Cytochemical gradients and mitochondrial exclusion in the apices of vegetative hyphae[1]

G. Turian

Laboratoire de Microbiologie générale, Université de Genève, Place de l'Université 3, CH-1211 Genève 4 (Switzerland), 21 May 1979

Summary. Apical maximal acidity (shown by subapically increasing pH) and maximal concentration of inorganic phosphate (shown by a subapically decreasing gradient of yellow phosphomolybdate) contrast with the subapical, mainly mitochondrial sequestration of calcium ions. Such gradiential control of calcium concentration appears to be the requisite for the Ca^{++} sensitive gelation of the apical hyaloplasm preventing the mitochondria from entering into this continuously elongating zone of growing hyphae.

In parallel with our recent finding of a high reducing power concentrated in the 'Spitzenkörper' of the amitochondrial tips of vegetative hyphae[2], we made another new observation, that of a maximum acidity at this apical site of hyphal elongation. The apico-basal gradient of increasing pH was found to span a range of up to 1 unit of pH (from 5.0–5.5 to 6.0–6.5) in hyphae of *Neurospora crassa* and *Sclerotinia fructigena.*

We have now confirmed such preliminary estimations, based on microscopical observation of colour changes in several standard pH indicators, in a wider range of filamentous fungi. In *Allomyces arbuscula,* the vegetative hyphae obtained from the advancing front of 2-day-old cultures at 25 °C on standard YpSs agar medium[3] showed yellowish apices and yellowish-green subapical zones (pH 6.2) when bathed in bromothymol blue (therefore $pH < 6.0$). With bromocresol purple, the domed apices were orange ($pH \sim 5.2$) and the enlarging subapical zones more and more purple including the nucleoli ($pH > 5.6$). Bromocresol green gave a general greenish hue in the apical zone, turning to bluish-green and therefore relative alcalinity (pH 5.8) in the nucleoli. In *Allomyces,* we can therefore estimate a pH gradient spanning values around 5.2–6.2. The relatively high acidity in its apices might be related to the lactic acid production known to occur during vegetative growth

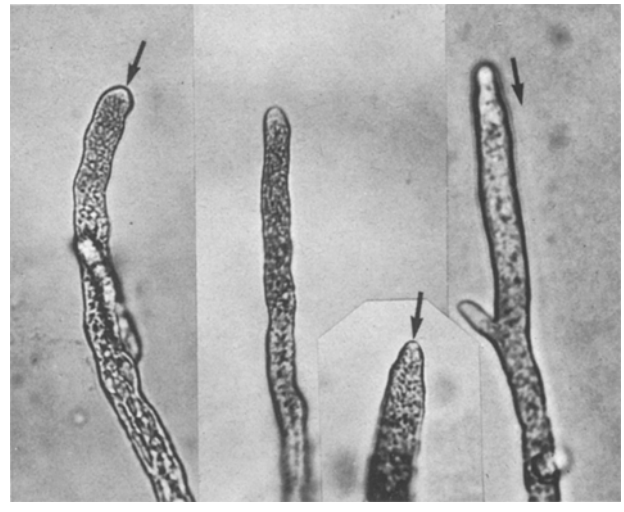

Fig. 1. Immediate (20 sec) yellow reaction (clearing in the black and white photo, arrows) of the NH_4-molybdate reagent with inorganic phosphate ($PO_4H_2^- + PO_4H^{--}$) pumped into the hyphal tips of vegetative hyphae of *Neurospora crassa* elongating on solid nutrient medium. × 800.

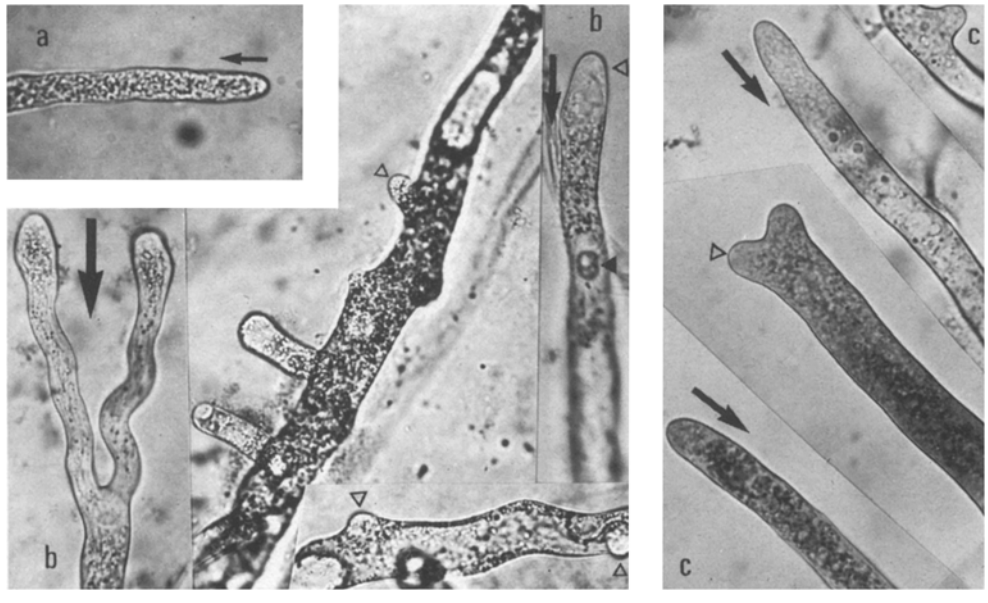

Fig. 2. Apico-basal gradients (long arrows) of the reddish to purple-red subvital reaction of alizarin (alizarin sulphonate, Merck) with Ca^{++} ions in vegetative hyphae of: *a N. crassa,* with vivid yellow 'Spitzenkörper'. × 800. *b Basidiobolus ranarum,* with a regular gradient from the yellowish homogenous apical zone (empty arrow) through the purple granular, mitrochondrially-rich zone to the large distal nucleus with its densely purple-stained nucleolus (black arrow); the yellowish tinge of alizarin is most conspicuous in the initial buds (empty arrows) and the tips of elongating side branches of the purple hyphal trunks. × 800. *c Allomyces arbuscula,* with orange-reddish tips, purple-red subapical zones and reddish-violet nucleoli; note the clear, yellowish spot on one of the early dichotomous branches (empty arrow). × 800.

of representatives of this genus[4]. In vegetative hyphae of *Basidiobolus ranarum* elongating on solid malt medium the pH gradient appeared less sharp, with a span from pH ~ 5.4 in the rounded apices (slightly pinkish with bromocresol purple) to around 6.2 (clearly purple from the first nucleus zone, inclusive of its large nucleolus).

The physiological basis of the lower apical pH might be found in the high glycolytic activity enforced in the highly reducing (NADH and -SH groups) amitochondrial tip zone[5]. As it is known that the glycolytic rate is enhanced by an increased availability of phosphate ions[6], we tried to check the hypothesis of a selective entrance of such ions (presumably mainly $H_2PO_4^-$) through the apical membrane of *Neurospora crassa.* As a preliminary technique we adopted as a micro-visual test the well-known yellow analytical reaction of inorganic phosphate in the presence of the molybdate reagent[7] ($(NH_4)_2MoO_4 + NH_3$ 14N, then mixed with HNO_3 5N). Due to the destructive action of this highly acidic reagent on the hyphal cytoplasm, the test had to be carried out as rapidly as possible on slides already placed under the microscope. After about 15 sec, the time required to pipette a drop of the reagent on the vegetative hyphae of *N. crassa* washed from liquid Vogel's medium[8], and to flatten them under a cover-slip, we could repeatedly observe a yellowing of their apical zones due to the localized formation of ammonium phosphomolybdate, especially noticeable in the tips of the wider hyphae which were photographed (figure 1). A diffuse phosphomolybdate reaction secondarily extended to the subapical and more distal zones of the hyphae while fading to a greenish hue (mixture with reduced bluish reagent) in these roughly coagulated zones; this secondary reaction presumably reflected the progressive hydrolysis of the various organic phosphate components of the cells, as was especially noticeable at the level of mitochondrial residues and ribosomal aggregates. Additional pipetting of a reducing agent such as ascorbic acid to switch artificially and quickly the yellow to the blue reduced product colorimetrically measured in analytical chemistry was of no advantage, as it was mostly the greenish and diffuse hue noticed in ordinary prolonged tests which could be observed. To date our most clear positive yellow reaction for phosphate ions in the apices has been obtained with wide and 'triangular-tipped' hyphae of *N. crassa* elongating at the front of young cultures on agarized Vogel's medium[8].

In domed tips of *Allomyces* vegetative hyphae, a yellowish reaction could sometimes be observed but the positive reaction was never as vivid as that seen in the more concentrated *Neurospora* apices with their intermingled 'Spitzenkörper' appearing to contribute to the enhanced yellow tinge. Phosphate localization in the hyphae of *Neurospora* was recently investigated by electron microprobe analysis and the increased concentration in the apices that we have cytochemically visualized could only be suggested by the extrapolation of the values measured on a mm scale[9].

The characteristic absence of mitochondria in the ultimate tips of vegetative hyphae[10] might be the consequence of an active prevention of the entrance of such organelles into this zone mainly filled with vesicles[11,12]. A phosphate-enhanced glycolytic activity at this level could play this role, as suggested by the enforcement of prolonged vegetative growth by high concentrations of sucrose (glucose) in *N. crassa*[13], which might provoke a mitochondrial repression in the tips. However, mitochondriogenetic repression by the glucose effect could not be shown in *Neurospora*[14], contrary to the well-known situation in *Saccharomyces.* We had then to turn primarily to a mechano-chemical explanation for the maintenance of the so-called apical restriction, or exclusion, amitochondrial zone in filamentous fungi. In this case, the increased acidity detected in this apical region might already be a clue to the explanation, if only by the hyaloplasmic gelation it could provoke there (low pH leads to gelation in extracts from myxamoebae[15]). Moreover, such a 'gelated apical cap' would imply some type of regulation of the Ca^{++} ion concentration, presumably lowering it (low Ca^{++} promotes gelation in the ectoplasm of myxamoeba)[15]. We got a first hint of this hypothesis from our previous observation of an increasing apico-basal gradient of the red reaction with alizarin in the vegetative hyphae of *Neurospora*[2,16]; this dioxyanthraquinone is known to react selectively to the ions Ca^{++} as first shown by Pollack who applied it in 1928 to obtain a 'red shower' of calcium in the pseudopodia of amoeba[17].

We have now confirmed this preliminary observation and extended it to the hyphae of *Allomyces arbuscula* and *Basidiobolus ranarum* grown respectively according to Emerson[3] and Robinow[18]. A purple to purple-violet reaction could only be localized in the mitochondrially rich subapical zone of all these fungi (figure 2), the purple reaction being indicative of a higher Ca^{++} concentration which appeared most densely localized in the mitochondria themselves as observed with sharp focussing and strong lighting. The apices of the hyphae clearly stained only yellowish in *Basidiobolus* (figure 2, b), at the most reddish-yellow in the domed tips of *Allomyces* (figure 2, c); suggestively, the yellow tinge was conspicuous in the newly formed apices of young side branches in both species (figures 2, b and c).

A yellowish spot has been observed at the top of the apical dome of actively enlongating hyphae of *Allomyces* (figure 2, c and unpublished microphotos). Such a presumably low Ca^{++} has a similar location to that of the previously-shown methylene blue reducing spot[5] as well as the star of microtubules found by electron microscopy in this 'clear spot'[19]. In the Ascomycetes *Neurospora* and *Sclerotinia,* the

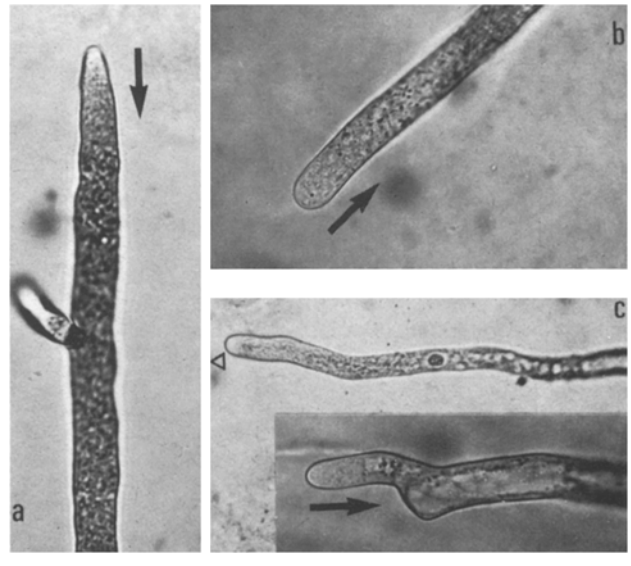

Fig. 3. Apico-basal gradients of Ca^{++} ion concentration (long arrows) as visualized by the increasing intensity of the violet Arsenazo III subvital staining: *a* Actively elongating, vegetative hypha of *N. crassa,* apices light pinkish. × 800. *b* The same from *Allomyces* cultures. × 900. *c* The same from *Basidiobolus* cultures with pinkish apices (pale tip, empty arrow) contrasting with subapical dark-violet mitochondrial granules as well as a dense nucleolus in the more distal zone. × 400. Pinkish tip in the side branch contrasting with the violet cytoplasm lining the vacuole in the main hyphal trunk. × 800.

low Ca^{++} spot roughly covers the area of microvesicles aggregated into the 'Spitzenkörper', which can look bright yellow by light reflection in the centre of the alizarin-stained hyphal tips (figure 2, a).

We have attempted to check the apico-basal correlation of purple alizarin-Ca^{++} concentration along the gradient in vegetative hyphae of the same fungi using a more specific reagent, the metallochrome indicator Arsenazo III (Merck), designed to complex Ca^{++} ions into a blue-violet complex absorbing maximally at 685 nm[17,20]. As with alizarin, increasing gradients of Arsenazo III staining, initiated by a pinkish reaction in the apices, were obtained in all cases (figure 3). Rounded mitochondria were most often more densely stained than their surrounding hyaloplasm, developing only a pinkish-violet hue. This last observation is in line with our previous assumptions of a homeostatic control of calcium concentrations along polarly elongating hyphae[16]. Subapical sequestration of Ca^{++} by respiratorily-active mitochondria could therefore lead to the continuous captation, in the subapical and distal zones of the hyphae, of the Ca^{++} ions pumped from their tips, maintaining the Ca^{++} in such tips at the low concentration compatible with a hyaloplasmic gelation able to prevent the entrance of mitochondria.

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Heterogeneity of DNA methylation in murine L5178Y lymphoblasts[1]

J. Sawecka, L. Kornacka and J. Malec

Department of Biochemistry, Institute of Haematology, Chocimska 5, PL-00-957 Warsaw (Poland), 27 October 1978

Summary. Comparison of the extent of methylation in mouse DNA fragments rendered $MgCl_2$ soluble after mild DNase II digestion of nuclei, with different reassociation rate and nucleoli-bound, revealed the existence of 3 regions of the genome particularly 5-methylcytosine-rich: the sequences considered to be related to the transcriptionally active chromatin with the highest content of this base and fast reassociating, as well as nucleolar DNA with somewhat lower proportion of the methylated cytosines.

5-methylcytosine (5MC) is known to be the only minor base in eukaryotic DNA. Some observations indicate that its intragenomic distribution is not random but there are regions more and less 5MC-rich[2-4]. The significance of such heterogeneity is, however, not understood. Moreover, the mutual quantitative ratio of the proportion of 5MC in the particular fragments of the genome is difficult to evaluate, as the data reported by various authors were obtained from various kinds of cell, in different fragments of DNA and by different methods of 5MC estimation. To get some further information on the nature of the intragenomic heterogeneity of DNA methylation in the present work, we compared the proportion of 5MC formation in several DNA fragments of mouse leukemic lymphoblasts cultured in vitro. Bearing in mind the presumed role of DNA methylation in the regulation of transcription[5], we concentrated first of all on DNA fractions considered to vary in their function in genetic transcription, i.e. transcriprionally active and inactive chromatin (prepared by the digestion of nuclei by DNase II), sequences differing in the reassociation rate and nucleolar DNA.

Materials and methods. L5178Y murine leukemia cells were grown in Eagle's medium supplemented with L-asparagine, folic acid and 10% calf serum[6]. For fractionation of chromatin by DNase II digestion, the procedure of Gottesfeld et al.[7,8] was followed. DNA was isolated by the procedure described by Butterworth[9]. For reassociation experiments, the resulting DNA was additionally purified by centrifugation for 30 min at 10,000 × g in the presence of acid-washed Norite and the final preparation ($A_{260}/A_{280} > 2.0$) sheared by sonication to the average length of molecules of about 500 nucleotides as determined by agarose gel electrophoresis. The solution of fragmented DNA was dialyzed against 0.01 M sodium phosphate buffer, heat denatured at 100 °C for 10 min, cooled in dry-ice acetone and brought to a suitable phosphate concentration by adding 1.0 M sodium phosphate buffer; reassociation was performed at 65 °C in 0.05 M (fraction reassociated to Cot = 0.01) and 0.12 M phosphate buffer; double stranded DNA fragments were separated from single stranded molecules on hydroxyapatite columns at 65 °C by elution with 0.4 and 0.12 M phosphate buffer, respectively. Following the separation of fraction reassociated to Cot 0.01, the subsequent fractions of decreasing repetitiveness were isolated by subjecting the previous fractions of non-reassociated DNA to another schedule of dialysis, heat denaturation and reassociation, as described by Church[10]. For the isolation of nucleoli, the procedure was based on the method of Muramatsu et al.[11] with some modifications described for the isolation of nucleoli from human leukemic lymphocytes[12]. The proportion of 5MC in DNA was determined on 12 N $HClO_4$ hydrolysates after separation of bases by 2-dimensional paper chromatography on Whatman no 1 paper with solvent systems propan-2-ol-12 M HCl-water (85:22:18) and methanol-12 M HCl-water (91:26:13)[13].

Results and discussion. The experimental design was to submit DNA of L5178Y cells prelabelled with deoxy-[U-C^{14}]-cytidine to 3 fractionation procedures: 1. Limited digestion of chromatin with DNase II. 2. Preparation of fractions with different reassociation rate. 3. Isolation of