SYNTHESIS OF HISTONES IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM DIRECTED BY A POLYRIBOSOMAL RNA FRACTION FROM SYNCHRONIZED HELA CELLS

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SUMMARY

A RNA fraction from HeLa cell polyribosomes derived from thymidine-synchronized cells in S-phase directs the synthesis in a rabbit reticulocyte cell-free system of acid-soluble polypeptides with the same electrophoretic properties as authentical HeLa cell histones. In sucrose gradients this RNA fraction sediments in the 5-10 S region.

In eukaryotic cells the synthesis of histones proceeds synchroneously with DNA-replication (1,2,3,4). In synchronized HeLa cells histones are synthesized on cytoplasmic polyribosomes (2,4,5). These proteins comprise a significant amount of the acid-soluble polypeptides completed in vitro on polyribosomes isolated from cells in S-phase (4,5). HeLa cell polyribosomes engaged in histone synthesis contain three RNA species with molecular weights between 1.5×10^5 and 2.2×10^5 daltons (6) which seem to represent histone messenger RNAs (mRNAs). Similar RNA fractions have been identified as presumptive histone mRNAs in sea urchins (7,8) and mouse L cells (9).

In studying the relationship between DNA and histone synthesis, especially the appearance in the cytoplasm of the individual RNA fractions presumably coding for different histones, we have used the rabbit reticulocyte lysate to functionally identify histone mRNA. We describe experiments here showing that a RNA fraction from HeLa cell polyribosomes derived from synchronized cells in S-phase directs the synthesis of some, probably of all, major histone fractions.

METHODS

Preparation of the rabbit reticulocyte cell-free system and incubations were

conducted as described by Lockard and Lingrel (10). The lysate was used immediately after preparation or after storage at -80°C. ^{3}H-4-L-arginine (spec. act. 11 C/mM) and ^{3}H-4,5-L-lysine (spec. act. 41.6 C/mM) were obtained from Schwarz Bio Research, ^{3}H-L-leucine (spec. act. 5 C/mM) from NEN.

HeLa S3 cells were grown in suspension culture in Eagle's minimum essential medium containing 5 % calf serum (2-6 x 10^{5} cells/ml) and synchronization of DNA synthesis was achieved by a double block with 2 x 10^{-3}M thymidine (11). Polyribosomes were prepared as described previously (4,5). RNA was extracted either with phenol and SDS at room temperature (6) or with 1 % SDS as described by Lockard and Lingrel (10) and modified by Stavnezer and Huang (12) and

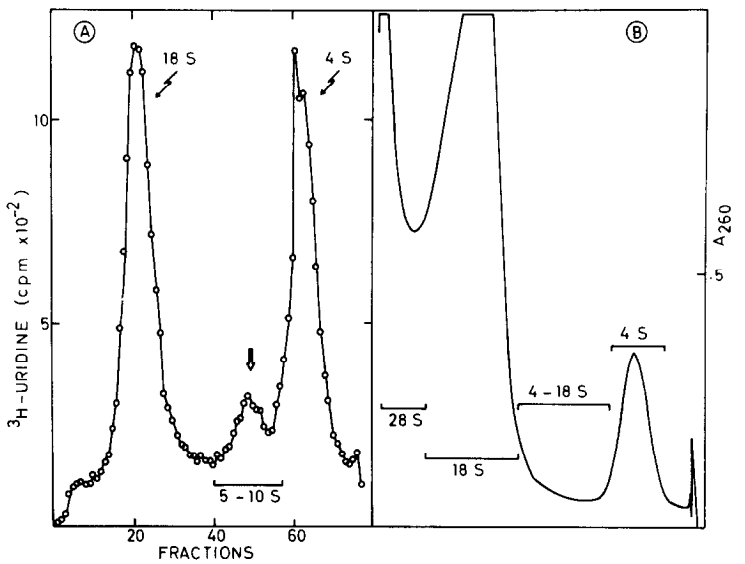

Fig. 1 - Sucrose gradient centrifugation of polyribosomal RNA. To polyribosomes suspended in 30 mM tris-HCl, pH 7.4, 15 mM KCl, 30 mM EDTA an equal volume of 150 mM NaCl containing 0.4 % SDS was added and the RNA extracted with water-saturated phenol for 15 min at room temperature. After reextraction with phenol of the aequeous phase the RNA was precipitated with 2.5 vol ethanol for 20 hrs at -20°C and reprecipitated once with ethanol from 100 mM NaCl, 1mM EDTA, pH 6.2 170 A_{260}units of RNA dissolved in NaCl-EDTA were centrifuged for 26 hrs at 27,000 rpm on a 15 - 30 % sucrose gradient in 20 mM tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA using a Beckman SW 27 rotor. The absorption profile was monitored at 260 nm in a Gilford 2400 spectrophotometer and fractions were pooled as indicated by the brackets (B). The RNA was precipitated twice with ethanol and dissolved in a small volume of water. RNA was labeled with 600 µC of ^{3}H-5-uridine (spec. act. 25 C/mM, obtained from NEN) in a 150 ml suspension culture (2.5 x 10^{5} cells/ml) during the first two hours after removal of the thymidine block. Centrifugation in a similar sucrose gradient as discribed above was performed for 20 hrs at 40,000 rpm using a Beckman SW 40 rotor (A). For experiments described in the text unlabeled RNA was pooled from the 5-10 S region as indicated.

subsequently fractionated on sucrose gradients in the manner described in legend to Fig. 1. Extreme care was taken to avoid RNA degradation, all glassware was autoclaved, buffers were prepared with glass-distilled, autoclaved water, the sucrose used was density gradient grade (ribonuclease-free) from Schwarz/Mann.

Acid-soluble proteins were extracted from the incubation mixture with 0.25 N HCl at + 1°C for 60 min and, after exhaustive dialysis in the cold against 0.025 N HCl, precipitated at - 20°C with 10 vol of acetone for 15 - 20 hours. After dissolving the dried proteins (1.5 - 2 mg) in 2 ml of 6 M urea in water they were passed over a 0.8 x 8 cm column of CM-cellulose equilibrated with 0.1 M tris-HCl, pH 7.4, containing 6 M urea. Under these conditions the bulk of globin passes through the column whereas essentially all histones are adsorbed to and eluted from CM-cellulose with 0.25 N HCl in 6 M urea as shown with ^{14}C-labeled HeLa cell histones. The proteins eluted from CM-cellulose were then precipitated with 10 vol of acetone as described above and dissolved in a small volume of 6 M urea in water for analysis by polyacrylamide gel electrophoresis at pH 2.8 as described by Panyim and Chalkley (13) or in the presence of SDS as described previously (14).

RESULTS AND DISCUSSION

RNA was extracted from polyribosomes of HeLa cells 4 hrs after the cells were released from a second thymidine block. At that time both DNA synthesis as well as the completion of histones *in vitro* on polyribosomes were close to their maxima. Confirming our earlier results using HeLa cells synchronized with amethopterin (5,6) polyribosomal RNA, pulse-labeled during S-phase, contained a highly labeled RNA fraction sedimenting between 7 S and 9 S (Fig. 1, A) and demonstrating many properties of histone mRNA (6). Only a very limited amount of this RNA was isolated from polyribosomes during thymidine blockade.

In a first attempt to identify histone mRNA we pooled the RNA from sucrose gradients as indicated in Fig. 1,B and tested the different RNA fractions in a reticulocyte lysate. 2.5 A_{260} units added to 0.5 ml of the cell-free system caused a significant inhibition of amino acid incorporation into total acid-precipitable material, an observation already described by others (10, 12, 15). Electrophoretograms at pH 2.8 of the acid-soluble *in vitro* products eluted with 0.25 N HCl from CM-cellulose showed two main radioactive peaks in the gels separating the endogeneous product, the second peak (fractions 64 - 67 in Fig. 2) being globin.

Addition to the cell-free system of the 4-18 S RNA fraction resulted in the formation of polypeptides co-migrating with the three histones f3, f2b and $f2a_2$ in parallel runs with authentical HeLa cell histones. Only a very small amount of such polypeptides was synthesized under the direction of the 18 S RNA fraction.

Next, polyribosomal RNA was prepared from cells 4 hrs after removal from the thymidine block and from a parallel culture in which DNA synthesis (and most of the histone synthesis) was prevented by 2 x 10^{-3}M thymidine. RNA in the 5 - 10 S region was pooled from sucrose gradients as shown in Fig. 1, A and tested in a freshly prepared reticulocyte cell-free system. A gel electrophoretic

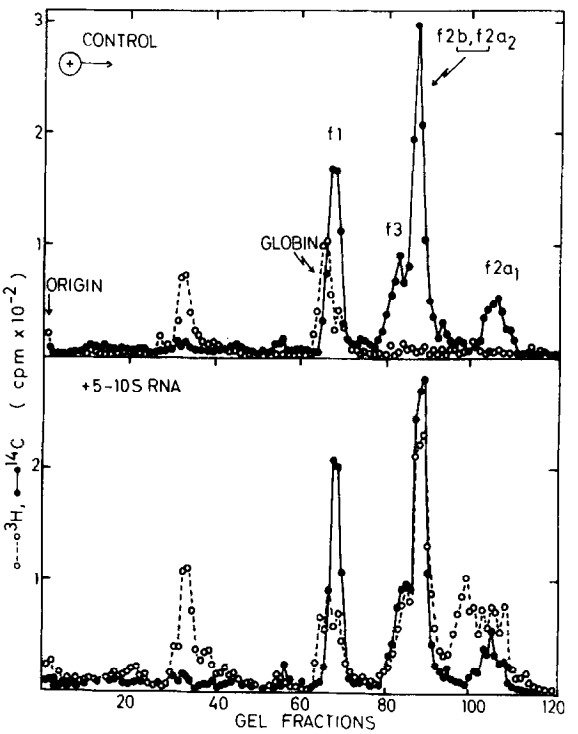

Fig. 2 - Gel electrophoresis of ^{3}H-labeled, acid-soluble in vitro products mixed with ^{14}C-labeled HeLa cell histones. The cell-free system contained in a final volume of 0.5 ml: 10 mM tris-HCl, pH 7.4, 100 mM ammonium acetate, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 30 µg creatine phosphokinase, 5 µC ^{3}H-4-L-arginine, 5 µC ^{3}H-L-leucine, 15 µC ^{3}H-4,5-L-lysine, between 4 and 50 µmoles of the other unlabeled amino acids and 0.2 ml reticulocyte lysate. Incubations were performed for 2 hrs at +26°C in the presence or absence of 0.7 A_{260} units of 5 - 10 S RNA and terminated by further incubation for 20 min with 20 µg of pancreatic ribonuclease. Proteins were prepared as described in the text and 100 µg were subjected to electrophoresis for 6 hrs on 0.6 x 8 cm 15 % acrylamide gels at pH 2.8 and 2 mA/gel (13). Gels were fractionated mechanically and the radioactivity was measured as described (14).

pattern of ^{14}C-histones mixed with ^{3}H-labeled *in vitro* products (Fig. 2) shows that the addition of such a RNA fraction from cells in DNA (and histone) synthesis directed the formation of acid-soluble polypeptides with the same electrophoretic properties as histones f3, f2b and $f2a_2$. Moreover, among the newly synthesized proteins some polypeptides showed the same mobility as the N-acetylated histone $f2a_1$. Since globin, which was not quantitatively removed from other acid-soluble proteins by CM-cellulose, migrates in the electrophoretic system at pH 2.8 close to histone f1 it could not be decided with certainty whether the latter histones were synthesized as well. SDS-gel electrophoresis in the presence of 6 M urea (5, 14) separated the endogeneous products from histone fraction f1 and, as seen in Fig. 3, there is little but definite ^{3}H-labeled material co-migrating with ^{14}C-labeled histone fraction f1. Unfortunately, in the SDS-gels used, globin migrates in the position of histone $f2a_1$ and the other acid-soluble endogeneous product (gel fractions 30-35 in Fig. 2) in the position of the two separated protein bands comprising histones f3, f2b and $f2a_2$. However, essentially all of the RNA-stimulated products (see Fig. 2) co-migrated with the ^{14}C-labeled histones lending further proof to the assumption that most of the newly synthesized products are indeed histones. This makes it likely that all major histone fractions can be synthesized in a rabbit reticulocyte lysate under the direction of a RNA fraction isolated from polyribosomes of HeLa cells in S-phase.

Compared to the polyribosomal 5 - 10 S RNA from cells in DNA synthesis, an equal amount of comparable RNA extracted from polyribosomes of thymidine-

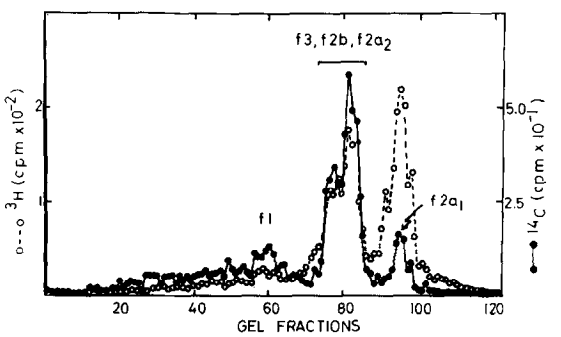

Fig. 3 - SDS-gel electrophoresis of ^{3}H-labeled, acid-soluble *in vitro* products mixed with ^{14}C-labeled HeLa cell histones. The same proteins as in the lower part of Fig. 2 were fractionated on 0.6 x 8 cm 10 % acrylamide gels containing 6 M urea (14) at 6 mA/gel for 10 hrs. Before electrophoresis the proteins were incubated for 5 hrs at 37°C in 0.02 M sodium phosphate buffer, pH 7.2, containing 0.1 % SDS, 0.14 M 2-mercaptoethanol and 6 M urea.

blocked cells resulted in the formation of only 10 - 15 % of acid-soluble proteins representing at least in part histone polypeptides. This suggests that in thymidine-blocked cells the synthesis of histones is not completely stopped. Indeed, this has been concluded from amino acid incorporation studies by Sadgopal and Bonner (16) and it is also evident from our own work showing that polyribosomes from cells blocked with 2×10^{-3}M thymidine are still able to complete some histone polypeptides in vitro (unpublished).

The rabbit reticulocyte cell-free system so successfully used to identify eukaryotic mRNAs (10, 12, 15, 17 and the presented report) renders feasible the characterization of the mRNAs coding for different histones and permits studies such as the timely relationship between the synthesis of these mRNAs and their appearance in the cytoplasm during the cell cycle.

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