Non-Histone Chromosomal Proteins

EVIDENCE FOR THEIR ROLE IN MEDIATING THE BINDING OF HISTONES TO DEOXYRIBONUCLEIC ACID DURING THE CELL CYCLE

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By selective dissociation of histones with the ionic detergent sodium deoxycholate, we have demonstrated that these basic chromosomal polypeptides, which are effective inhibitors of transcription, are more tenaciously bound to DNA in mitotic than in S-phase chromatin. Evidence is presented which suggests that cell-cycle-stage-specific non-histone chromosomal proteins can account for such variations in the association of histones with DNA. When chromatin is reconstituted with DNA and histones are pooled from S-phase and mitotic cells and either S-phase or mitotic non-histone chromosomal proteins, a preferential extraction of histones with sodium deoxycholate from chromatin reconstituted with S-phase rather than mitotic non-histone chromosomal proteins is observed. In contrast, the extractability of histones with sodium deoxycholate from nucleohistone complexes reconstituted with DNA pooled from S-phase and mitotic cells and either S-phase or mitotic histones is identical. Since non-histone chromosomal proteins rather than histones are responsible for the differences in chromatin template activity during S-phase and mitosis, we propose that non-histone chromosomal proteins may modify gene expression during the cell cycle by mediating the binding of histones to DNA.

The eukaryotic genome exists in the form of a complex nucleoprotein structure referred to as chromatin, consisting primarily of DNA and chromosomal proteins. Although histones restrict the capacity of DNA for RNA synthesis (Huang & Bonner, 1965; Allfrey *et al.*, 1963; Marushige & Bonner, 1966; Paul & Gilmour, 1966*a,b*) evidence is accumulating that suggests that non-histone chromosomal proteins may play a key role in the regulation of gene expression in general (Wang, 1968; Paul & Gilmour, 1968; Spelsberg & Hnilica, 1969; Gilmour & Paul, 1970; Spelsberg & Hnilica, 1970; Kostraba & Wang, 1972; Teng *et al.*, 1971; Stein & Baserga, 1972; Stein *et al.*, 1974; Spelsberg *et al.*, 1972; Shea & Kleinsmith, 1973), and specifically in the control of transcription during the cell cycle (Stein & Baserga, 1972; Stein & Farber, 1972; Stein *et al.*, 1972). Such a regulatory function for non-histone chromosomal proteins in continuously dividing cells, as well as in quiescent cells which are stimulated to proliferate, is supported by significant variations in their rates of synthesis (Stein & Baserga, 1970; Rovera & Baserga, 1971; Stein & Borun, 1972; Borun & Stein, 1972; Stein & Matthews, 1973; Stein & Thrall, 1973; Levy *et al.*, 1973; Tsuboi & Baserga, 1972), turnover (Borun & Stein, 1972) and phosphorylation (Platz *et al.*, 1973) during defined periods of the cell cycle. The synthesis and phosphorylation of cell-cycle-

stage-specific non-histone chromosomal proteins has also been observed (Stein & Borun, 1972; Stein & Matthews, 1973; Stein & Thrall, 1973; Levy *et al.*, 1973; Tsuboi & Baserga, 1972). Further, unlike the histones whose synthesis is restricted to S phase and is tightly coupled to DNA replication (Stein & Borun, 1972; Robbins & Borun, 1967; Spalding *et al.*, 1966), non-histone chromosomal proteins are synthesized throughout the cell cycle, independent of concomitant DNA synthesis (Stein & Borun, 1972; Stein & Thrall, 1973). Additional and more direct evidence that non-histone chromosomal proteins are involved in the regulation of DNA-dependent RNA synthesis during the cell cycle comes from studies which show that chromatin reconstituted with cell-cycle-stage-specific non-histone chromosomal proteins exhibits a template activity characteristic of the native chromatin from which it is isolated (Stein & Farber, 1972; Stein *et al.*, 1972). However, the specific manner in which chromosomal proteins interact with the genome is not clear.

The present studies demonstrate that in HeLa S₃ cells, consistent with the restricted transcriptional capacity of mitotic compared with S-phase chromatin, histones are more tenaciously associated with DNA during mitosis than during S-phase and that non-histone chromosomal proteins may be responsible for mediating the binding of histones to DNA.

Materials and Methods

Cell culture and synchronization

Exponentially growing HeLa S₃ cells maintained in suspension culture in Joklik-modified Eagle's Minimal Essential Medium supplemented with 3.5% each of calf and foetal calf serum (Grand Island Biological Company, Grand Island, N.Y., U.S.A.) were synchronized as previously described (Stein & Borun, 1972). S-phase cells were obtained 3 h after release from two cycles of 2 mM-thymidine block, and mitotic cells were collected by treatment of thymidine-synchronized G₂-phase cells with Colcemid (CIBA Pharmaceuticals, Basle, Switzerland). In one experiment, cells were synchronized by mitotic selective detachment (Stein & Borun, 1972).

Preparation of chromatin and DNA

Chromatin was isolated from nuclei obtained by washing cells four times in 80 vol. of Earle's Balanced Salt Solution (Grand Island Biological Company) and four times in 60 vol. of 80 mM-NaCl–20 mM-EDTA–1% Triton X-100, pH 7.2. The nuclei were washed twice with 60 vol. of 0.15 M-NaCl–0.01 M-Tris-HCl, pH 8.3, and lysed in water by gentle homogenization. The chromatin was allowed to swell in an ice bath for 30 min, then pelleted by centrifugation at 20 000g for 15 min, resuspended in water, and again pelleted at 20 000g for 15 min. DNA was prepared by the method of Marmur (1963) and treated with ribonuclease for 30 min at 37°C (50 µg/ml), Pronase for 2 h at 37°C (50 µg/ml), and phenol before use. Enzymes were purchased from Sigma Inc., St. Louis, Mo., U.S.A.

Chromatin dissociation, fractionation and reconstitution

Chromatin was dissociated in 3 M-NaCl–5 M-urea–0.01 M-Tris-HCl, pH 8.3, and the DNA was pelleted by centrifugation at 100 000g for 48 h. The supernatant containing the chromosomal proteins (histones and non-histone chromosomal proteins) was extensively dialysed against 300 vol. of 5 M-urea–0.01 M-Tris-HCl, pH 8.3, and fractionated into histones and non-histone chromosomal proteins as follows (Gilmour & Paul, 1970; Stein & Farber, 1972; Stein *et al.*, 1972; Bekhor *et al.*, 1969). The protein was added to QAE-Sephadex (Pharmacia, Piscataway, N.J., U.S.A.) previously equilibrated with 5 M-urea–0.01 M-Tris-HCl, pH 8.3, and the histone fraction was collected by vacuum filtration of the slurry. After the Sephadex was washed extensively with 5 M-urea–0.01 M-Tris-HCl, pH 8.3, to remove any remaining histones, the non-histone chromosomal proteins were eluted with 3 M-NaCl–5 M-urea–0.01 M-Tris-HCl, pH 8.3. The histone and non-histone

chromosomal proteins were dialysed against 3 M-NaCl–5 M-urea–0.01 M-Tris-HCl, pH 8.3. The yields of chromosomal proteins (histones and non-histone chromosomal proteins) from S phase and mitotic chromatin were similar.

Chromatin was reconstituted by procedures described previously (Gilmour & Paul, 1970; Stein & Farber, 1972; Stein *et al.*, 1972; Bekhor *et al.*, 1969). DNA pooled from S-phase and mitotic HeLa cells was mixed with histones pooled from S-phase and mitotic chromatin and with non-histone proteins from either S-phase or mitotic chromatin. NaCl was removed by gradient dialysis for a minimum of 4 h against 5 M-urea–0.01 M-Tris-HCl, pH 8.3, containing successively 3.0, 2.5, 2.0, 1.5, 1.2, 1.0, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 M-NaCl. The chromatin was then dialysed against 5 M-urea–0.01 M-Tris-HCl, pH 8.3, and pelleted at 30 000g for 30 min. In one experiment chromatin was reconstituted with DNA pooled from S-phase and mitotic HeLa S₃ cells and histones from either S-phase or mitotic chromatin. Evidence for fidelity of chromatin reconstitution has been provided by several investigators (Paul & Gilmour, 1968; Gilmour & Paul, 1970; Stein & Farber, 1972; Stein *et al.*, 1972; Bekhor *et al.*, 1969; Paul & More, 1972).

Selective dissociation of histone from chromatin

Histones were selectively dissociated from chromatin by using the ionic detergent sodium deoxycholate by the method of Smart & Bonner (1971). Chromatin suspended in 0.0025 M-Tris-HCl, pH 8.0, was diluted with the same buffer so that a final volume of 10 ml and a final concentration of 10 E₂₆₀ units would be obtained after the addition of sodium deoxycholate. The required amount of 0.25 M-sodium deoxycholate–0.0025 M-Tris-HCl, pH 8.0, was added dropwise while stirring vigorously on a Vortex mixer. Each 10 ml chromatin sample was gently layered on 2 ml of 1.2 M-sucrose–0.0025 M-Tris-HCl, pH 8.0, and centrifuged in a 50 Ti Spinco rotor for 16 h at 50 000 rev./min. The pellet containing the partially dehistonized chromatin was then analysed for DNA and chromosomal-protein content. In agreement with Smart & Bonner (1971) the order of histone extraction with sodium deoxycholate was slightly-lysine-rich, arginine-rich and then very-lysine-rich histones. This was observed in S-phase as well as in mitotic chromatin. An identical order of histone extraction was observed in reconstituted chromatin suggesting additional evidence for fidelity of reconstitution.

Analytical procedures

Histones were extracted from chromatin with three washings with 2 ml of 0.1 M-H₂SO₄. Nucleic acids were then extracted with 5% (w/v) trichloroacetic acid for 15 min at 90°C, followed by extraction with an equal

volume of 1M-HClO₄ at the same temperature, and the nucleic acid extracts were pooled. The residual pellet (non-histone chromosomal proteins) was solubilized in 1M-NaOH. The amount of protein present in the histone and non-histone chromosomal protein fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Binding of histone to DNA in S-phase and mitotic chromatin

To assess the manner in which histones are associated with DNA in S-phase and mitotic HeLa S₃-cell chromatin, the extractability of these proteins with the ionic detergent sodium deoxycholate was determined. Fig. 1 demonstrates that the probe provides a valid indication of histone binding of DNA, since increased concentrations of sodium deoxycholate between 0.005M and 0.1M selectively

Fig. 1. Effect of sodium deoxycholate on release of DNA and chromosomal proteins from chromatin

Exponentially growing HeLa S₃ cells were labelled for 24h with L-[³H]tryptophan (0.2μCi/ml) or [³H]thymidine (0.1μCi/ml). The cells were harvested, nuclei were isolated and chromatin was prepared. The chromatin was then treated with sodium deoxycholate (0–0.15M), pelleted by centrifugation, and histones, nucleic acids and non-histone chromosomal proteins were extracted. Histone content was assayed by the method of Lowry *et al.* (1951) (▲, histones released). DNA and non-histone chromosomal proteins associated with chromatin after sodium deoxycholate treatment are reflected by the [³H]thymidine (○) and L-[³H]tryptophan (●) radioactivity respectively. Each point represents a minimum of six determinations, and the range of values did not exceed 5%.

extract progressively increased amounts of histone polypeptides without releasing DNA or non-histone chromosomal proteins from chromatin.

Fig. 2(a) shows that a higher concentration of the detergent is required to dissociate a given amount of histone from mitotic than from S-phase chromatin, suggesting that these proteins are more tenaciously bound to DNA during mitosis. Specifically, 0–0.08M-sodium deoxycholate releases 1.2- to three-fold greater quantities of histones from S-phase than from mitotic chromatin. A similar variation in dissociation by sodium deoxycholate of histones from S-phase and mitotic chromatin prepared from cells synchroni-

Fig. 2. Effect of sodium deoxycholate on release of histone from native chromatin, reconstituted chromatin and nucleohistone complexes

(a) Dissociation of histones from native S-phase (●) and mitotic (○) chromatin with sodium deoxycholate. (b) Dissociation of histone from chromatin reconstituted with DNA and histones pooled from S-phase and mitotic chromatin and non-histone chromosomal proteins from either S-phase (●) or mitotic (○) chromatin. (c) Dissociation of histone from nucleoprotein complexes reconstituted with DNA pooled from S-phase and mitotic chromatin and histones from either S-phase (●) or mitotic (○) chromatin. Each point represents a minimum of six determinations, and the range of values did not exceed 5%.

Fig. 3. Sodium dodecyl sulphate-polyacrylamide-gel electrophoretic profiles of non-histone chromosomal proteins synthesized during S phase (a) and mitosis (b). S-phase and mitotic HeLa S_3 cells were labelled for 30 min with L-[^3H]tryptophan ($10\mu\text{Ci/ml}$), and chromatin was isolated from purified nuclei dissociated in 3M-

zed by mitotic selective detachment was observed, indicating that the utilization of thymidine and Colcemid to produce cell synchrony in the present studies does not influence histone release.

Regulation of histone binding by non-histone chromosomal proteins

Plate 1 shows the similarity of histones associated with S-phase and mitotic chromatin, suggesting that these basic chromosomal polypeptides by themselves are not responsible for the observed cell-cycle-stage-specific variations in their binding to DNA. Although differences in the state of S-phase and mitotic histone phosphorylation may exist (Lake & Salzman, 1972) they are not resolved in these polyacrylamide gels. In contrast, Fig. 3 suggests that there are differences in the molecular-weight classes of non-histone chromosomal proteins synthesized and associated with chromatin during S phase and mitosis. Other studies indicate variations in the phosphorylation of specific S-phase and mitotic non-histone chromosomal protein fractions (Platz *et al.*, 1973). Taken together, these findings implicate non-histone chromosomal proteins as mediating the binding of histones to DNA. To test this hypothesis directly chromatin was reconstituted with DNA and histones pooled from S-phase and mitotic chromatin and non-histone chromosomal proteins from either S-phase or mitotic chromatin. A regulatory function from non-histone chromosomal proteins within this context should be reflected by an increased extractability of histones from chromatin reconstituted with S-phase rather than mitotic non-histone chromosomal proteins. Fig. 2(b) indicates that a lower concentration of sodium deoxycholate dissociates a given amount of histone from chromatin reconstituted with S-phase non-histone chromosomal proteins than from chromatin reconstituted with mitotic non-histone chromosomal proteins. Further, the extractability of histones from the reconstituted chromatin preparations is similar to that of native chromatin from which the non-histone chromosomal proteins are derived.

To establish further that histones themselves are not responsible for cell-cycle-stage-specific variations in their binding to DNA, nucleohistone complexes were reconstituted with DNA pooled from S-phase

NaCl-5M-urea-0.01M-Tris-HCl, pH 8.3. Chromosomal proteins ($75\mu\text{g}$) were electrophoresed on 7.5% (w/v) polyacrylamide gels ($0.6\text{cm}\times 15\text{cm}$) containing 0.1% sodium dodecyl sulphate (Maizel, 1966). A 3% (w/v) polyacrylamide stacking gel ($0.6\text{cm}\times 2\text{cm}$) was used. Since histone polypeptides do not contain tryptophan residues, the distribution of radioactivity in these gels solely reflects the synthesis of non-histone chromosomal proteins.

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of S-phase (a) and mitotic (b) histones

Histones were extracted from chromatin with 0.1 M-H₂SO₄ and 50 µg samples were electrophoresed by the method of Panyim & Chalkley (1969).

and mitotic cells and either S-phase or mitotic histones. Fig. 2(c) clearly demonstrates that (a) there are no significant differences in the amounts of histone polypeptides extractable with 0–0.1 M-sodium deoxycholate from the DNA–histone complexes reconstituted with S-phase or mitotic histones, and (b) higher concentrations of sodium deoxycholate are required to extract a given amount of histone from such complexes than from native S-phase and mitotic chromatin or from chromatin reconstituted with DNA and histone pooled from S-phase and mitotic cells and either S-phase or mitotic non-histone chromosomal proteins. The apparent increased binding of histone to DNA in the absence of non-histone chromosomal proteins is in agreement with a decreased template activity for RNA synthesis under these conditions (Paul & More, 1972).

Discussion

We have shown that histones are more tenaciously bound to DNA in mitotic than in S-phase chromatin. The binding of histone polypeptides was assayed by selective dissociation with the ionic detergent, sodium deoxycholate. Since histones appear to repress genome transcription (although perhaps in a non-specific fashion) the tight binding of these proteins to DNA during mitosis is consistent with the restriction of mitotic RNA synthesis *in vitro* (Stein & Farber, 1972; Johnson & Holland, 1965) and *in vivo* (Taylor, 1960; Baserga, 1962; Prescott & Bender, 1962). However, the similarity of S-phase and mitotic histones makes it unlikely that they alone can account for cell-cycle-stage-specific variations in their binding to DNA. The latter point is further supported by the inability to distinguish between the binding of histones to DNA in reconstituted nucleohistone complexes consisting of DNA pooled from S-phase and mitotic cells and either S-phase or mitotic histones.

Differences in the synthesis (Stein & Baserga, 1970; Rovera & Baserga, 1971; Stein & Borun, 1972; Borun & Stein, 1972; Stein & Matthews, 1973; Stein & Thrall, 1973; Levy *et al.*, 1973; Tsuboi & Baserga, 1972), turnover (Borun & Stein, 1972), and phosphorylation (Platz *et al.*, 1973) of non-histone chromosomal proteins associated with the genome during S-phase and mitosis, as well as the preferential extraction of histones with sodium deoxycholate from chromatin reconstituted with S-phase rather than with mitotic non-histone chromosomal proteins, implicates non-histone chromosomal proteins as possibly mediating the binding of histones to DNA. The present results, along with evidence that non-histone chromosomal proteins rather than histones are responsible for the differences in chromatin template activity during S phase and mitosis (Stein & Farber, 1972), suggested that non-histone chromo-

somal proteins may modify genome expression during the cell cycle by reversing some of the non-specific inhibition of transcription caused by the binding of histones to DNA. Such reasoning is consistent with the model for gene regulation by non-histone chromosomal proteins proposed by Paul & Gilmour (1968). An alternative although less viable explanation would be that non-histone chromosomal proteins alone are responsible for the condensation of mitotic chromosomes. Under these conditions, histones might be less accessible to extraction by sodium deoxycholate.

A critical question that remains unresolved is the precise relationship between the various components of chromatin. Although a number of recent studies have been aimed at the resolution of this problem, it is clear that at present considerable controversy exists as to the distribution of chromosomal proteins along the DNA molecule and the functional implication of protein–DNA interactions. Definitive answers to these questions are essential for an understanding of the control of gene expression in eukaryotic cells, the regulation of DNA-dependent RNA synthesis during the cell cycle being an example of such a phenomenon.

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