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Translation of Messenger RNA for Histones from HeLa Cells by a Cell-Free Extract from Mouse Ascites Tumor

(7–9S RNA/histones/protein synthesis/acrylamide gel electrophoresis)

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ABSTRACT **7–9S RNA has been isolated from polyribosomes of HeLa S-3 cells in S phase. These RNA species direct the synthesis *in vitro* of all five classes of human histones when they are added to a protein-synthesizing system derived from mouse ascites tumor cells. The histones synthesized *in vitro* have been identified by two kinds of acrylamide gel electrophoresis, and by paper electrophoresis of tryptic digests. By use of the ratio of tyrosine to tryptophan incorporation as an index of contamination, it was determined that less than 10% of the 7–9S RNA is translated into proteins other than histones.**

The mRNA species that are thought to carry the information directing the synthesis of histones have many unique properties. Robbins and Borun (1) showed that histones are synthesized on small cytoplasmic polyribosomes during the S phase of the HeLa cell cycle. Subsequently, Borun *et al.* (2) detected rapidly-labeled species of 7–9S RNA associated with polyribosomes synthesizing histones that had many characteristics to suggest that they were histone mRNA. These RNA species are associated with polyribosomes only while histones are being synthesized (S phase of the cell cycle), and they rapidly disappear from polyribosomes if DNA replication is interrupted and histone synthesis ceases (2). These observations were confirmed by Gallwitz and Mueller (3), and similar observations in sea urchin embryos were subsequently reported (4–5). In sea urchin, 7–9S RNA represents the major informational RNA species synthesized just after fertilization, a time at which DNA is being replicated and histones comprise a significant fraction of the proteins synthesized in the embryos (4).

These observations suggest a unique, tight coupling between histone synthesis and DNA replication, and there is evidence that histone synthesis may be regulated at both the translational and transcriptional level (6). Kedes and Birnstiel (7) reported evidence that the DNA complements of 7–9S RNA of sea urchin are reiterated and closely clustered, in contrast to a lack of reiteration found for genes coding for duck globin (8). Another rather unique feature of the putative histone mRNA species is their rapid transfer to cytoplasmic polyribosomes after synthesis (9). This rapid transfer may be related to lack of the polyadenylic acid tracts detected in other eukaryotic mRNA species (10).

The evidence that the 7–9S RNA described above carries the information directing the synthesis of histones is circumstantial, though convincing. Recently, Borun *et al.* (11) have been able to isolate milligram quantities of these RNA species from polyribosomes of HeLa S-3 cells in S phase, and it has become possible to conclusively prove that 7–9S RNA directs the synthesis of histones. To that end, we have translated this RNA in a heterologous protein synthesizing system from mouse ascites tumor (12).

Abbreviation: SDS, sodium dodecyl sulfate.

METHODS

Isolation of 7–9S RNA

RNA was obtained from HeLa S-3 cells that had been synchronized by a double thymidine block, as described by Stein and Borun (13). 3.5 Hr after reversal of the second thymidine block, the cells were collected by centrifugation, washed with Earle's saline solution, and resuspended in buffer (10 mM NaCl–10 mM Tris·HCl, pH 7.4–1.5 mM $MgCl_2$), at a concentration of 3.8×10^7 cells/ml. The cells were homogenized in a Dounce homogenizer, and the postmitochondrial supernatant was prepared by centrifugation for 10 min at 27,000 $\times$ *g*. Polyribosomes were pelleted by a 75-min centrifugation at 120,000 $\times$ *g*, resuspended in 2.5% sodium dodecyl sulfate–0.05 M Tris·HCl, pH 7.3–1 mM $ZnCl_2$–0.1 mM naphthalene disulphonic acid, and extracted with phenol (11). The RNA was precipitated from the aqueous phase with two volumes of ethanol, and reprecipitated twice with ethanol after resuspension in 0.1 M NaCl–1mM EDTA–10 mM Na acetate pH 5.4. 300 mg of RNA was dissolved in this buffer and fractionated on an 800-ml 5–30% sucrose gradient in a Ti-15 zonal rotor at 33,000 rpm for 24 hr. The RNA sedimenting between the 18S and the 4S peak was precipitated with ethanol; about 10 mg of RNA was recovered in this fraction. This RNA was fractionated by preparative acrylamide gel electrophoresis in a Canalco apparatus, by the procedure outlined by Moriyama *et al.* (14). 2 mg of RNA was run on an 8% acrylamide gels (4.5 cm long) at 40 mA gel (constant voltage). The flow rate of the elution buffer was 2 ml/min, and the effluent was run through an ISCO A_{260} monitor (Fig. 1). The fractions corresponding to each peak were pooled, and the RNA was precipitated with ethanol and its size was determined (2) on 2.4% acrylamide gels. The identity of the RNA in each peak was thus established, with the exception of peak X (Fig. 1); this peak is eluted in variable positions and appears to be an artifact (11). About 0.2% of the total polyribosomal RNA was recovered in the 7–9S peak.

A more complete description of the preparation of 7–9S RNA will be given elsewhere (11).

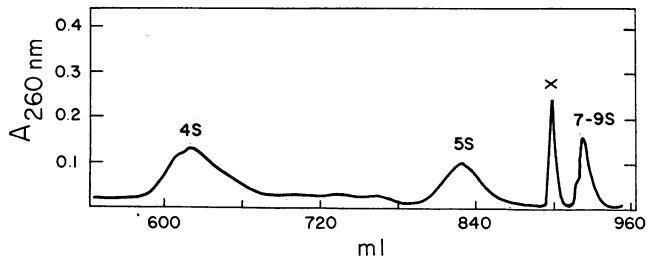

FIG. 1. Elution pattern of HeLa-cell RNA from preparative acrylamide electrophoresis. The RNA was prepared as described in *Methods* and fractionated on 8% acrylamide gels. The A_{260} of the eluate was monitored continuously; the first peak (4 S) was eluted after about 4.5 hr. The identity of the RNA in each peak has been established as described in the text.

Cell-free protein synthesis

The cell-free system from mouse ascites described by Housman *et al.* (15) was used, except that the Mg^{2+} concentration was 3 mM. The samples were incubated 60 min at 30° unless otherwise indicated. Aliquots from the reaction mixtures were counted as described (16), except that the filters were heated for 20 min at 90°.

Analysis of the cell-free product

After incubation, DNase was added to a final concentration of 50 μg/ml in 50 mM Tris, pH 7.4–8 mM Mg-acetate; this mixture was incubated for 30 min at 30°. Marker histones were added, and samples were processed as described below.

10% Acrylamide Gel Electrophoresis. Samples were dialyzed against 10 mM Na-phosphate buffer, pH 6.8–0.1% sodium dodecyl sulfate (SDS),‡ and applied to 0.6 × 18-cm gels containing 10% acrylamide, 0.2% bis-acrylamide, 0.1% SDS, 0.1 M Na-phosphate, pH 6.8, and 0.5 M urea. The gels were run 10 hr at 7.5 mA/gel, and fractionated in a Savant Autogeldivider. The fractions were counted after addition of 6 ml of a solution of 1 volume of Triton-x 100 and 2 volumes of 1.2% butyl-PBD (CIBA) in toluene.

15% Acrylamide Gel Electrophoresis. Samples were dialyzed against 0.9 M acetic acid‡ and applied to gels containing 15% acrylamide, 0.1% bis-acrylamide, 6.25 M urea, and 0.9 M acetic acid (17). The gels were run 10 hr at 2 mA/gel, fractionated, and counted as described above.

Paper Electrophoresis of Tryptic Peptides. Samples were dialyzed against distilled water and digested (18) with trypsin. The tryptic peptides were separated by electrophoresis at pH 3.5 at 4.5 kV (19) for 4.5 hr on a 110 cm long sheet of Whatman 3 MM paper, which was cut into 1-cm strips and counted as described (20).

Preparation of uniformly labeled histones

A spinner culture of HeLa cells (2.5×10^5 cells/ml) was incubated under sterile conditions for 20 hr with 0.1 μCi/ml of [^{14}C]phenylalanine or [^{14}C]lysine. The cells were collected by centrifugation, washed, and lysed with 80 mM NaCl–20 mM EDTA, pH 7.2–1% Triton X-100. The nuclei were washed twice with 0.15 M NaCl, then extracted twice with 0.25 N H_2SO_4. The extracted histones were precipitated with 3 volumes of ethanol and resuspended in water. To prepare [^{14}C]phenylalanine–labeled extracts of histones that did not contain significant amounts of *F1* polypeptides, labeled nuclei were processed as described until the acid-extraction stage. At this point, the nuclei were first extracted with 5% $HClO_4$ to remove the *F1* histone, then were extracted with 0.25 N H_2SO_4 to remove the other histones. To prepare [^{14}C]phenylalanine-labeled histone *F1*, the cells were labeled and processed as described until the acid-extraction stage. Histone *F1* was then extracted according to method B of Johns (21).

‡ Heated for 2 min at 80° in the presence of 5 M urea–0.1 M 2-mercaptoethanol.

RESULTS

7–9S RNA, isolated from polyribosomes of HeLa cells synchronized in S phase, stimulates protein synthesis in an ascites cell-free system. The rate of protein synthesis in these extracts is shown in Fig. 2. Labeled amino acids are incorporated linearly for at least 30 min, and most of the synthesis is completed by 60 min. The extract responds linearly (Fig. 3) to added RNA in the range tested (up to 80 μg/ml of reaction mixture). The stimulation varies somewhat, depending on the particular ascites extract used; 4- to 11-fold stimulation has been obtained in several experiments with 50–100 μg of 7–9S RNA per ml of reaction mixture.

The product of cell-free synthesis has been characterized in three different ways: (*i*) by electrophoresis on 10% acrylamide gels in the presence of SDS (SDS gels)—a procedure that separates proteins on the basis of their molecular weight (22); (*ii*) by electrophoresis on 15% acrylamide gels at acid pH (acetic acid gels), a procedure that separates proteins mainly on the basis of their charge (17); and (*iii*) by paper electrophoresis of the tryptic peptides.

The pattern obtained by electrophoresis on SDS gels is shown in Fig. 4. Marker histones uniformly labeled with [^{14}C]-lysine show two distinct peaks. The slow-migrating one corresponds to the lysine-rich histones *F1*, which have a molecular weight of 21,000 (23). The fast-migrating peak corresponds to the remaining classes of histones, which have molecular weights between 11,000 and 15,000 (23). This peak shows a shoulder that migrates faster than the main peak; it separates

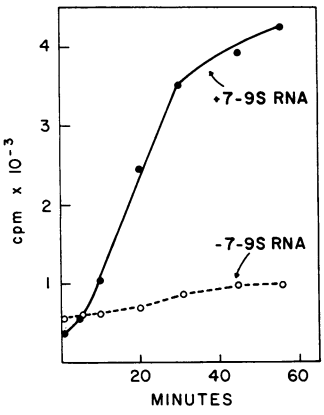

FIG. 2. Rate of protein synthesis in ascites cell-free extract with 7–9S RNA. In a final volume of 265 μl were present 160 μl of ascites extract, 80 μl of 7–9S RNA solution (8.0 A_{260} /ml) [or buffer in the control], 0.2 mCi of [^{3}H]aminoacids, and the components of the cell-free system described under *Methods*. Incubation was at 30°, and 5-μl samples were withdrawn at the times indicated and processed for counting.

into two distinct peaks when histones uniformly labeled with [^{3}H]phenylalanine are analyzed (Fig. 4*C*). However, very small amounts of *F1* histones are observed, possibly due to the low content of phenylalanine in this class of histones (23).

The product of synthesis *in vitro* directed by 7–9S RNA corresponds to the marker histones. Lysine-rich histones (*F1*) are more effectively labeled, as expected, when [^{3}H]-lysine is used (Fig. 4*A*). When no 7–9S RNA is added only a small amount of radioactive material is recovered and no distinct peaks are observed (Fig. 4*A* and *B*). The product has also been analyzed on gels at low pH (17). These gels separate the five classes of histones into four bands (peaks 1–4 of Fig. 5*A*): a well-defined band on the anode side (*F1*), followed by two closely migrating bands (*F3* and *F2a2* plus *F2b*) and a band on the cathode side (*F2a1*). This is indeed the pattern observed with the cell-free product of 7–9S RNA (Fig. 5*A*). No peak corresponding to the markers is observed in the control (Fig. 5*B*), even though five times more material was applied to the gel.

For analysis of tryptic peptides of the cell-free product, we wanted to obtain a simple and characteristic pattern. Since phenylalanine has been reported to be relatively rare but present in all histones (23), we used [^{3}H]phenylalanine to label product proteins, digested the product with trypsin and separated the tryptic peptides by paper electrophoresis. The resulting peptides show a pattern almost coincident with that of peptides of marker histones labeled with [^{14}C]phenylalanine (Fig. 6*A*). The specific activity [^{3}H/^{14}C] of most of the peptides is quite similar (Fig. 6*A*), except for peptide *6*, which has a specific activity lower than that of the other peptides. A control incubation (minus 7–9S RNA) shows no peak corresponding to those of the marker histones, except for the material at the origin and one peak close to peak *1* that is due to free phenylalanine (Fig. 6*C*). The material at the origin contains undigested protein and insoluble peptides. *F1* histones extracted from nuclei of HeLa cells uniformly labeled with [^{14}C]phenylalanine show only one tryptic peptide, migrating close to the position of peak *7* (Fig. 6*C*). These histones have been analyzed on acetic-acid gels and have shown only one major peak in the position of *F1* histones.

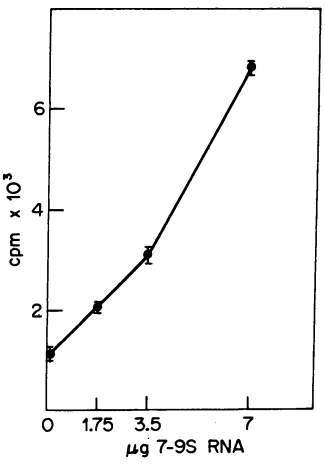

FIG. 3. Stimulation of ascites cell-free system by 7–9S RNA. For each incubation 65 μl of complete ascites cell-free system containing 5 μCi of [^{3}H]aminoacids were used. Different aliquots of 7–9S RNA solution (8.0 A_{260}/ml) or buffer were added, to a final volume of 85 μl. Two 40-μl aliquots were sampled from each incubation mixture after 60 min of incubation at 30°.

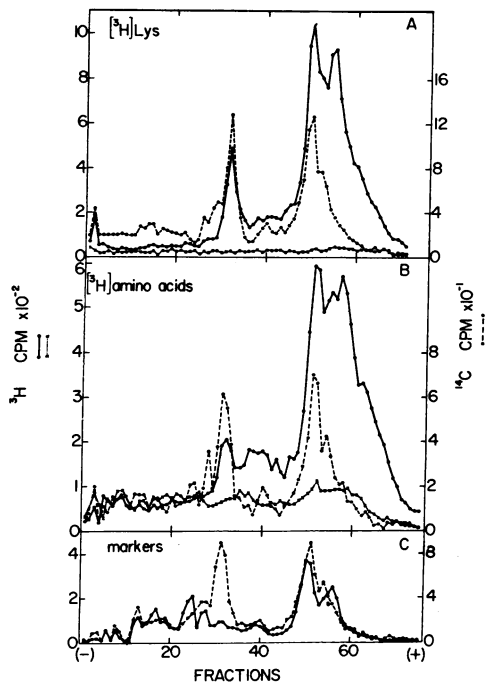

FIG. 4. Electrophoresis on 10% acrylamide–SDS gels of the product of the ascites cell-free system. For each incubation 30 μl of 7–9S RNA solution (15.1 A_{260}/ml), 210 μl of ascites extract, and radioactive amino acid(s) were incubated for 60 min at 30° in a final volume of 350 μl. The samples were digested with DNase and dialyzed; 33% (*A*) or 5% (*B*) of the mixture was analyzed by electrophoresis on acrylamide gels (see *Methods*). Histones uniformly labeled with [^{14}C]lysine were added as markers to all gels (O—O). (*A*) 30 μCi of [^{3}H]lysine (2.1 Ci/mmol); (B) 140 μCi of [^{3}H]aminoacids (New England Nuclear L-aminoacid mixture). These two panels show superimposed the patterns obtained with the cell-free product of 7–9S RNA (●——●) and with controls (×···×) without any added RNA. (*C*) Histones uniformly labeled with [^{3}H]phenylalanine (●——●).

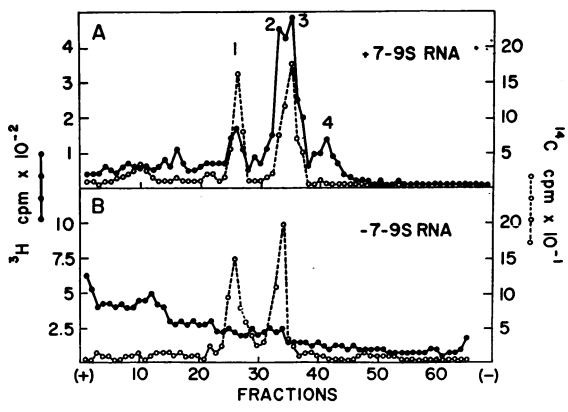

FIG. 5. Electrophoresis on 15% acrylamide–acetic acid gels of the product of the ascites cell-free system. 10% of the reaction mixture described in Fig. 3*A* with added 7–9S (*A*) and 50% of the reaction mixture without added 7–9S RNA (*B*) were dialyzed and analyzed by electrophoresis on 18-cm acrylamide gels after addition of marker histones labeled with [^{14}C]lysine. *Solid line*, cell-free product; *dotted line*, marker histones.

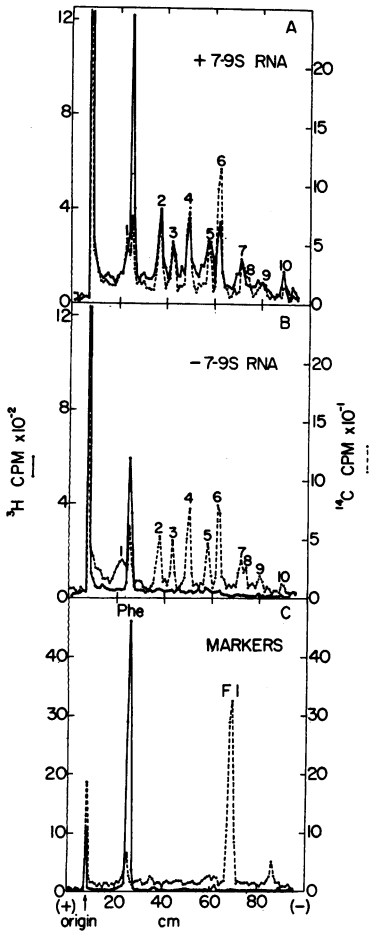

FIG. 6. Electrophoretic analysis of a tryptic digest of the product of the ascites cell-free system. (*A*) 20 μl of 7–9S RNA solution (15.1 A_{260}/ml), 120 μl of ascites extract, and 30 μCi of [^{3}H]phenylalanine (50.4 Ci/mmol) were incubated 60 min at 30° in a final volume of 200 μl. (*B*) An incubation was run as in *A*, except that the mRNA was omitted and water was added. The stimulation by 7–9S RNA over the control (*B*) was 4.8-fold. The samples were digested with DNase, and histones uniformly labeled with [^{14}C]phenylalanine were added; after dialysis against distilled water, the samples were digested with trypsin and analyzed by paper electrophoresis at pH 3.5. (*C*) Free [^{3}H]phenylalanine and a tryptic digest of [^{14}C]phenylalanine-labeled *F1* histones were analyzed in the same way. The papers were cut into 1-cm strips and counted as described (19). *Solid line*, cell-free product (*A* and *B*) and [^{3}H]phenylalanine (*C*); *dotted line*, marker histones.

Thus, we have shown that 7–9S RNA contains information to direct the synthesis of histones. We have also tried to establish whether this RNA codes for proteins other than histones. Taking advantage of the fact that histones do not contain tryptophan (23), we compared the incorporation of this amino acid into the cell-free product of 7–9S RNA with that of tyrosine, which is present in all histones (23). 7–9S RNA does not significantly stimulate the incorporation of tryptophan over that of a control incubation, whereas it markedly stimulates the incorporation of tyrosine (Table 1). As a positive control, we added mRNA for rabbit globin. This mRNA may be translated in the ascites cell-free system to give globin (15). Rabbit globin contains three tryptophan and six tyrosine residues (24–25); globin mRNA stimulates the incorporation of these two amino acids in the anticipated ratio.

TABLE 1. *Stimulation of tryptophan and tyrosine incorporation by 7-9S RNA and globin mRNA*

	cpm [^{3}H]-tyrosine	cpm [^{3}H]-tryptophan	Tyrosine/tryptophan (net molar ratios)
− RNA	912	132	— —
+ 7–9S RNA	12,100	158	44.3
+ Globin mRNA	21,300	1200	1.98

Each incubation mixture (15) contained, in a final volume of 10 μl: 1 μg of 7–9S RNA, 0.65 μg of globin mRNA (27) or no added RNA; 2.3 nCi of [^{3}H]tryptophan (4.5 Ci/mmol) or 17.5 nCi of [^{3}H]tyrosine (43.6 Ci/mmol); and 0.125 mM 19 unlabeled amino acids minus tryptophan or tyrosine. Each incubation was run in duplicate, and two 4-μl aliquots were sampled after 60 min at 30°, and counted (16). The results shown are the average of each duplicate experiment. The tyrosine to tryptophan molar ratio has been calculated by subtraction of the values obtained without added RNA, and calculation of the pmol of each amino acid incorporated with added RNA.

DISCUSSION

We have shown that human 7–9S RNA can be translated by an ascites cell-free system from mice and that the major cell-free products coded for by 7–9S RNA are histones. The identification of histones is based on electrophoretic analysis of these proteins and of their tryptic peptides. 7–9S RNA contains different species of mRNA, coding for all the histones we could separate electrophoretically.

The ratio of tyrosine to tryptophan incorporated into the cell-free product of 7–9S RNA (Table 1) has been used to estimate the contamination of this RNA with other mRNAs that code for proteins that contain tryptophan. Assuming a tyrosine to tryptophan ratio of 3 to 1 for an average protein, and knowing the tyrosine content of histones, we calculate that less than 10% of the mRNA in 7–9S RNA codes for proteins other than histones. However, we have no way of estimating contamination with mRNAs that code for tryptophan-poor proteins. In any case, since all the tryptic peptides of the cell-free product coded for by 7–9S RNA coincide with marker peptides (Fig. 6), it is unlikely that 7–9S RNA is contaminated with a major species of mRNA coding for a nonhistone protein.

It is difficult to establish whether 7–9S RNA contains other RNA species besides mRNA. It may seem possible to investigate this point by determination of the activity of 7–9S RNA in the cell-free system relative to another well-defined mRNA. In the experiment shown in Table 1, 7–9S RNA is at least three times less active than rabbit globin mRNA; this finding has been confirmed in several independent experiments. However, different mRNAs may be translated with different efficiency of the cell-free system, as in the case of the α and β chains of rabbit globin (5, 20). Moreover, the methods used to prepare 7–9S RNA and globin mRNA are quite different, and may lead to the isolation of RNAs with different biological activity. It seems possible that 7–9S RNA

contains a species of RNA that does not code for protein in the ascites cell-free system; we have demonstrated (20) a 7S RNA of this kind in rabbit reticulocytes, and an RNA sedimenting at 7 S has also been seen by Berns *et al.* (26) in calf-lens polyribosomes. It is not clear whether this 7S RNA is related to ribosomal RNA, though this seems quite possible.

In conclusion, we now have available a cell-free system that allows us to study the translational control of histone synthesis.

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