

ABSENCE OF TRANSLATIONAL CONTROL OF HISTONE SYNTHESIS DURING THE HE_LA CELL LIFE CYCLE

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ABSTRACT

The cell-free synthesis of histone-like polypeptides has been achieved using a selected class of small polyribosomes as the only particulate fraction. This synthesis is prevented if the deoxyribonucleic acid (DNA) inhibitor, cytosine arabinoside, is added to the cells prior to disruption, and it is not detected when the cytoplasm used is derived from postmitotic (G_1) cells. When the 100,000 g supernate from pure metaphase populations was compared with that from S phase cells, the cell-free synthesis of histone-like polypeptides in the presence of S phase polyribosomes remained unchanged. These data suggest that, except for the histone messenger RNA-ribosome complex, the cytoplasmic factors requisite for histone synthesis are present throughout the cycle, and that the shut-off of this synthesis is not under translational control.

INTRODUCTION

Histones are synthesized only when deoxyribonucleic acid (DNA) is replicated, and their synthesis on small cytoplasmic polyribosomes has been reported (1). Recent experiments (2) with a cell-free (nucleus-free) system have supplied techniques for investigating whether the restriction of histone synthesis to the S phase of the cell cycle is contingent on inhibitory factors in mitotic, G_1 and G_2 cells, and also whether the cytoplasm of these cells possesses the capacity to support histone synthesis if the proper ribosome-messenger complex is present. In this report we have used a refined system to localize *cell-free* histone synthesis on a specific group of small polyribosomes. In addition, we have been able to show that when these small polyribosomes are added to supernatant fractions from cells that are not synthesizing histones, such as those in mitosis, the reconstituted system supports histone synthesis as effectively as one derived en-

tirely from S phase cells. The implications of this observation are noted.

MATERIALS AND METHODS

Cells and Synchronization

Cells derived from the S_3 strain of HeLa were routinely maintained either as monolayers or in suspension culture using Eagle's medium (3) supplemented with 3½% each of calf and fetal calf sera. Synchronization was effected either by treatment of suspension cultures with 2 mM thymidine for 9 hr (4) or by selective detachment of mitotic cells from monolayer cultures grown in low Ca^{++} medium (5). Thymidine-treated suspension cultures were preferable for studying populations synchronized in the S phase of the cell cycle both because of the relatively large numbers of cells easily obtained and because of the sharp peak of DNA synthesis which occurs predictably 3½ hr after removal of the metabolite; how-

ever, the gradual loss of synchrony in these preparations as they proceed into mitosis reduces their usefulness for the study of G₁. Populations in this phase were obtained by selective detachment of mitotic cells from partially confluent monolayers (5). Cells so collected passed from mitosis into G₁ within 60 min. In some experiments the DNA synthesis inhibitor, cytosine arabinoside (Sigma Chemical Co., St. Louis, Mo.), 40 μg/ml, was added to S phase cells for 3 hr.

S phase cells were harvested at the peak of DNA synthesis and G₁ cells at 2 hr after selective detachment.

Cell-Free System

A 100,000 *g* supernatant fraction was prepared from populations that were in either metaphase, G₁, or S. Approximately 4 × 10⁷ cells were swollen in 2 ml of hypotonic buffer (6) and then disrupted in a Dounce type homogenizer (25 μ clearance). Nuclei were removed by centrifugation at 1600 *g* for 5 min, and the supernatant fraction was spun in the Spinco Type-40 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 60 min at 39,500 rpm.

The histone-synthesizing polyribosomes were prepared from S phase cells at the peak of DNA synthesis. About 2 × 10⁸ cells were swollen in 8.0 ml of buffer and disrupted as above. The postnuclear supernate was layered onto 15–30% linear sucrose gradients, and spun at 26,000 rpm for 2 hr in the Spinco SW27 rotor (Beckman Instruments, Inc.). The upper two-thirds of the gradients were collected in 18 1-ml fractions using a continuous flow cell and a Gilford recording spectrophotometer to monitor the optical density at 260 mμ. Fractions 17–27, known to contain the histone-synthesizing polyribosomes (1), were pooled and sedimented in the Type-40 rotor at 39,500 rpm for 4 hr. Fractions 1–11 were treated similarly and used for comparative purposes. The polyribosome pellets were gently resuspended in 2.5 ml of incubation buffer (0.05 M NH₄Cl; 0.005 M MgCl₂·6H₂O; 0.005 M 2-mercaptoethanol; 0.05 M Tris-HCl, pH 7.4), and three strokes of a Dounce homogenizer were applied to disperse clumps. To this were added: 0.5 ml of 100,000 *g* supernate, 0.05 ml of Eagle's 20 × essential and nonessential amino acids (3) minus lysine and tryptophan, 0.50 ml of 2-phosphoenolpyruvic acid (50 mg/ml), 0.02 ml of pyruvate kinase (2000 enzyme units/ml), 100 μCi of L-tryptophan-³H (specific activity 6.6 Ci/millimole) and 20 μCi of L-lysine-¹⁴C (specific activity 0.25 Ci/millimole). Adenosine triphosphate (ATP) (36 mg) and guanosine triphosphate (GTP) (20 mg) were both dissolved in 1.0 ml of incubation buffer immediately before use, and 0.10 ml of this solution was added to the cell-free system. Incubation was carried out at 37°C for 30–60 min and the reaction was

stopped by adding ribonuclease to 50 μg/ml (20 min, 37°C). The histone polypeptides were extracted with 0.25 N HCl and precipitated with acetone according to Gallwitz and Mueller (2). The precipitate was redissolved in 1% sodium dodecyl sulfate (SDS) in 0.01 M phosphate buffer, pH 7.0, and electrophoresed on 7.5% polyacrylamide gels containing 0.1% SDS as previously described (1). Gels were mechanically fractionated into glass vials (7) and diluted with Bray's fluid for liquid scintillation counting. Since it was consistently observed that a significant and selective loss of ¹⁴C counts occurred over the 24 hr after addition of the scintillation fluid, counting was carried out immediately after dilution and each vial was counted for only 1 min.

Experiments were performed to determine whether polypeptides isolated as above included complete and released histone chains. After incubation, the system was chilled to 4°C and centrifuged at 39,500 rpm in the Type-40 rotor for 60 min. The polyribosome-free supernate was then made 0.25 N in HCl for 30 min; the resulting cloudy suspension was spun at 39,500 rpm for 30 min, and the supernate, containing the HCl-soluble polypeptides, was diluted with 10 volumes of acetone. The precipitate was redissolved in 1% SDS, 0.01 M PO₄ and analyzed on SDS-polyacrylamide gels as above.

RESULTS AND DISCUSSION

We have previously localized histone synthesis on a specific group of polyribosomes by virtue of several criteria (1). These include: the lysine-rich, tryptophan-deficient character of the nascent polypeptides isolated from these polyribosomes following a short pulse—a pattern consistent with the amino acid composition of histones (8, 9); the coelectrophoresis of polypeptides isolated from these specific polyribosomes with histones isolated from nuclei; the disappearance of these polypeptides from the polyribosomes when histone synthesis is arrested with specific DNA synthesis inhibitors and their presence only during the S phase of the cell cycle which is the only time that histones are made.

Fig. 1, which is a composite of several experiments, illustrates that the same criteria used to identify histones synthesized on polyribosomes in the intact cell may be successfully applied to a polypeptide product synthesized by a specific group of polyribosomes in a cell-free system. Panel A shows the polyribosome profile (OD 260) from histone-synthesizing HeLa cells. The region *Y* delineates those fractions of the gradient where histone synthesis occurs in the intact cell and those which were utilized in the present cell-free system experiments. The region *X* was used in a separate

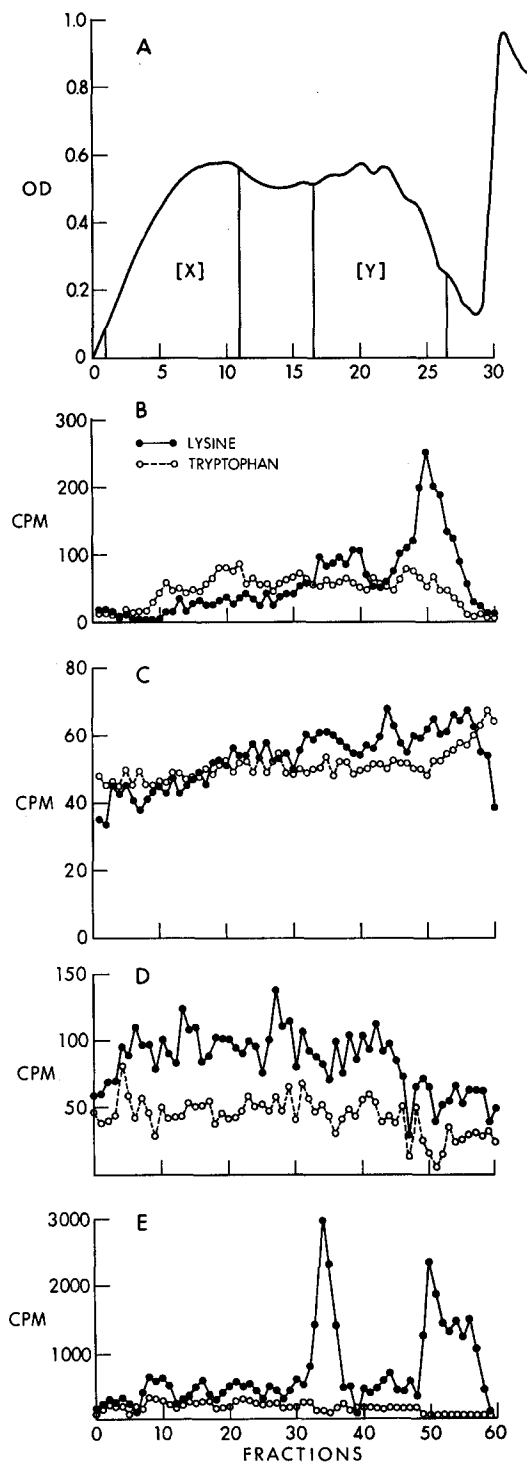


FIGURE 1 (A) OD₂₆₀ profile of polyribosomes from S phase HeLa cells. Cytoplasmic extract was prepared as described in Materials and Methods and centrifuged

cell-free system and served as a control. Panel B is a radioelectropherogram of the polypeptides extracted from the cell-free system containing polyribosomes from region Y and 100,000 g supernatant fraction, both from S phase cells. In contrast to the system of Gallwitz and Mueller (2), no membranes or other polyribosomes were present. Two distinct peaks of rapidly migrating, relatively lysine-rich material are noted (fractions 35-40 and 48-53). The ratio of lysine to tryptophan under these peaks is on average 2.5, while in the earlier fractions of the gel this ratio is 0.5. Histones contain no tryptophan, and therefore the presence of this isotope under the lysine-rich peaks indicates that, if these do indeed represent histones or histone-like polypeptides, they must include other polypeptides as well. This is consistent with the fact that the polyribosome fraction containing the histone-synthesizing polyribosomes is not pure, and numerous other polypeptides, encompassing non-histone proteins, are in various stages of completion when released by the ribonuclease treatment. Some of these are soluble in HCl and cannot readily be separated from histones. Panel C shows that in a cell-free system, as in the intact cell (1), lysine-rich peaks are selectively eliminated when the polyribosomes of region Y are derived from cells treated with a DNA inhibitor. In contrast, those polypeptides containing tryptophan are not affected. This change in the pattern of lysine incorporation without a concomitant effect on the tryptophan pattern indicates that cytosine arabinoside treatment of the intact cell has inhibited the cell-free synthesis of lysine-rich, tryptophan-deficient polypeptides, i.e., histones or related macromolecules.

through a 15-30% linear sucrose gradient for 2 hr at 26,000 rpm in a Spinco SW27 rotor. Direction of sedimentation is from right to left. Region X contains heavier polyribosomes; region Y includes histone-synthesizing polyribosomes. (B-E) Gel electropherograms of lysine-C¹⁴ (●—●) and tryptophan-H³ (○—○)-labeled polypeptides extracted from (B) cell-free system containing S phase small polyribosomes (region Y of gradient), (C) a cell-free system containing small polyribosomes from S phase cells treated with the DNA inhibitor cytosine arabinoside prior to disruption, (D) a cell-free system with heavy polyribosomes (X) from S phase cells, and (E) S cell nuclei labeled in the intact cell. Electrophoresis was performed on 7.5% polyacrylamide gels containing 0.1% SDS and 0.01 M phosphate buffer, pH 7.0 (5 ma/gel for 10 hr at 23°C). Direction of migration is from left to right.

Addition of cytosine arabinoside directly to the cell-free system rather than to the intact cell had no effect on the synthesis of the lysine-rich peaks shown in Panel B.

Although not illustrated, we have also found that the labeling pattern of polypeptides synthesized in a cell-free system derived from G₁ cells is identical with that obtained from cytosine arabinoside-treated cells, confirming that when histones are not being synthesized, the small polyribosomes do not yield lysine-rich peaks. Likewise, in panel D we note that there are no lysine-rich peaks synthesized by polyribosomes derived from region X of the gradient. This is true irrespective of whatever life cycle phase the contributory cells may be in at the time the polyribosomes are prepared. It should be emphasized that, while the ratio of lysine to tryptophan in panel D averages about 2, there are no selectively lysine-rich peaks as in panel B. The absolute ratios are of no consequence, since they are determined by the amino acid pools in the system as well as the amount of isotope added in any given experiment. The values of critical importance are the comparative lysine:tryptophan

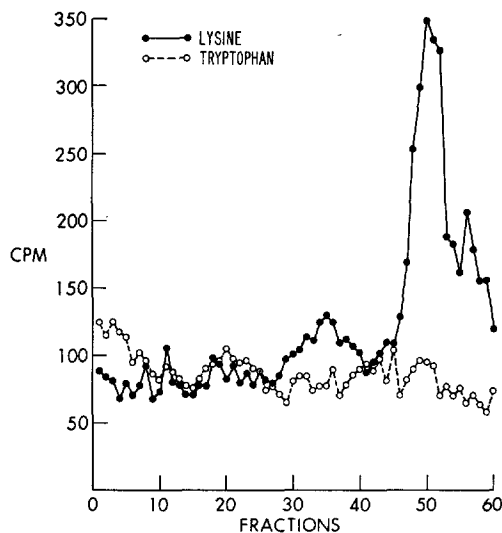


FIGURE 2 Release of histone-like polypeptides from polyribosomes in the cell-free system. S phase polyribosomes from region Y in Fig. 1 A were incubated in the system for 60 min, and the released polypeptides were prepared by extracting the polyribosome-free 100,000 g supernate with 0.25 N HCl and precipitating with acetone as described in the text. Electrophoresis was as in Fig. 1.

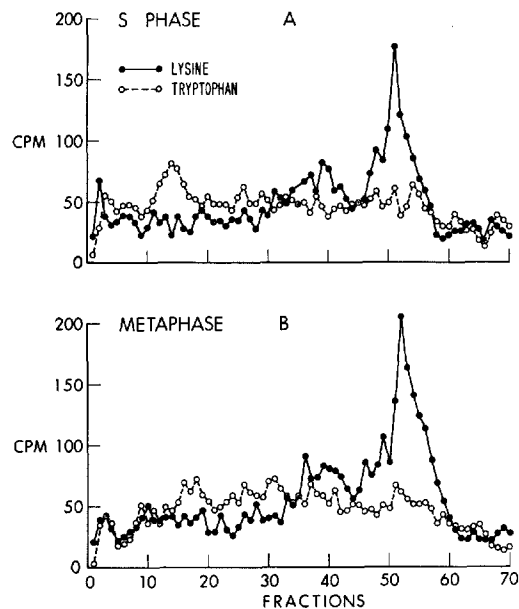


FIGURE 3 Comparison of mitotic and S phase supernates with respect to their support of histone-like polypeptide synthesis on S phase polyribosomes. Small polyribosomes from S phase cells (Y in Fig. 1 A) were combined with the cell-free system containing 100,000 g supernate from either (A) S phase or (B) metaphase cells. After 60 min of incubation, the acid-soluble polypeptides were extracted, precipitated with acetone, redissolved in 1% SDS, and electrophoresed as in Fig. 1.

ratios in one part of the gel with respect to another, and, as we have noted, the lysine:tryptophan ratio in panel B is high in the rapidly migrating fractions and low in the early fractions, while in panel D there is little variation throughout the gel.

Finally, panel E illustrates that the lysine-rich peaks synthesized by small polyribosomes in a cell-free system coelectrophorese with histones isolated from S phase nuclei by HCl extraction; however, relatively less of the slower migrating lysine-rich peak is made in the cell-free system. These data together strongly support the hypothesis that the lysine-rich peaks synthesized by a system containing a specific group of polyribosomes as the only particulate fraction include histones or histone-like polypeptides. The fact that the slowly migrating fraction (F₁ of Johns et al. [10]) is quantitatively less prominent in products of the cell-free system than in nuclear extracts maybe an *in vitro* artifact or may indicate that, in contrast to the majority of histones, at least some of the F₁ fraction is synthe-

sized in the nucleus. In at least one special case, that of the thymocyte, Reid and Cole (11) have in fact observed that isolated nuclei synthesize the F_1 fraction in vitro.

Although the data presented in the previous section clearly show that histone-like polypeptides are synthesized in the cell-free system, they do not indicate whether any of these polypeptide chains are completed and released from the polyribosomes, as they are in the intact cell. To examine this point, intact polyribosomes and their associated nascent polypeptides were pelleted at 100,000 g after 60 min of incubation in the cell-free system; the resulting supernatant material was extracted with 0.25 N HCl and acetone-precipitated as above. The electropherogram of such HCl-soluble proteins from the cell-free system of S cells (Fig. 2) shows a pattern that is grossly similar to that obtained with the polypeptides extracted from the system still containing the polyribosomes. It thus appears that, besides polypeptide chain elongation, the present cell-free system carries out chain termination and release as well.

We have previously established that histone-synthesizing polyribosomes are present only during S phase, and this would seem a sufficient reason for the absence of histone synthesis during mitosis. However, considering the profound morphological transformations that accompany passage of the cell into metaphase, it was appropriate to determine whether, at this time, factors other than the

histone-synthesizing polyribosomes might also be responsible by virtue of either their presence or absence. Fig. 3 makes this possibility unlikely, in that the 100,000 g supernatant fluid from mitotic cells effectively supports the synthesis of histone-like polypeptides when it is combined with the histone-synthesizing polyribosomes from S phase cells. This result does not rule out any potential defect in chain initiation within the mitotic cells' protein-synthesizing machinery, since we do not measure initiation in our system; it does, however, establish that, with respect to histone chain elongation as well as chain termination, the only difference between S phase and mitotic cells is the presence in the cytoplasm of a specific group of small polyribosomes. Comparable results were obtained with G_1 and G_2 100,000 g supernates. Correlatively we may state that, at least in vitro, no translational control of histone synthesis by cytoplasmic factors is demonstrable during the cell cycle.

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