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Regulation and Timing of Deoxyribonucleic Acid Synthesis in Hyphae of *Aspergillus nidulans*

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Pulse labeling of deoxyribonucleic acid (DNA) and radioautography have been used to study the effect of growth rate on nuclear replication in *Aspergillus nidulans*. When conidia were germinated in media supporting a fast growth rate, the radioactive pulse labeled either all of the nuclei in a cell or none of them. At slower growth rates, hyphae contained both labeled and unlabeled nuclei. Altering the growth rate thus changed nuclear replication from simultaneous to sequential. The time taken to duplicate the DNA in a nucleus, estimated from the ratio of labeled to total nuclei, remained constant at the different doubling times. The distribution of label showed that nuclei in the same hypha spent unequal times in both the postmitotic gap (G1) and the premitotic gap (G2) periods when grown at slow rates. These unequal G1 and G2 periods are considered to cause asynchrony. Once DNA synthesis was out of phase through growth on a poor medium, transferring the hypha to a rich medium did not resynchronize the nuclei. To interpret the data, two initiator mechanisms, one starting DNA synthesis and the other mitosis, are postulated to control nuclear replication in *A. nidulans*.

We have previously shown (14) that the nuclei in individual hyphae of *Aspergillus nidulans* can replicate in synchrony but only do so at fast growth rates. At slow growth rates, nuclei in the same hypha divide at different times, and we suggested that this is the way the organism fits the rate of chromosome duplication to the overall growth rate. How slow growth leads to an out-of-phase replication of neighboring nuclei is not known.

Eukaryotic nuclei generally exhibit a resting period after division (G1), a period of deoxyribonucleic acid (DNA) synthesis (S), a resting period after DNA synthesis (G2), and mitosis in the course of their replication (16). In the present work, we have used pulse labeling of DNA and radioautography to investigate how changes in the growth rate affect these stages in *A. nidulans*. In such radioautograms, the proportion of labeled nuclei to total numbers of nuclei indicates the approximate length of S. The distribution of labeled nuclei in individual hyphae will show whether DNA synthesis is synchronous and whether changes in growth rate affect G1 and G2. Our results suggest that slow growth does not alter the length of S, but leads to nuclei in the same hypha having both G1 and G2 periods of different lengths.

MATERIALS AND METHODS

Strain. A mutant, 46 NX, isolated after nitrosoguanidine treatment of an adenine- and biotin-requiring strain of *A. nidulans* (strain 46, Fungal Genetics Stock Center, Dartmouth College, Hanover, N.H.) was used throughout this work. Strain 46 NX differs from the parent strain in not being able to utilize hypoxanthine and nitrate as sole nitrogen sources. It can utilize uric acid and appears to correspond to the cnx mutants (lacking xanthine dehydrogenase activity) described by Pateman et al. (12). We introduced this mutation to prevent breakdown of the tritiated adenine used to label the nucleic acids.

Growth conditions. The media, conditions of incubation, and preparation of conidial suspensions were as described previously (14), with the following exceptions: 0.3% (w/v) $(NH_4)_2SO_4$ was substituted for $NaNO_3$, and all media contained 0.2% (w/v) histidine dihydrochloride to minimize the utilization of adenine for histidine biosynthesis.

Determination of growth rate. In addition to estimating growth rates by direct weighing (14), we measured the rate of ^{3}H-adenine incorporation into the nucleic acids of germinating conidia. Conidia were inoculated into medium containing 2 μc of adenine-*2,8*-^{3}H per ml (Radiochemical Centre, Amersham, England) and 60 μg of unlabeled adenine per ml. Samples, taken at intervals, were pipetted into an equal volume of cold 10% (w/v) trichloroacetic acid, kept at 0 C for 30 min, and collected on a mem-

brane filter (Millipore Corp., Bedford, Mass.). After washing with distilled water, the filter plus cells was transferred to 2 ml of 10% trichloroacetic acid and boiled for 15 min to extract the nucleic acids. After filtration, the radioactivity in the filtrate was determined in a Packard Tri-Carb liquid scintillation counter. The doubling times found by this technique agreed closely with those found by weighing.

Labeling with ^3H-adenine and preparation of radioautograms. Cultures in complete medium were filtered onto a membrane filter, washed, and resuspended in the same medium with no adenine. These operations were completed within 30 sec. The cells were then reincubated with shaking for 3 to 5 min to exhaust the internal purine pools. Adenine-8-^3H (Schwartz BioResearch Inc., Orangeburg, N.Y.) with a specific activity of 3 to 8 c/mmole was added to give a final concentration 20 μc/ml. Incubation was continued for either 5 min (glucose medium) or 10 min (arabinose and xylose media), and labeling was terminated by adding an equal volume of cold 10% trichloroacetic acid to the cultures. After standing at 0 C for 30 min, the cells were collected on a membrane filter, washed thoroughly, resuspended in distilled water, spread on slides, and air-dried.

Since ^{3}H-adenine labels ribonucleic acid (RNA) as well as DNA, we removed the cell RNA by a modification of Williamson's technique (17). The hyphae on the slides were incubated for 2 hr at 37 C with three times crystallized ribonuclease (1 mg/ml, 0.05 M phosphate buffer, *p*H 6.3) which had previously been heated to 90 C for 4 min. Control slides were incubated under the same conditions with a mixture of ribonuclease and deoxyribonuclease (1 mg of ribonuclease plus 0.5 mg of deoxyribonuclease per ml) in phosphate buffer, *p*H 7.0, containing 10^{-3} M $MgSO_4$. All slides were then treated according to the following schedule: washed in running water; 30 min at room temperature in 0.3% (w/v) formaldehyde in 0.05 M phosphate buffer, *p*H 7.0; washed in running water; 1 hr at 25 C in 1 N NaOH; washed in running and distilled water; and air dried. The slides were covered with Ilford K5 Nuclear Emulsion and stored in light-tight boxes at 4 C. The radioautograms were developed with Kodak D19b developer, fixed in 30% (w/v) $Na_2S_2O_3 \cdot 5H_2O$, and stained with Mayer's hematoxylin containing, per 100 ml of water: hematoxylin, 0.1 g; $NaIO_3 \cdot H_2O$, 0.02 g; and potassium alum, 5.0 g.

Storage times before development were adjusted so that among those nuclei showing grains at least 90% had two or more grains directly above the nucleus (approximate area, 3 μ^2). Parallel control slides treated with deoxyribonuclease as well as ribonuclease showed negligible grain densities compared with that of two or more grains per 3 μ^2; the density of the background was also negligible (Fig. 2–7). All nuclei with two or more grains directly above the nucleus were scored as labeled, others, as unlabeled. Although two grains per nucleus was taken as the lower limit indicating labeling, the great majority of nuclei scored as radioactive showed more than two grains (Fig. 2–7).

RESULTS

Growth rate and relative frequency of hyphae with two to eight nuclei in media containing different carbon sources. The growth of 46 NX conidia in shaken, liquid culture was similar to that of the parent strain 46 (14) and exhibited an exponential phase lasting for the first four dry-weight doublings. With glucose as carbon source, the doubling time (t_D) during exponential growth (average of three determinations) was 87 min; with L-arabinose, t_D was 135 min, and with L(−) xylose, 196 min. In the same media, containing NH_4^+ as N source, strain 46 grew at closely corresponding rates.

The synchrony of nuclear replication in strain 46 NX changed with the growth rate as it did with the strains previously studied (14). Populations of conidia germinating in glucose medium consisted almost entirely of hyphae with two, four, or eight nuclei, indicating close synchrony. At the slower growth rates, synchrony was lost, and progressively more hyphae with three, five, six, or seven nuclei were found (Fig. 1).

Effect of growth rate on the synchrony of DNA synthesis. Germinating conidia in the exponential phase were labeled with ^{3}H-adenine for 5 min when growing on glucose (1/17 t_D) and for 10 min when growing on arabinose (1/13 t_D) or xylose (1/20 t_D). Radioautograms of glucose-grown cells showed that the majority of hyphae with labeled nuclei had radioactivity in all of the nuclei. However, when arabinose or xylose was the carbon source, the majority of hyphae with radioactivity contained both labeled and unlabeled nuclei (Table 1; Fig. 2–7). The absence of grains above the nuclei in slides treated with deoxyribonuclease as well as ribonuclease showed that we were indeed measuring DNA synthesis. At all growth rates, synchrony was better in the second generation (two nuclei) than at later stages (three to seven nuclei). Thus, differences in growth rate alter the phasing of DNA synthesis in the same way that they change the synchrony of overall nuclear replication (14).

In hyphae having three to seven nuclei, some labeled and some not, no correlation appeared between the position of the nucleus in the hypha and synthesis of DNA. However, the majority of longer hyphae had more than one labeled nucleus, and the labeled nuclei almost always occupied neighboring positions (Table 1).

Effect of growth rate on the G1 period. The proportion of hyphae containing two nuclei, only one of which was labeled, increased markedly at the slow growth rates (Table 1; Fig. 4). Since the first two nuclei in a hypha are formed

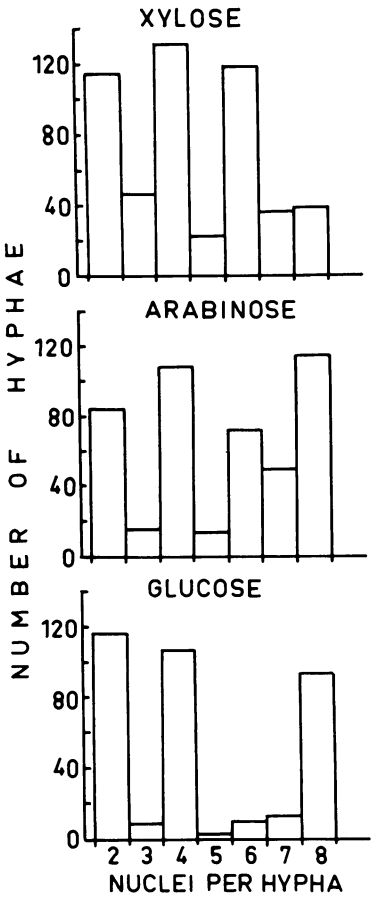

FIG. 1. *Frequency of hyphae with two to eight nuclei in samples taken during the germination of strain FGSC 46 NX conidia in media supporting different growth rates. Doubling times in media were: glucose, 87 min; arabinose, 135 min; xylose, 196 min.*

simultaneously, this must be due to differences in either their respective G1 periods or their S periods. The data in Table 2 indicate that S does not vary with growth rate, and the effect thus appears to be on G1.

Time taken for the synthesis of nuclear DNA at various doubling times. We prepared radioautograms after pulse-labeling conidia growing in the three different media and examined microscope fields chosen at random. From the ratio of labeled to total numbers of nuclei and the t_D, an approximation of S, the time taken to duplicate the nuclear DNA, was calculated. The values of S for the three growth conditions were almost identical (Table 2).

Effect of growth rate on the G2 period. In the absence of methods which, in our system, can measure G2 directly, we used an indirect approach. During growth in a glucose medium, nuclear replication in individual hyphae is synchronous and this synchrony is progressively lost when hyphae are washed and resuspended in a xylose medium (Table 3). Within 60 min after the transfer to xylose, the number of hyphae with three, five, six, or seven nuclei had already increased significantly. Since neither the length of S (Table 2) nor of mitosis (14) is affected by a change in growth rate, the change in carbon source must have altered either G1 or G2, or both. A comparison of cultures pulse-labeled immediately prior to transferring from glucose and some time after the transfer to xylose can distinguish between these alternatives.

Suppose that after change to the slow growth rate G2 becomes unequal for nuclei in the same hypha. A pulse given in glucose medium will label nuclei in S which, after an unequal G2, will give rise to hyphae with three, five, six, or seven labeled nuclei (N_L hyphae). N_L hyphae will therefore appear at an earlier stage than if the pulse is given after the transfer to xylose. However, if G2 remains constant under all growth conditions, nuclei labeled in S at the time of transfer will divide synchronously to give hyphae with two, four, or eight nuclei. Only after a further complete round of replication will these become N_L hyphae. If G2 is constant and the interval between labeling in glucose and xylose is less than the time taken for a whole round of replication (i.e., completion of S, a constant G2, mitosis, an unequal G1, and initiation of S), N_L hyphae should appear first

TABLE 1. *Radioactivity in the nuclei of individual hyphae pulse-labeled during growth on various carbon sources*

Carbon source	Labeled hyphae containing					
	Two nuclei		Three to seven nuclei			
					$^3H + {}^1H$ with > one ^3H nucleus	
	No. of hyphae	$^3H + {}^1H$ nuclei[a]	No. of hyphae	$^3H + {}^1H$ nuclei[a]	^3H nuclei adjacent	^3H nuclei not adjacent
					%	%
Glucose...	84	6	97	25	100	0
Arabinose.	114	30	112	86	65	4
Xylose....	117	53	71	93	52	3

[a] Hyphae containing both labeled and unlabeled nuclei as percentage of total labeled hyphae.

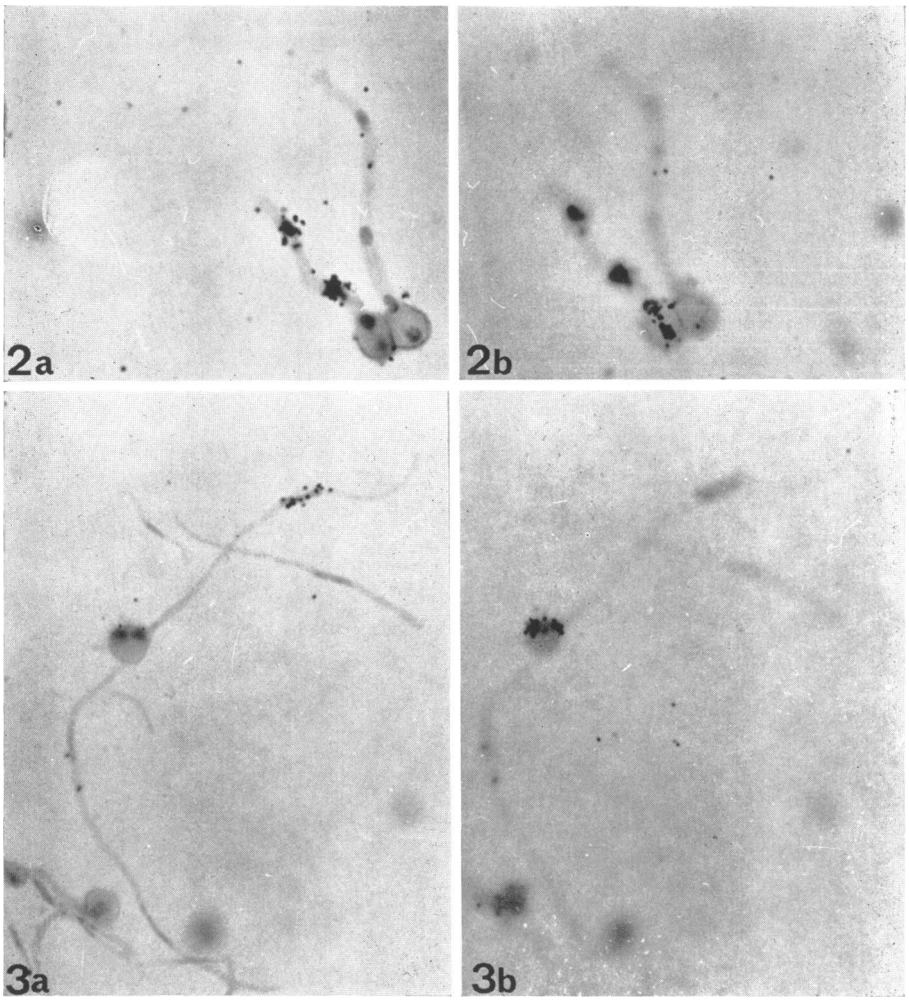

FIG. 2. *Radioautograph of a germinating spore grown with glucose as carbon source and treated with ribonuclease. All four nuclei are synthesizing DNA and lie alongside a non-DNA-synthesizing sporeling. The photographs are in different planes of focus to demonstrate the grains above all the nuclei.* × *1,100.*

FIG. 3. *Radioautograph of a germinating spore grown in* L(−)*xylose and treated with ribonuclease. Only three of five nuclei are synthesizing DNA, and these lie adjacent to one another. The very thin hyphae are typical of growth in xylose with the nuclei elongated to fit the narrow dimension of the hypha.* × *1,100.*

of all in the xylose-labeled culture. From Table 3, it appears that either a whole round of replication was finished in less than 15 min, which seems extremely unlikely, or that G2 for nuclei in the same hypha was unequal.

Synchrony of DNA synthesis after transfer from xylose to glucose. To test whether growth on glucose is in itself sufficient to synchronize DNA synthesis within a single hypha, we germinated conidia in xylose medium, washed them, and resuspended them in glucose. Samples were taken at intervals, labeled for 5 min, and radioautographed. The lack of synchrony found immediately after the transfer and characteristic of xylose-grown cells was maintained for 135 min of growth on glucose (Table 4). Thus, once the nuclei are out of phase in their replication cycle, growth on glucose does not bring about a realignment.

DISCUSSION

Radioautograms showed that DNA synthesis in individual multinucleate hyphae was synchronized at fast but not at slow growth rates. This confirms directly our previous predictions based on the relative frequency of hyphae with

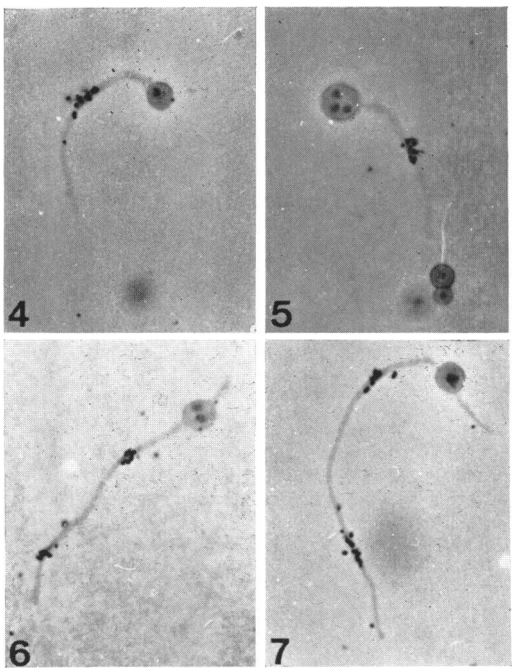

FIG. 4–7. *Radioautographs of spores grown in* L(−)*xylose and treated with ribonuclease. These are typical examples of labeled and nonlabeled nuclei present in the same hypha. Note that when more than one nucleus is labeled they lie adjacent to one another.* × *1,100.*

two to eight nuclei (14). Lark (8) has suggested that a change from simultaneous to sequential chromosome duplication occurs when cells of *Escherichia coli* having two chromosomes grow at slow rates. Lark's interpretation of the *E. coli* data has, however, been seriously criticized (6). Germinating conidia of *A. nidulans* thus appear to be the first system where changes in nutrient supply clearly result in asynchronous DNA synthesis by nuclei in the same cell.

The interpretation of these results will depend in the first place on whether the cytoplasmic environment in individual hyphae can be considered as homogeneous or not. The germinating conidia we have studied have short coenocytic hyphae ranging in length from approximately 2 to 20 μ. There is vigorous protoplasmic streaming, and this, combined with the short distances, makes it unlikely that differences in solute concentrations are maintained in the cytoplasm. In the very much larger multinucleate cells of *Physarum*, the cytoplasm is sufficiently homogeneous to ensure strict synchrony of nuclear replication (4). If it is accepted that the cytoplasm has a uniform constitution, then the mechanisms controlling DNA synthesis should have the general features discussed by Lark (7) and Maaløe and Kjeldgaard (10). These are the existence of initiators required to start some process in nuclear replication and the coupling of the rate of initiator formation to the rate of overall cell synthesis.

Determining the length of S from the ratio of labeled to unlabeled nuclei gives approximate values only, unless the age distribution of the nu-

TABLE 2. *Ratio of labeled to total nuclei in pulse-labeled cultures growing on various carbon sources*

Carbon source	Total no. of nuclei	Labeled nuclei	S[a]
			min
Glucose.....	2,086	455	19
Arabinose....	1,738	296	23
Xylose.....	1,637	163	20

[a] Calculated from labeled nuclei/total nuclei times t_D.

TABLE 3. *Distribution of hyphae with three, five, six, or seven nuclei (N_0 hyphae) after transfer from glucose to xylose*

Time after transfer	N_0 hyphae[a]	Label in N_0 hyphae	
		Pulsed at −5 min	Pulsed at 10 min
min	%		
0	5	—	—
60	15	21.7[b]	12.6[c]
105	23	—	—
165	33	—	—

[a] Percentage of 200 hyphae chosen at random.
[b] Percentage of 166 N_0 hyphae examined.
[c] Percentage of 108 N_0 hyphae examined.

TABLE 4. *Asynchrony of nuclear replication in xylose-grown hyphae transferred to glucose*

Time after transfer[a]	No. of labeled hyphae with three to seven nuclei	
	Total examined	Containing $^3H + {}^1H$ nuclei
min		
0	50	47
45	50	41
90	50	45
135	50	43

[a] Samples taken at indicated times, pulsed with ^{3}H-adenine for 5 min, and radioautographed.

clei and the position of S in the replication cycle are taken into consideration (1). We have not been able to correct for these factors. A further assumption, basic to the calculation, is the ability of all nuclei in the population to replicate. This, although plausible, has not been proven. In spite of these reservations, the values for S indicate that it is not a change in the rate of nucleotide polymerization which fits chromosome replication to growth rate. For twofold variations in growth rate, the range that we examined, the same appears to be true of *E. coli* (7, 10). The constant length of S and of the mitosis time (14) support the view that *A. nidulans* controls the frequency with which the phases in nuclear replication are initiated rather than their rate.

Changes in growth rate altered the length of both the G1 and the G2 periods. This is in contrast to the widely held view that the length of G1 is the only point of control and that, once DNA synthesis has started in eukaryotic nuclei, replication continues to the next G1 according to a fixed schedule (2, 9, 13, 15). Moser (11) has recently pointed out that the evidence for this view is not sound and has shown that G2 in mammalian cells can vary within wide limits. In *A. nidulans*, not only can the growth rate alter G2 but nuclei in the same hypha can spend different times in G2. This indicates that an initiator mechanism for mitosis, analogous to that controlling DNA synthesis, may exist. It is relevant in this connection that mitosis in eukaryotic cells requires concomitant protein synthesis (3).

At the slower growth rates, nuclear replication in individual hyphae was not strictly sequential (Table 1). The majority of the longer hyphae had label in more than one nucleus. This is perhaps not surprising since the slowest growth rate tested was only half of the maximal. At this rate, a hypha containing four or six nuclei should be producing sufficient initiators for more than one nucleus. The labeled nuclei almost always occupied neighboring positions, and this could indicate that replication was sequential for whole regions of a hypha rather than for individual nuclei. Alternatively, neighboring nuclei may be the products of the same nuclear division and therefore much closer in age to each other than to the other nuclei.

According to a widely held view, the control of chromosome replication differs from that of other macromolecules in being positive and depending on the supply of initiators (5). This leads to the prediction that, once nuclei in a hypha are out of phase, transferring the hypha to a fast growth rate will not resynchronize them. The fast growth rate will allow each nucleus to replicate at a constant, maximal rate and perpetuate the asynchrony. We verified this prediction experimentally by transferring conidia from xylose to glucose medium. During 1.5 generations in glucose medium, there was no sign of synchrony being reestablished.

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