*RAPIDLY LABELED, POLYRIBOSOME-ASSOCIATED RNA HAVING THE PROPERTIES OF HISTONE MESSENGER**

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Histone synthesis and DNA replication (S phase) begin simultaneously in the HeLa cell life cycle. While these macromolecules are made, the cytoplasm contains a class of small polyribosomes which are absent in postmitotic (G_1) cells and which disappear following exposure to cytosine arabinoside, an effective inhibitor of both DNA and histone synthesis. These polyribosomes incorporate a relative excess of lysine over tryptophan into their nascent polypeptides, supporting the hypothesis that they are the cytoplasmic site of histone synthesis.[1] The present report describes: (*a*) the isolation of both histone-like polypeptides and 7–9*S* RNA species from these polyribosomes, and (*b*) the relationship between this 7–9*S* RNA and histone synthesis with specific reference to the possibility that it is messenger RNA.[2]

Materials and Methods.—Most of the techniques used in the present work have been described in previous publications. These include maintenance of HeLa S_3 cells in suspension culture,[3] preparation of cytoplasmic polyribosomes with the detergent Nonidet P-40 (NP-40),[4] analysis of polyribosomes in sucrose gradients,[5] and extraction of histones and their separation into different electrophoretic groups on acrylamide gels.[1]

Cell synchronization: Although cells were usually synchronized by selective detachment of mitotic cells from monolayers,[3] such populations become asynchronous late in the DNA-synthetic (S) and premitotic (G_2) phases of the cell life cycle. For studies at those times, cells were synchronized by treatment in suspension with 2 mM thymidine[6] for 16 hours, resuspension in fresh medium for 8 hours, followed by a second exposure to the drug for 16 hours. After a second resuspension in fresh medium, 90 per cent of the cells divided within 9 to 12 hours.

Identification of polyribosome-associated polypeptides: To label nascent polypeptides with nearly equivalent amounts of radioactivity from each amino acid, 10^8 S-phase cells, resuspended in 10 ml of tryptophan, lysine, and serum-free growth medium,[3] were incubated for one minute with 50 μc C^{14}-lysine and 500 μc H^3-tryptophan. Cytoplasmic extracts were prepared with NP-40,[4] centrifuged through sucrose gradients, and analyzed for optical density at 260 mμ and for acid-precipitable radioactivity.[3] Appropriate fractions from the gradients were pooled, and polyribosomes were pelleted at 104,000 $\times$ *g* for one hour. Nascent polypeptides were released by treatment with 100 μg/ml of ribonuclease at 37°C .[7] The samples were dissolved in 1% sodium dodecyl sulfate (SDS), 0.5 *M* urea, 0.1% 2-mercaptoethanol (ME); dialyzed against 0.1% SDS, 0.1% ME in 0.01 *M* phosphate at pH 7.4; and electrophoresed on 7.5% acrylamide gels.[1] G_1- and S-phase cells pretreated for one hour with 40 μg/ml cytosine arabinoside were similarly processed.

Analysis of rapidly labeled, polyribosome-associated RNA: Synchronized cells at

specific stages of the cell life cycle were concentrated to 4×10^6/ml and 25 ml were incubated with 300 μc uridine-6-H^3 (9.3 c/mM) for 30 minutes at 37°C. Cytoplasmic extracts were centrifuged through sucrose gradients, appropriate fractions pooled, and RNA was released with 1% SDS-0.1% ME for 30 minutes at 37°C. The RNA was precipitated with two volumes of ethanol at −20°C for 24 hours, pelleted by centrifugation, and resuspended in 0.01 *M* phosphate buffer (pH 7.4) containing 1% SDS, 0.1% ME, and 2×10^{-3} *M* EDTA. Samples were electrophoresed for 16 hours at 50 volts on 2.4% acrylamide gels containing 2×10^{-3} *M* EDTA.[8]

In some experiments, cytosine arabinoside (40 μg/ml) or actinomycin D (5 μg/ml) was added after the pulse and aliquots were removed subsequently for gel electrophoresis. Radioactive precursors were purchased from New England Nuclear, Boston, Mass. Actinomycin D was the gift of Merck, Sharp and Dohme; Nonidet P-40 was the gift of the Shell Chemical Co.

Results.—Analysis of HeLa cell histones on SDS-acrylamide gels resolves two major groups of histone polypeptides which we have arbitrarily termed *C* and *A*.[1] When compared to histone fractions from HeLa cells prepared according to the method of Johns and Butler,[9] group *C*, a single peak, coelectrophoreses with the lysine-rich (f_1) fraction (mol wt calculated[10] as approx. 20,000), while group *A*, a multiple peak, coelectrophoreses with the slightly lysine-rich (f_{2a},f_{2b}) and arginine-rich (f_3) fractions of Johns and Butler (mol wt approx. 13,000).[10] None of these fractions contain tryptophan.[11, 12] When synchronized cells, making DNA and histones in S phase, were pulse-labeled with H^3-tryptophan and C^{14}-lysine, only the small polyribosomes showed the high ratio of lysine to tryptophan incorporation characteristic of histones (Fig. 1, top panel). This and other data[1] suggested that small polyribosomes were the site of histone synthesis. More direct evidence has now been obtained by acrylamide gel analysis of nascent polypeptides released from polyribosomes (Fig. 1, panels *A*–*C*). All sizes of polypeptides released from large

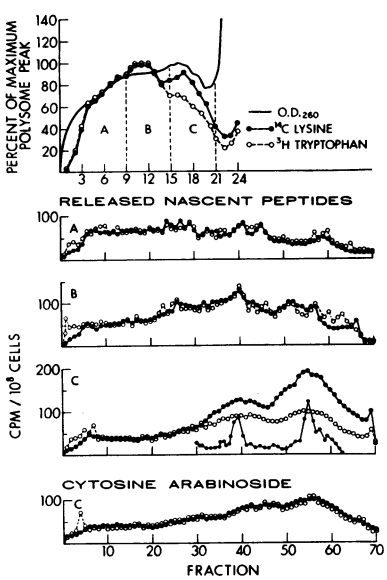

FIG. 1.—*Top panel:* Polyribosome profile (OD_{260}) and incorporation of acid-precipitable H^3-tryptophan and C^{14}-lysine into nascent polypeptides. Synchronized HeLa cells were pulse-labeled with these amino acids for 1 min, 2.5 hr after the beginning of DNA synthesis. Polyribosomes were analyzed on 15–30% sucrose gradients centrifuged at 24,000 rpm for 2 hr.

Middle three panels: Gel electropherograms (7.5% acrylamide gels, electrophoresed at 90 volts for 12 hr) showing the distribution of tryptophan and lysine radioactivity in different sizes of polypeptides released from (*A*) large, (*B*) medium, and (*C*) small polyribosomes of gradient as indicated in top panel. In panel *C* marker electropherogram of HeLa cell nuclear histones, labeled with C^{14}-lysine, has been superimposed for comparison.

Bottom panel: Electropherogram of polypeptides from (*C*) small polyribosomes, isolated from cells pretreated for 1 hr with cytosine arabinoside (40 μg/ml).

(panel *A*) and medium-sized (panel *B*) polyribosomes show a uniform ratio of lysine:tryptophan incorporation, essentially 1:1 under the conditions of these experiments (cf. *Materials and Methods*). In contrast, polypeptides released from small polyribosomes show a significantly increased ratio of lysine to tryptophan incorporation in two diffuse peaks, the migration of which approximates that of histones extracted from cell nuclei and simultaneously analyzed on marker gels (Fig. 1, panel *C*). Following treatment with cytosine arabinoside only these lysine-rich, tryptophan-poor nascent polypeptides disappear, consistent with a selective inhibition of histone synthesis. These findings, and the absence of comparable differences in peptides isolated from G_1 cells, offer additional support for the hypothesis that small polyribosomes of S-phase cells are the site of histone synthesis.

Rapidly labeled, polyribosome-associated RNA: Exposure of S-phase HeLa cells to cytosine arabinoside not only inhibits DNA and histone synthesis but also causes a significant decrease in the amount of small polyribosomes (Fig. 2, top panel). When rapidly labeled, polyribosome-associated RNA is extracted from treated and untreated cells and analyzed on acrylamide gels (Fig. 2, panels *A–C*), a drug effect is seen only in the RNA isolated from small polyribosomes (panel *B*). All samples contain heterogeneous 45–12*S* and 5–4*S* peaks; the small polyribosomes from cells in S contain, in addition, a rapidly labeled and sharply defined peak migrating at about 7–9*S* which is absent in both treated S cells and G_1 cells (Fig. 2, bottom panel). These small polyribosomes are disrupted on treatment with EDTA and the 7–9*S* RNA disappears with them from the 120–200*S* area of the gradient. The sedimentation value of 7–9*S* for this RNA has been confirmed in sucrose gradients.

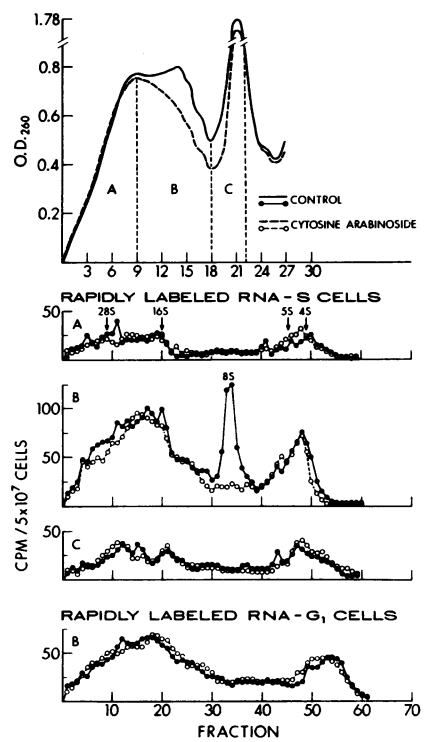

FIG. 2.—*Top panel:* Polyribosome OD_{260} of HeLa cells pulsed with H^3-uridine for 30 min, 2.5 hr after the beginning of DNA synthesis. Polyribosomes were analyzed on 7.5–45% sucrose gradients centrifuged at 24,000 rpm for 3.25 hr. Control cells, ——; cells pretreated 1 hr with cytosine arabinoside (40 μg/ml), —.

Middle three panels: Gel electropherograms (2.4% acrylamide gels, electrophoresed at 50 volts for 16 hr) showing the distribution of H^3-uridine radioactivity in different species of rapidly labeled RNA extracted from the (*A*) large plus medium-sized polyribosomes, (*B*) small polyribosomes, and (*C*) single ribosomes of gradients as indicated in top panel. In each panel cytosine arabinoside (O——O) and control cells (●——●) are compared.

Bottom panel: Rapidly labeled RNA species associated with the small polyribosomes of similarly prepared postmitotic (G_1) cells.

When electrophoresed on 5% (instead of 2.4%) acrylamide gels it resolves into 9*S* and 7*S* peaks, indicating the presence of at least two RNA species. These RNA's are sensitive to RNase, but not DNase, and do not incorporate H^3-thymidine. This material is not an RNA-protein complex since its rate of migration is not affected by prior digestion with pronase and it is not labeled with radioactive amino acids. Since these 7–9*S* RNA species are absent from the single ribosome area of the gradient (Fig. 2, panel *C*), they are not low-molecular-weight contaminants from the top of the gradient. Finally, small "histone" polyribosomes and 7–9*S* RNA can be quantitatively recovered from cytoplasmic extracts prepared by osmotic shock and Dounce homogenization[5] without the addition of sodium desoxycholate, indicating that neither is membrane-associated. These characteristics, together with the selective sensitivity of both 7–9*S* RNA and "histone" polyribosomes to cytosine arabinoside treatment, suggest that this RNA may be histone messenger.

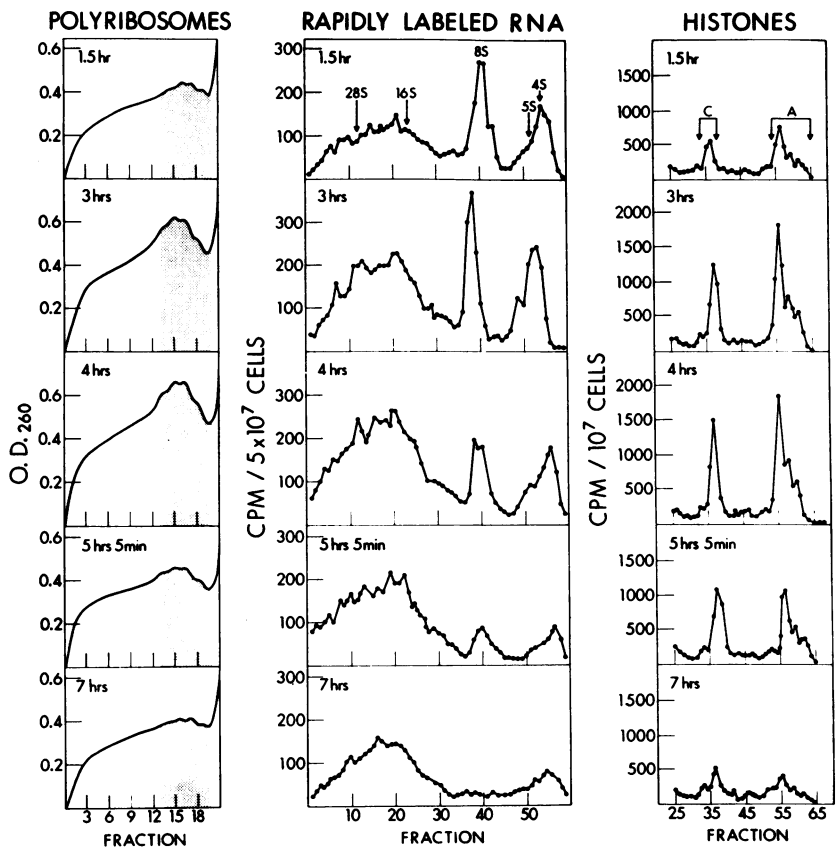

FIG. 3.—Polyribosome profiles, associated rapidly labeled RNA, and synthesis of histones at various stages of the DNA synthetic (S) phase. At the indicated stage in the S phase of the cell life cycle, 10^8 thymidine synchronized cells were pulsed with H^3-uridine for 30 min. Polyribosomes were analyzed in sucrose gradients as described for Fig. 1 and the uridine radioactivity in RNA extracted from the small polyribosomes (shaded area, column 1) was analyzed on acrylamide gels (column 2) as described in the legend of Fig. 2. Simultaneously, 2×10^7 cells were pulsed with 5 μc C^{14}-lysine for 20 min, histones were extracted from the nuclei with 0.1 *N* HCl and analyzed on acrylamide gels (column 3) as described in *Materials and Methods*.

Temporal relationships of polysomal 7–9S RNA, DNA replication, and histone synthesis: Cells obtained by thymidine synchronization were analyzed during the S and G_2 phases of the cell life cycle for small polyribosomes, associated 7–9*S* RNA, and histone synthesis. Cells selectively detached in mitosis were used for similar experiments during the G_1 phase and the transition to S. The results of these experiments are shown in part in Figure 3 and are summarized in Figure 4. The synthesis of group *C* and *A* histones begins at the onset of DNA replication (taken as time 0 in Fig. 3) and simultaneously newly synthesized 7–9*S* RNA becomes associated with small polyribosomes. The optical density of the histone polyribosome region (shaded area, Fig. 3, column 1) increases to a maximum four hours into S, as do the rates of DNA and histone synthesis (Fig. 4). The amount of newly synthesized, polyribosome-associated 7–9*S* RNA is maximal two hours earlier and has declined about 20 per cent when histone synthesis has peaked. It should be emphasized that the electropherograms (Fig. 3, column 2) represent only RNA made and associated with polyribosomes during a 30-minute uridine pulse, and not the total amount of RNA present. The actual quantity of 7–9*S* RNA probably reached a maximum at the same time as histone polyribosomes and histone synthesis. By seven hours into S, *newly synthesized* 7–9*S* RNA is no longer detectable on polyribosomes, but histone synthesis remains measurable at a rapidly declining rate for about two additional hours. The optical density of the histone polyribosome area declines in parallel with histone synthesis.

Half life of 7–9S RNA on polyribosomes as measured by the effects of actinomycin D and cytosine arabinoside: To estimate the time that 7–9*S* RNA remains associated with polyribosomes, cells $2^1/_2$ hours into S were pulse-labeled with H^3-uridine for 30 minutes and treated with actinomycin D to inhibit further RNA synthesis (Fig. 5*A*). The half life of labeled 7–9*S* RNA on polyribosomes is one hour, in marked contrast to other heterogenous, rapidly labeled and polyribosome-associated RNA species which have a half life of three hours in actinomycin D-treated cells. The synthesis of group *A* histones and DNA decrease in parallel with the amount of

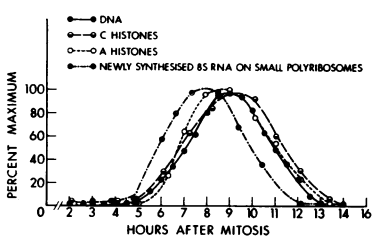

FIG. 4.—Rates of DNA replication, group *A* and *C* histone synthesis, and the amount of newly synthesized 7–9*S* RNA (called 8*S* in the figure) associated with polyribosomes throughout the HeLa cell life cycle. DNA synthesis was estimated with 20-min H^3-thymidine pulses and synthesis of the two histone groups with 20-min C^{14}-lysine pulses. 7–9*S* RNA was determined as described in the legend of Fig. 2 and in *Materials and Methods*.

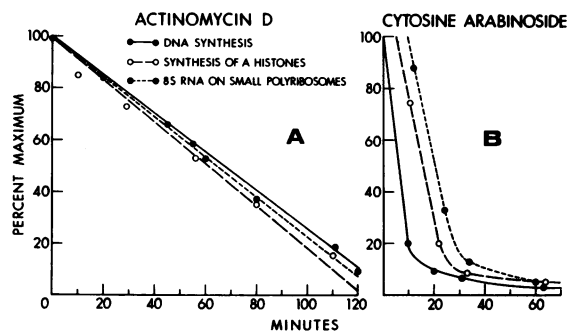

FIG. 5.—(*A*) Effects of 5 μg/ml actinomycin D on (1) prelabeled 7–9*S* (called 8*S* in the figure) polyribosomal RNA, (2) the rate of DNA replication, and (3) synthesis of the *A* group of histones. These were determined as described in the legends of Figs. 1, 2, and 4 and in *Materials and Methods*.

(*B*) Effects of 40 μg/ml cytosine arabinoside on labeled 7–9*S* polysomal RNA, the rate of DNA replication, and synthesis of the *A* group of histones.

7–9S RNA. Under the same conditions, synthesis of group C histones (not shown in Fig. 5) has a half life of about 90 minutes.

Cytosine arabinoside (Fig. 5B) induces its effects more rapidly than does actinomycin D. The synthesis of DNA, A-group histones, C-group histones, and the amount of labeled 7–9S RNA associated with polyribosomes all fall to background levels within 30 minutes. All other RNA and protein species appear unaffected under these experimental conditions.

Discussion and Summary.—Several properties of the polysomal 7–9S RNA here described suggest that it is messenger RNA for group A histone polypeptides. (*a*) It associates with polyribosomes more rapidly than ribosomal RNA, and maintenance of this association is Mg^{++}-dependent. (*b*) It is associated only with polyribosomes of the size responsible for histone synthesis, and only when histones are made, i.e., during the DNA-synthetic phase (S) of the cell life cycle. (*c*) It rapidly and selectively disappears from polyribosomes when histone synthesis is inhibited with cytosine arabinoside. (*d*) Its one-hour half life on polyribosomes after treatment with actinomycin D is the same as the half life of group A histone synthesis under the same conditions. (*e*) The size of the smallest of the RNA peaks is consistent with that expected of messenger RNA for the polypeptides of group A histones, which have molecular weights of about 13,000.

The kinetics of 7–9S RNA synthesis as measured by its association with polyribosomes imply that the HeLa cell controls synthesis of group A histone polypeptides principally at the level of messenger transcription. Thus, at the transition of cells from G_1 to S, newly synthesized 7–9S RNA becomes associated with polyribosomes and histone synthesis begins. Two hours before it ends, messenger transcription apparently ceases and the remaining messenger decays with a one-hour half life, effectively accounting for the absence of histone synthesis in the subsequent mitotic and G_1 phases of the cell life cycle.

After treatment with cytosine arabinoside, histone synthesis and the amount of 7–9S RNA associated with polyribosomes decline about four times faster than after exposure to actinomycin D. This suggests that there may also be a translational control of histone synthesis, conceivably functioning as a fine adjustment of the amount of histones made during chromosomal replication.[13]

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[13] We regret that in a previous paper (these PROCEEDINGS, **57**, 409 (1967)) we misquoted the design of an experiment performed by Spalding, Kajiwara, and Mueller (these PROCEEDINGS, **56**, 1535 (1966)).