ASSOCIATION FRANCAISE CONTRE LES MYOPATHIES
Conseil Scientifique/Scientific Board
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APPEL D'OFFRES "DEVELOPPEMENT DES THERAPEUTIQUES"

DEMANDE D'AIDE AUX ETUDES / APPLICATION FOR FELLOWSHIP

✯ **1ère année /** First year
❑ **2ème année /** Renewal (1)

Nom / Name

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Prénoms / First name :Jean-Patrick

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Appartenance administrative du laboratoire / Administrative institution :

❑ **INSERM** ✯ **CNRS** ❑ **UNIVERSITE** ❑ **HOPITAL** ❑ **AUTRE** / OTHER

Directeur du laboratoire / Laboratory director : Mme Portier **Signature /** Signature (2) :

Titre du projet de recherche / Title of research project (3)

State of the telomeres in genetically modified satellite cells.

Date du dépôt de la demande / Date application sent : **2000**

(1) préciser l'année (Give year previously funded)
(2) signature obligatoire/Obligatory
(3) en 100 caractères maximum et en majuscules/100 characters max ; capital letters

n° d'enregistrement (AFM) : .....
Date de saisie (AFM) : .....

Titres et diplômes / Qualification

Titres obtenus (indiquer les dates d'obtention) et joindre obligatoirement un C.V.
Degrees (enclose a C.V.)

1999 : Doctorat de l'université de Paris VI, spécialité : Biologie du vieillissement. Titre de la thèse :« *Dynamique de la longueur des télomères au cours de la sénéscence et de l'immortalisation de cellules humaines.* »
Directrice de thèse : Dr Laure Sabatier, Laboratoire de Radiobiologie et d'Oncologie, CEA Fontenay-aux-Roses, France.
1994 : DESS de Biologie et de pharmacologie du vieillissement de l'université de Marne-la-vallée.
1993 : Maîtrise de biochimie de l'université Paris VI.
1992 : Licence de biochimie de l'université Paris VI.

Diplômes en préparation / Degrees in preparation

Le candidat a t-il sollicité une bourse ou aide aux études dans un autre organisme ?
Has the applicant applied for a fellowship in an other institution ?
Dans l'affirmative / if yes : **. lequel ?** / Which one ?

. date à laquelle il obtiendra une réponse / Date of decision :

Ressources diverses du candidat en cas d'attribution de l'aide aux études et pendant la durée de cette aide / Other income during the year of fellowship

Date souhaitée d'attribution de l'aide aux études / When would you want the fellowship to start
1er Janvier 2001

Perspectives d'avenir du candidat / Future prospects of the applicant

Présentation aux organismes de recherche

Inscription sur liste d'aptitude pour l'enseignement supérieur

Présentation aux organismes de recherche / Applying for an institutional position

Nombre de présentations / How many time did you apply :

Dernier classement obtenu / Last placing

Résumé du Projet / Abstract

Les capacités prolifératives des cellules somatiques sont sous le contrôle d'une structure nucléoprotéique, les télomères, localisée à l'extrémité de chaque chromosome. A chaque cycle de division cellulaire, la quantité de motifs TTAGGG diminue. Ainsi chaque télomère d'une cellule jouerait le rôle d'un sablier comptant le nombre de division cellulaire L'absence de tels motifs d'ADN déclenche l'arrêt et la sénescence cellulaire.

La thérapie cellulaire est fondée sur une greffe de cellules autologue qui ont été modifiée génétiquement pour compenser une perte de fonction. Cette approche nécéssite une expension cellulaire ex-vivo qui va consommer une part importante du potentiel de division cellulaire, ce potentiel ne sera plus disponible lorsque les cellules seront réinjectées chez le patient. Cette perte de potentiel explique sans doute en majeure partie l'échec de la thérapie cellulaire avec les cellules satellites.

Pour dépasser la barrière du potentiel proliférati des cellules satellites, une stratégie consiste à rallonger la longueur des télomères de ces cellules. L'élongation des télomères, dans les cellules satellites, est possible en introduisant le gène codant pour une sous unité d'une enzyme capable de rallonger les télomères. Contrairement à ce qui se passe dans les fibroblastes, les cellules satellites ainsi modifiées ne voit pas leur potentiel de prolifération augmenter.

Le but de mon projet est de déterminer le devenir des télomères dans les cellules satellites modifiées. L'analyse sera effectuée par hybridation in-situ en fluorescence. Cette technique puissante permet d'analyser le devenir de chacun des télomères présent dans une cellules.

Travaux antérieurs et Publications du demandeur

Precedent works and publications of the applicant

(Souligner le nom du demandeur / Underline applicant's name**)**

Publications

Pommier, J.P. and Sabatier L.
Analysis of the Telomere Length Distribution: Digital Image Processing and Statistical Analysis
Methods in Molecular Medicine (submitted).

Ducray, C., Pommier, J. P., Martins, L., Boussin, F. D., Sabatier, L.
Telomere dynamics, end-to-end fusions and telomerase activation during the human fibroblast immortalization process
Oncogene (1999) 18, 29, 4211-4223

Pommier, J. P., Gauthier, L., Livartowski, J., Galanaud, P., Boue, F., Dulioust, A., Marce, D.,Ducray, C., Sabatier, L., Lebeau, J., Boussin, F. D.
Immunosenescence in HIV pathogenesis
Virology (1997) 231 1, 148-154

JP Pommier, J Lebeau, C Ducray, L Sabatier
Chromosomal instability and alteration of telomere repeat sequences.
Biochimie (1995) 77, 817-825

Exposé du projet de recherche / Description of the research programme

See document

.../...

Avis du directeur de laboratoire / Laboratory director's opinion :

Nom / Name :

Avis du responsable du programme / Opinion of the leader of the programme

Nom / Name :

Compte-rendu d'activité / Progress report

(en cas de renouvellement de l'aide aux études / In case of renewal**)**

.../...

Mots-clefs

1	**Génothérapies** **Cytothérapies** **Pharmacothérapies** **Essais pré-cliniques** **Essais cliniques**	❒ ✯ ❒ ❒ ❒
2	**Groupe de maladies** Dystrophies musculaires, myopathies congénitales, et cardiomyopathies Maladies inflammatoires du muscle et maladies de la jonction neuromusculaire Myotonies, myopathies métaboliques et anomalies des canaux ioniques Maladies du motoneurone et neuropathies sensitivo-motrices Autres (préciser)	 ✯ ❒ ❒ ❒ ❒
3	**Modèle utilisé** (ex : homme, souris mdx, rat wistar, drosophile...) en précisant le stade de développement (oeuf, foetus, adulte, ...)	Homme (adulte)
4	**Organes ou tissus, cellules, systèmes subcellulaires** (ex : muscle strié squelettique, myocyte cardiaque, mitochondrie,	Cellules satellites
5	**Constituant, gène transféré et/ou cellule modifiée** (ex : canaux potassium, facteurs neurotrophiques, gène smn, utrophine, organoïdes, ...)	Télomères télomérase
6	**Discipline** (ex : myologie, biologie cellulaire, génétique moléculaire, ...)	Biologie cellulaire, Cytogénétique moléculaire
7	**But de l'étude** (ex : métabolisme cellulaire, fibrose, dégénérescence nerveuse, essais cliniques, vectorologie, ciblage, immunologie thérapeutique, ...)	Sénescence réplicative immortalisation
8	**Pour la thérapie génique et la thérapie cellulaire** - Méthodes (préciser le type d'injection et/ou la voie d'administration (transfection, infection, lipofection, intramusculaire, ...) - Vecteurs (préciser le type de vecteur)	Construction hTERT

Keywords

1	**Gene therapy** **Cell therapy** **Pharmacology** **Pré-clinical trials** **Clinical trials**	✯ ✯ ❒ ❒ ❒
2	**Group of diseases** Muscular dystrophies, congenital myopathies and cardiomyopathies Muscle inflammatory diseases and diseases of neuromuscular junction Myotonies, metabolic myopathies and anomalies of ionic channels Diseases of the motoneuron and other neuropathies Others (precise)	 ✯ ❒ ❒ ❒ ❒
3	**Used models** (ex : human, mdx mouse, wistar rat, drosophila...). Precise the stage of development (egg, fœtus, adult, ...)	Human (adult)
4	**Organs or tissues, cells subcellular systems** (ex : skeletal muscle, cardiac myocyte, mitochondria, ...)	Satellites Cells
5	**Constituent, transfered gene, and/or modified cell** (ex : K channels, neurotrophic factors, SMN gene, utrophin, X chromosome, intracellular Ca, ...)	Telomeres
6	**Subject** (ex : myology, cellular biology, molecular genetics, ...)	Cellular Biology, Molecular cytogenetic
7	**Aim of the study** (ex : cellular metabolism, fibrosis, nervous degenerescence, clinical trials, vectorology, targeting, therapeutic immunology, ...)	Replicative senescence immortalization
8	**For gene therapy and cellular therapy** - Methods (precise the type of injection and the administration way (transfection, infection, lipofection, intramuscular...) - Vectors (precise the type of vector)	Construction hTERT

Research Program

State of the telomeres in genetically modified satellite cells.

Aim of the project

Satellite cells are the only cells which are able to efficiently regenerate muscle fibres. For this reason they have become one of the favorite targets for cell mediated gene therapy, aimed either at replacing a missing gene in muscle or at producing a circulating factor. The extensive vascularisation of skeletal muscle will ensured the distribution of the circulating factor. Since the pioneering work of the group of T. Partridge, numerous trials achieved in the mouse have shown some promising results. It is now also possible to isolate human satellite cells, and to modify them genetically. However, no clinical trial has fullfilled the promises raised by these experimental facts. Two main obstacles have been uncovered. One is the rejection of the injected cells when they are not isolated from the patient, and the other is the massive cell death (originally evaluated at 99% in the mouse) following injection. Initial results obtained from the laboratory I will join have shown that human satellite cells have a limited proliferative capacity which is related to the age of the subject. These results have allowed us to predict the consequences of this phenomenon on the progression of various muscular dystrophies (see table 1 in next section) as well as on the possible use of these cells as a vector in therapeutic strategies.

Two possible approaches can overcome this limit : extending the proliferative capacity of the satellite cells isolated from patients, e.g. by transduction of the telomerase gene, or using cells with a myogenic potential which present a large proliferative capacity ("stem cells" or "progenitor cells") as vectors. One should note that in both cases, the analysis of telomeres status and telomerase activity will be extremely informative. Since I have some experience with the analysis of telomeres in situ from my work in the laboratory directed by L. Sabatier, I will join the laboratory directed by G. Butler-Browne and V. Mouly to use this knowledge in order to better understand the effect of telomerase expression on the telomeres. Since some collaboration already exist between the two laboratories, I will be able to use all expertises from both groups, in accordance with their respective directors.

In the optic to produce normal satellite cells with an extented or indefinite proliferative potential, the major aim of my project is to understand the precise consequences of hTERT expression on telomeres by monitoring the state of the telomeres in genetically modified satellite cells expressing ectopic copies of the hTERT gene. For this purpose, I will use fluorescent *in-situ* hybridization (FISH) at the resolution of the individual telomere, a technical approach I have developed in the group of L. Sabatier. Additionaly, the fluorescent in-situ hybybridzation (FISH) technique will be performed to determine the proliferative history of transplanted cells, and in dystrophic cells with a reduced division potential. Finally, it will be applied to analyse the telomere status of putative stem cells with a myogenic potential, if a source of these cells becomes available in the near future.

Scientific background

As stated above, the proliferative capacity of human satellite cells decreases with age. This decrease is particularly important during muscle growth, while it is limited in the adult. One can assume that the 15-25 divisions left on average in the adult satellite cell population is sufficient to achieve the few regeneration cycles that occur in the normal individual. However, this might not be the case after more important trauma, and it becomes certainly a limiting factor in cases of muscle degenerative diseases, such as Duchenne Muscular Dystrophies (DMD). Table 1,presents some examples of proliferative capacity measured in vitro by the group of G. Butler-Browne and V. Mouly .

	infants				adults				DMD	
Age	5 days	5 mo.	9 yrs	15 yrs	26 yrs	28 yrs	81 yrs	86 yrs	7 yrs	11 yrs
n divisions	55-60	46	30	28	19	21	15	15	19	14

Table 1 : Proliferative capacity measured in vitro of human satellite cells isolated from donors of various ages as well as from two patiens suffering from DMD.

The proliferative capacity of the human cells is under the control of a nucleoproteic structure called the telomere. The telomeres are located at the end of all the chromosomes (figure1), they are composed of hundred to thousands double stranded TTAGGG DNA motifs associated with specific proteins (1, 2, 3, 4, 5). The very end of the telomeres is composed of single stranded TTAGGG DNA motifs (The G strand). The protruding G strand is present at the telomeres of human chromosomes (6, 7, 8).

One of the function of the telomeres is to ensure the stability of the chromosomes in the proliferating cells. Due to the properties of the DNA replication machinery, the telomeres become shorter at each cellular division in somatic cells. Once the telomeric DNA becomes too short, this triggers a signal for growth arrest, and cells become senescent. It is thought that pathological processes accelerating the cellular turn over will also lead to senescence *in-vivo* (9, 10), and telomere measurements carried out on muscle biopsies from children suffering from muscular dustrophies have shown a minimum length which is much smaller than the one measured on all adults, including subjects in their eighties (9). The presence of large T antigen from SV40 has been shown to extend the proliferative potential of satellite cells (11, 12), but only by 20-25 divisions, and its expression has been known for a long time to be oncogenic. Therefore, one solution to the problem of the limited proliferative capacity of DMD cells could be to elongate their telomeres.

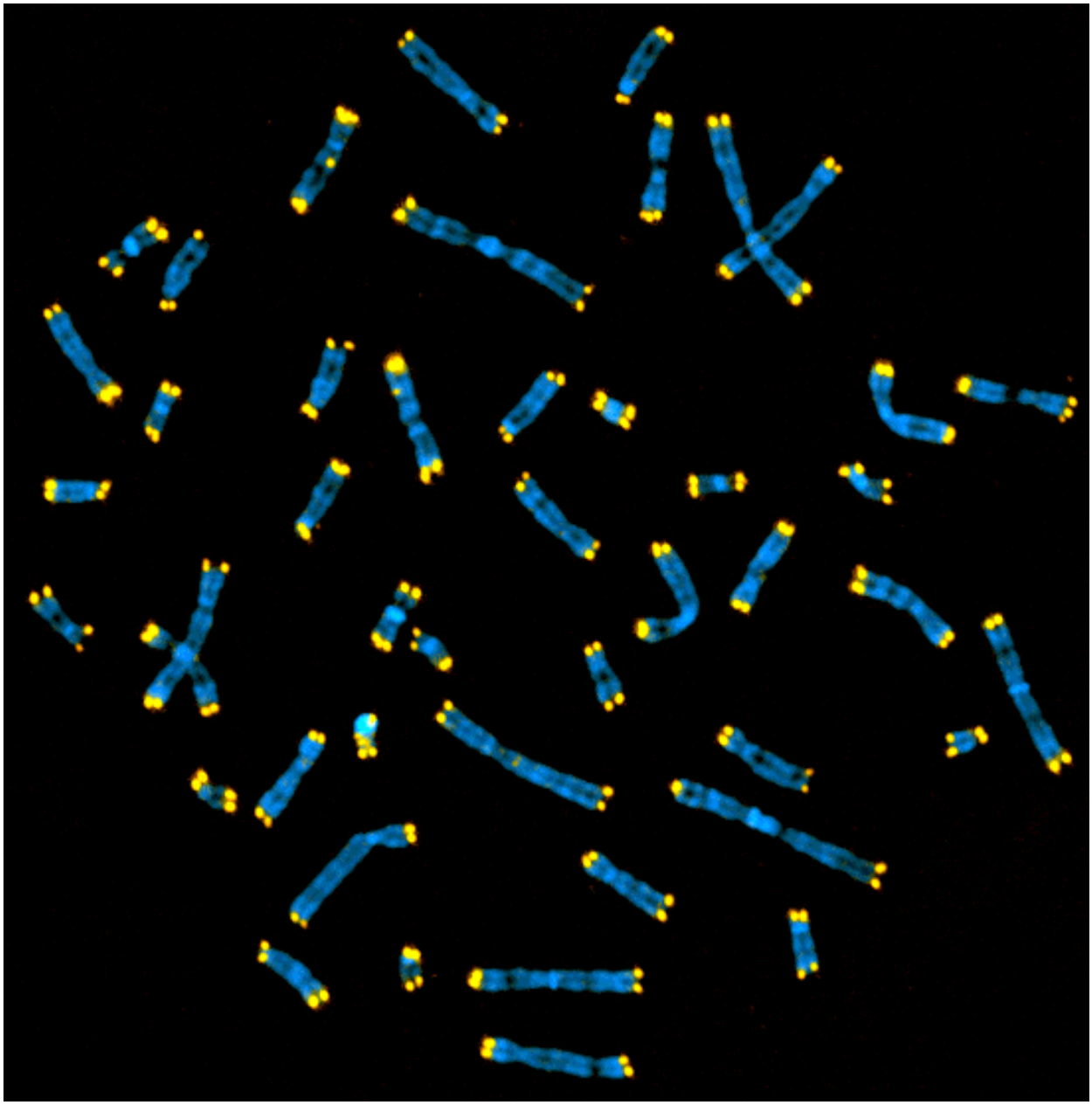

Figure 1

Hybridization of human chromosomes, counterstained with DAPI (blue) from white blood cells with a telomeric PNA probe (yellow).

Telomerase is a ribonucleoproteic enzyme capable of adding TTAGGG DNA motifs on the telomeres. In human cells, the core enzyme is composed of two subunits: the RNA matrix (hTR) and a catalytic subunit (hTERT). The hTR RNA is expressed in all the cells but expression of the catalytic subunit hTERT is restricted to a subset of proliferating cells where a *bona fide* telomerase activity can be detected. These cells include the germ line, stem cells from the bone marrow, differentiated white blood cells, and some cells located in the basal layer of the dermis. Telomerase activity is also detected in embryos during early stages of development (13, 14, 15, 16). Telomerase activity is not detected in other somatic such as human fibroblasts or satellite cells, and telomeres shorten regularly during their lifespan before reaching proliferative senescence. However the introduction of ectopic copies of the hTERT gene into fibroblasts has been shown to greatly extent their proliverative potential, eventually giving rise to immortal clones. This extented potential is due to a lengthening of the telomeres. Extension of proliferative capacity of the satellite cells is currently a priority in our research program, using telomerase in collaboration with the University of Dallas and Geron.

Research project

Cell-mediated gene therapy is based on the graft of autologous cells which have been genetically modified to compensate a loss of function. The genetic modifications require an *ex-vivo* (*in-vitro*) expansion of the cells from the patient. However, the proliferative potential of the satellite cells is limited and the satellite cells encounter an erosion of their telomeres during *in-vitro* expansion (17). The finite proliferative potential of the satellite cells is certainly a key factor limiting the success of a cell-mediated gene therapy based on these cells.

Immortalization of somatic cells allowing the cells to conserve a normal youthful phenotype have been performed in fibroblasts after transfection of the hTERT gene (18, 19, 20). The transfection of the satellite cells by hTERT has been performed. Preliminary results show that these cells have longer telomeres than the parental cells, with an interclone variability in mean telomere length. In contrast with the fibroblasts, the $hTERT^{+}$ satellite cells do not become immortalized, a phenomenon reminiscent from some epithelial cells (21, 22). Therefore, the general aim of my project will be to understand why hTERT expression does not immortalise human satellite cells, as it does with fibroblasts. The behaviour of the hTERT+ satellite cells, raises several questions as to the state of their telomeres (figure2), and some of these questions will be relevant concerning cells isolated from patients suffering from degenerative diseases such as muscular dystrophies :

Amount of TTAGGG DNA motifs at each telomere :
For each hTERT clone, the telomeric FISH signal will be quantified at different passages of the cellular culture (see annexe). This approach allows to know if the ectopic telomerase is able to elongate correctly all the telomeres of the cells (particurlarly chromosomes devoided of TTAGG DNA motifs must be searched). The rate of elongation at individual telomere (on metaphasic chromosomes) will be determined and rapid variations of telomere length will suggest recombination events.

Dynamics of the telomere length at specific homolog chromosome and intracellular heterogeneity of the telomere length and telomeric :
The aim of this part of the project is to determine if the telomerase acts identically on short and long telomeres in each cell (intracellular heterogeneity). For that purpose, it is necessary to use a method to test if there are long and short telomeres in each individual cell, as I observed in primary fibroblasts (unpublished data), telomeres being classified by their telomere length. For that purpose it is necessary to distinguish the homolog chromosomes with additionnal probes producing an heteromorphic FISH signal.

Total amount of TTAGGG in each cell :
To test if there are hTERT cells with big telomeres and cells with small telomeres (intercellular heterogeneity), analysis of the amount of telomere on interphasic cells will be performed by FISH (23) or possibly by flow-FISH. This approach allows to indirectly test the efficiency and the stability of the expression of the ectopic copies of the hTERT transgenes. The presence of a mixture of cells (i.e. cells with big telomered and cells with shorter telomeres) should signal a problem in stability of the long termed expression of the transgenes. This approach will be also

informative about the possible heterogeneity of cell populations isolated from patients, even though these have not received hTERT. Such an heterogeneity, if it is observed, will imply more proliferation from the cells with long telomeres to replace the lost proliferative capacity from the other cells.

Anti-fusion function of the telomeres in senescent hTERT$^+$ satellite cells. :
Chromosomal damages and particularly the formation of terminal dicentric chromosomes (ter-ter) are an hallmark of the senescent cells at the cytogenetic level *in-vitro* (24, 25, 26). In a senescent cellular culture, cells capables of entering the G_2/M phase, where the chromosomes can be identified by their morphology, are very rare. The observed ter-ter dicentric chromosomes might represent the rare end joining events between short or fully eroded telomeres allowing the senescent cells to escape the G1/S blockage.
In order to explore the complete set of events occuring at the telomeres of senescent cells, it is necessessary to monitor the state of the telomeres in interphasic cells which represent the majority of the senescent cells. To test the hypothesis of the senescence induced by a failure of the telomere function in the satellite cells transfected by the retroviral hTERT construction, the premature chromosome condensation (see annexe) coupled with in situ hybridization with a cyanine$_3$ PNA $(CCCTAA)_3$ probe will be used. Here again, this approach will also be applied to cells isolated from patients suffering from muscular dystrophy, for the same reasons as above.

Relationship between hTERT copy number and proliferative potential :
The different clones of satellites cells obtained after transfection of the hTERT gene (e.g. by a retroviral Cre-lox construction) will also be characterized, by FISH, for the number and the location of integration sites which could be multiple as in the case of the integration sites of the HIV provirus (27). The number of integration sites will be determined in order to see if there is a relationship between the copy number of the hTERT transgene and the replication potential of the clones and the distribution of telomere lengths.

Failure of the G strand function :
The senescence of the hTERT$^+$ satellite cells might not be linked to the amount of doubled stranded telomeric sequences, since cells with long telomere are able to enter senescence, but by a disfunction of the G strand making long telomeres equivalent to double strand DNA breaks (This could be the case if strong telomeric signals will be observed at the fusion point of two chromosomes). The analysis of the length of the G tail will be performed by FISH in living cells. Telomeres devoided of G tails might be a signal inducing the senescence of the cells.

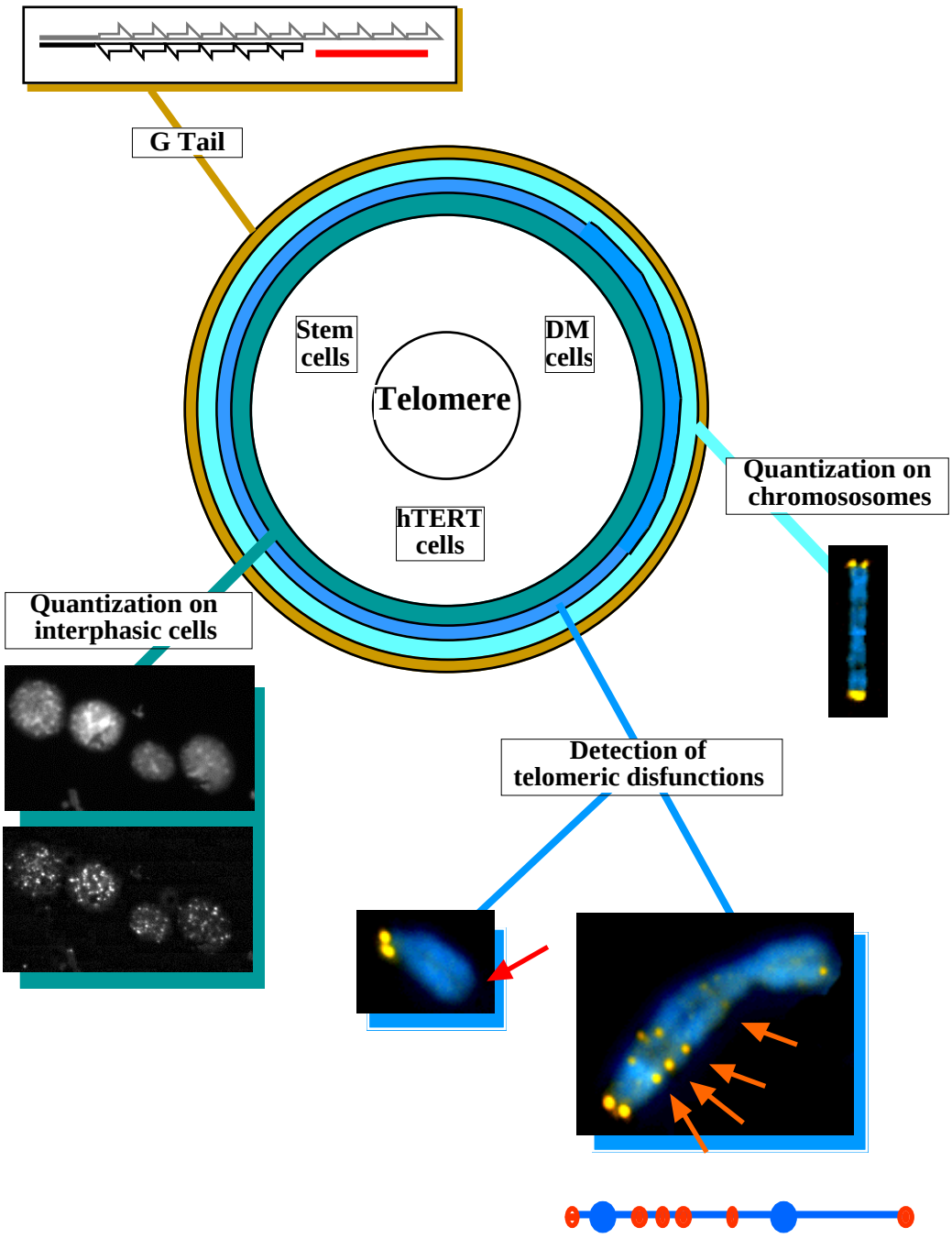

Figure 2

The analysis of the state of the telomeres will be applied in different cellular contexts: in interphasic cells (DAPI:upper panel, telomere:lower pannel); in cycling or in senescent cells. To detect telomeric dysfunction, analysis of the telomeric signals will be performed on chromosomes (exemple from SV40 transformed cells is given, showing a chromosome with multiple chromosomal anomalies at its telomeres).

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Annexe

Quantification of the telomere length at the resolution of the individual telomere

To measure the telomeric FISH signals by epifluorescence microscopy, hardware (band pass filters, highly sensitive CCD camera, …) and specialized software for image processing and image understanding are required. For that reason we decide, dr Laure Sabatier from the Laboratoire de Radiobiologie et d'Oncologie (CEA, France) and myself decided to integrate the methods developped at the LRO for the quantification of the telomeric FISH signals in a new software package. This new software called CytoGenetic Assistant (figure3, a screenshot is visible at http://www.aai.com), has been developped for the LRO by ADCIS from the image processing library of their Aphelion software. CGA integrates a user and files manager system, a module driving a high resolution 12 bits CCD camera (KAF 1600 CCD), a module for the segmentation of the images and for the measurement of the FISH signals, a karyotyping module, and a database browsing module (manuscript in preparation). The main differences between the way I analyse the telomeric FISH signal (28) and the methodology designed by Lansdorp's team in their TFL-Telo software (29, 30, 31) are the following : **Firstly** in the TFL-Telo software the telomeric signals are converted into kilobase unit using an external calibration curve obtained with telomere sequences of known length. The length of these model sequences is far from the length of true telomeres, this problem has been discussed by Krejci and Koch (32). This external calibration, even if the telomere length values are not real, has the advantage to give measures in a unit independent from the conditions of experiments (fluorochrome used for the labelling of the probe, microscope, filters set, spectral sensitivity of the CCD, …). The external calibration has also the drawback not to take into account the variability of the intensity of the signals due to the inherent variations of the rate of hybridization (due to the variation of chromosome spreading from one metaphase to another one (33, 34, 35)). This problem can be circumvented using an internal standardization of the measures of telomeric signals with a simple mathematical transformation. This tranformation provides a dimentionless unit for the telomeric intensities, but the price to pay is that it is no more possible to compare absolute variations of the telomere length, nevertheless this methods has allowed a robust comparison of the relative telomere length in fibroblasts during *in-vitro* senescence, combined with multivariate statistical analysis different subclasses of telomere length have been identified, systematic telomeric heteromorphism have been searched in order to distinguish the telomeres of homolog chromosomes in the analysis, and rapid telomere length variations have been detected during the senescence of fibroblasts. Furthermore this method allows to compare telomeres from experiments performed with different fluorochromes, filters, and cameras (Telomere-Telomerase congress, Cold Spring Harbor 1999 ; manuscript in preparation). External calibration remains possible in absence of model molecules with a fluorescent slide such a GG17 fluorescent standard (Karl Zeiss), the measure unit is simply expressed as a percentage of the mean grey levels flow of the flatfield image obtained with the GG17 slide. **The second difference** between Cytogenetic Assistant software and the TFL-telo sofware, is that the measurments of the telomeric signals related to each homolog chromosome are coded in a 2D vector while the results obtained with TFL-Telo were presented has unidimentional distributions. The use of 2D vectors can be used to analyse the telomere length distribution of one specific homolog chromosome when a heteromorphism is available. I plan to extend this analysis to higher dimentional vector by the quantization of other repeated sequences.

Multiple hybridization scheme

Additional FISH probes will be used with the telomeric probe. Pancentromeric alphoïd probes will be used to increase the robustness of the identification of possible dicentric chromosomes, and other repeated sequences will be used to give an internal reference in order to compare the telomere length from one cell to an other one. This internal calibration of the telomeric signals measurements will allow to compensate the variability of the FISH signals inherent to this methodology and which cannot fully overcome by the use of an external calibration.

Oligonucleotidic probes recognizing all of the chromosomes or specific chromosomal alphoïd motifs, microsatellites repeats (CCG_n, CTG_n, $AATGG_n$), 5S-2.2 sequences, and probes recognizing some minisatellites sequences will be used in conjunction with the telomeric $(CCCTAA)_3$ cyanine3 labelled PNA probe. This work will be performed in collaboration with the Laboratoire de Radiobiology et d'Oncologie where the cytogenetic experiments will be performed. Since microscopes with four positions wheel filters are available at the LRO, the fluorochromes labelling the probes will be restricted to the FITC, Cyanine3 and Cyanine5. DAPI will be used to counterstain the chromosomes. There are multiple aims for such kind of hybridization scheme : it will allow to distinct more homolog chromosomes, it will allow to perform internal and absolute calibration of telomeric FISH signals, it will allow to karyotype easier without performing sequential hybridization with whole chromosome painting probes.

Analysis of the telomere length by FISH in senescent hTERT transfected satellite cells after premature chromosome condensation.

The premature chromosome condensation (PCC) technique has been used to detect the chromosomal rearrangement in cell lines (36) and to study the kinetic of chromososomal reparation after irradiation (37, 38, 39). This technique allows one to reveal the chromosomal damage in the interphasic cells which normally escapes the conventional cytogenetic analysis, by forcing the condensation of the chromosomes of cells blocked in interphase. Different protocols have been used to induce the PCC, the induction can be performed by fusing the blocked cells with cycling cells (CHO cell line or Hela cells) or by chemical treatment (40, 41, 42). The chemical treatment, compared to the method by cellular fusion, overcomes the difficulty to distinguish between the chromosomes issuing from the inducing cells and the chromosomes to be analysed. Nevertheless, even using cellular fusion PCC, it is still possible to detect the PCC inducing cells without ambiguities : PCC inducing cells can be cultured to incorporate BrdU. A lymphoïd cell line, with a high mitotic index, can be chosen as the PCC inducer. After cellular fusion, the chromosomes of the inducing cells can be easily recognized by hybridization with an FITC labelled anti BrdU monoclonal antibody, whereas the chromosomes of the target cells will not be labelled. Both treatments (*i.e.* by cellular or chemical induction) will be tested since the PCC response of senescent satellite cells is unknown.

Detection of chromosomal instability at the telomeres

Hybridization of $(CCCTAA)_3$ on metaphases preparation from SV40 T antigen transformed fibroblasts has already allowed to detect rearranged chromosomes with strong telomeric signals at interstitial location as well has chromosomes devoided of signal at their telomere (23), thus this approach will be capable to detect events, in senescent hTERT satellite cells, which cannot be observed by a simple analysis of the telomeric restriction fragments length distribution performed on genomic DNA by Southern blot, particularly the absence of TTAGGG

motif at a telomere or recombination between telomere leading to interstitial telomeric sequence (this event could theorically observed by Southern blot after digestion of the genomic DNA with the nuclease Bal31, but the fusions should occur at a high rate to be detectable).

The use of subtelomeric probes (43) can also allows to distinguish true telomeric dicentric chromosomes from other possible dicentric chromosomes generated implicating other sequences of the genome.

Analysis of the function of the protruding TTAGGG DNA strand in living cells by fluorescent (CCCTAA)n PNA probes.

The PNA molecules mimics the structure of DNA oligonucleotids, they are stable *in-vivo* (44) making them suitable for antisens drug therapy. Until now, only $(TTAGGG)_n$ PNA probes have been used for antitelomerase strategies in the optic of the cancer therapy. The $(TTAGGG)_n$ PNA probe inhibits the telomerase activity *in-vitro* (45, 46, 47), as well as in cell lines (48).

Since the amount of double strand telomeric sequence might not be the only factor determining the life span of the cells, if the double strand telomeric motifs serves as a stock of G tail, binding the G tail with a $(CCCTAA)_n$ PNA probe in living cells is interresting for different reasons. **Firstly** a $(CCCTAA)_n$ PNA probe might interfere with other intracellular G tail binding proteins which could have effects on the equilibrium between elongation and shortening of the telomeres. Such interaction might favor the equilibrium towards elongation and may be to an extend life span of the cells which would be reminiscent of results of oligonucleotids effects on the life span of telomerase$^-$/telomerase$^+$ hybrid cells (49). **Secondly** treatments of the hTERT transgenic satellite cells might present differential effects beyond the different clones (on their chromosomal stability for example), which might gives insight into the dynamic of the G tail which is important in the life span of telomerase negative cells (50). A differential effect beyond the clones might help in identifying G tail binding factors as the A1 and the UP1 ribonucleoproteins (51). **Thirdly** a treatment of living cells with a fluorochrome labelled $(CCCTAA)_n$ PNA probe might reveal the organization of the telomeres in a living cell. Organization of the chromosomes in living cells have been performed by micro injection of cyanine3-dUTP (52). Since a telomeric signal can be detected in certain donnor at the 2q13-q14 location (Pommier, unpublished data), a region containing a few non degenerated TTAGGG motifs (53), fluorescent $(CCCTAA)_n$ PNA probe bound to G tails might produce a signal strong enough to analyse the topography of the telomeres as in the case of the effect of $p21^{WAF-1}$ in the topography of alphoid sequences (54), and finally might estimate the length of the G tail chromosome per chromosome. A differential effect might be observed between the hTERT clones of the satellites cells which do not have an immortal phenotype but also between the satellites cells and hTERT immortalized fibroblasts.

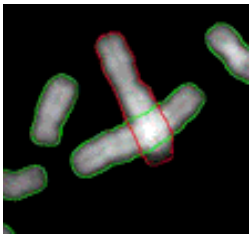
Figure 3 :

Graphical user interface of the *CytoGeneticAssistant* software package. **A** : modules bar used for calling the different modules of CGA. **B** : Images manager bar. **C1** :Detection of the chromosomes counterstained with DAPI, **C2** :Resolution of overlapping chromosomes. **D** : Detection of the telomeric FISH signal (the spots located on the short arm of the chromsomes are labelled in red, the spots located on the long arm of the chromosomes are labelled in yellow). **E1** :tools for the chromosomal component of the image, **E2** :tools for the telomeric component of the image. **F** : detail of the karyotyping module. **G** :settings of the CGA modules, different algorithms are available for the segmentation of the chromosomes, of the telomeres ; the number of chromosomes in the karyotype can be set by the user allowing to analyse the telomeres in different species. **H** :module for the extraction of the measures.

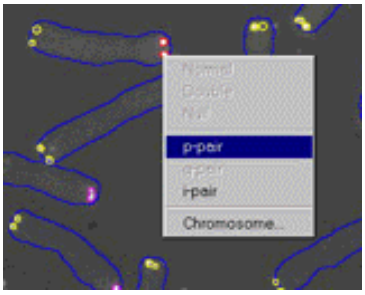
A

B

C1



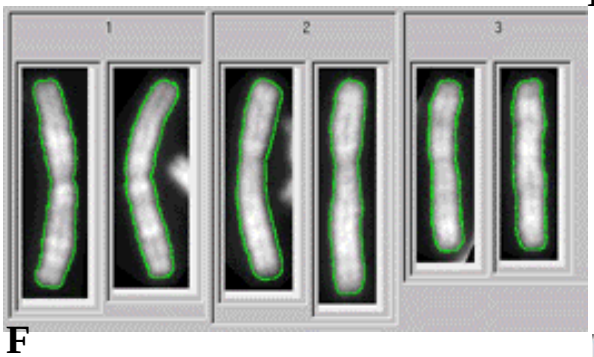
C2



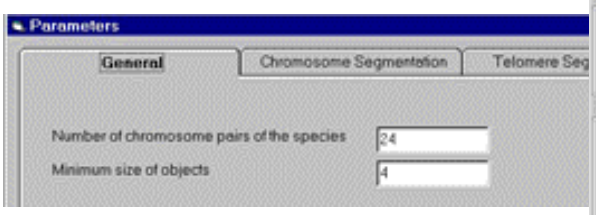
D

E1

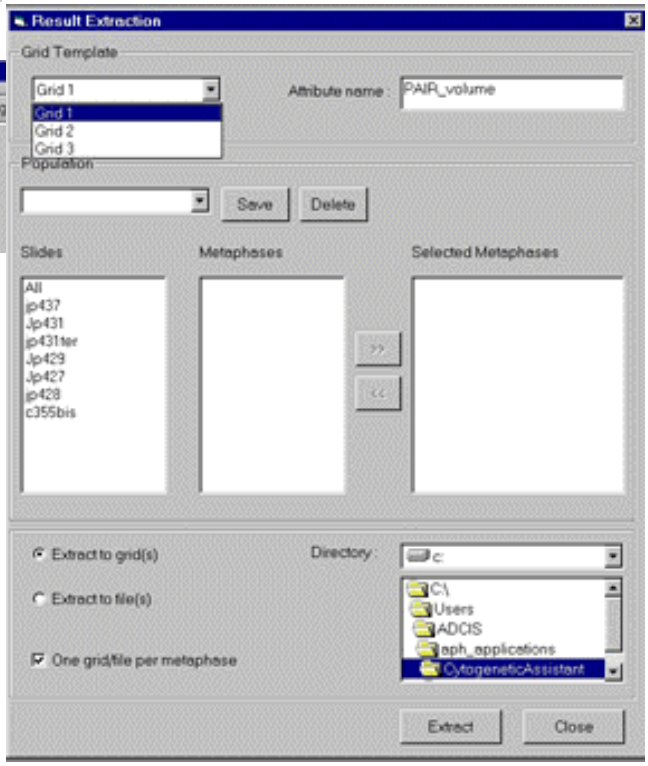
E2



F



G



H

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Formation

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