CELLULAR EVENTS OCCURRING IN GROWING HYPHAE
OF *ASPERGILLUS ORYZAE* AS STUDIED BY
AUTORADIOGRAPHY¹

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The duration of nuclear cycle and activities of RNA and protein syntheses along the length of growing hyphae of *Aspergillus oryzae* were estimated by the techniques of autoradiography. The labeling of DNA was effected by incubating the hyphae in the presence of adenine-³H followed by the removal of the labeled RNA with ribonucleases I and T₁. For the estimation of the duration of the nuclear cycle, two methods were presented. Estimations of activities of RNA and protein syntheses were carried out by the short term labeling with adenine-³H and L-leucine-³H, respectively. The statistic analysis of the data on the labeling of nuclear regions showed that, in short hyphae of several hundreds microns in length, the generation time of the end nuclei was 1.5–2 times longer than that of the head nuclei. Activities of RNA and protein synthesis, so far observed by autoradiography, were apparently less active in the apical region than in regions behind the apex. It is discussed that the latter observation may not necessarily gives the real picture, since the presence of the nucleotide and amino acid pool highly concentrated in the apical region possibly causes the dilution of radioactive precursors taken up by hyphae.

The growth of fungal hyphae is confined to their apices. This causes in necessity a gradual differentiation of cellular activities along the growing hyphae. Cytochemical observations of ZALOKAR (1) on growing *Neurospora* hyphae clearly demonstrated that distributions of various enzyme activities vary even in an apical cell compartment. Our recent experiments also showed that this seems to be the case over the whole length of growing hyphae of *Aspergillus niger* and that various enzyme activities change from one region to another in developing colony, *e.g.*, in growing hyphae (2, 3).

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The present paper describes autoradiographic experiments on *A. oryzae* hyphae. The results showed that synthetic activities of DNA, RNA and protein varied characteristically along the growing hyphae.

MATERIALS AND METHODS

Organism and cultivation. Organism used was *Aspergillus oryzae* strain I obtained from Kojiya Sanzaemon Co., Kyoto.

Short and separate hyphae with a few branches were obtained by shaking culture at 30°. Conidia (25 mg) were inoculated to 100 ml of TERUI's medium (4) contained in a 500-ml shaking flask.

Longer hyphae were obtained by standing culture using the same medium. Dry conidia were suspended in water and, after 1 hr, 0.1 ml of unsedimented suspension containing ca. 1,000 non-aggregated conidia per ml was inoculated to the liquid medium (ca. 5 mm in depth) placed in a petri dish. Most conidia grew adhering to the bottom. A long hypha was then scraped off with a fine glass needle from the surface, sucked into a capillary and transferred onto a cover slip (60×24 mm).

Autoradiography. Adenine-³H (250 mCi/mmole) and L-leucine-³H (144 mCi/mmole) were purchased from the Daiichi Pure Chemicals Co., Tokyo. The X-ray-sensitive emulsion, Sakura NR-M1, was a gift of Konishiroku Photo Industries Ltd., Tokyo. Autoradiography was performed essentially by the method described earlier (5).

When the sample of longer hyphae was used, a single hypha was placed gently on a cover slip as described above. An excess medium was decanted and a drop of fresh medium containing adenine-³H was placed on the sample. Incubation was carried out in a moist chamber at 30° for a given period. When the sample of shorter hyphae was employed, the labeling experiment was carried out in a small test tube using a small amount of hyphal suspension. The labeling condition was the same as described above. These hyphal samples were then fixed by successive treatments with 30, 50, 75, 90 and 100% ethanol.

For the examination of DNA synthesis, a hyphal sample labeled with adenine-³H was treated with a mixture of RNase I (1 mg/ml) and RNase T₁ (100 µg/ml) for 15 min at 30°. The sample was then washed with cold 5% trichloroacetic acid for 90 min and again treated successively with increasing concentrations of ethanol as described above.

The coverslips mounted with the treated samples were dipped in the emulsion, dried and stored at 5° in a dry chamber. After the development of autoradiographs, cell samples were stained with Giemsa solution (E. Merck AG, Darmstadt) for the observation of nuclei.

In an autoradiographic experiment in which RNA synthesis was studied, the developed samples bearing silver grains were treated with RNases and then stained with Giemsa solution to visualize nuclear regions.

RESULTS

Growth characteristics of A. oryzae hyphae

When 25 mg of dry conidia were inoculated into 100 ml medium and shaken cultured at 30°, a linear growth was obtained for about 16 hr after a lag of about 4 hr. Microscopic examinations revealed that most conidia protruded polarly two germ tubes (rudimentary hyphae) from a single conidium and as growth of the hyphae proceeded only a few branches were formed. An average length of main hyphae was about 120 μ at the 15th hr of cultivation. On the other hand, the standing culture in the same medium resulted in the formation of longer hyphae of about 660 μ in length with many branches after 15 hr.

Fig. 1. Schematic representation of *Aspergillus oryzae* hyphae.

Table 1. Cytological characteristics of *Aspergillus oryzae* hyphae.

Aspergillus oryzae conidia (25 mg) were inoculated into 100 ml medium and cultured with shaking at 30° for 15 hr. Average length of hyphae at the end of the culture was about 120 μ .

Characteristics	Number
Hyphae/conidium	2.1
Cells/hypha ^a	3.3
Nuclei/hypha ^a	20.7
Nuclei/cell ^b	6.3
Nuclei/tip cell	9.4
Nuclei/end cell	4.6

^a The hypha represents a single hypha protruding from a conidium and includes branches, if any.

^b The cell represents a part of a hypha separated with two cross walls.

Cytological characteristics of growing hyphae in the shaking culture are shown in Table 1 and schematically presented in Fig. 1. Nuclei are arranged almost one-dimensionally in a hypha except in the very tip where they were sometimes densely populated. In the apical region of the hypha, where growth proceeds, the number of nuclei per cell was much greater than that in older regions, suggesting the delayed formations of cross walls after nuclear division. In fact, in the stationary phase, in which nuclear division had already been ceased, the number of cells in a hypha was found to increase.

Fig. 2. Autoradiogram of *Aspergillus oryzae* hyphae which incorporated adenine- ^3H into DNA.

Hyphae were labeled with $30\text{ }\mu\text{Ci/ml}$ of adenine- ^3H for 30 min, treated with RNases I and T_1 to remove RNA, and autoradiographed.

Nuclear division cycle in different regions of hypha

It is generally known that the labeling of nuclei in fungal cells with tritiated thymidine cannot be achieved. This is also the case in *A. oryzae*. Therefore, the autoradiographic analysis of DNA synthesis was carried out by labeling the hyphae with tritiated adenine followed by the removal of labeled RNA with RNases I and T_1 . As shown in Fig. 2, silver grains deposited only over nuclear regions. When the hyphae were further treated with DNase, no grain was detected on the autoradiogram. This indicates that DNA synthesis has occurred in each labeled nucleus.

Characteristics of nuclear cycle in a growing hypha was analyzed autoradiographically by two methods. In the first method (A), growing hyphae were transferred to a fresh medium containing radioactive adenine- ^3H and samples for autoradiography were taken at intervals. Then the course of change in the percentage of labeled nuclei (with grains) was analyzed statistically. In the second method (B), hyphae which had been totally labeled with adenine- ^3H were transferred to the cold medium and samples for autoradiography were taken at intervals. Then the time for the half-decay in the cold culture of average grain counts per nucleus was estimated. It has been known that in fungal hyphae nuclei are actively dividing in growing region and become resting in older regions. Taking this fact into account for the

estimation of nuclear activity by these methods, we observed the behavior of two separate nuclei located at two extreme regions of the hyphae, the head and end nuclei (see Fig. 1).

In method A, the analysis of labeled nuclei in a nuclear population was carried out in principle by the method of STANNERS and TILL (6), who analyzed characteristics of cell division cycle of cultured mammalian cells. It should, however, be considered that the age distribution of nuclei in question is quite different from that of cultured mammalian cells. When we observe the nuclei located at extremities in hyphae, the birth rate of daughter nuclei should be invariable. Therefore, the age distribution of these nuclei is uniform and the percentage of nuclei belonging to each stage in the nuclear cycle is proportional to the duration of each stage.

Let us assume that the nuclear cycle can be divided into four stages, G_1 , S , G_2 and D (nuclear division), and that DNA synthesis occurs only in the S phase. In a nuclear population, whose age distribution is uniform as mentioned above, the frequency of labeled nuclei, η , after the incorporation of adenine- ^3H for time t is expressed by the following equations:

$$\eta = (S+t)/T \quad \text{when } 0 \leq t < (G_2 + D + G_1) \quad (1)$$

$$\eta = 1 \quad \text{when } (G_2 + D + G_1) \leq t \quad (2)$$

where G_1 , S , G_2 and D represent the duration (in min) of respective stages and T that of whole nuclear cycle. Accordingly, when we analyze the course of nuclear labeling by method A, the percentage of labeled nuclei would increase linearly with time and finally attain the maximum level. The time required for reaching the maximum should be the duration all way through

Table 2. Nuclear division cycle of *Aspergillus oryzae*. Figures in A and B were obtained by methods A and B, respectively (for details see text).

Method of Estimation	Culture condition	Average length (μ)	Location of nucleus	$G_2 + D + G_1^a$ (min)	S^b (min)	T^c (min)	Active nuclei (%)
A	Shaking	120	Head	55	42	97	95
			End	90	73	163	91
	Standing	660	Head	55	48	103	87
			End	85	78	163	85
B	Shaking	180	Front			130	
			End			180	

^a $G_2 + D + G_1$: observed.

^b S : $0.41 T/f_a$, where f_a represents rate of active nuclei in a nuclear population and 0.41 is ratio of S/T as obtained from Fig. 3A by extrapolating the curves to time zero.

^c T : $(G_2 + D + G_1)/(1 - 0.41/f_a)$.

Figs. 3. Course of incorporation and decay of adenine- ^3H in nuclear DNA of *Aspergillus oryzae* hyphae.

A: Hyphae were transferred at time 0 to radioactive medium containing $30 \mu\text{Ci/ml}$ of adenine- ^3H and cultured at 30° . Samples for radioautography were taken at intervals. Percentages of labeled nuclei located at the head and end regions in hyphae were plotted against time. Broken line represents the course of change as calculated from Equation A-3 in APPENDIX using necessary parameters obtained by approximation.

B: Conidia were inoculated into radioactive medium containing $0.3 \mu\text{Ci/ml}$ of adenine- ^3H and cultured by shaking for 15 hr. The labeled hyphae were transferred to non-radioactive medium at time 0 and change in the radioactivities of head and end nuclei was estimated by autoradiography.

stages G_2 , D and G_1 . Fig. 3A shows the time course of the increase in labeled head and end nuclei. As seen in this figure, by extrapolation of the curve to time zero, we may obtain a value representing the ratio, S/T (see Equation 1; for the implications of this explanation, see APPENDIX).

Thus, we can estimate the duration of T and S from the value of

G_2+D+G_1 . The results of the estimation of nuclear cycle by method A are listed in Table 2A. In such short hyphae, more than 80% of both head and end nuclei were actively synthesizing DNA. The duration of the whole nuclear cycle of the end nuclei was about 1.5–2 times longer than that of the head nuclei. It is interesting that the durations of G_2+D+G_1 and S phases were equally elongated in the former.

In method B, from the curve of grain-count decay caused by the isotopic dilution during the chase culture, the half-life was taken as the duration of nuclear cycle, T . The decay curves of radioactivities of the head and end nuclei are shown in Fig. 2B and the duration of the nuclear cycle estimated from these curves is listed in Table 2B. In this case also the nuclear cycle of the end nuclei was much longer than that of the front nuclei.

As seen in Table 2, those two estimation methods gave somewhat different results for the hyphae cultured under the same condition. In method B, the estimation was based on an assumption that all nuclei were active in synthesizing DNA. This assumption is, however, not true as indicated in Fig. 2A and Table 2A. Assuming that inactive nuclei occupies 15% of the population, the duration of nuclear division cycle of the head and end nuclei is calculated to be 110 and 150 min, respectively. A nucleotide pool in hyphal cells is also considered to affect the course of radioactivity decay in the chase culture. From these reasons, it may be concluded that method A is more reliable than method B.

RNA synthesis in different regions of hypha

When hyphae were pulse-labeled for less than 3 min with 30 μ Ci/ml of adenine- 3 H, no grain was detected in hyphal cells. This may probably be caused by the dilution of the isotope by the nucleotide pool in hyphae. On incubation of hyphae with the precursor for 3 to 6 min, silver grains appeared only on and around nuclei. Since these grains did not appear when the labeled hyphae were treated with RNases prior to autoradiography, it is highly possible that DNA had not been labeled during such a short contact with the precursor. The incubation for more than 6 min caused the formation of abundant grains all over the hyphal cells. In the present experiment, hyphae were incubated for 3 to 6 min with adenine- 3 H and the grains appearing in a nuclear region were considered to represent RNA synthesized in each nucleus. As summarized in Table 3, in the apical region, up to about 10 μ toward the apex, less grains per nucleus were observed than in the subapical and distal regions. Such difference appeared to be marked when the duration of the pulse-labeling was short.

Protein synthesis in different regions of hypha

After the pulse-labeling of hyphae with leucine- 3 H, the number of grains in an unit length of hyphae was counted and assumed to represent the activity of protein synthesis in that region. Results shown in Table 4 apparently

Table 3. Incorporation of adenine-³H into RNA of *Aspergillus oryzae*.

Duration of labeling (min)	Average grain counts/nucleus		
	Apical region ^a	Subapical region ^b	Distal region ^c
4	0.1	0.28	1.98
5	0.3	0.89	2.62
6	1.07	2.48	2.93

^a Mean value of grain counts observed in 5 nuclei from the head.

^b Mean value of grain counts observed in 15 nuclei from the 6th to the 20th.

^c Mean value of grain counts observed in 5 nuclei from the end.

Table 4. Incorporation of leucine-³H into hyphae of *Aspergillus oryzae*.

Duration of labeling (min)	Average grain counts/10 μ length		
	Apical region ^a	Subapical region ^b	Distal region ^c
3	7.8	10.5	11.3
4	13.3	14.0	17.2

^{a, b, c} Refer to the footnote to Table 3. In this case, grains appearing in hyphal regions including these groups of nuclei were counted.

indicate that the activity of protein synthesis in the apical region was rather lower than those in the subapical and distal regions.

DISCUSSION

The present experiments revealed that, when the fungus was cultured by standing, the rate of nuclear cycle of the head nuclei is almost twice that of the end nuclei located at ca. 600 μ distal to the apex. In a shaking culture, however, the rate of nuclear cycle seemed to decrease along hyphae from the apex to the distal end much more abruptly than in a standing culture. This might probably be due to a heavy inoculum in the shaking culture, which resulted in the rapid consumption of limiting nutrients by a crowded hyphal mass.

If we assume that the rate of nuclear cycle in a standing culture drops linearly along the hypha as mentioned above, that of end nuclei located 2 mm distal to the apex may be about one-tenth that of the head nuclei. Although this has not been proved on long hyphae by the autoradiographic experiment because of technical difficulties, nuclear activities with respect to the chromosomal replication may become decreased in distal regions as hyphae elongate and, thus, resting nuclei may appear in hyphal regions far behind the apex.

The activity difference in RNA and protein syntheses along growing hyphae seemed apparently to be somewhat distinct from that of DNA synthesis.

This has already been observed by ZALOKAR (7) in *Neurospora* hyphae. RNA and protein syntheses were rather less active in the apical region than in distal regions as deduced from the results of autoradiographic experiments. It should, however, be noted that the size of amino acid pool in *Aspergillus* hyphae is rather high. The observation that the shorter the duration of labeling, the greater was the difference in grain counts between the apical and distal regions suggests the presence of amino acid or nucleotide pool of a larger size in the apical than in the distal region. If this is the case, the dilution effect on the incorporated radioactive precursors may cause an apparent reduction in the synthetic activities in the apical region when examined by autoradiography.

ZALOKAR (7) pointed out that an active cytoplasmic streaming proceeds toward the growing region along a hypha through septal pores and the hyphal tip is thus constantly supplied with cytoplasm. He also stated that the formation of vacuoles in distal regions results in the displacement of cytoplasm through the hypha in the direction of least resistance. Such a view on the growth of fungal hyphae has conventionally been accepted by mycologists. It should, however, be mentioned that any positive evidence for such a view has not been presented.

YANAGITA and KOGANÉ (8, 9) demonstrated employing growing *A. niger* colonies that, when those prelabeled with radioactive precursors were grown in the cold media, no shift of radioactivity toward the periphery occurred in the substrate mycelium during the chase culture, but did in the aerial mycelium. So far as the substrate mycelium is concerned, the above conclusion was deduced from observations on the shift of labeled regions, in an accuracy of the order of magnitude of a few millimeters or less. Therefore, we attempted to demonstrate more precisely the positive or negative migration of prelabeled cytoplasm toward the apical region. Hyphae were labeled for a short period with adenine- ^{14}C just after the protrusion of germ tubes. Thus, we obtained autoradiographic samples bearing an uncountably large number of silver grains. Such prelabeled hyphae were further cultured in the cold medium and then autoradiographed. The result showed that grains were evenly distributed along the whole hyphae at the density of about 5 grains per $100\ \mu$, which were of the same order of magnitude of the background count. This result strongly suggests that the turnover of RNA proceeds very actively, and thus we failed to chase the fate of the prelabeled RNA in growing hyphae.

Unpublished data of Yanagita showed that, when *A. niger* hyphae grown on Cellophane placed on a medium were cut into fragments at varied lengths from the tip, apical cell fragments retaining septa at the closest site to their tips grew as fast as intact hyphae. Since cytoplasm of the neighboring cell to the apical cell leaked out in such cell fragments, it can safely be concluded that the streaming force in the neighboring cell does not affect the growth rate of the apical cell.

From these observations, it may be stated that in the substrate hyphae the growth of apical cells is supported largely by biosynthetic activities *in situ* but not or at least only partially by the supply of cellular materials from cells distal to the apex. By contrast, the growth of the aerial hyphae is most probably dependent on the supply from distal cells driven by an active cytoplasmic streaming and also by vacuolation. ZALOKAR (7) stated that the rate of streaming at a particular point of aerial hyphae increases with the number of growing tips distal to that point and that in substrate hyphae the rate of flow far from the tips is low influenced by the absorption of nutrients on the way. Such considerations strongly support the view that apparently reduced activities of RNA and protein syntheses in the apical region of substrate hyphae as observed by autoradiography do not represent the real picture but are due to the dilution of radioactive precursors by amino acid and nucleotide pools.

APPENDIX

In the estimation of the number of "labeled" nuclei, the distribution of grain counts per nucleus should be taken into account. However, the number of labeled nuclei may be approximated in practice by the number of nuclei with grains for the following reasons.

In a nuclear population, whose average grain counts per nucleus is λ , the probability of obtaining grainless nuclei can be expressed by $e^{-\lambda}$, since the grain count distribution is of Poisson type. Accordingly, the probability of obtaining grainless nuclei, $P(0)$, in a nuclear population, whose age distribution is uniform, is expressed by the following equation:

$$P(0) = \frac{1}{T} \int_0^T e^{-\lambda} d\tau, \quad (\text{A-1})$$

where T stands for the duration of whole nuclear cycle. When average grain counts per nucleus which incorporated adenine-³H for time t are calculated for each nucleus in respective stages in the nuclear cycle (see Fig. 4), the probability of obtaining nuclei with grains may be derived from Equation A-1 as follows:

$$\begin{aligned} \xi &= 1 - P(0) \\ &= [S + t + \varepsilon(t)] / T. \end{aligned} \quad (\text{A-2})$$

In this equation, $\varepsilon(t)$ represents the following:

$$\varepsilon(t) = -\frac{2}{\alpha} + \left(\frac{2}{\alpha} - S + t\right) e^{-\alpha t} \quad \text{when } 0 \leq t < (G_2 + D), \quad (\text{A-3})$$

and

$$\varepsilon(t) = -\frac{3}{\alpha} + \left\{ \frac{2}{\alpha} - S - \frac{1}{\alpha} e^{\alpha(G_2+D)} + t \right\} e^{-\alpha t} + \frac{2}{\alpha} e^{\alpha(G_2+D)/2} \cdot e^{-\alpha t/2}$$

when $(G_2+D) \leq t < S \leq (G_2+D+G_1)$, (A-4)

where α stands for the mean rate of increase in grain counts (per min) of nuclei in the S stage.

If we assume as prospective values $\alpha=1$ grain/min and $(G_2+D)=30$ min, and substitute S and T in Equation A-2 with the values obtained by the above approximation (see Table 2), the course of change as represented by a broken line in Fig. 3A may be obtained for the end and head nuclei. It is seen in the figure that the approximation obtained by Equation 1 can be employed in practice.

Fig. 4. Diagrams for calculation of $P(0)$

Possible courses of changes in average grain counts per nucleus (λ) during the nuclear cycle under different labeling conditions (labeling period, t) are shown in A and B. Duration and λ for each section (indicated by Roman numerals) as conditioned by the duration of t are shown in the following table:

Section	A when $0 \leq t < (G_2+D)$		B when $(G_2+D) \leq t < S \leq (G_2+D+G_1)$	
	Duration	λ	Duration	λ
I	$0 \sim G_1$	0	$(t-G_2-D) \sim G_1$	0
II	$G_1 \sim (G_1+t)$	$\alpha(\tau-G_1)$	$G_1 \sim (G_1+t)$	$\alpha(\tau-G_1)$
III	$(G_1+t) \sim (G_1+S)$	αt	$(G_1+t) \sim (G_1+S)$	αt
IV	$(G_1+S) \sim (G_1+S+t)$	$\alpha(t+G_1+S-\tau)$	$(G_1+S) \sim T$	$\alpha(t+G_1+S-\tau)$
V	$(G_1+S+t) \sim T$	0	$0 \sim (t-G_2-D)$	$\alpha(t-G_2-D-\tau)/2$

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