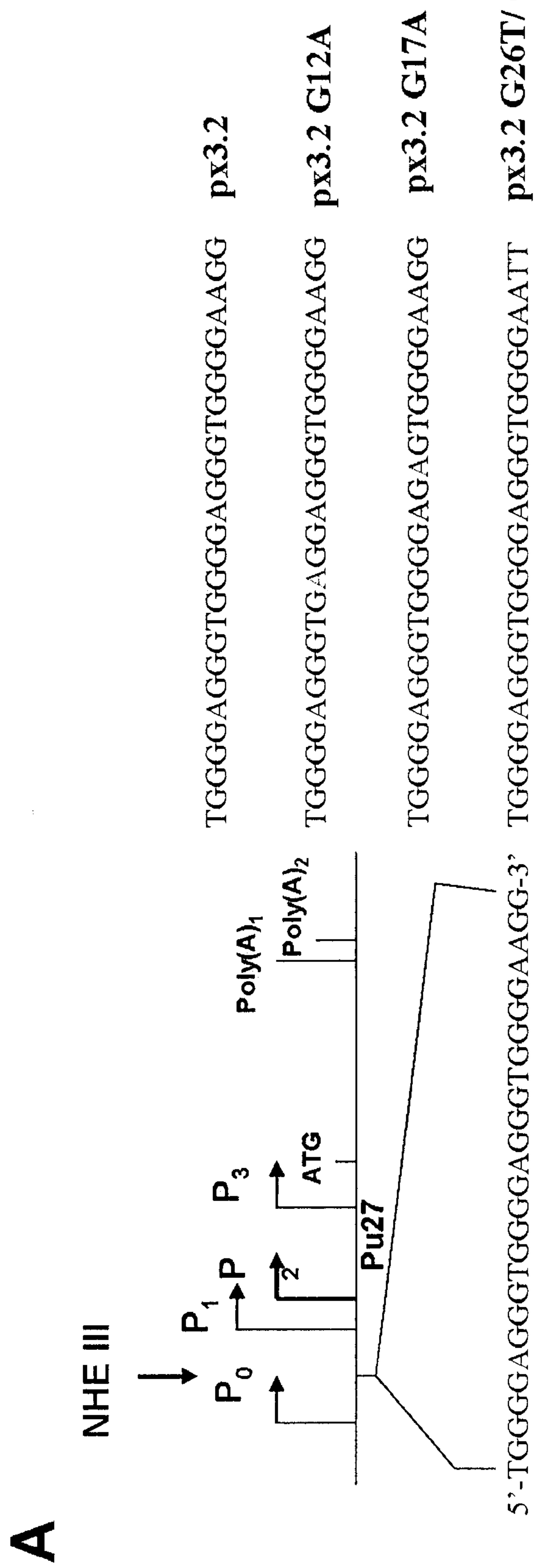


Fig 13

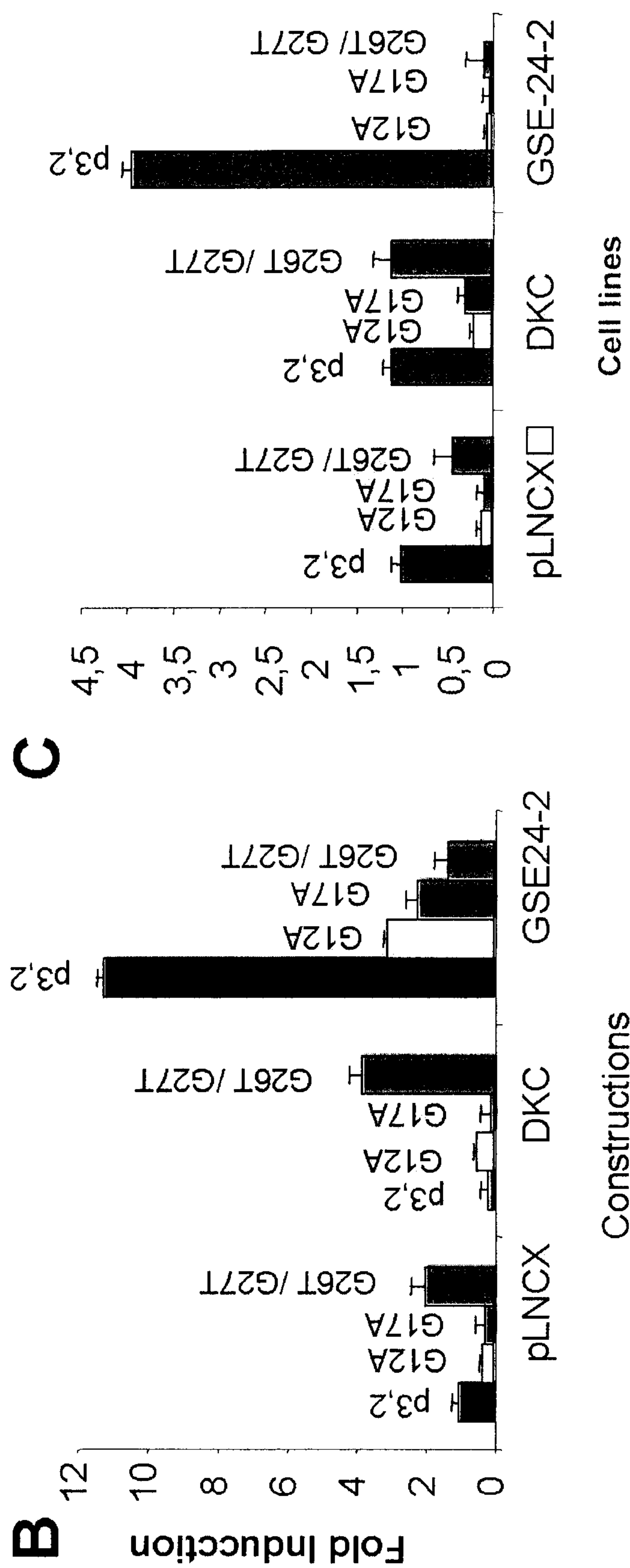


Fig 13