Changes in Nuclear Protein during the Cell Cycle in Cultured Mast Cells Separated by Zonal Centrifugation

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1. Exponentially grown mouse mast cells (cell line P815, strain Y) were separated by zonal centrifugation on a Ficoll gradient. Fractions were allocated to different phases of the cell cycle according to the specific radioactivity of their DNA. 2. Histones were extracted and their thiol content was analysed. The proportion of reduced thiol increased in S phase, decreasing subsequently. 3. The phosphate content of histone F1 and of the other histones reached a peak in early and later S phase respectively. The incorporation of ^{32}P into these fractions showed a corresponding increase. 4. The timing of histone synthesis was examined. Incorporation of ^{14}C -labelled amino acids into the histone fractions took place at the same times as phosphorylation. 5. Acid nuclear proteins differ from the histones in incorporating labelled amino acids and ^{32}P fairly constantly through the cell cycle.

In a number of systems phosphorylation of nuclear histones is associated with the period of DNA synthesis (Gutierrez & Hnilica, 1967; Stevely & Stocken, 1968; Cross & Ord, 1970; Shepherd *et al.*, 1971). The increase in the amount of phosphate is most pronounced in histone fraction F1, and has been shown in regenerating liver to be accompanied by elevated histone F1 kinase activity (Pawse *et al.*, 1971). Histone synthesis also occurs at this time (Robbins & Borun, 1967; Gurley & Hardin, 1968) and it is not clear whether phosphorylation takes place on old or new histone. The relationship between histone synthesis and histone phosphorylation was therefore investigated

To compare the precise timing of these events in S phase it was necessary to have a homogeneous and synchronous cell system. The separation of cultured mast cells by zonal gradient centrifugation (Warmesley & Pasternak, 1970) satisfied these requirements and provided enough cells at different stages in the cell cycle for biochemical analysis.

In addition to changes in phosphate content the oxidation state of thiol on histone fraction F3 (Phillips, 1965) is modified during the period of DNA synthesis (Ord & Stocken, 1968; Cross & Ord, 1970). The timing of this modification was also investigated in separated mast-cell fractions.

For comparison with the behaviour of the histones, acidic nuclear proteins were isolated and their synthesis and phosphorylation examined during the cell cycle.

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Methods

Preparative methods

Cell culture and zonal centrifugation. The procedure was essentially that described by Warmesley & Pasternak (1970). P815Y neoplastic mouse mast cells were grown in Fischer's medium (Fischer & Sartorelli, 1964), supplemented with 10% (v/v) horse serum (Tissue Culture Services, Slough, Bucks., U.K.). Large numbers of cells for centrifugation were grown in 4-litre spinner culture flasks. Zonal centrifugation was done at 4°C in an MSE Mistral 6L centrifuge with an MSE type A zonal rotor. Exponentially growing cells, pre-labelled with the appropriate radioisotope, were separated by centrifugation through a linear gradient of 2–10% (w/v) Ficoll dissolved in 50 mM-tris-HCl buffer, pH 7.4, containing 0.9% (w/v) NaCl. Optimum separation was obtained at 500 rev./min for about 20 min, by which time separating cell bands were visible. Cell fractions were collected and analysed for cell number, mean cell volume and specific radioactivity. Up to 10^9 cells were pumped on to the gradient and about 40 25 ml fractions were collected. Of these about 15 contained cells. Those at the beginning and end of the range were pooled to increase the cell numbers in these fractions, so that eight to 12 samples were analysed, containing approx. 10^8 cells each. Cell fractions were allocated to a particular phase of the cell cycle, primarily on the basis of the specific radioactivity of their DNA.

Preparation of nuclei and extraction of nuclear proteins. Nuclei were separated by homogenization and histone fractions extracted and characterized as

described by Cross & Ord (1971). After removal of histone and nucleic acid, acidic nuclear protein was extracted from the nuclear pellet by incubation in 0.05M-NaOH for 2h at 38°C, by the method of Steele & Busch (1964).

Extraction and determination of nucleic acids. Nucleic acids were extracted by the method of Schmidt & Thannhauser (1945), as described by Munro & Fleck (1966). DNA was measured by the diphenylamine assay of Burton (1956). RNA was determined as described by Cross & Ord (1971).

General methods

Determination of protein. The method of Lowry *et al.* (1951) was used, with histone F3 as a standard.

Determination of phosphate. The total phosphorus content of histone preparations was measured by the method of Bartlett (1959), with the omission of H₂O₂ and with 60% (w/v) HClO₄ instead of 5M-H₂SO₄.

Determination of thiol groups. Reduced thiol groups were determined by a modification of the micro-Saville method, developed by Todd & Gronow (1969).

Reduction of disulphide groups. Disulphide groups were reduced by alkaline borohydride (Marsh *et al.*, 1964) for determination as thiol by the micro-Saville method.

Preparation and assay of histone kinase. Histone F1 kinase activity was measured on cytoplasmic extracts, after the removal of nuclei. The assay procedure was described by Cross & Ord (1971). Enzyme activity was expressed as ³²P radioactivity (c.p.m.) incorporated/mg of histone F1 per 10000 cells.

Labelling of histone thiol groups with [1-¹⁴C]-N-ethylmaleimide. A modification of the procedure described by Marsh *et al.* (1964) was used. Extracts of total histones after removal of histone F1 were neutralized with NaOH. Dimethylglutarate (0.1M) was added to a final concentration of 0.02M and the solution was made 5M with respect to urea. The pH was readjusted to between 6 and 6.5. [1-¹⁴C]-N-Ethyl maleimide labelled in the ethyl group (Schwarz Bio-Research, Orangeburg, N.Y., U.S.A.) was added in 1.5-2-fold excess of the required amount and the mixture was left at room temperature for 1.5h. Excess of N-ethylmaleimide was removed by dialysis, first against 100vol. of 50mM-HCl for 4h, and then against water for 12h. After polyacrylamide-gel electrophoresis (Reisfeld *et al.*, 1962) the labelled histone bands were dissolved in 60% (w/v) H₂O₂, by incubation at 37°C for 5h, and their radioactivity was measured.

Counting of ³²P radioactivity in gel bands. Gels were sliced and dissolved as described in the previous

section. Radioactivity in the extracts was measured as Čerenkov radiation.

Incorporation of radioisotopes and measurement of radioactivity. Exponentially growing cells were concentrated by centrifugation and resuspension (Warmsley & Pasternak, 1970) (approx. 10⁹ in 40ml), and incubated with label at 37°C for 30min before washing and resuspension in 25ml of 50mM-tris-HCl buffer, pH 7.4, containing 0.9% NaCl. DNA synthesis was measured by determining the incorporation of [6-³H]thymidine (22Ci/mmol) into DNA after exposure to the radioisotope at a concentration of 1μCi/ml. The extent of synthesis of histones and acidic nuclear proteins was determined on cultures that had been exposed to DL-[1-¹⁴C]valine (20-40mCi/mmol) or DL-[1-¹⁴C]leucine (50mCi/mmol), or ¹⁴C-labelled protein hydrolysate (52mCi/mmol of amino acid). Cultures were labelled with ³²P by exposure to ³²P_i at 5μCi/ml.

Incorporation was measured with a Beckman liquid-scintillation counter (CPM 200), by counting in a toluene-Triton X-100-based scintillant described by Patterson & Greene (1965). In this scintillant ¹⁴C was counted with about 80% efficiency and ³H with about 40%. ³²P was counted as Čerenkov radiation, in water on the tritium channel, with about 50% efficiency. In double-labelling experiments the contribution of ³²P, measured as described above, to the ¹⁴C and ³H counting was calculated and appropriate corrections were made.

Radioactive compounds were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), except where otherwise specified.

Results

Pre-labelled cells were separated by zonal centrifugation before extraction and analysis. Suspension cultures of P815Y mast cells in exponential growth were exposed to [³H]thymidine at 1μCi/ml for 30min, and then washed free of isotope and separated on a 2-10% (w/v) Ficoll gradient, since this has no adverse osmotic effects on cells. Cell number and cell size were estimated on the collected cell fractions. Since the separation depends on the increase in mean cell volume as cells progress from the G₁ phase to the G₂ phase, changes in cell size gave a preliminary indication of the success of the separation. The criterion principally used to allocate fractions to a given phase of the cell cycle was that of DNA synthesis. On the basis of DNA specific radioactivity results from different experiments were compared and positioned within each phase of the cell cycle.

Changes in histone thiol content

The thiol content of histone extracted from cells in different phases of the cell cycle was measured and

the results are shown in Table 1. In this set of experiments the separation of cell types was less satisfactory; the values for the specific radioactivity of

Table 1. *Changes in histone thiol content in P815 Y mast cells during the cell cycle*

Cultures were exposed to [³H]thymidine for 30 min and then separated by zonal centrifugation on a Ficoll gradient. The specific radioactivity of the DNA in the separated fractions was measured and from these values cells were allocated to a given phase of the cell cycle. Histone thiol was determined in the cell fractions. The results are the average of at least two experiments, varying by less than 15%. The total amount of oxidized and reduced thiol remained constant at 73±10nmol (SH+S-S)/mg of total histone.

Cell-cycle phases	10 ⁻³ × Specific radioactivity of DNA (³ H c.p.m./mg of DNA)	Thiol content (nmol/mg of histone)
G ₁	9.7	22
	9.5	33
	13.0	45
S	21.0	57
	18.0	47
G ₂	15.5	42
	13.7	32

DNA indicate contamination between fractions which will tend to reduce the apparent significance of the increase in thiol content in S phase. In some experiments histone was labelled with [¹⁴C]N-ethylmaleimide and after separation by polyacrylamide-gel electrophoresis the gel bands were solubilized and counted for radioactivity. Labelled thiol groups were present in the F3 fraction, in agreement with Phillips' (1965) results. The labelling results confirmed that the amount of reduced histone thiol increases during S phase, reaching a peak when DNA synthesis is maximal and decreasing subsequently to a lower value in G₂ phase (Table 1). The total amount of thiol present remained constant at 73±10nmol (SH+S-S)/mg of total histone.

Histone phosphorylation

Phosphate was determined in histone fraction F1 and on the histones remaining after extraction of fraction F1, as it was not practicable with the quantities available to isolate all the histones. The phosphate content of the two fractions increased during the period of DNA synthesis and decreased again in G₂ phase (Table 2). There was, however, a significant difference between the fractions in the timing of this change. The phosphate content of histone F1 began to increase at the G₁/S transition, and had doubled by early S phase. It declined thereafter. The amount of phosphate on the other histones remained constant in early S phase, increasing only in late S phase and returning to a low value in G₂ phase.

Table 2. *Changes in the phosphate content of histones in P815 Y mast cells during the cell cycle*

Cell fractions were allocated to a given position in the cell cycle according to the specific radioactivity of their DNA after a 30 min incubation with [³H]thymidine at 1 µCi/ml, and results from different experiments were compared on this basis. Measurements of the phosphate content of the histone fractions are expressed as nmol of phosphate/mg of histone and represent the average of at least two experiments, results varying by ±10%. ³²P radioactivity was determined on the histone solutions, after pre-labelling with ³²P_i for 30 min. Results are expressed as c.p.m./mg of protein and are the average of at least three experimental points, varying by ±14%.

Cell-cycle phases	Phosphate content (nmol of phosphate/mg of histone)		³² P radioactivity (³² P c.p.m./mg of histone)		10 ⁻³ × Specific radioactivity of DNA (³ H c.p.m./mg of DNA)
	Histone F1	Other histones	Histone F1	Other histones	
G ₁	33	34	190	320	3.9
	33	34	180	310	4.2
	38	35	190	310	3.8
	45	38	220	315	7.5
S	58	40	330	310	13.0
	84	40	410	300	25.0
	47	42	220	330	30.6
	47	46	200	390	20.0
G ₂	48	62	190	500	9.8
	49	43	190	320	7.0

In some experiments cultures were exposed to $^{32}\text{P}_i$ in addition to ^3H thymidine for 30min before the zonal separation. Changes in ^{32}P incorporation into the two histone fractions during the cycle corresponded in timing and relative extent to the

Table 3. *Histone F1 kinase activity during G_1 and S phases in the P815 Y cell cycle*

Cultures were exposed to ^3H thymidine at $1\mu\text{Ci/ml}$ for 30min before zonal centrifugation, which was timed to achieve optimum separation in the G_1/S region of the gradient. The specific radioactivity of the DNA was determined. Kinase activity was measured on cytoplasmic extracts by using calf thymus F1 as substrate, under the assay conditions described in the Methods section. The kinase results are the mean of two or three experiments, varying by $\pm 15\%$.

Cell-cycle phase	$10^{-3} \times$ Specific radioactivity of DNA (^3H c.p.m./mg of DNA)	$10^{-4} \times$ Kinase activity (^{32}P c.p.m./mg of histone F1 per cell)
G_1	4.5	68
	4	70
	4	76
	5	89
S	9	90
	14	87
	24	79
	30	72
	32	68

increase in phosphate content (Table 2). In G_1 phase the incorporation of labelled phosphorus into the total histones after the removal of fraction F1 was higher than that into fraction F1, although the amount of phosphate present in these histones is about the same, suggesting a higher rate of turnover of phosphate in some component of the mixed histones. Counting of ^{32}P radioactivity in solubilized gel bands indicated that histones F1, F2b and possibly F3 were phosphorylated and confirmed that the phosphate content of these fractions undergoes the changes described.

To complete the description of histone phosphorylation during S phase, the activity of the histone kinase first described by Langan (1968) was investigated. Cells were centrifuged for a time that resulted in optimum separation in the G_1/S region of the gradient. Enzyme activity was determined on cytoplasmic preparations, by using calf thymus F1 as substrate. It was found that histone F1 kinase activity increased at the beginning of S phase, decreasing thereafter (Table 3). The change in activity suggests that the enzyme effects histone F1 phosphorylation in early S phase.

Histone synthesis

The phosphate assays indicate that phosphorylation of histone fractions occurs at specific times in S phase. Since histone synthesis also takes place in cells in S phase, the timing of histone synthesis was compared with that of the phosphorylation of histone F1 and the histones remaining after F1 extraction in mast-cell fractions. An amino acid pre-

Table 4. *Histone synthesis in P815 Y mast cells during the cell cycle*

Experimental points were assigned to a position in the cell cycle according to the accompanying value for DNA specific radioactivity. Cells were exposed to ^3H thymidine and ^{14}C -labelled amino acids as described in the Methods section. The results are the average of at least two experimental points, varying by $\pm 13\%$.

Cell-cycle phases	Histone F1 synthesis (^{14}C c.p.m./ μg)	Other histones synthesized (^{14}C c.p.m./ μg)	$10^{-3} \times$ Specific radioactivity of DNA (^3H c.p.m./mg of DNA)
G_1	200	60	3.3
	200	50	3.0
	190	55	3.6
	220	60	5.2
S	310	50	14.8
	400	50	29.3
	220	65	19.0
	185	130	11.9
G_2	185	150	6.8
	185	70	5.5
	200	50	6.5

cursor such as valine or leucine, which was a common constituent of both histone fractions, or in some experiments a ^{14}C -labelled protein hydrolysate, was used for labelling newly synthesized histones. After exposure to the ^{14}C -labelled precursor for 30 min, cells were separated by zonal centrifugation and cell fractions collected. The specific radioactivity of the histone fractions was determined. Histone F1 is synthesized in early S phase, and the other histones in late S phase (Table 4). It is noteworthy that incorporation of label into histone F1 is higher than that into the other histones during the remainder of the cell cycle. This is consistent with the observations on histone F1 turnover made by Gurley & Hardin (1970).

The timing of synthesis corresponds closely with that of phosphorylation (Table 2). To confirm the coincidence of the two, a number of double-labelling experiments was performed in which cells were exposed for 30 min to both a ^{14}C -labelled amino acid and to $^{32}\text{P}_i$. The results confirmed for both histone fractions that histone synthesis and histone phosphorylation occur at the same time in S phase. This is illustrated in Fig. 1.

Phosphorylation and synthesis of acidic nuclear proteins

In addition to examining histone synthesis and modification, changes in acidic nuclear protein

Fig. 1. *Histone synthesis and phosphorylation during the cell cycle*

Mast-cell cultures were exposed to $[^3\text{H}]$ thymidine, $^{32}\text{P}_i$ and ^{14}C -labelled amino acids for 30 min before the zonal separation. Cell cycle phases were distinguished on the basis of the specific radioactivity of DNA, and fractions allocated accordingly. ^{32}P and ^{14}C radioactivities were determined on histone extracts. $\circ$, Histone F1; Δ , histones remaining after extraction of histone F1. The phases are indicated by the arrows.

Table 5. *Synthesis and phosphorylation of acidic nuclear proteins in P815 Y mast cells separated by zonal centrifugation*

Fractions were allocated to a phase of the cell cycle according to the specific radioactivity of their DNA. Cells were exposed to [^3H]thymidine, and to ^{14}C -labelled amino acids and $^{32}\text{P}_i$ as described in the Methods section. In some experiments both ^{14}C -labelled amino acids and $^{32}\text{P}_i$ were used, in others only one. The results are the average of two or three experiments, with an error of about 5% on the protein values and about 9% on the counting results.

Cell-cycle phase	$10^{-8} \times \text{Protein}$ ($\mu\text{g}/\text{cell}$)	^{14}C incorporated (c.p.m./ μg of protein)	^{32}P incorporated (c.p.m./ μg of protein)	$10^{-3} \times \text{Specific}$ radioactivity of DNA (^3H c.p.m./mg of DNA)
G_1	153	820	610	4.5
	161	980	602	5.0
	149	860	670	5.3
S	169	820	860	10.8
	161	710	870	19.5
	167	750	710	31.0
		805	940	15.3
G_2	142	545	590	7.0
	127	660	630	7.5
	133	525	530	6.9
	127	665	640	7.4

content were investigated briefly during the mast-cell cycle. After removal of histone, non-histone acidic protein was extracted from washed nuclei with 0.05M-NaOH (Steele & Busch, 1964). After digestion for 2h at 38°C with 0.3M-NaOH–0.5M-HClO₄, RNA was removed from the residue with cold, and DNA with hot 0.5M-HClO₄ at 70°C for 15 min. The relative proportions of these constituents were as follows: DNA, 1.00: histone, 1.17: acidic protein, 0.67: nuclear RNA, 0.045. The amount of acidic nuclear protein per cell was found to increase slightly in S phase and to decrease in G₂ phase (Table 5). Incorporation of labelled amino acids into protein was highest in G₁ phase, decreased slightly in S phase, and was lowest in G₂ phase. ^{32}P incorporation was greatest in S phase.

Discussion

In separated mast cells it was found that the histones are synthesized in S phase, in agreement with the report of Robbins & Borun (1967). The appearance of increased labelling in the nucleus did not significantly precede the onset of DNA synthesis, as has been reported in some other systems (Orlova & Rodionov, 1970; Shepherd *et al.*, 1971). Labelled histone F1 appeared in the nucleus before the other histones; the timing of this in relation to the appearance of the other histones is of interest in chromosomal assembly. Histone F1 is further distinguished by a higher rate of turnover, in keeping with the

reports of Stellwagen & Cole (1969) and Gurley & Hardin (1970).

By contrast with the histones, label was incorporated into the acidic nuclear proteins continuously in G₁, S and G₂ phases. This is in agreement with the consistently high rate of turnover reported for these proteins (Bustos-Valdes *et al.*, 1968). The metabolic activity of these proteins coincides more with the extent of template activity during the cycle than with the period of DNA synthesis. Stimulation of their synthesis (Stein & Baserga, 1970) and phosphorylation (Turkington & Riddle, 1969) is observed in situations where RNA synthesis is increased. In the results reported here the rate of phosphorylation appears maximal in S phase.

Modification of the proportion of reduced thiol groups present on histone F3 takes place during the cell cycle, reaching a peak in S phase. The timing of this increase does not appear to bear a significant relation to the timing of histone synthesis in S phase. No direct precursor species nor enzyme system has been described for the oxidation and reduction of histone thiol and the question of whether its variation reflects general cellular changes remains open.

Histone phosphorylation of both histone F1 and of the remaining histones reached its greatest extent at different times in S phase. Phosphorylation of histone F1 is a well characterized modification (see e.g., Langan, 1972). It is not clear, however, which of the other histone fractions are phosphorylated. The specificity of histone kinase *in vitro* suggests that

histones F1 and F2b are the fractions significantly phosphorylated by the enzyme (Langan, 1968).

The timing of the appearance of phosphorylated histone F1, and, later, of the other histones, corresponds exactly with that of newly synthesized histone. This might suggest that phosphorylation takes place on newly synthesized material, and is similar to the finding of Shepherd *et al.* (1971) in Chinese hamster ovary tumour cell cultures, synchronized by isoleucine depletion. The experiments of Marushige *et al.* (1969), however, in a system in which little synthesis of new histone is occurring suggest that phosphorylation of old histone may also take place. Preliminary experiments on the effects of inhibitors of DNA, RNA and protein synthesis on the association between histone synthesis and phosphorylation in stimulated lymphocytes (M. E. Cross & M. G. Ord, unpublished work) indicate that the relationships between the two are complex, some phosphorylation probably taking place on old histone, as well as on newly synthesized material.

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