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Abstract

In recent years, growing evidence has shown that mutations of mitochondrial DNA (mtDNA) are an important cause of mitochondrial disorders in humans, and have been associated with common neurodegenerative disorders, aging and cancers. In line with this, it has been proposed that those mutations could genetically predispose an individual to some environmental factors thereby initiating the disease process. To test such a hypothesis in Parkinson's disease we proposed to: 1) develop an animal model with accumulated mtDNA mutations in catecholaminergic neurons by creating a transgenic mouse containing a tyrosine hydroxylase (TH) promoter-driven transgene encoding a proofreading-deficient mouse mtDNA polymerase (Pol γ), 2) testing whether those animals are more susceptible to mitochondria-specific environmental toxins and aging 3) investigating the underlined molecular mechanism.

In this annual report, we describe the characterization of a transgenic mouse line which produces at high level a proofreading-deficient mouse mtDNA polymerase (pol γ) in TH expressing tissues at different developmental stages (from embryogenesis to adult). Then, we describe the strategy that we are currently using to perform a sequence analysis of the mtDNA isolated from the ventral mesencephalon of transgenic animals. This study should tell us whether mtDNA mutations accumulate in catecholaminergic tissues in this experimental model.

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I) Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. While present worldwide and in all populations, its prevalence is however the highest in Western world including North America and Europe (estimated at around 160/100.000) (de Rijk et al., 2000). Given that the incidence of PD rises with age, it is expected that the number of individuals suffering from this devastating disease (e.g. more than one million in USA alone) will increase significantly in the future because of the aging character of society. The cardinal clinical features of PD include resting tremor, rigidity, slowness of movements and postural instability (Fahn et al., 2000). These motor manifestations are the consequence of brain dopamine depletion, which result from the progressive and selective loss of dopaminergic (DA) neurons in the mesencephalon. Although many efforts have been made to unravel the origin of PD, its etiology still remains a mystery. Among different hypothesis, it has been proposed that genetic, aging, as well as environmental factors may contribute significantly to PD (Olanow and Tatton, 1999). However, based on our present knowledge, it is unlikely that in the majority of cases, PD can be explained by a single cause. Instead, another conceivable explanation is that PD may result from a more complex interaction between genetic mutations and/or a mutant gene and an environmental toxin. The present research project will aim at testing, *in vivo*, this "double hit" hypothesis for PD.

Our central hypothesis is that, in patients with PD, accumulated mtDNA mutations in dopaminergic neurons may act as an important vulnerability factor to environmental toxins. This hypothesis is inspired by several observations: 1) a specific deficiency in complex I activity is observed in parkinsonian patients, 2) mitochondrial gene-transfer experiments (cybrid technique) demonstrate that mitochondrial dysfunction in PD has a genetic basis in mitochondria, 3) it has been shown recently that mutations in ND5 mitochondrial was associated with idiopathic PD (Parker and Parks, 2005), 4) mitochondrial dysfunction in PD is associated with increased oxygen radical production and susceptibility to mitochondrial toxin-induced cell death as shown by *in vitro* assays.

To provide, *in vivo*, functional mechanistic evidence for such a hypothesis, we first need to develop a rodent model in which mtDNA mutations are induced to accumulate in dopaminergic neurons, the neuronal population that degenerate in PD (see technical objective 1). For this purpose, we hypothesize that mice expressing a proofreading-deficient mitochondrial polymerase (pol γ) will accumulate mtDNA mutations due to reduced fidelity of the mtDNA replication. This strategy has been successfully employed recently by Peter Zassenhaus's group in generating mice with tissue-specific acceleration of mtDNA mutagenesis (Zhang et al., 2000). By driving transgene expression using a tyrosine hydroxylase (TH) promoter (the rate limiting enzyme in dopamine synthesis and specific marker of dopaminergic neurons), we expect to induce accumulation of mtDNA mutations specifically in dopaminergic neurons in these animals.

STATEMENT OF WORK

The proposal sets out to demonstrate *in vivo* whether dopaminergic neurons with high mtDNA mutational burden have increased sensitivity to mitochondrial toxin-induced cell death or aging and to define the underlined mechanism. The proposed timeframe includes specific objectives for three years.

Development of a mouse model with accumulated mtDNA mutations in dopaminergic neurons. (months 1-24)

- a) create a transgenic mouse containing a tyrosine hydroxylase (TH) promoter-driven transgene encoding a proofreading-deficient mouse mtDNA polymerase (Pol γ) (months 1-6).
- b) Establishment of transgenic mouse colonies (months 6-11).
- c) Investigate the transgene expression pattern temporally and regionally (months 8-18)
- d) Investigate the mutagenic status of those animals (months 9-24).

Evaluate the sensitivity of proofreading-deficient Pol γ transgenic mice to mitochondrial toxins and aging. (months 13-36)

- a) Adapt the annonacin model developed in the rat to the mouse (months 13-18).
- b) Compare the toxic effect of two well-characterized complex I inhibitors (MPTP and annonacin) on dopaminergic neurons between transgenic and non transgenic mice (months 13-24).
- c) Investigate the effect of accumulated mitochondrial DNA mutations in catecholaminergic neurons during normal aging (months 13-36).

Analyze the mechanism of mtDNA mutation-induced dopaminergic cell vulnerability. (months 24-36)

- a) Analyze the respiratory function (measured by oximetry using a Clark-type electrode) (months 25-26).
- b) Characterize oxidative stress (oxyblot and AGEs detection) (months 25-26).
- c) Study alteration in protein processing (immunohistochemical detection of intracellular inclusions and proteasome activity measurements (months 26-29).
- d) Investigate the expression level of pro-apoptotic signaling pathways (expression levels and localization of apoptosis-related factors) (months 30-36).

In this first annual report we describe the development of a mouse model genetically designed to accumulate mtDNA mutations in catecholaminergic neurons. For

this purpose, we created a transgenic mouse containing a tyrosine hydroxylase (TH) promoter-driven transgene encoding a proofreading-deficient mouse mtDNA polymerase (pol γ). Furthermore, we investigated the transgene expression pattern temporally and regionally and have selected a transgenic founder which displays a correct spatio-temporal transgene expression. From this founder a mouse colony has been established. Finally, we started to investigate the mutagenic status of those animals

II) Body

A. Production and characterization of a proofreading deficient-pol γ expressing mouse line

1) Production

The development of transgenic mice with TH promoter-driven expression of mutant Poly has been performed in collaboration with Dr. Hans Peter Zassenhaus (Saint Louis University, MO, USA) and Pr. Richard A. Flavell (Yale University, CT, USA). A 4.5 kb 5' flanking sequence of the rat TH gene containing vector was kindly provided by Dr. Dona Chikaraishi (Duke University Medical Center, NC, USA). This rat TH promoter (accession # AF069036) has been previously used and characterized in transgenic mice expressing an alkaline phosphatase reporter gene (Schimmel et al., 1999). It contains essential consensus sites for transcription factors which are required for early differentiation of mesencephalic neurons. The mutated version of the mouse Pol γ cDNA was obtained from Dr. Zassenhaus and has been previously characterized both *in vitro* and *in vivo* (Zhang et al., 2000).

To avoid any effect of the cloning strategy on transgene expression we decided to make two different expression vectors based on alternative insertion of either the promoter or the mutated Pol γ cDNA cassettes.

The first strategy consisted in inserting a *EcoRV/SmaI* restriction fragment from the 4.5 kb rat TH promoter containing vector by blunt end ligation into the *FseI* cloning site of the mutant Pol γ cDNA vector. The transgene cassette was excised from the vector by *NruI* digestion to get rid of bacterial vector sequence, purified and injected into fertilized B6C3HF1 eggs

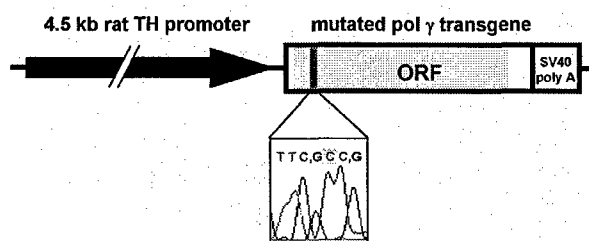


Figure 1: Schematic representation of the TH-mutated pol γ construct. The arrow represents the 4.5 kb rat TH promoter. The box represents the mutant pol γ cDNA followed by an SV40 splice poly A cassette. The light grey box represents the transgene open reading frame. Site-directed mutagenesis (dark grey line) changed a A to C nucleotide (yellow box)

located in a codon for an aspartate residue at position 181. This nucleotide change replace the aspartate residue by an alanine and also, converts a preexisting *TaqI* restriction site to a *DsaI* site.

A first set of 9 founders (named Pol-TH) has been obtained using this construction. Four of these founders failed to give offsprings and, therefore, were not analyzed further. The five remaining founders were characterized for transgene expression in different organs (brain: cortex, striatum, cerebellum, ventral mesencephalon, dorsal mesencephalon; liver; spleen; gonads; heart, see below).

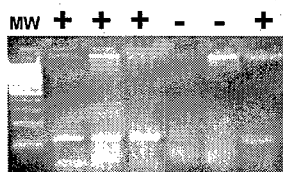


Figure 2: Identification of positive (+) transgenic animals by PCR performed on DNA isolated from tail. The primer pair used for PCR amplify a 117 bp product from the transgene whereas from the endogenous gene, the product is much larger due to the presence of an intron. (MW: DNA ladder).

For the second construction, the transgene (see figure 1) was constructed by inserting a *MluI-BclI* restriction fragment from the mutant Pol γ cDNA clone by blunt-end ligation into the *SmaI* cloning site of the 4.5 kb rat TH promoter containing vector. The transgene cassette was excised from the vector by a double *Sall-EcoRV* partial digestion to get rid of bacterial vector sequence, purified and injected into fertilized C57Bl/6 eggs.

Using this construct, a second set of 20 new founders (named TH-Pol) has been produced and bred in order to produce offspring which have not been obtained so far.

2) Characterization of transgene expression :

In the initial proposal, we suggested to use the strategy described by Zhang et al, 2000, to analyze transgene expression. Using this approach, cDNA obtained by mRNA retrotranscription, is PCR amplified using radiolabeled nucleotides. Then, PCR products are digested with *DsaI* or *TaqI* restriction enzymes (in order to identify Pol γ cDNA produced by the transgene and the endogenous gene, respectively) and resolved on acrylamide gels followed by quantification using a phosphor imager. Yet, recent technical developments in our laboratory, now allow to increase the sensitivity of PCR fragment detection by using fluorescent-labeled nucleotides coupled with Snap shot analysis.

Thus, we first isolated mRNA from different organs (brain: cortex, striatum, cerebellum, ventral mesencephalon, dorsal mesencephalon; liver; spleen; gonads; heart) using trizol extraction method (Invitrogen). Purified mRNA was then treated with DNase to get rid of DNA contamination (RNA easy mini kit, Qiagen). Retrotranscription was performed using thermoscript RT-PCR system (Invitrogen) and cDNA was amplified using primer pairs flanking the mutation and giving rise to a 117 bp product. We used two different pairs of primer (see figure 3). Within each pair, one of the primer was fluorescent-labeled with FAM. PCR products were digested by *DsaI* and *TaqI*. The A to C nucleotide change in the transgene replaces the aspartate residue by an alanine and also, converts a preexisting *TaqI* restriction site (endogenous) to a *DsaI* site (transgene). Before Snap shot analysis, we pre-screened founder lines by resolving PCR products on 4% agarose gel to grossly check for possible ectopic transgene expression. Two major criteria of selection were chosen: 1) a high transgene expression in the ventral part of the mesencephalon in which the SN locates, and 2) a correct spatial transgene expression within the transgenic mice. Thus, mice displaying a high ectopic transgene expression were excluded from further analysis (table 1). Preselected founders were then qualitatively analyzed by fluorescent measurement (GA3720 DNA analyser, applied biosystem) (figure 5).

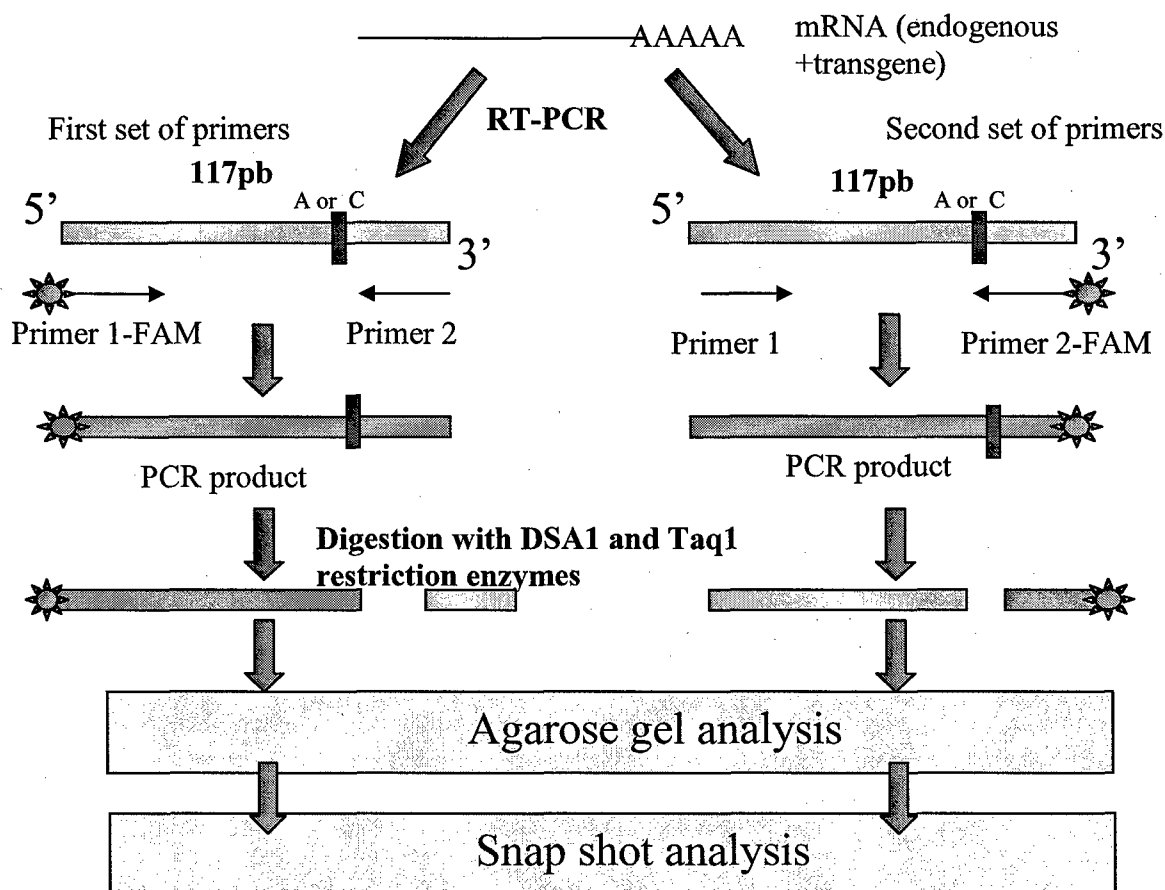


Figure 3 : Schematic representation of the strategy used to determine transgene expression levels. Isolated total mRNA contains both endogenous Pol γ and transgenic mutated Pol γ copies. After PCR amplification of cDNA using FAM labeled primers, PCR products are digested by either *Dsa1*, or *Taq1*. *Dsa1* only digests transgenic PCR product, while *Taq1* digests the endogenous PCR product. This differential identification allows to measure the proportion of each copy. * : FAM labeled-nucleotide. Blue box: non labeled DNA; green box: labeled DNA; Red box: putative site of mutation (A: nucleotide for the endogenous form; C: nucleotide of the mutated form)

A total of 5 Pol-TH (12 month old) were analyzed using this strategy (table 1).

Our results showed that **founders # 1, 2, 4 and 5** displayed high transgene expression in all analyzed regions of the central nervous system (Table 1). Therefore these lines were excluded from the study. In addition, founders # 2, 4 and 5 presented high ectopic expression in peripheral organs such as heart and liver. This ectopic expression could be explained by the topology of the transgene insertion site where specific enhancers may modulate such expression.

In contrast, Founder # 3 displayed an expected expression pattern in the CNS (Table 1). In this founder line, a mild expression was observed in the striatum and the dorsal mesencephalon, together with a strong expression in the ventral part of the midbrain containing the SN.

All other tissues, but the spleen, were devoided of transgene expression. Interestingly, TH activity has been previously described within lymphocytes (Qiu et al., 2004), which could explain such splenic expression.

	Founder 1	Founder 2	Founder 3	Founder 4	Founder 5
Cerebellum	++	++	-	++	+
Striatum	++	+++	+	++	+
Mesen. Ventral	+++	+++	++	++	++
Mesen. Dorsal	++	+++	+	++	+
Cortex	++	++	-	++	+
Gonads	ND	ND	-	-	-
Heart	ND	++	-	++	+
Liver	ND	+	-	-	+
Spleen	ND	ND	+	ND	ND
Kidney	ND	+	-	-	-
Muscle	ND	++	ND	ND	ND

Table 1: Transgene expression study of Pol-TH founders by agarose gel analysis. +: positive for transgene expression; -: negative for transgene expression; ND: not determined

Altogether, these data show that founder #3 (named Pol-THL3) displays the correct transgene expression profile. This line was therefore analyzed using Snap shot technique (Figure 4).

Carreful spatial analysis of transgene expression in the Pol-TH L3 confirmed our results obtained with agarose gel analysis. Importantly, a strong transgene expression (80%) could be observed in the ventral part of the mesencephalon, together with a mild expression in the striatum (27%), the projection site of nigral dopaminergic neurons (Figure 4). In the dorsal mesencephalon, which contains the catecholaminergic nucleus A8, transgene expression could also be detected (24%). Only very weak expression was observed in the cerebellum and cortex which was not detected by agarose gel analysis. This expression probably reflects transgene mRNA content in dopaminergic nerve terminals.

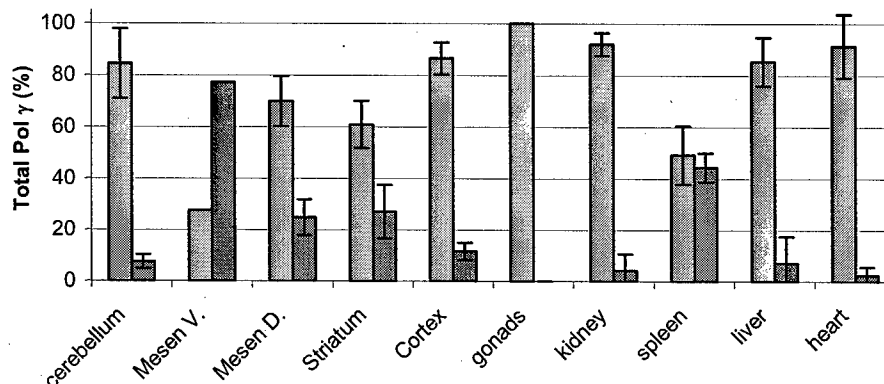


Figure 4 : Founder # 3 : Quantitative analysis by fluorescence measurement of the *DsaI* -(Pol γ mutated form, purple boxes) and of *TaqI*-digested (endogenous Pol γ form, blue boxes) fragments in the cerebellum, ventral mesencephalon (Mesen V.), dorsal mesencephalon (Mesen D.), striatum, cortex, gonads, kidney, spleen, liver and heart.

In peripheral organs, a strong expression was observed in the spleen confirming our previous data. Moreover, a very weak expression could also be detected in kidney, liver and heart. Schimmel et al., 1999 reported that the rat TH promotor was active in the adrenal medulla but also in kidney mesonephric tubules. Therefore, transgene expression in the kidney may be explained by such TH promotor activity. Transgene expression in the heart and liver although very weak, is more puzzling but could result from possible expression leakage. Yet, there is an important sympathetic innervation of the liver parenchyma and heart tissues (Nyquist-Battie et al., 1994 ; Berthoud, 2004). Therefore, such a weak expression could also result from the presence of transgene-derived mRNA content at the synaptic level.

Interestingly, low transgene expression (cortex, cerebellum, liver, heart) was not observed in our first screening using agarose gel. Therefore, the fluorescence measurement method was much more sensitive since it has allowed to detect very small amounts of transgene mRNA.

In summary we have identified a founder carrying the transgene with correct spatial expression of mutated Pol γ .

A mouse line has been established from this founder. Although the first offsprings were quite difficult to obtain for unknown reasons, now the reproduction rate is normal and we have obtained a pool of transgenic animals. Animals are backcrossed with C57/B6J in order to obtain a pure genetic background suitable for further experiments using mitochondrial toxins (see proposal).

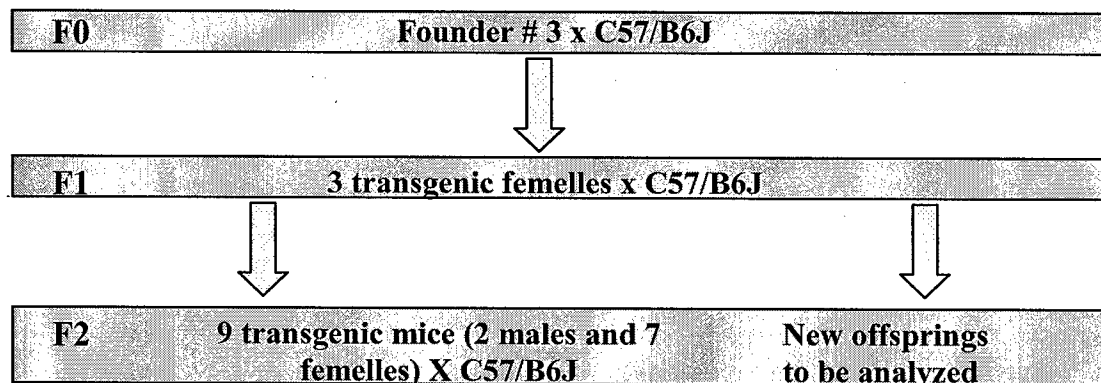


Figure 5: Schematic representation of the mouse line established from Pol-TH founder # 3.

Our second goal was to analyze the temporal transgene expression pattern. Indeed, it is important to appreciate such parameter to make sure that the transgene is expressed early during development when neural progenitors proliferate. From a theoretical point of view, early transgene expression is a guaranty to get high mutational burden within mitochondrial DNA. The rat TH promotor is active as soon as E10,5 and is expressed throughout the development (shimmel et al, 1999).

In order to characterize transgene expression during the development, we used the same strategy as described in figure 3. At E10,5, E14,5, and P0 stages, the entire mesencephalon was dissected. At 0.5, 2 and 12 months, only the ventral part of the mesencephalon was recovered. Total mRNA was extracted and processed as described in figure 3.

So far, only E14,5, P0 and 12 months stages have been analyzed. The other stages are under investigation.

Our data revealed that the transgene is strongly expressed as soon as embryonic day 14,5. In addition this expression appears stable over time (70% in average, Figure 6).

It is important to note that, because the whole mesencephalon has been dissected at E14,5 and P0 stages, the expression level of the mutated Pol γ in TH expressing cells is virtually much higher than that detected here since these cells only represent a small part of total cells within this tissue (other neurons, glial cells, etc...).

Moreover, a stable and high transgene expression from E14,5 to 1 year stages indicates that the mutated Pol γ is predominantly expressed in TH-positive cells throughout animal life and that transgene positive cells are likely to accumulate mutations in the mtDNA.

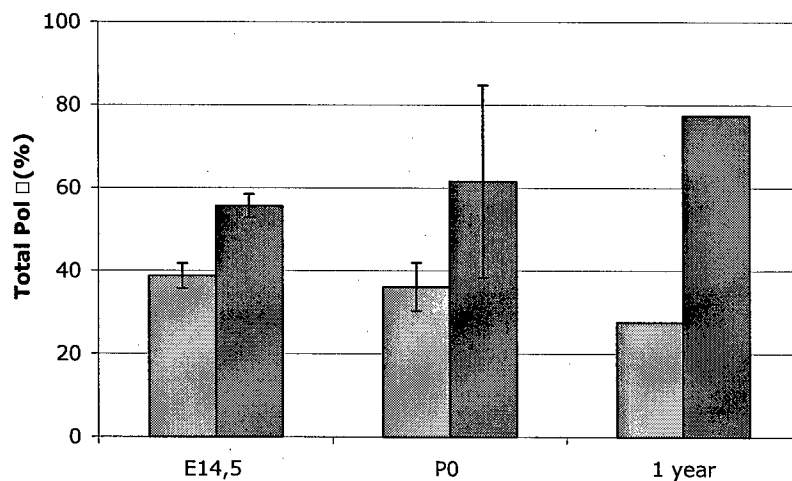


Figure 6: Temporal transgene expression in the mesencephalon. Blue boxes: endogenous Pol γ . Purple boxes: mutated Pol γ .

Altogether, these results confirm that the TH-Poll3 mouse line expresses high level of transgene in a correct spatio-temporal manner and represents therefore a good candidate for further studies. The next step of the project will be to perform sequencing analysis of the mitochondrial genome isolated from transgenic and wild type animals.

B. Mitochondrial DNA Sequencing Strategy

In the initial proposal we suggested to use the same strategy as described by Zhang et al., 2000 to determine the rate of mtDNA mutations. In this approach, mtDNA is extracted and purified from target tissues and then digested by *Bst**I* restriction enzyme (figure 7). The resulting restriction fragments are directly cloned into a *Bam**HI* restriction site from a convenient plasmid.

However, after careful examination of this strategy, we realized that some fragments were underrepresented in the final sequence analysis.

Therefore, we decided to adapt this strategy in order to perform a more complete sequencing analysis of mtDNA. In addition, we chose to perform this study using homozygous transgenic mice in order to increase the expression level of the mutated Pol γ and, thus, to amplify the mutational rate. Such strategy should allow to overcome the dilution effect that arises from non-mutated mtDNA derived from TH-negative cells (glia and neurons) and TH expressing cell (heteroplasmic phenomenon).

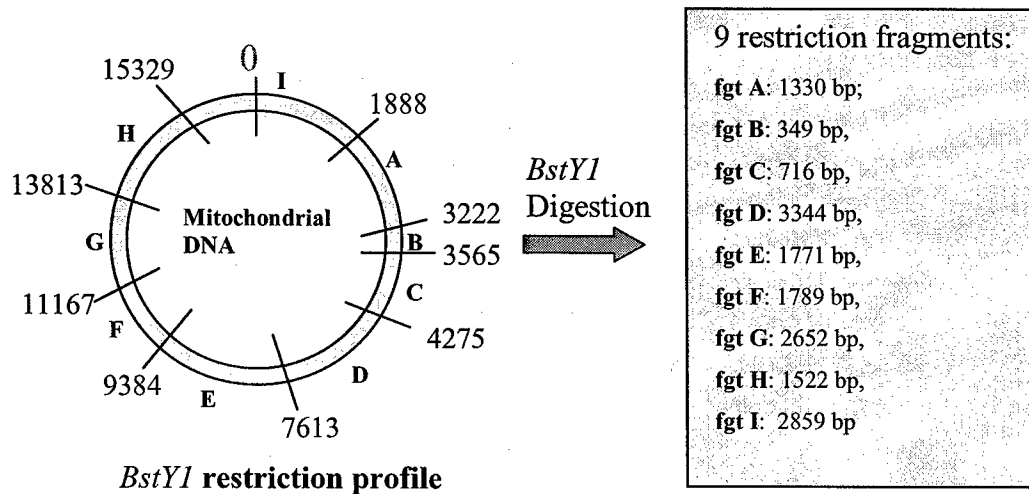


Figure 7: BstYI restriction profile. Fgt: Digestion fragment. **Black line:** BstYI restriction site.

Our cloning strategy was as followed:

Analyzed tissues:

3 different tissues have been dissected:

- 1) Ventral mesencephalon from 5 transgenic mice (2 month old): It is expected that this tissue will display a high mutation frequency since strong transgene expression was found throughout the life of the animal. (figure 6).
- 2) Heart from the same transgenic mice : This tissue is expected to have a low mutation frequency, since the transgene is almost absent in this tissue (figure 4).
- 3) Ventral mesencephalon from 5 wild type mice: This tissue is expected to have a very low mutation frequency since the transgene is not expressed at all.

Isolation of mtDNA:

Tissues have been homogenized in a glass potter. A first centrifugation at 1000g for 10 minutes has been conducted in order to remove heavy material. The supernatant was then centrifuged in a ficcol gradient (7,5% to 10%). The pellet containing mitochondria was recovered and mtDNA was then extracted using the mtDNA extractor CT kit (Wako).

First cloning step:

MtDNA from the 3 tissues was digested with *Bst*YI. Digestion products were resolved on agarose gel and each expected band, (see figure 7) was isolated using a DNA gel extraction kit (Qiagen), and cloned into a BamHI linearized pBluescript SK(+) sequencing vector.

The pBluescript SK(+) contains a convenient multicloning site for further cloning experiments (see below), and allows to perform white/blue screening for positive selection.

Tissue 1: Ventral mesencephalon from transgenic mice

In order to sequence the equivalent of 25 mtDNA molecules, 25 independent clones from each fragment (A, B, C, D, E, F, G, H, I, see figure 7) have to be analyzed.

Tissue 2 and 3: In order to sequence the equivalent of 12 mtDNA molecules from each tissue, 12 independent clones from each fragment (A, B, C, D, E, F, G, H, I, see figure 7) have to be analyzed.

At this step, 441 plasmids have been obtained (245 plasmids from the mutated Pol γ expressing tissue). Among these plasmids, 98 (from B and C fragments, 349bp and 716bp respectively) are directly sequenced. Because all other fragments (A, D, E, F, G, H, I) are too long for direct sequencing, a second step of cloning has been designed.

Second cloning step :

Within each fragment (A, D, E, F, G, H, I), convenient restriction sites were chosen for easy cloning of short restriction fragments into the pBluescript SK(+) multicloning site. Each 25 independent clones from each fragment is cloned using the same strategy. After white/blue screening and clone analysis, a total of 1421 plasmids were ready for DNA sequencing.

Therefore a total of 1519 (98+ 1421) plasmids will be sequenced

Sequencing and bioinformatics analyses:

All sequencing and bioinformatic analyses are performed by Genoscreen company (Lille, France).

A DNA sequence change will be scored as a mutation (as opposed to a naturally occurring polymorphism) if all four of the following criteria are met:

- (1) all mutations should be confirmed by sequencing of the complementary strand,
- (2) mutations must not have been previously reported in GenBank,
- (3) at least three independent clones of that region must have given a wild type sequence.
- (4) if a mutation is overrepresented, this mutation should not be found in control tissues.

Following these criteria, a mutational rate will be calculated for each tissue and then compared.

III) Key Research Accomplishment

- Devevelopment of a transgenic mouse containing a tyrosine hydroxylase (TH) promoter-driven transgene encoding a proofreading-deficient mouse mtDNA polymerase (Pol γ).
- The Spatio-temporal transgene expression pattern was succesfully determined.
- A transgenic mouse colony has been established.
- We established the mtDNA sequencing strategy.

IV) Reportable outcomes

N/A

V) Conclusions

During the first awarded year, we identified a transgenic mouse carrying the tyrosine hydroxylase (TH) promoter-driven transgene encoding a proofreading-deficient mouse mtDNA polymerase (pol γ) This mouse presents a strong transgene expression in TH expressing tissues without ectopic expression in other critical tissues including the heart. Furthermore, this transgenic mouse displays a correct spatio-temporal expression of the transgene. Importantly, the mutated Pol γ is strongly expressed in the SN, and therefore mtDNA mutation should accumulate in nigral dopaminergic neurons. To confirm this contention, we are now trying to determine by sequencing analysis whether high mutational rate in catecholaminergic neurons can be generated in this experimental model. Among future developments, we will test the susceptibility of those transgenic mice to mitochondrial toxins and aging.

Overall, all major aims of the project for the first awarded year have been accomplished.

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VII) Appendices

N/A