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Biochemistry

Synthesis of yeast histones in the cell cycle

(*Saccharomyces cerevisiae*/nuclei/histone characterization/gel electrophoresis)

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ABSTRACT **The yeast, *Saccharomyces cerevisiae*, contains four types of histones resembling histones H3, H2b, H2a, and H4 of animal cells. These proteins are synthesized primarily, if not exclusively, in the S-phase of the cell cycle. This result is discussed with reference to the insensitivity of ongoing DNA replication in yeast to inhibitors of protein synthesis.**

Replication of the genetic material in higher organisms is a complex process which involves not only the duplication of DNA, but also the synthesis of nuclear proteins, especially the histones, and the assembly of these constituents into chromatin. The production of DNA and histones in higher eukaryotes is highly coordinated and occurs in the S-phase of the cell cycle. Inhibition of the synthesis of one component influences the formation of the other (1–5).

Whereas in most eukaryotes DNA replication requires protein synthesis throughout the S-phase, in yeast the situation turns out to be different. In this organism, inhibition of protein synthesis interferes with DNA replication only at the beginning of the S-phase, whereas ongoing replication is not affected by inhibitors of protein synthesis, such as cycloheximide (6–8). This raises the question whether, in yeast, DNA and histone formation are less stringently coupled, and DNA replication can continue without concomitant formation of these nuclear proteins, or whether histone synthesis is not restricted to the S-phase. In this latter case, a pool of histones would exist which could supply these proteins for the assembly of chromatin even in the absence of protein synthesis.

The results described in this paper show that the four types of yeast histones which resemble histones H3, H2b, H2a, and H4 of animal cells, are synthesized primarily, if not exclusively, in the S-phase of the cell cycle.

MATERIALS AND METHODS

Growth and Labeling of Synchronized Yeast Cells. Cells of the diploid wild-type strain A 1160 of *Saccharomyces cerevisiae* were grown at 25° in complete medium (0.5% peptone, 0.3% yeast extract, 1% glucose), well into stationary phase, until only unbudded cells were present. These were used for synchronization according to published procedures (9, 10). Synchronized cells were inoculated into the medium described above to a cell density of about 10^7 cells per ml and grown at 25°. Such cultures divide synchronously for at least three generations. Synchronous growth was monitored by determining the bud index (9) and the DNA content of aliquots of the culture. Labeling was carried out in 350 ml samples of cells at different stages in the cell cycle by adding [^{3}H]lysine to a final concentration of 2 μCi/ml. Experiments were started at the beginning of the second division cycle in order to allow the cells to recover from the synchronization treatment. After a 20 min pulse at 25°, further protein synthesis was stopped with the addition of cycloheximide (200 μg/ml) to the medium. A 2 ml aliquot was removed from the culture to determine the incorporation of [^{3}H]lysine into total trichloroacetic acid-insoluble material. Cells were then rapidly cooled and harvested by centrifugation. They were washed twice with water containing 200 μg/ml of cycloheximide and used for the preparation of histones.

Isolation of Histones. Cells were converted into spheroplasts by a two-step procedure: The preincubation buffer contained 0.66 M 2-mercaptoethanol, 2.6 mM EDTA (pH 7.4), and 200 μg/ml of cycloheximide. Two milliliters of this medium were used per g wet weight of cells. After incubation for 30 min at 30°, cells were harvested and washed once with water followed by 1 M sorbitol (both containing cycloheximide). The cell wall was digested by an incubation in 1 M sorbitol containing 2.5% "Glusulase" (see below) and 50 μg/ml of cycloheximide. Five milliliters of this solution were used to suspend 1 g of cells. Incubation was carried out at 30° until most of the cells were converted into osmotically labile spheroplasts. These were collected by centrifugation and washed twice with 1 M sorbitol. Nuclei were prepared from spheroplasts as described earlier (11) except that the lysis buffer contained 20% rather than 18% Ficoll and 0.2 mM of the protease inhibitor, phenylmethylsulfonylfluoride. The first centrifugation of the homogenate was at 30,000 × *g* in order to completely remove residual cells and spheroplasts. Purified nuclei were washed once with 0.025 M NaCl, 0.01 M EDTA (pH 8.0), 0.2 mM phenylmethylsulfonylfluoride to remove Ficoll and histones were extracted with 0.2 M H_2SO_4 and precipitated overnight with 9 volumes of cold acetone. The same procedure was used to isolate histones from unlabeled or from [^{3}H]lysine- or [^{14}C]tryptophan-labeled yeast cells asynchronously growing in logarithmic phase.

Electrophoresis of Histones. This was carried out in 15% polyacrylamide gels at pH 2.8 in the presence of 2.5 M urea as described by Panyim and Chalkley (12). Gels were stained for 30 min with 0.5% amido black in 7% acetic acid and destained with a solution containing 7% acetic acid and 35% ethanol. To determine the radioactivity of [^{3}H]lysine in electrophoretically separated histones, we sliced gels into 2 mm slices using a Mickle gel slicer. Slices were placed in scintillation vials and dried for 2 hr at 100°. They were then dissolved by overnight incubation at 60° with 0.5 ml of Soluene 350 containing 10% of water. After the samples were cooled, 10 ml of toluene based scintillator were added. Radioactivity of the samples was determined in a Nuclear Chicago Mark I scintillation counter. Recovery of radioactivity from the gels always was nearly 100%.

Other Methods. DNA was determined according to a modified Burton procedure (13) using calf thymus DNA as standard. Protein was measured according to Lowry *et al.* (14) using crystalline bovine serum albumin as standard.

Materials. L[4,5-^{3}H]lysine monohydrochloride (specific activity between 12 and 19 Ci/mmol and D,L-[*methylene*-^{14}C]tryptophan (specific activity 57 mCi/mmol) were from the Radiochemical Centre, Amersham, England. Ficoll was pur-

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chased from Pharmacia, Uppsala; the snail gut juice "Glusulase" from Endo Laboratories, Garden City, N.Y.; cycloheximide, hydroxyurea, phenylmethylsulfonylfluoride, and amido black were from Serva, Heidelberg; Soluene 350 from Packard; yeast extract and peptone from Difco. All other chemicals were of reagent grade purity.

RESULTS

Earlier reports indicated that yeast nuclei contain basic proteins migrating in acrylamide gels in the region of the histones H3, H2b, H2a, and H4 from animal cells (11, 15). Three distinct peaks and a shoulder can be distinguished in the electropherograms of histones isolated by Wintersberger, Smith, and Letnansky (11) from yeast nuclei or chromatin, whereas three proteins were purified, separated, and characterized by Franco, Johns, and Navlet (15). For a determination of the timing of histone synthesis in the yeast cell cycle, a reliable method was necessary for the estimation of the amounts of histones present in the cell at various stages of growth. We have therefore reexamined the electrophoretic pattern of yeast histones. Our experiments show that these proteins can be reproducibly separated into four components by electrophoresis in 2.5 M urea at pH 2.8 (see the densitometer tracing of Fig. 1). Separation of the most slowly moving histones (corresponding to H3 and H2b) is, however, incomplete and overloading of the gel frequently results in the appearance of a single large peak with a shoulder (11). Like the earlier investigators (11, 15, 16) we also failed to detect a histone migrating like H1 of animal cells. Acid-soluble proteins from yeast chromatin, on the other hand, always contain minor fractions moving more slowly than the histones in polyacrylamide gels, and it could therefore be suspected that histone H1 might be among these proteins. When tritiated lysine is incorporated into yeast cells, these less basic proteins also become highly labeled (Fig. 1). Because all histones isolated so far lack the amino acid tryptophan (17), we have labeled yeast cells with tryptophan and tested for the incorporation of this amino acid into the acid-soluble nuclear proteins. The result of this experiment is also included in Fig. 1, and two important conclusions can be drawn therefrom: (*i*). There are no distinct peaks with tryptophan label at positions where the four types of histones migrate in the gel, providing another criterion for the identification of these proteins as histones. (*ii*) All proteins with a lower mobility than the histones contain tryptophan. If we assume that the putative histone 1 from yeast lacked tryptophan, like the other yeast histones, its presence among these more slowly moving proteins becomes less likely, although we cannot yet exclude that a yeast histone 1 present in low concentration comigrates with the nonhistone proteins.

The time at which histone synthesis takes place in the cell cycle of *Saccharomyces cerevisiae* was determined by pulse labeling synchronously growing cells with tritiated lysine at various times of the division cycle, and isolating histones from purified nuclei by acid extraction. Protein synthesis was completely stopped at the end of the pulse period by cycloheximide addition to the cultures as well as to the solutions used for the preparation of spheroplasts. The concentration of cycloheximide used completely inhibits histone synthesis (see Table 2). As is evident from Fig. 1, acid extracts of yeast nuclei in addition to histones contain a heterogeneous population of nonhistone proteins into which lysine is incorporated. Thus, it is impossible to directly equate acid-extractable protein with histones. It proved necessary to separate proteins extracted at each time point by electrophoresis in polyacrylamide gels in the presence

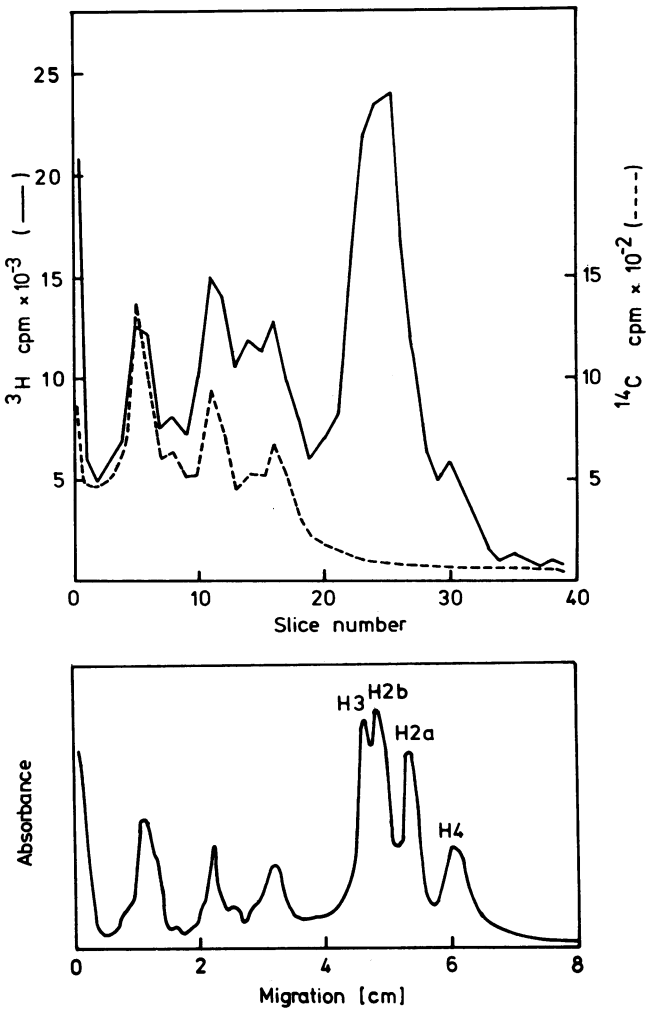

FIG. 1. Analysis of acidic extracts of purified yeast nuclei by electrophoresis in polyacrylamide gels in the presence of urea. Each 350 ml of medium was inoculated with yeast cells from a preculture to a final cell density of 5×10^6/ml. Cells were grown for three generations in the presence of either 1 μCi/ml of [^{3}H]lysine or 0.2 μCi/ml of [^{14}C]tryptophan. Histones were isolated as described in *Materials and Methods* and analyzed in 15% polyacrylamide gels in the presence of 2.5 M urea at pH 2.8. Two gels of each sample were run in parallel; one was stained with amido black, the other one was sliced to determine the radioactivity incorporated into the acid-extractable nuclear proteins. The lower part of the figure shows a typical densitometer tracing as it is obtained by scanning stained gels at 550 nm. The designation of the histones (H3, H2b, H2a, and H4) rests on the similarity of the electrophoretic mobility of these yeast histones with those from rat liver (11) or calf thymus. The upper part of the figure shows the distribution of label in the gels.

of urea and to sum up the radioactivity under the main peaks of the histones. The varying recovery of spheroplasts from cells and of nuclei from spheroplasts was corrected for by DNA measurements in aliquots of cells, spheroplasts, and nuclei during the preparative procedures. In the experiments summarized in Figs. 2 and 3, equal amounts of synchronous cells were inoculated under identical conditions into five different flasks which contained the same amount of medium and were grown for different times. Tritiated lysine was then added and cells were labeled for 20 min. Histones extracted from nuclei of cells at the different stages of the cell cycle were then separated by electrophoresis in polyacrylamide gels. Duplicate gels of each preparation were run in parallel; one gel was stained with amido black to determine the position of the histones, the

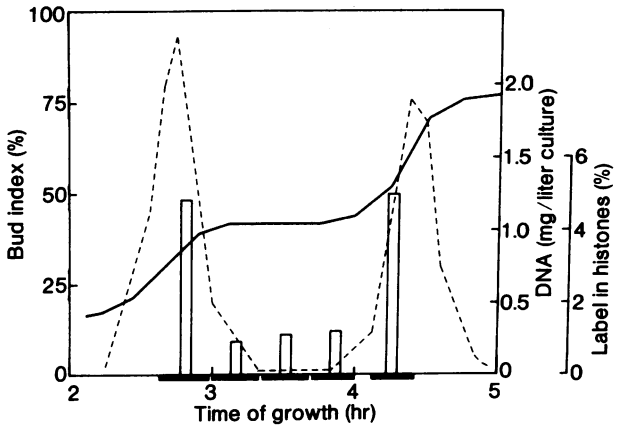

FIG. 2. Synthesis of yeast histones in the cell cycle. Each 350-ml culture of synchronously growing yeast cells was pulse labeled for 20 min with [^{3}H]lysine at the times indicated by the heavy lines below the abscissa. Details of the procedure as well as the method used to isolate labeled histones are given in *Materials and Methods*. From analyses in polyacrylamide gels (see Fig. 3), the percentage of label in histones relative to the total radioactivity incorporated into cellular protein was estimated. This is presented as vertical bars. Also included in this figure are bud index (- - - -) and DNA content (——) of the synchronous culture.

second one was sliced and radioactivity was determined. Incorporation of lysine into histones occurs predominantly during the S-phase. There is also some labeling of histones at other periods of the cell cycle but this amounts to less than 20% of that measured in the S-period. This low rate of histone synthesis outside the S-phase could be due to a less stringent coupling of histone and DNA synthesis in yeast compared to higher organisms. It is more likely, however, that it is simply caused by an incomplete synchrony of the cell population used. By labeling cells at early, mid, or late S-phase it was demonstrated that histone synthesis takes place throughout the entire S-period.

Because all our measurements were carried out on histones extracted from isolated nuclei, the possibility remained that we actually had determined the capacity of cells to transport labeled histones from the cytoplasm into nuclei. If this transport were coupled to active DNA synthesis, it might occur only in the S-phase; cytoplasmic histone synthesis could also take place at other stages of the cell cycle which would result in the establishment of a cytoplasmic pool. To exclude this possibility, cells were pulse labeled at a time when histones isolated from nuclei were poorly labeled. A great excess of unlabeled lysine was then added and cells were further grown into the S-phase, nuclei were prepared, and histones analyzed. For these conditions, we found a similarly low percentage of radioactivity in the histones as in those isolated immediately after the pulse (Table 1). If the assumption of a regulated histone transport from a cytoplasmic pool into nuclei was correct, then we would have expected histones isolated in this experiment to contain as much radioactivity as those pulse labeled in the S-phase. This was clearly not the case; newly synthesized histones therefore seem to appear rapidly in nuclei and there is no evidence for an accumulation of these proteins in the cytoplasm.

These results suggest one should expect that DNA synthesis takes part in the control of histone formation in yeast. If this were the case, then inhibition of DNA replication should block

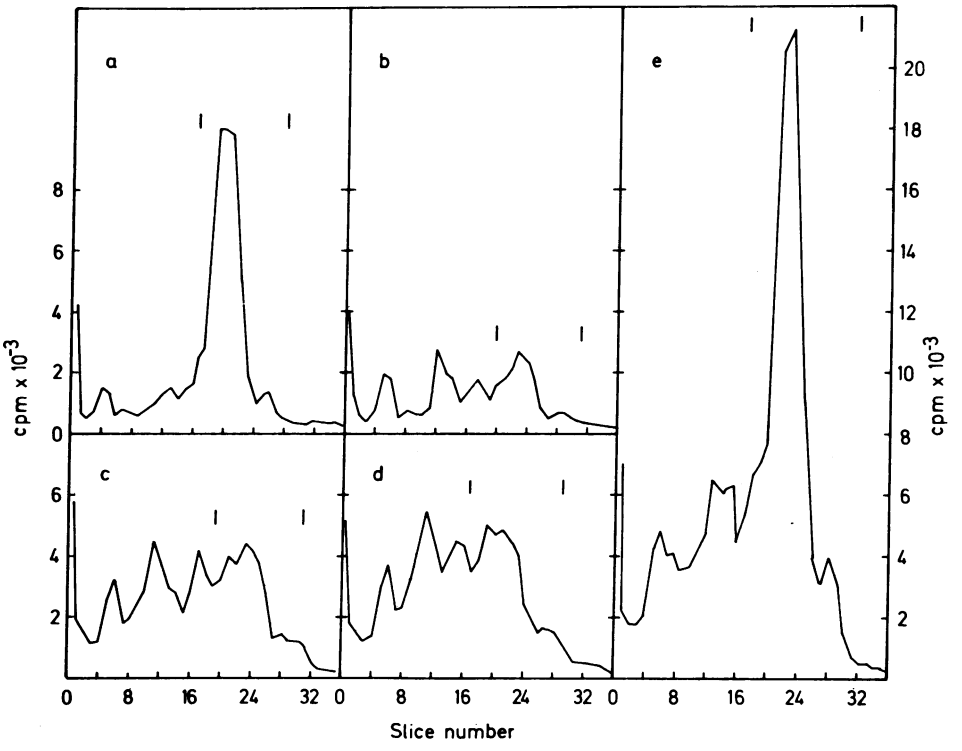

FIG. 3. Analysis of histones labeled at various times in the cell cycle. Synchronously growing cultures were labeled with [^{3}H]lysine at the times indicated by the heavy lines in Fig. 2. (These were: a = 2 hr and 40 min —3 hr; b = 3 hr—3 hr and 20 min; c = 3 hr and 20 min—3 hr and 40 min; d = 3 hr and 40 min—4 hr; e = 4 hr and 7 min—4 hr and 27 min. Histones isolated after the pulse labeling periods were analyzed by electrophoresis in polyacrylamide gels in the presence of 2.5 M urea at pH 2.8. The total amount of acid-extractable nuclear protein obtained at each time point was applied to two gels which were run in parallel. One gel was stained with amido black; the other one was sliced and radioactivity was determined. Only the distribution of radioactivity is shown, for clarity. Data were corrected for the slightly varying recovery of nuclei from cells at different stages in the cell cycle. Histones are present in the fractions between the vertical lines. Total radioactivity in this area of the gels was used to calculate the percentage of label in histones (see Fig. 2).

Table 1. Pulse-chase labeling of histones

	% Label in histones
Pulse started at 3 hr and 20 min of synchronous growth	1.24
Pulse started at 3 hr and 20 min followed by chase into the following S-phase	1.38
Pulse started at 4 hr and 7 min of synchronous growth (S-phase)	5.24

Cells were grown synchronously as described in *Materials and Methods*. At the indicated times (see Fig. 2) the cells were pulse labeled for 20 min with [^{3}H]-lysine. For the chase experiment, a thousandfold excess of unlabeled lysine was added after the pulse and cells were grown into the next S-phase. Histones were isolated and characterized as described in *Materials and Methods*.

Table 2. Inhibition of histone synthesis by hydroxyurea and cycloheximide

	% Histone synthesized
Control	100
Plus hydroxyurea	29
Plus cycloheximide	6

Three 500-ml cultures of yeast cells were grown asynchronously to mid-logarithmic phase (10^7 cells per ml). One culture received hydroxyurea (final concentration 0.1 M) 2 hr prior to labeling with [^{3}H]lysine. To the second culture, cycloheximide (200 μg/ml) was added 10 min before labeling and the third culture served as a control. All three cultures were labeled for 60 min with 1 μCi/ml of [^{3}H]lysine. Cells were then collected by centrifugation and histones isolated as described in *Materials and Methods*. Equal quantities of protein were applied to polyacrylamide gels and separated in the presence of 2.5 M urea (pH 2.8). The radioactivity under the histone bands was determined, and used to calculate the amounts of histone synthesized relative to the control without inhibitor.

histone synthesis. We have studied this postulate by using the inhibitor of DNA replication, hydroxyurea. It was found (Table 2) that histone synthesis is indeed inhibited as soon as DNA replication has come to a complete stop. In a control experiment, the effect of hydroxyurea on total protein synthesis as measured by the incorporation of [^{3}H]leucine into cellular proteins was studied. It was found that with the conditions employed hydroxyurea did not interfere with the capacity of cells to synthesize protein. This excludes an unspecific and general effect of the drug on protein synthesis in yeast and allows us to conclude that DNA replication indeed exerts a control over histone synthesis in yeast similar to that known to operate in higher organisms.

DISCUSSION

Four histones can be demonstrated to occur in the yeast *Saccharomyces cerevisiae*. With regard to the electrophoretic mobility in acrylamide gels these proteins correspond to the histones H3, H2a, H2b, and H4 of higher cells. As long as amino acid sequences and functional properties of the yeast histones are unknown, it is impossible to draw any further analogy to the histones of animal cells.

Histone H1 has not previously been found in yeast (11, 15, 16) and this study also cannot provide any evidence for its existence. On the other hand, it is difficult to prove that a protein having the function of histone H1 is indeed absent from yeast. This histone is known to be the most sensitive one to proteolytic attack. Possibly, the elaborate procedures that are required for the isolation of yeast nuclei, chromatin, and histones might lead to a complete digestion of this protein despite the fact that the protease inhibitor phenylmethylsulfonylfluoride was present in all isolation buffers. The electrophoretic mobility of histone H1 varies in different organisms. Particularly in lower eukaryotes, this protein can migrate more slowly or considerably faster than histone H1 from animal cells (18, 19). We have no evidence for basic proteins moving faster than histone H4 in acrylamide gels. The tryptophan labeling experiment described here makes it unlikely—although it does not exclude the possibility—that histone H1 is present among the proteins moving more slowly than histone H3. The only other remaining alternative is that histone H1 comigrates with one of the four histones described so far. This possibility can only be tested by separation of the four yeast histones and analysis in acrylamide gels in the presence of sodium dodecyl sulfate.

Yeast histones are synthesized nearly exclusively in the S-phase of the cell cycle and synthesis is dependent on DNA replication occurring simultaneously. On the other hand, several groups have shown that inhibition of protein synthesis (and thereby histone synthesis) in synchronously growing yeast cells stops DNA replication only if applied early in the S-phase (6–8). Apparently, DNA can be replicated in the absence of concomitant histone synthesis; protein synthesis, at least in yeast, is required only for the initiation step of DNA replication. It is known that each one of the small yeast chromosomes contains one or several replication units which are initiated at different times in the S-phase (20). From the results of the experiments on inhibition of protein synthesis (6–8) it could be predicted that all, or at least the great majority, of replicons in yeast are initiated in the initial one-third of the S-phase. This was, in fact, demonstrated recently by fiber autoradiography (21). Thus, there is a clear difference between yeast and higher eukaryotes in which initiation of the many replicons takes place throughout the entire S-phase. Because these initiation events at any rate require protein synthesis (22), it is not surprising to find dependence of DNA replication on simultaneous protein synthesis in these higher organisms. The data obtained with yeast likewise suggest that the inhibition of protein synthesis, first of all, interferes with the initiation step of DNA replication. These considerations are important for a judgement of a possible role of histones in DNA synthesis (3). Several investigations (3–5) have shown that in the presence of cycloheximide, DNA synthesis in HeLa or in chick red blood cells initially continues at about 20%–50% of its normal rate. The chromatin thereby formed is more susceptible to nucleases than chromatin made in the presence of histone synthesis. This indicates the capability of these cells to also synthesize some DNA in the absence of concomitant histone formation. It is interesting to note that an uncoupling of histone and DNA synthesis in the reverse sense, namely, histone synthesis in the absence of DNA replication, likewise can occur under certain conditions, as demonstrated, for instance, in early amphibian development (23).

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