Hypothesis

Introns, protein syntheses and aging

Marguerite Picard-Bennoun

Laboratoire de Génétique, Université Paris XI, Bâtiment 400 (UA CNRS no. 086), 91405 Orsay Cédex, France

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In the fungus *Podospora*, a correlation has recently been established between the presence of circular DNA molecules arising from the mitochondrial genome (SEN-DNAs) and the senescence syndrome. Here, I propose a hypothesis which accounts for the initial event which leads to the first SEN-DNA. A molecule in the most frequent situation where the SEN-DNA is an intron which might code for a maturase. This hypothesis is based upon several observations made either in *Podospora* or in the yeast *S. cerevisiae*. It assumes that mitochondrially synthesized maturases are unspecific nucleases able to work at the level of RNA and DNA molecules. Their specificity for RNA splicing instead of DNA is given by cytoplasmic proteins. Therefore, if the balance between cytoplasmic and mitochondrial protein syntheses is disturbed in favour of the mitochondrial compartment, the maturase would be accumulated and allowed to splice introns from DNA instead of RNA molecules. This hypothesis can account for aging of higher eucaryotic cells by postulating analogous processes in their nuclear compartment.

Cell compartment *RNA maturase* *Senescence* *Translation*

Aging has been very difficult to study in higher eucaryotes because of the complexity of these organisms. Therefore, this phenomenon has been investigated with the use of simplified cell models [1]. Among such experimental models, the fungus *Podospora anserina* appears as one of the most convenient for two main reasons. First, this organism is well characterized genetically. Second, the senescence phenomenon (arrest of vegetative growth of the mycelium) has been well characterized in *Podospora* [2,3] and can be controlled by genetic manipulation as well as by changing the culture conditions. Recently, a strict correlation has been established between the presence of circular DNA molecules arising from the mitochondrial genome (SEN-DNAs) and the senescence syndrome [4–8]. In spite of this important discovery, two main questions remain to be answered. First, what is the molecular nature of the primary event which leads to the first SEN-DNA molecule and what are the physiological conditions which favour this primary event? Second, how does the presence of SEN-DNA molecules lead to the death of the fungus? A partial response to the second question has been proposed [9]. The assumption that the amplification of SEN-DNA could be the prime cause of senescence is supported by several lines of data. The most striking is the rejuvenation caused by ethidium bromide which is associated with the loss of SEN-DNA molecules [8]. Here we propose a working hypothesis which tries to answer the two parts of the first question, at least in the cases where the aging event is associated with the α-SEN-DNA which is the most frequent and the best known situation [7,10]. Based upon 6 relevant sets of data which will be discussed below, this hypothesis can be summarized as follows. The primary event which leads to the production of the α-SEN-DNA is that an intron-encoded maturase works erroneously, using DNA instead of RNA as a substrate. The physiological conditions responsible for this event arise when the equilibrium between cytoplasmic and mitochondrial protein syntheses is disturbed in favour of the mitochondrial compartment, and allows the maturase to be accumulated. According to this hypothesis, the

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translational apparatus plays a key role in the triggering of the senescence process because the dynamics of translation and the energy cost associated with protein synthesis determine the balance between the cytoplasmic and mitochondrial compartments. This hypothesis accounts for the initial event leading to the formation of SEN-DNA in the mitochondrial compartment of *Podospora*. It will be shown that the hypothesis can be extended to account for aging of higher eucaryotic cells by postulating analogous processes in their nuclear compartment.

The 6 sets of data which support this hypothesis are the following.

(1) Three non-overlapping fragments of the mitochondrial DNA can be recovered from senescent cultures of *Podospora* [7,10]. The region which is most frequently amplified (α-SEN-DNA) is a sequence of 2540 bp [10]. This sequence corresponds exactly to a class II intron [11,12] which lies close to the beginning of the gene coding for subunit 1 of cytochrome oxidase (gene CO1) [13]. This intron shows an open reading-frame (2367 bp). Numerous mitochondrial introns have been shown to contain ORFs [14,15]. There is no clear-cut evidence that these ORFs code for proteins except in a few cases where genetic data are available. In these cases, excision of introns has been demonstrated to rely, at least in part, on an intron-encoded protein which has been named RNA-maturase [14–19]. *Therefore, the first key observation which has led to the model is that the α-SEN DNA is an intron which might code for a maturase.*

(2) Reversion of the senescent state is not usually observed under normal conditions in *Podospora*. However, mutants resistant to senescence have been selected as sectors of growth that developed after several weeks of incubation of senescent cultures [20]. Although they are altered in several phenotypic properties, these mutants, which escaped from senescence, never showed symptoms of senescence again. Preliminary analysis [21] reveals that they display deletions of the mitochondrial genome which overlap partially or totally the intron which corresponds to the α-SEN-DNA. *Therefore, the second relevant observation is that the integrity of the α-intron is necessary for senescence to occur.*

(3) In the yeast mitochondria, Gargouri et al. [22] looked for reversion of intron mutations which cause defects in RNA splicing. They observed that such defects can be eliminated by intron deletions. Such clean *DNA-splicing of introns* is a general phenomenon which occurs quite frequently and which can involve one, two or three introns. A role for RNA-maturases has been postulated in these gene processing events [22]. Therefore, these data led to the first aspect of the model which proposes that *the DNA excision yielding the first α-SEN-DNA molecule in* Podospora *is performed by a RNA-maturase working erroneously, using DNA instead of RNA as a substrate.*

(4) Chloramphenicol and cycloheximide are considered to act, respectively, on mitochondrial and cytoplasmic protein syntheses. Chloramphenicol greatly increases the life spans of all the strains studied, whereas cycloheximide decreases them. The results were obtained in many independent experiments carried out on different races of *Podospora*, which display different life spans, and using concentrations of drugs that were just sufficient to cause a slight decrease in the elongation rate of the mycelia [23]. The effect of chloramphenicol which inhibits the mitochondrial ribosomes and increases the life span can be correlated with the effects of the mitochondrial mutation cap_1^R. This mutant, which is chloramphenicol resistant, seems to be altered at the level of the mitochondrial protein synthesis because it produces less cytochrome oxidase than the wild-type. The cap_1^R mutant exhibits a 5-fold increased life span when compared to the isogenic cap_1^S strain [23]. Therefore, it appears that *a decrease in the efficiency of the cytoplasmic protein synthesis diminishes the life span while a decrease in the efficiency of the mitochondrial protein synthesis increases the life span of* Podospora.

(5) Mutations which increase (*su*) or decrease (*AS*) the error rate in cytoplasmic translation have been described in *Podospora* [24]. Most of them are point mutations and, in several cases, it has been shown that they lie in genes coding for ribosomal proteins [25]. In a preliminary study, the life spans of a few *su* and *AS* strains have been compared to the wild-type life span (A. Raynal, personal communication). Among the 4 *su* muta-

tions used, 3 do not modify the life span while the fourth strongly increases it. Among the 4 *AS* mutations studied, two increase the life span and two lead to a premature senescence phenomenon. These data, connected with the genetical and biochemical analysis of these mutants [25], clearly lead to a striking conclusion: *the error rate per se does not influence the cellular life span.* In fact, the senescence phenomenon can be delayed by either an increase or a decrease of the misreading level. A detailed discussion of these data will be presented elsewhere in the light of our present knowledge concerning the dynamics of translational accuracy in *E. coli* [26]. However, the relevant parameters may be briefly presented here. The two *AS* mutations which reduce the life span do that likely in a way which is reminiscent of the effect quoted above for cycloheximide. In fact, both of them are cold-sensitive mutants. A slight defect of ribosome assembly might still be retained at the permissive temperature in such a way that the efficiency of cytoplasmic protein synthesis would be reduced. The effect of the 3 other mutants which delay the senescence state might be explained in terms of speed [27] and (or) energy cost for protein synthesis [28,29]. If these mutants are high demand mutants, the extra cost needed to synthesize proteins in the cytoplasmic compartment has to be paid through a loss in another compartment, likely the mitochondrial one.

These mutations would deplete the mitochondrial protein synthesis indirectly as chloramphenicol does directly. Therefore, *the cytoplasmic translational apparatus appears to play a key role in the triggering of the senescence process because any change in its dynamics disturbs the balance between cytoplasmic and mitochondrial protein syntheses.*

(6) In the yeast *S. cerevisiae*, it has been shown that mitochondrial RNA splicing needs two kinds of proteins: the maturases which are encoded in part by specific mitochondrial introns and synthesized inside the mitochondria [14–19] and proteins encoded by nuclear genes and synthesized in the cytoplasmic compartment [30–32]. Nuclear mutants which are defective in mitochondrial RNA splicing have also been described in the fungus *Neurospora crassa* [33]. The data which are most relevant to our model are those concerned with the NAM_2 mutants of the yeast [34]. In the yeast *S. cerevisiae*, the mitochondrial maturase coded by the b14 intron is involved in the splicing of two introns located in two split genes: the COB and the CO1 genes [16,35]. When the b14 maturase is deficient, none of these two introns is spliced. Suppressors of this defect have been obtained as mitochondrial (MIM_2) or nuclear (NAM_2) mutations [36]. The MIM_2 mutation corresponds to a single base substitution in the a14 intron of the CO1 gene. The mutation activates the latent maturase which is coded by this intron and which is inactive in the wild-type strain [37]. Genetic and biochemical analysis of the nuclear suppressor NAM_2 leads to two main conclusions. First, the mutated gene codes for a protein which is able to activate the latent a14 maturase [38]. Therefore, it seems likely that the mitochondrial maturases perform splicing of specific introns with the help of proteins coded by the nucleus and synthesized in the cytoplasm. Second, when the NAM_2 gene is destroyed most of the mitochondrial genome is lost [34]. This result suggests that the product of this gene plays a key role in mitochondrial functions. Therefore, at least in yeast, *specific proteins synthesized on cytoplasmic ribosomes are needed in addition to the mitochondrially synthesized maturases for mitochondrial RNA splicing.* In one case, the *loss of the cytoplasmically synthesized protein is lethal for the mitochondrial DNA.*

These 6 sets of data lead us to the following interpretation. Maturases are unspecific nucleases able to work both at the level of DNA and RNA molecules. Their specificity for RNA splicing instead of DNA is given by cytoplasmic proteins. Maturases are therefore dangerous enzymes which can kill the mitochondrial genome when they are allowed to splice introns from DNA instead of RNA molecules. Normally maturases cannot be accumulated because they destroy their own message [14]. It was supposed that these enzymes were made in such a low amount because the cell needed only a few molecules of them. We can now imagine that these proteins are synthesized in low amounts because they are so dangerous. The potential menace associated with maturases is controlled by the way they are synthesized and by the relative amount of their effectors which are made in the cytoplasm. If the equilibrium between the

two compartments is disturbed in such a way that the mitochondrial protein synthesis is favoured, then the maturase will be accumulated without enough effectors. The enzyme will be allowed to use the DNA as a substrate. The first intron will be spliced from the DNA and the first α-SEN-DNA molecule will appear.

We used the α-SEN-DNA event of *Podospora* as a model system because it is the most frequent and the best known situation. However, the other situations (β- and γ-SEN-DNAs) might be explained in similar ways. New data about maturases and about the relationships between the protein synthesis systems of the eucaryotic cell are needed for a better understanding of senescence. However, we can ask whether our hypothesis might be applied to certain aging phenomena in higher eucaryotes. If introns also play a key role in such processes, the interest has to be turned from the mitochondrial genome (which is devoid of introns in higher eucaryotes) to the nuclear one. *Splicing of introns at the level of nuclear DNA* seems to occur when the macronucleus is generated from the micronucleus in a protozoan [39]. Furthermore, *nuclear maturases have been postulated* [40] although the existence of a protein synthesis apparatus inside the nuclear compartment remains to be demonstrated. If such intron-encoded maturases exist in the nucleus, the hypothesis would have to be formulated in terms of the equilibrium between the nuclear and the cytoplasmic compartments for protein synthesis (nuclear maturases and cytoplasmic effectors). If there is no nuclear translation, the problem would be transferred at the level of the protein complexes which perform the processing of messengers. A small change in the cytoplasmic translational apparatus could disturb the relative amounts of these proteins and allows some of them to become DNases. Structural changes at the level of DNA have been reported in some aging situations [41]. However, if the primary event concerns a few key sequences, it will be very difficult to identify, owing to the complexity of the nuclear genome and to the lack of genetic tools in higher eucaryotes.

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