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NUCLEIC ACIDS IN HAPLOID AND DIPLOID CELLS OF *PROTOMYCES INUNDATUS*

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Abstract

In both haploid and diploid strains of *Protomyces inundatus* Dangeard, grown in liquid culture, DNA content per cell remained constant irrespective of age of culture. The RNA content were very high in the early stages of growth but declined to fairly constant value at later stages of growth, and at all stages the RNA content of diploids were around 25 per cent higher than haploids; the behaviour of protein closely resembling that of RNA. The DNA after X-press extraction had a molecular weight of around 1×10^6, in both haploids and diploids. The GC content of DNA from all the strains were 55 per cent. A melting transition profile analysis of the DNA from various strains showed that proportion of the total DNA contained in the two satellite peaks in diploids was nearly half that found in haploids. It is suggested that some DNA stretches in diploids, on which RNA is transcribed, are deleted

Introduction

Nucleic acids behaviour in haploid and diploid cells of *Protomyces inundatus*, Dangeard has been described (Valadon *et al*, 1962) and DNA and RNA content per cell were observed to decrease with increase in age although there was constancy of RNA to DNA ratio of 2:1 for haploid and diploid cells; thus RNA produced per unit DNA in diploid cell was one half to that produced in haploid cell. This result was in contrast to the studies made on the nucleic acid content of the polyploid series of *Saccharomyces* (Ogur *et al*, 1952). It is demonstrated that dry weight, RNA, metaphosphate apart from DNA, are ploidy dependent in yeast. The diploid yeast cells have DNA as well as RNA two times more in amount as compared to that of haploid cells.

An examination of the behaviour of RNA in conjugating haploid cells of *P. inundatus* has revealed that both DNA and RNA are contributed from each of the conjugant to zygote and increase in RNA is not conserved in the normal division cycle (Venitt *et al*, 1968). Nutritional physiology could not be implicated to the differences in RNA to DNA ratio because haploid and diploid cultures had similar growth rate constant, glucose yield constant and glucose saturation constant (Ahmad, 1973a). Also nucleotide composition for total RNA, rRNA and sRNA from haploid and diploid cells was identical (Ahmad, 1973b).

In this study, DNA as well as RNA content per cell basis in haploid and diploid cultures of *P. inundatus* has been re-examined. Evidence has been obtained suggesting that differences in RNA to DNA ratio in haploid and diploid cells lie in the differences in their structure of DNAs.

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Materials and Methods

(a) *Strains:*

The haploids 18+ and 2— and diploids 2nB and F2 were the same strains that have been described by Ahmad, 1973a. The cultures were maintained on basal medium and it was found that culture remained true to type for long periods on this medium. Sub-cultures were made every two months and slants were incubated at 20°C. Each strain was sub-cultured at least three times before being used for experimental work to ensure that the strains employed were stable and true to type.

(b) *Procedure for experiments on age variation and nucleic acids content in haploid and diploid cells.*

In some experiments the cultures were grown in one litre of medium in two litre dropping flasks, subjected to forced aeration with addition of 10-30 ppm silicone to check foaming. In other experiments the cultures were grown in 350 ml Erlenmeyer flasks each containing 100 ml medium. on a reciprocating shaker at 50 strokes per min. The cultures were incubated at 20°C.

(c) *Cell harvesting and counting:*

The cultures were harvested for cell counts and estimation of nucleic acids, at different times of growth, in a manner that has been described previously (Ahmad, 1974).

(d) *Estimation of nucleic acids and proteins:*

Nucleic acids were estimated by method similar to those of Ogur *et. al.*, (1952). After preliminary washing with spectroscopic ethanol at 85°C and 0.2 per cent perchloric acid ($HClO_4$) at O°C, the RNA was extracted in 10 per cent $HClO_4$ at 4°C and estimated spectrophotometrically. DNA was extracted in 10 per cent $HClO_4$ at 65°C and estimated by the improved diphenylamine method of Giles & Myers (1965). Proteins were extracted in IN NaOH at 37°C and determined by the method of Ellman (1962), modified by measuring absorbance at 300 nm.

(e) *Growth procedure for experiments on DNA extraction:*

All strains were grown at 20°C in a litre of medium pH 4.5, under forced aeration; and cells were harvested from late log phase of growth. Usually the cell yield was 8-10g packed wet weight per litre culture. The cells were harvested by centrifugation at 7,000 *g* in an MSE 'Super Speed 40' ultracentrifuge, washed with ice cold water and suspended in buffer. The cells were either used immediately or stored at —20°C.

(f) *DNA extraction:*

The cell wall of *P. inundatus* could not be removed with detergents or digested by the gut enzyme of *Helix pomatia* or egg white lysozyme; therefore the cells were broken by passage through an X-press (BIOX, Sweden). The DNA was then extracted by the method of Marmur (1961) with the modification that the polysacchar-

ides were removed after the ribonuclease step by differential centrifugation. Solid sodium chloride to 1M concentration + 1/9 vol. of 5 per cent sodium dodecyl sulphate in 45 per cent ethanol was added to the RNA free and deproteinized DNA. The mixture was shaken, allowed to stand for 15 min at 4°C and centrifuged at 40,000 *g* for 3 hr. The clean supernatant was then removed and treated with chloroform-isoamyl alcohol twice; the DNA was precipitated with 2 vol. of ethanol and spooled around a glass rod. The DNA was then dissolved in standard saline citrate (SSC). dialysed against two changes of 100 ml SSC and kept at —20°C at a concentration of 2 mg. per ml.

(g) *Determination of DNA molecular weight:*

A comparison was made of the molecular weights of DNA samples from *Escherichia coli* B and from *P. inundatus* extracted under similar conditions. The sedimentation coefficient (S_{20}, w) of DNA was determined in an analytical ultracentrifuge (Spinco Model E) at 20 μg per ml DNA in SSC at 35,600 rev/min. Molecular weights were calculated from the values of S_{20}, w, using the relationship established by Doty, McGill & Rice (1958).

(h) *Determination of DNA base composition:*

Base composition was determined by three methods, 1: the paper chromatographic method (Bendich, 1957; Wyatt, 1951), 2: the calculation of GC content from the ratio of optical densities at 260 nm and 280 nm in 0.1 N acetic acid at pH 3 (Fredericq *et al*, 1961), 3: estimation of GC content from the change in absorbance at 270 nm after bromination of DNA solution in 1N H_2SO_4 (Wang & Hashagen. 1964).

(i) *Density gradient centrifugation of DNA.*

Buoyant density determinations of DNA were made by the method of Meselson *et al*, (1957); and Schildkraut *et al*, (1962), using a 12 mm Kel F Single Sector Cell in Spinco Model E. Since preliminary studies had revealed interaction between *E. coli* B DNA and *P. inundatus* DNA, no reference DNA was added, and therefore care was taken to refill the same cell to the same weight for each run so that accurate comparison might be possible. *E. coli.* B DNA and *Micrococcus lysodeikticus* DNA were run separately in the same cell under the same experimental conditions, as reference DNA.

(j) *Thermal denaturation profiles of DNA:*

Thermal denaturation profiles were determined both for calculations of base composition (Marmur & Doty, 1962) and for the recognition of any satellites which might be present. The melting behaviour of DNA was studied in 0.1 SSC. DNA concentration being adjusted to give absorbance of 1 unit at 260 nm. The DNA sample and a blank consisting of adenine in glycol were heated slowly in a Hilger and Watts Ultrascan and increase in A_{260} nm was traced. Temperature was recorded by a thermocouple immersed in the cuvette containing DNA. After recording the temperature transition profile, the differential A_{260} nm for 0.5°C was tabulated serially; nearest neighbour readings were then added and an overlapping differential A_{260} nm for 0.5°C was plotted against the temperature. The

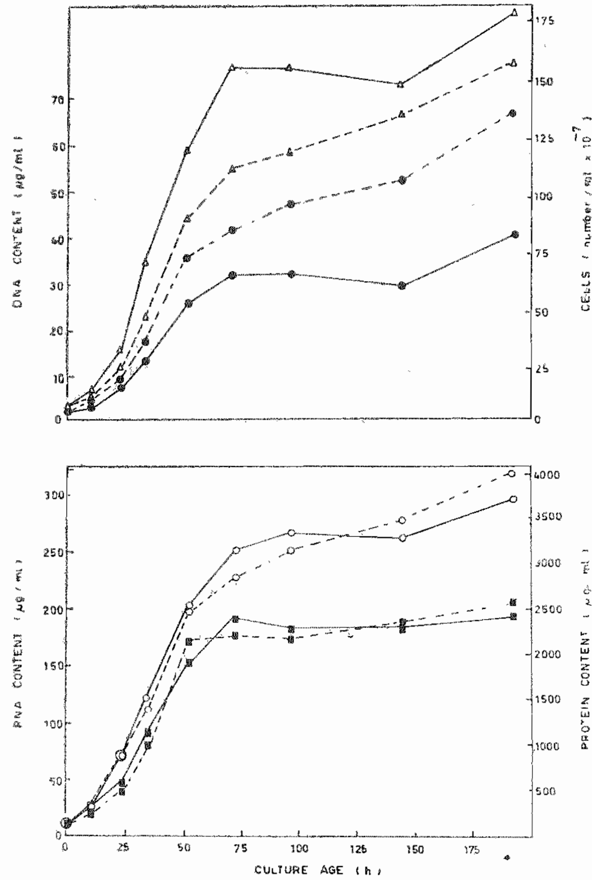

Fig. 1. Variation with time of cell number (△), and of RNA (O), DNA (●) and protein (■) content per culture of 2—(———), a haploid strain, and 2nB (- - -), a diploid strain of *P. inundatus*.

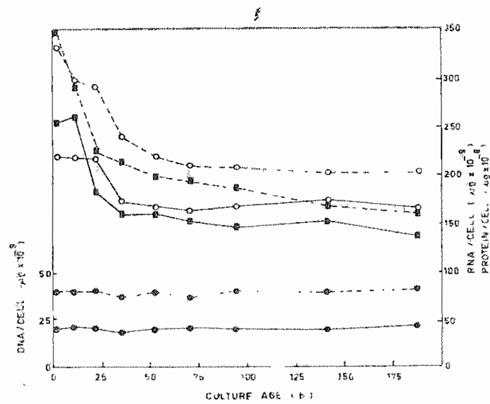

Fig. 2. Variation with time of RNA (◯), DNA (●) and protein (■) content per cell of 2—(———), a haploid strain, and 2nB (- - -), a diploid strain of *P. inundatus*.

quantities of different DNA species were calculated from the areas under the various peaks assuming that for all the species of DNA the hyperchromatic effect is numerically the same. Instrument noise and electronic disturbance were substracted from the apparent areas under the peaks by taking into account the A_{260} nm per unit area in the region 45-55°C where no peaks were observed. Tm was corrected for SSC ionic strength: the value for bacterial DNA was lowered by 17.5°C in 0.1 SSC.

(k) *Assessment of purity of DNA preparations:*

The ultraviolet absorbance spectra for DNA, A_{260}/A_{260}nm and A_{260}nm/A_{230}nm ratios, confirmed that the preparations were of a satisfactory degree of purity. Protein in DNA and RNA samples determined by the method of Lowry *et al.*, (1951), was less than 1 per cent. The purified DNA samples contained about 1 per cent RNA, determined by the method of Savitsky & Stand (1965), and the increase in A_{260} nm after heating in SSC was 37-39 per cent.

Results and Discussion

(a) *Variation in DNA, RNA and protein content with age.*

Before comparing the nucleic acids of haploids with those of diploids, variation with age of culture in both type of strains was examined. Valadon *et al*, (1962) had shown, in rather old cultures, that DNA as well as RNA content decreased with age, though RNA to DNA ratio remained constant. It was desirable to examine younger cultures, in which cell division was still occurring and prior to onset of sensescence and to use more modern methods of nucleic acids determination. In this experiment the fungus was grown in a 2 litre flask with forced aeration at 20°C.

To assist in the interpretation of the 'per cell' results, variation on a 'per culture' basis will first be considered. Changes with age in nucleic acids and protein content of strain 2-(haploid) and strain 2nB (diploid) were studied (Figs. 1 & 2). On a 'per ml' basis, all components increased; for the first 36 hr. after the start of observable growth, there was logarithmic increase of cell contents; thereafter, upto 72 hr. increase was still rapid, then falling off sharply to a quite slow rate. The cell number of the diploid was consistently somewhat below that of the haploid; the DNA values for the haploid were about two thirds those of the diploid. The values per culture for RNA and protein, however, were virtually identical for haploid and diploid, due to the lower cell numbers in the latter being balanced by greater content per cell.

On a 'per cell' basis, DNA content were very constant throughout the experiment, the values for the diploid being almost exactly double those for the haploid. RNA values were very high in the early stages, dropping to a fairly constant level 34 hr. after the start of observable growth, at a time when growth rate, previously exponential, was starting to decline. At all stages the RNA content of diploids were around 25 per cent greater than that of haploids. The behaviour of protein closely resembling that of RNA.

DNA content were constant irrespective of age of culture, even in the early stages when there was a rapid transition from active cell division to a stationary state. This suggests that the phase of DNA doubling prior to nuclear division occurs more or less simultaneously with the production of a visible bud, since a

dividing cell was counted as two cells if a visible bud had appeared. The decline with age found in various studies (Valadon *et al*, 1962) was most probably due either to the considerable age of the culture employed, or to the use of less specific methods of estimation than those available now. The variation in RNA content per cell with age conforms that reported by Valadon *et al*, (1962) bearing in mind the different conditions employed.

(b) *Differences between haploid and diploid strains:*

The experiment described above, although designed piimarily to investigate variation with age, gave useful indication of differences between haploid and diploid strains. In addition to strains 2— and 2nB, a haploid strain 18+; and a diploid strain F2 derived from a mating in culture between strain 2— and 18+ were studied. The results of several experiments are summarized in Table 1. In all experiments incubation was at 18-22°C. The DNA and RNA figures represent the mean values for around ten harvests at different stages of growth. The RNA means were calculated from values obtained when the cultures were old enough for RNA values to have become relatively stable.

TABLE 1. Nucleic acids content per cell in cultures of various strains of *Protomyces inundatus*; **the values expressed as $\mu g \times 10^{-9}$.**

Experiment	Strain	DNA/cell	RNA/cell	RNA/DNA
1	2—	21.2	170	8.0
	2nB	39.8	213	5.4
2	18+	25.3	218	8.6
	2nB	48.6	278	5.7
3	2—	23.7	172	7.3
	18+	26.5	220	8.3
	2nB	46.2	223	4.8
	F2	42.0	235	5.3

The strains fall clearly into two groups with respect to DNA content per cell, all the values for 2— and 18+ being around 23 × 10^{-9} ug/cell. These values for 2nB and F2 being around 43 × 10^{-9} ug/cell. It is reasonable to assume that these approximate to the haploid and diploid values respectively. The RNA values are less easy to interpret, there is clear strain difference between the two haploids, the value for 18+ being considerably higher than those for 2— and a suggestion that the values for the diploids are higher than those for the haploids. The RNA to DNA ratio for haploids and diploids are around 8.4 and 5.4, respectively.

(c) *Molecular weight:*

The molecular weight values for the preparations of *E. coli* B and *M. lysodeikticus* DNA obtained by the Marmur method were very close to those reported by Marmur (1961). After passage through the X-press all the *P. inundatus* DNA samples, like the *E. coli* B X-press sample, had similar molecular weight at around 1 million daltons, (Table 2.) The values, however, are probably those for disrupted molecules, making it impossible to draw any conclusions regarding possible differences in sizes of the native DNA molecules of the *Protomyces* strains examined.

TABLE 2. Sedimentation behaviour of *Escherichia coli, Micrococcus lysodeikticus* and *Protomyces inundatus* DNA preparations.

Strain	$S_{20,w}$	$Mw \times 10^{-5}$
E. coli B (Marmur)	27.0	130
E. coli B (X-press)	10.3	9.9
M. lysodeikticus (Marmur)	24.1	94
P. inundatus (X-press)		
18+	9.7	9.5
2–	8.1	8.4
2nB	9.4	9.4
F2	10.2	9.1

(d) *Base composition:*

The results obtained by the various methods employed are given in Table 3. The values for DNA GC content of the reference materials were independent of the method and agreed well with those reported by previous workers.

There appears to be no differences between the GC content of the DNA of the four *Protomyces* strains, the slight differences observed in some cases are probably not significant. The values obtained by hydrolysis and acid denaturation, taken together, are lower than those obtained by the other three methods. The reason for this difference is obscure, but the hydrolysis method, being direct, is likely to be the most reliable.

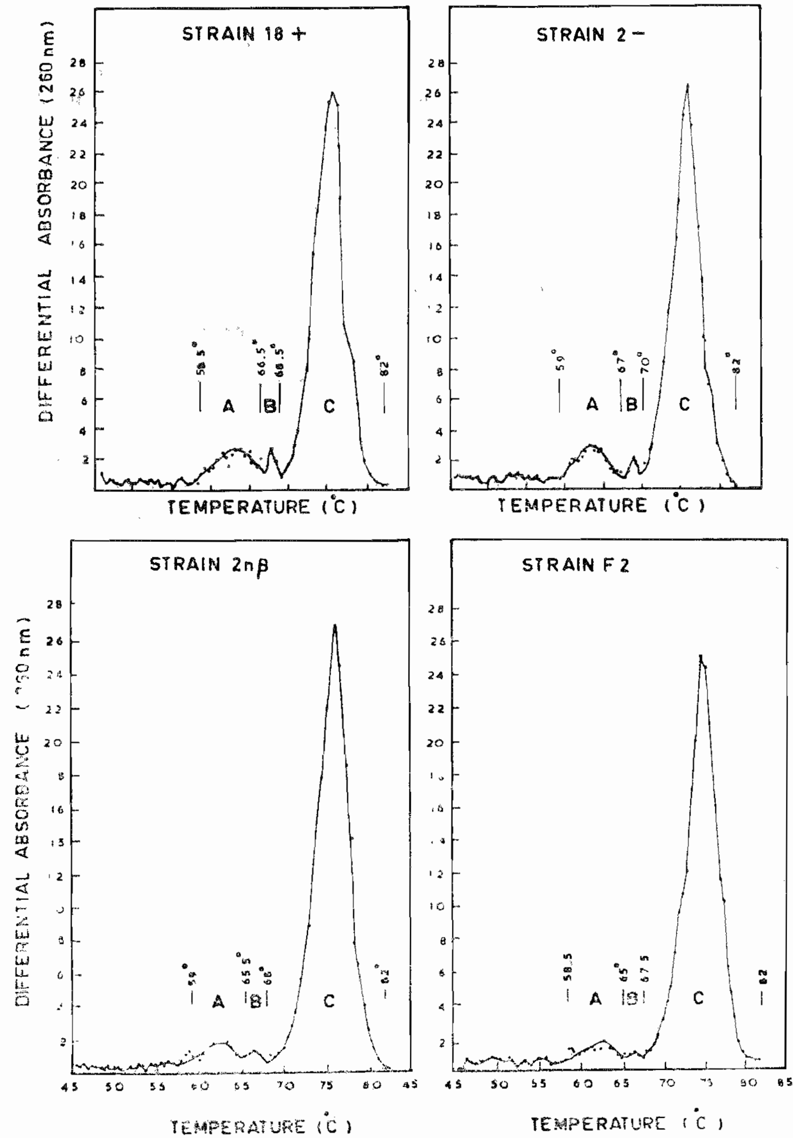

Fig. 3. Melting transition profiles of two haploid strain (18 and 2 −) and two diploid strains (2nB and F2) of *P. inundatus*. A, B and C indicate the positions of the three peaks (see also Table 4); areas beneath peaks were measured between the limits indicated.

TABLE. 3. Base composition of DNA preparations expressed as GC content per cent.

Strain	Hydrolysis	Acid denaturation	Bromination	Buoyant density	Melting behaviour
E. coli B (Marmur)	—	50.3	—	51.0	50
E. coli B (X-press)	—	50.2	50.5	51.0	—
M. lysodeikticus (Marmur)	—	—	71.0	72.5	72
Calf thymus (Sigma)	—	44.7	46.0	—	—
P. inundatus (X-press)					
18+	50.9	49.4	55.4	58.2	55.0*
2—	50.0	49.4	56.6	58.2	55.2*
2nB	51.5	48.1	55.1	66.3	57.7*
F2	47.1	48.6	55.3	53.1	54.4*

* Weighted means of values for each of the three peaks.

Of the methods employed for the determination of GC content only the buoyant density and melting behaviour method could be expected to reveal any differentiation of the DNA into fractions with different base content. The buoyant density method revealed no such fractions, but evidence for their presence was obtained in melting profiles (Table 4, Fig. 3). The hyperchromatic effect for different DNA preparations varied between 37 and 39 per cent indicating that the preparations were pure and double stranded. The melting plots of the DNA preparations showed two satellite peaks A and B, and a main peak C. The proportion of the total DNA contained in the satellites in diploids, as indicated by the relative areas contained under satellite and main peaks, was less then half of that in haploids. It seems likely that satellite DNA remained undetected by buoyant density because DNA is sheared during preparation and it is affecting the sharpness of the peaks.

Genetic experiments on *Xenopus laevis* and *Drosophila melanogaster* indicate that rRNA which is about 90 per cent of total RNA is under DNA control. Brown & Gurdon (1964) have demonstrated in embryos of X. *laevis* that lack the ability to form nucleoli are incapable of synthesis of rRNA and homozygote of this nutant dies at the tail-bud stage, and further Wallace & Birnstiel (1966) have concluded

that DNA stretches complementary to rRNA are directly proportional to the number of nucleolar organizers and consequently to the amount of rRNA is directly proportional to the number of nucleolar organizers (Ritossa & Spiegelman, 1965). The size of the nucleolar organizer in *Bufo marinus* is polymorphic (Miller & Brown, 1969), giving a highly variable content of DNA complementary to rRNA for individuals taken at random from wild type population.

TABLE 4. Melting transition profile analysis of DNA from *Protomyces inundatus* strains.

Strain	Hyperchromatic effect at 260nm $h_{max}=(A_{max}/A_{20})-1$	Tm(0.1 SSC) $Tm=\frac{1}{2}(h_{max}+1)$	GC content %	Area under peak (% of total)
E. coli B (Marmur)	40	73.0	50	—
M. lysodeikticus (Marmur)	40	82.0	72	—
P. inundatus (X-press)				
18+	39	A*63.0	27.3	7.9
		B 67.5	38.3	1.9
		C 75.5	57.8	90.3
2—	38	A 63.0	27.3	7.3
		B 68.5	40.7	2.0
		C 75.5	57.8	90.7
2nB	38	A 62.5	26.1	3.3
		B 66.5	35.9	1.1
		C 76.0	59.0	95.6
F2	37.5	A 62.5	26.1	2.9
		B 66.5	35.9	0.7
		C 74.5	55.4	96.4

*A.B.C. are the three peaks (see Fig. 3).

The difference between the two types of DNA samples is of interest, since it was the only property in which the nucleic acids of diploids differed from those of haploids and it may be the basis for differences in RNA produced per unit DNA in *P. inundatus* strains. It is likely that in diploid strains of *P. inundatus* some of DNA stretches, on which RNA is transcribed, are deleted.

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