Non-conventional infectious elements in filamentous fungi

Old data (most often in French) described phenomena involving non-conventional infectious factors in filamentous fungi. Recently, it was shown that two yeast cytoplasmic determinants are similar to known mammalian prions, in that their different states are attributed to conformational changes of normal cellular proteins. In the light of this discovery, fungal elements are now being reconsidered. This review presents four elements that affect vegetative incompatibility, conidiogenesis, morphology and cell growth. Recently, one element has been shown to be a prion analogue. The status of the others is not clear. We consider the view that non-conventional inheritance might be initiated by the appearance, in the cytoplasm, of a metabolite or a macromolecule whose production involves a positive regulatory loop.

During the 1950s and the 1960s, cases of non-mendelian cytoplasmically mediated inheritance were reported in many organisms[1]. The detection of mutations located in the mitochondrial or chloroplastic genomes, as well as the presence of episomic nucleic acid has clarified the nature of many of the genetic determinants involved. However, some are still mysterious. In many cases, their highly infectious properties suggest that they are not of a truly genetic nature, but of an epigenetic nature. Recent findings showing that two such yeast elements, [PSI] and [URE3], bear close resemblance to mammalian prions[2–4] has prompted a novel interest in these phenomena. In the 'protein-only' prion hypothesis, the infectious element is an abnormal conformational state of a cellular protein, which can trigger the conversion from the normal to the abnormal form. This has clearly been demonstrated for the [PSI] element[4].

Because of their coenocytic structure (Box 1), filamentous fungi stand as very good models to detect and study such cytoplasmic and infectious particles (Fig. 1). Cellular continuity is a main characteristic of these organisms. Hyphal septae have pores and hyphae can be connected by anastomosis. Therefore, a single infectious element is able to contaminate a large area of mycelium, and so its effects are detectable without the need of selection. In this review, we focus on phenomena observed in filamentous fungi that do not seem to be connected with any kind of nucleic acid. Hence, senescence phenomena, in which mitochondrial dysfunction has been observed, will not be discussed, despite the fact that the real nature of the responsible determinants is still mysterious[5].

BOX 1. Glossary

Anastomosis (pl. anastomoses)
Process by which two hyphae fuse to create a network, resulting in the exchange of cytoplasmic constituents and in some cases of nuclei.

Ascospore
A spore produced in an ascus and resulting from meiosis in Ascomycetes.

Coenocytic (or syncytial)
Characteristics of organisms for which a cellular continuity exists. In filamentous fungi, continuity is ensured by perforated hyphal septae and anastomoses.

Conidium (pl. conidia)
Specialized non-motile cell involved in asexual dispersion.

Conidiogenesis
The process of conidium formation.

Hypha (pl. hyphae)
One filament constituted of successive cells separated by septae.

Hyphal septum (pl. hyphal septa)
Cell wall between two cells of a hypha. In most fungi, it possesses a special structure that ensures a cytoplasmic junction between two contiguous cells.

Mycelium (pl. mycelia)
A mass of hyphae.

Propagule
Any kind of cell that is involved in the dispersion of the fungus.

Thallus (pl. thalli)
The vegetative body of a thallophyte. In filamentous fungi, it is equivalent to the mycelium.

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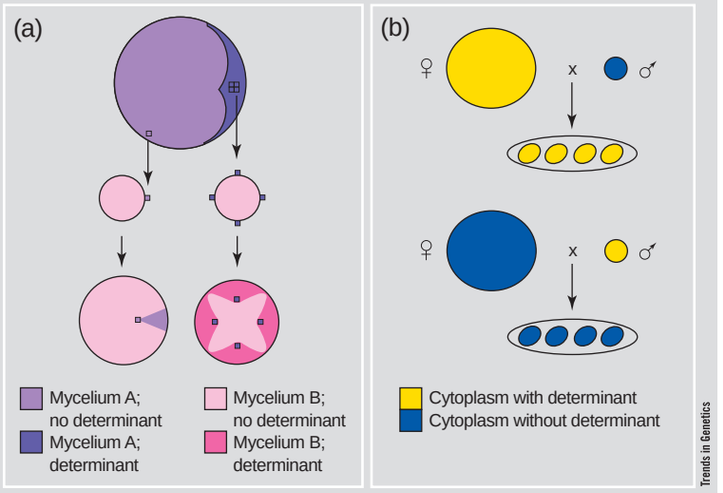
FIGURE 1. Diagnostic for cytoplasmic and infectious elements in filamentous fungi



In fungi, the cytoplasmic and/or infectious nature of determinants is defined by two characteristics: (a) their transmission into a recipient mycelium following anastomosis and (b) their non-mendelian segregation during meiosis. (a) To demonstrate transmission through anastomoses, one usually performs 'contamination experiments' (as exemplified by the 'Secteur' of *N. haematococca*). When a small inoculum of the donor mycelium (that contains the determinant) is put at the periphery of a growing culture of the recipient mycelium (that lacks the determinant), the growing recipient mycelium usually presents the characteristic phenotype promoted by the determinant. During the contamination process, the donor nuclei do not invade the recipient mycelium and hence the determinant is cytoplasmic and infectious. This can be observed if different alleles mark the donor and recipient nuclei. (b) Non-mendelian segregation is observed following a cross between a mycelium that contains the determinant with one that does not. Usually, ascus analysis shows that the nuclear markers, if present, do exhibit the classical 2:2 segregation in each ascus, whereas the determinant does not. It might be inherited by all the progeny, lost in all the progeny or present with any of the segregation intermediates between these two extremes but always without any clear mendelian proportion. If crosses involve a male and a female partner, transmission, if any, occurs only through the female partner (as exemplified by the [Het-s*] → [Het-s] transformation of *P. anserina*).

The barrage phenomenon in *Podospora anserina*

Rizet described the first instance of fungal non-conventional inheritance[6] when studying the 'barrage' reaction at the contact area between the two strains S and s (renamed [Het-S] and [Het-s], Ref. 9), of *Podospora anserina*. This 'barrage' is the result of a vegetative incompatibility reaction promoting cell death. [Het-S] and [Het-s] strains differ by the nature of the polymorphic allele that is present at a locus, now called *het-s*. When [Het-S] or [Het-s] strains are self-crossed, the resulting progeny are uniformly reactive with [Het-s] or [Het-S], respectively. Upon crossing a [Het-S] strain with a [Het-s] strain, the progeny are composed of two [Het-S] strains and two unexpected [Het-s*] (formerly denoted s^S) strains that are not reactive with either [Het-S] or [Het-s]. Interestingly, from these [Het-s*] strains, one can spontaneously obtain true [Het-s] strains at low frequency. Anastomoses between [Het-s*] and [Het-s] strains invariably promote the transformation of the [Het-s*] mycelium into a [Het-s] strain independent of nuclear transmission[7]. Anastomoses are required to promote the transformation[7]. Additionally, crosses between [Het-s] and [Het-s*] yield only [Het-s] strains when [Het-s] is the female partner, and mostly [Het-s*] strains when the female partner is [Het-s*] (Ref. 7). Involvement of mitochondria was excluded when the first mitochondrial mutant was obtained, because the transformation and the mitochondrial mutation did not propagate similarly in heterokaryons[8]. To account for these properties, it was suggested that the protein encoded by *het-s*, which is able to induce and regulate its own synthesis, is present in strain [Het-s] and absent in strain [Het-s*] (Ref. 7).

Recent observations suggest that the transformation of [Het-s*] into [Het-s] is a prion phenomenon[9]: (1) the *het-s* allele is necessary for the propagation of the transformation from [Het-s*] to [Het-s]. Indeed, a strain containing the null allele *het-s*° (which therefore lacks the pHET-s polypeptide) is unable to transmit the [Het-s] state to a [Het-s*] culture; (2) overexpression of the pHET-s polypeptide increases the probability of the transition; (3) the transition occurs in the absence of translation; and (4) the pHET-s polypeptide is present in similar amounts and has similar electrophoretic mobility in [Het-s] and [Het-s*] strains. However, the polypeptide present in the [Het-s] strain is more resistant to proteinase K digestion. In both types of strain, the pHET-s polypeptide is present either as monomers or multimers (di-, tri- and tetramers). Interaction between the pHET-s monomers is confirmed in yeast with the two-hybrid system; in addition, interactions between the pHET-S polypeptides or between the pHET-s and pHET-S polypeptides are also detected. All these properties are shared with [PSI] and [URE3], the two yeast prions[2].

The [Het-S], [Het-s] and [Het-s*] system is interesting for three reasons. First, it adds a fourth member to the repertoire of proteins suspected to be able to catalyse their own modification of structure. Comparison of the four proteins does not reveal any clear sequence similarity. It remains to be determined whether some structural motifs are conserved in all these polypeptides and whether the same mechanism accounts for all the self-propagating conformational changes. Second, it suggests that the ability of protein to catalyse or 'seed' their own conformational modification is a property of proteins more widespread than presently thought. Pre-existing 'seeds' could be needed to perform proper folding, as observed for the self-folding of hsp60 proteins[10] and the conformational changes of the SUP35p in the [PSI] phenomenon or the PrP protein of mammals[4]. In the case of *Podospora anserina*, crosses with the *het-S* allele result in absence of the 'seed', thus revealing the phenomenon. However, if the protein is essential, like hsp60, 'seeds' are always present and continuously transform the newly synthesized proteins. The third reason is the particular properties of the pHET-S polypeptide, namely, (1) its ability to interact with pHET-s, and not pHET-s*,

FIGURE 2. Morphological modifications

The same strain of *N. haematococca* can display two different morphological modifications caused by two specific cytoplasmic and infectious factors, the 'Anneau' (A; left) and the 'Secteur' (S; middle). A morphological modification very similar to the 'Secteur' has been described in several fungal species, including *C. pallescens* (right).

to start the incompatibility reaction and (2) its effect during meiosis in converting [Het-s] to [Het-s*]. The pHET-S and pHET-s/pHET-s* proteins differ by 14 amino-acid substitutions[11]. Only one of these is responsible for the incompatibility reaction. None has yet been analysed for the [Het-s] to [Het-s*] converting effect of the *het-S* allele. Analysis of these two properties via a combination of genetic and biochemical approaches should yield interesting data.

The closed system of cytoplasmic variation in *Aspergillus glaucus*

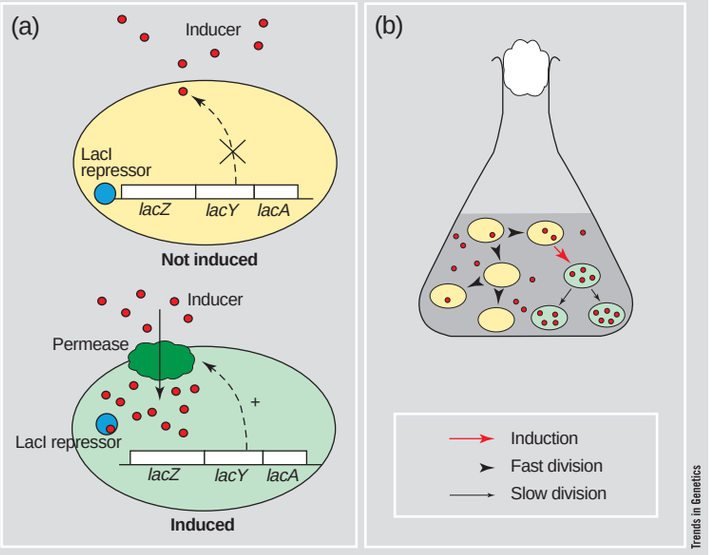
Subak Sharpe[12] reported the second instance of a cytoplasmic and infectious factor, in *Aspergillus glaucus*. It involved sectors that differ from the surrounding mycelium by several characteristics, particularly enhanced conidiogenesis. It was readily infectious through anastomoses, but it was not clear whether it could infect resting mycelium like the *Podospora* transition from [Het-s*] to [Het-s], or only the cells of the growing margin like the elements described in the next section. It was shown that a mosaic of cells that do or that do not contain the determinant formed the sectors. This could be transmitted via the conidia or the ascospores and curiously segregated in nearly a 1:1 manner in sexual crosses. In this system, it was not clear what prevented the factor from invading the totality of the mycelium. No further studies were carried out and we are left with conjectures about its nature. Interestingly, this phenomenon was not the sole report on the role of cytoplasm in conidiation of *Aspergilli*[13].

The morphological modifications of numerous species

The third example that we discuss is that of the mycelium morphological modification present in numerous ascomycetous and basidiomycetous species. When grown on appropriate media, their reproductive propagules (being either asexual or sexual spores) generate thalli with dense aerial hyphae. Often, at the periphery of the growing cultures, sectors with a modified morphology develop randomly (Fig. 2). These are characterized by a reduced amount (or absence) of aerial hyphae and an intense pigmentation that diffuses in the medium. Four fungal species have been carefully looked at, *Curvularia pallescens*[14] (M.J. Vicariot-Hugonnet, 1965, PhD thesis, pp. 1–58, Université de Paris-Sud, Centre d'Orsay), *Pestalozzia annulata*[15,16], *Hypomyces ipomoeae*[17,18] and, especially, *Nectria haematococca*[19,20] (M.J. Daboussi-Bareyre, 1979, PhD thesis, pp. 1–163, Université de Paris-Sud, Centre d'Orsay).

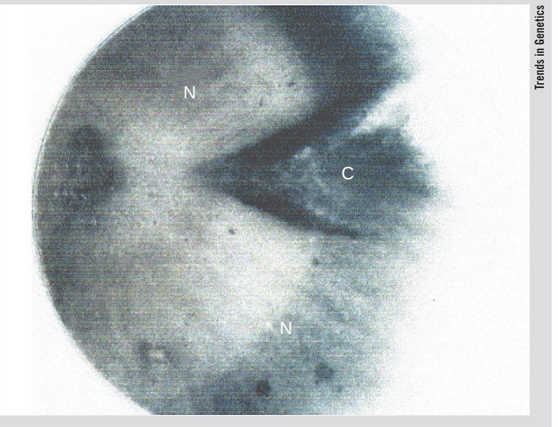
In this latter species, two types of modifications can appear in the same culture, the 'Secteur' and the 'Anneau' (Fig. 2). The frequency of these modifications varies substantially with culture conditions, especially temperature. Once initiated, these modifications spread through anastomoses, but only in the cells of the growing edge. The modifications are characterized by a speed that dictates their form. The 'Secteur' determinant travels at twice the growth rate and generates a typical sector. The 'Anneau' determinant travels at 20 times (around 4 mm h^{-1}) the rate of the growing hyphae and the resulting 'Secteur' is a ring that surrounds the culture. Contamination experiments have shown that each determinant is transmissible to undifferentiated cultures (Fig. 1) and is specific to one modification. Upon subculture with mass hyphal transfer, the modified morphology can be continually maintained. However, fragmentation of a modified culture allows the restoration of normal morphology in a varying proportion

FIGURE 3. Alternate metabolite states as hereditary determinants



(a) The first description, but not proposition[31], that a positive regulatory loop can generate a hereditary determinant is that of Novick and Weiner[27]. They showed that, when grown in a medium that contains a limiting, constant and specific concentration of a gratuitous inducer of the lactose operon, an *E. coli* population is composed of cells that are either in a non-induced state (top) or a fully induced state (bottom). It happens because entry of lactose in the cell is greatly facilitated by the permease encoded by the *lacY* gene. This generates a self-maintained regulatory circuit, by which induced cells stay induced (because of efficient entry of lactose) and non-induced cells remain so, until a stochastic activation of the lactose operon occurs. They also showed that a dynamic equilibrium is reached (b) despite the constant induction of cells. Indeed, non-induced cells divide faster than induced ones allowing for a constant replacement in the population of the cells that have made the transition towards the fully induced state. In this lactose operon system, a single positive loop is sufficient to generate the alternative states. A double reciprocal negative action might achieve the same generation of alternative states[31,32]. This has been demonstrated in the case of the lytic/lysogenic choice of phage λ (Ref. 33). In view of the simplicity of the elements required to initiate such a system, and the numerous feedback loops and cross-talk events encountered in regulatory and metabolic pathways, it would not be surprising if several cryptic loops, potentially able to generate alternative states, exist in the cell.

FIGURE 4. Growth modification



Some mutant strains of *Podospora anserina* (here the *AS6-5* strain) might spontaneously present sectors with an altered morphology, in which hyphal growth rate and morphology are modified (C) or normal (N). Unlike the 'Secteurs' depicted in Fig. 2 that develop in wild type, this 'Crippled Growth' is restricted to mutant strains that have elevated translation accuracy.

of subcultures. This suggests that the modified mycelia are mosaics of cells that may or may not contain the determinant and might, therefore, be in a state of dynamic equilibrium (Fig. 3). Asexual conidia can transmit both factors; ascospores never do so. From all the above data, it was concluded that each modification is caused by a specific cytoplasmic and infectious factor.

Both 'Secteur' and 'Anneau' are under the control of nuclear genes[20–22]. Two kinds of mutations have been detected. Some do prevent expression of both modifications and these map to at least four loci. Expression of both determinants seems thus to involve a common pathway. The other mutations prevent specifically the formation of either one of the modifications. They are located at a unique locus for the 'Secteur' (the S locus) and at another unlinked single locus for the 'Anneau' (the A locus). Two kinds of mutant alleles were detected, those that completely abolish the production of the determinants (*a* or *s* alleles) and those that promote its constitutive expression (*a** or s*). Interestingly, in the latter cases, the mutant mycelia present a red colour but are able to produce aerial hyphae to the same extent as wild type. It is supposed that these loci are directly involved in the generation of the determinants. Cloning of the *A* and *S* genes is now under way (S. Graziani, P. Silar and M.J. Daboussi, unpublished). It would not be surprising if a membrane component is involved, in view of the amazing speed (an order of magnitude higher than that of the barrage phenomenon in *P. anserina*) at which the 'Anneau' factor travels through the anastomoses.

The similar phenomena observed in the other three fungi have not been studied thoroughly, but it seems that development of the sectors follows roughly the same rules as above. However, optima of temperature for appearance of the modification are variable and it was suggested that two events are required to allow the formation of sectors in *P. annulata* and *C. pallescens*[23] (M.J. Vicariot-Hugonnet, 1965, PhD thesis, pp. 1–58, Université de Paris-Sud, Centre d'Orsay). Therefore, despite a similar morphology (Fig. 2), these sectors might be generated by different mechanisms.

The Crippled Growth in *Podospora anserina*

The final case that we discuss of cytoplasmic and infectious fungal element is the recently discovered *C* determinant that causes growth impairment (Crippled Growth as opposed to Normal Growth, Fig. 4) in some mutant strains of *Podospora anserina*[24]. Unlike the above determinants, its propagation through anastomoses is not very efficient since only half the contamination experiments succeed in the best cases. The responsible element is efficiently transmitted through mitosis but not through meiosis. It is induced in stationary phase and cured by various stresses. Whereas the latter property is well established for the yeast element [PSI] (Ref. 25), the former one is unique to the *C* element.

Presence of *C* can be detected during stationary phase in all strains that have been tested, including the wild type, but it propagates during the growth phase only in strains that display increased translation accuracy (AS strains). Two models can be proposed to account for the role of translation fidelity. First, translation accuracy might be directly involved in the generation of the element. It could increase during stationary phase accounting for induction of *C* during this period, but during active growth, fidelity would be too low, except in AS strains, and hence the element would disappear. Alternatively, a translation error might control the production of a factor involved in the elimination of the determinant. In AS strains, this hypothetical factor might be under-produced, thus allowing the propagation of the *C* determinant. It is clear that transition from Normal Growth to Crippled Growth displays very different properties from the barrage phenomenon and is thus likely to caused by a somewhat different mechanism.

Biological significance

It is worth remembering that stable alternative metabolic states can be heritable in some cases (Fig. 3)[26]. This was first proved and discussed for the all or none β-galactosidase induction observed at low inducer concentrations in *E. coli*[27]. In truth, a positive regulatory loop, at any level of a metabolite or macromolecule production could be sufficient to promote with some stability alternative states[26]. In fact, the conformational changes undergone by prion proteins is formally identical to such processes because, in a sense, it can be viewed as a loop at the level of the protein folding.

Are these phenomena mere exceptions or of general occurrence in living cells? Two lines of reasoning suggest that the latter proposition is probably correct. First, in view of the complexity of the cell regulatory network, it is likely that unwanted positive loops could arise during evolution with high frequency along with mutations in the components of the network. Second, in many of the organisms that were genetically studied for cytoplasmic inheritance, like yeast, *Aspergilli*, *Podospora* or paramecia[1], several of these phenomena have been described.

Once the loop has appeared, two possibilities follow. The phenomenon might be positively selected by natural selection. It would thus commit the cell to engage in a primitive 'differentiation' process[28]. Appearance of the sectors in many fungal strains isolated from nature (J. Chevaugeon, unpublished) strongly indicates that they are positively selected and are thus akin to a true differentiation. This proposal is confirmed by the fact that several differentiation pathways, like sex determination in *Drosophila melanogaster*[29] or dauer formation in *Caenorhabditis elegans*[30], do present a regulatory circuitry that can generate alternative states. The process might be deleterious, at least in some conditions. Control systems as the one postulated for Crippled Growth might then be set up. If such systems are not present, we have proposed that activation of the loops could lead in part to ageing[24].

Genetics has so far been mostly concerned with the inheritance of the individual constituent of the cell (e.g. 'one gene = one enzyme' paradigm). The phenomena we have discussed show that it is also important to study the inheritance of structure and of regulatory circuitry. Sudden and stable phenotypic change, especially in a mitotic lineage, might not always be due to a nucleic acid mutation.

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The Ure2 protein in yeast has now been shown to form amyloid structures *in vitro*, which provides further evidence that Ure2 is a prion protein because amyloid structures are a key characteristic of the so-called prion diseases.

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Signaling specificity

the RTK/RAS/MAP kinase pathway in metazoans

The molecular basis by which commonly used signaling pathways are able to elicit tissue-specific responses in multicellular organisms is an important yet poorly understood problem. In this review, we use the receptor tyrosine kinase (RTK)/RAS/MAP kinase signaling cascade as a model to discuss various hypotheses that have been proposed to explain signaling specificity. Specificity can arise at the level of the receptor, through the modulation of signaling kinetics, through the interaction of different signaling pathways, and at the level of downstream signaling components. Mechanisms of specificity used by the RTK/RAS/MAP kinase signaling pathway might apply to other signaling pathways as well, and might help explain how multicellular organisms are able to generate tissues of diverse forms and functions from a small set of common signaling pathways.

A great mystery in biology is the question of how equipotent cells subsequently acquire distinct tissue-specific properties. In recent years, it has become clear that cellular signaling processes play an integral role in the process of tissue differentiation, and one striking finding is that a handful of conserved signaling pathways seem to be used reiteratively to specify a wide variety of tissues. However, the pleiotropic nature of these pathways has prompted the question of how they can preserve the specificity of a signal in order to ultimately elicit distinct tissue-specific responses. This review focuses on one such pleiotropic signaling pathway: the receptor tyrosine kinase (RTK)/RAS/MAP kinase signaling cascade. Activation of this cascade is mediated by growth factor ligands (e.g. EGF, NGF), that bind to and activate specific RTKs (Refs 1, 2). Receptor activation results in the initiation of a RAS/MAP kinase signaling cascade, culminating in the regulation of nuclear transcription factors by MAP kinase[3]. A wide variety of biological decisions are mediated by this signaling pathway. For instance, RAS proteins are essential for embryonic development in vertebrates[4], as well as neuronal and adipocytic differentiation in cell culture assays[5,6]. Furthermore,

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