ISOLATION AND CHARACTERIZATION OF TWO mRNAs FROM HeLa S_3 CELLS CODING FOR HISTONE H4

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Summary: Histone mRNAs from S phase HeLa S_3 cells were fractionated electrophoretically in a 6% acrylamide slab gel. We have isolated two RNA species, both of which code for H4 histone when translated in a wheat germ cell-free protein-synthesizing system. Using oligo dT cellulose chromatography we have shown that neither of the RNA species contains poly A. Electrophoretic fractionation under denaturing conditions in 98% formamide-8% acrylamide gel indicates that the two H4 histone mRNAs differ in molecular weight.

Histone genes have been extensively studied in sea urchins and in HeLa cells, largely because these genes and the mRNAs for which they code have properties that are unique for protein-coding RNAs. Histone mRNAs are transcribed from reiterated genes (1,2) which in sea urchins are organized as a cluster of repeat units containing the sequences for all five histones (3,4). It has been shown in continuously dividing mammalian cells and in such cells following stimulation to proliferate that DNA synthesis and histone synthesis occur concomitantly during the cell cycle, and that inhibitors of DNA synthesis such as hydroxyurea and cytosine arabinoside block histone synthesis (5-9). Evidence has been presented which suggests that in HeLa cells and in human diploid cells following stimulation to proliferate, initiation of histone mRNA synthesis parallels the onset of DNA synthesis (10,11), although recent results suggest that in HeLa cells inhibition of DNA replication does not block histone gene transcription (12). The relationship between histone mRNA synthesis and DNA replication appears to differ in sea urchins during early stages of development. Several laboratories have reported that prior to fertilization sea urchin eggs contain histone mRNA in a nontranslated storage

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form (13-15) and that after fertilization DNA replication can occur in the absence of histone mRNA synthesis (16).

The fact that histone mRNAs in both HeLa cells and sea urchins are transcribed from multiple gene copies raises an important consideration regarding the identity of the sequences. Have all copies of the genes coding for individual histones been conserved? Grunstein et al. (17) have shown that in sea urchins there are several different mRNA species which code for histone H4. In this paper we report evidence for two distinct mRNA species in S phase HeLa S_3 cells which code for histone H4. The two HeLa cell H4 histone mRNAs are not present on polysomes of S phase cells treated with an inhibitor of DNA synthesis, translate H4 histone in vitro, do not contain poly A, and differ in molecular weight.

MATERIALS & METHODS

HeLa S_3 cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum. Unlabeled total polysomal RNA was isolated from $5x10^9$ S phase cells (18,19) synchronized by a single 2 mM thymidine block (7). ^{32}P labeled RNA was prepared from one liter of S phase HeLa cells synchronized by 2 cycles of 2 mM thymidine block (7). Following release from the second thymidine block the cells were resuspended in 1 liter of thymidine-free growth medium for 1 h, followed by incubation in phosphate-free growth medium containing 7% dialyzed calf serum and 50 mCi of ^{32}P ($2x10^6$ cells/ml) at 37°C for 3.5 - 4 h. Polysomal RNA was fractionated on 5-30% sucrose gradients in either a Beckman Ti 14 zonal rotor or a Beckman SW 27 rotor as described (19).

RNAs were electrophoresed on a 0.3x16x30 cm slab apparatus (Bio-Rad Laboratories, Richmond, CA) using the gel and buffer system of Grunstein et al. (17) which consists of a separating gel of 6% acrylamide-0.2% bisacrylamide and a 3 cm, 3% acrylamide stacking gel. Both gels are polymerized in 36 mM Na_2HPO_4-30 mM Tris·HCl (pH 7.7)-2 mM EDTA. The electrode buffer was the same but contained 0.2% SDS[a]. The gel was run at 60 mA (constant current), at 68 to 75 volts, at 20°C for 37 h; the buffer was recirculated between the upper and lower reservoirs to avoid excessive pH changes. After removal of one glass plate wet gels were covered with plastic wrap and Kodak RP-54 X-ray film was placed on the gel, covered with a glass plate, and exposed at 4°C. After varying periods the film was developed in a Kodak X-O-Mat and used to locate ^{32}P labeled RNA. Bands were located and excised with a scalpel according to the method of deWachter and Fiers (20). RNA was electrophoretically eluted from gel slices as described by Grunstein et al. (17). Excised gel slices were placed in glass pipettes with the constricted end plugged with glass wool, and the RNA was electrophoresed into small dialysis bags which had been boiled three times in EDTA and pushed over the end of the pipettes. The dialysis bags and the electrode chambers contained 40 mM Tris·HCl (pH 7.7)-20 mM Na acetate-2 mM EDTA-0.2% SDS and electrophoresis was carried out at 2 mA p tube, usually overnight. The contents of the dialysis bag were extracted once wi

[a] SDS: sodium dodecylsulphate

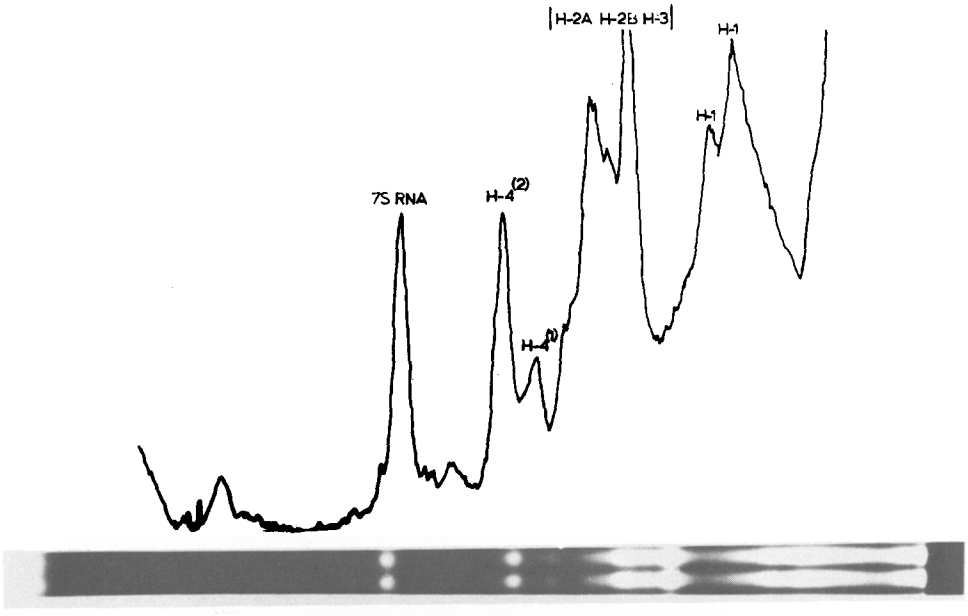

Figure 1 Preparative acrylamide gel electrophoretic fractionation of 4-18 S polysomal RNA from S phase HeLa S_3 cells. 75 µg of unlabeled RNA were combined with 700,000 cpm of ^{32}P-labeled, 4-18 S RNA from S phase HeLa cells, loaded on 0.3 x 0.4 cm wells of a 6% acrylamide gel and electrophoresed as described in Materials and Methods. The gel was analyzed autoradiographically and bands were excised as described in Material and Methods. A densitometric tracing of one of the wells is shown along with a positive contact print of the negative showing two of the wells.

water-saturated phenol, NaCl was added to the aqueous phase to a final concentration of 0.25 M and RNA was precipitated with two volumes of ethanol at -40°C. RNA was prepared for *in vitro* translation by ethanol precipitation twice from 0.25 mM LiCl and once from 0.25 M potassium acetate, and then dried in vacuo and resuspended in distilled water (18).

In vitro translation was carried out in the presence of 200 µCi/ml [^3H]lysine (38 Ci/mmole) using a cell-free protein synthesizing system derived from wheat germ according to a modification of the method of Roberts and Patterson (21). Details of the procedure have been reported (18). Samples from the *in vitro* translation mixture were dialyzed overnight against 0.9 M acetic acid-6 M urea-0.4% β-mercaptoethanol and electrophoresed on acetic acid-urea polyacrylamide gels polymerized in a 0.15x14x10 cm slab gel electrophoresis apparatus essentially according to the method of Panyim and Chalkley (22); however, polymerization was catalyzed by 10 µl of 10% ascorbic acid, 10 µl of 0.25% $FeSO_4$ and 2 µl of 30% H_2O_2 per 24 ml of gel solution, and a 1 cm stacking gel of 10% acrylamide-0.325% bis acrylamide was used (23). After electrophoresis at 100 V for 7.5 h, the gel was stained with amido black and prepared for fluorography according to Bonner and Laskey (24) except that 3% PPO in dimethylsulfoxide was used. Kodak RP-54 X-ray film was pre-exposed by a single exposure to an electronic flash shielded by a Kodak Wratten series red filter (#25) according to the method of Laskey and Mills (25).

Formamide slab gel electrophoresis was carried out according to Maniatis et al. (26). A 0.15x16x30 cm gel containing 8% acrylamide-0.4% bis acrylamide-98% formamide-16 mM Na_2HPO_4-4 mM Na_2HPO_4 (pH 7.4) was at 300 volts for 25 h. The running buffer, 16 mM Na_2HPO_4-4 mM Na_2HPO_4 (pH 7.4), was recirculated. Oligo dT-cellulose chromatography was carried out according to the method of

Aviv and Leder (27) except that the 0.1 M KCl was omitted and the 0.5 M KCl flow-through was reapplied to the column before the 0.5 M KCl wash.

RESULTS & DISCUSSION

Preparative scale electrophoresis of unlabeled 4-18 S RNA in the presence of ^{32}P labeled 4-18 S tracer RNA gives the pattern shown in Fig. 1. The individual bands were excised and the RNAs were eluted as described in Materials and Methods. RNAs were then translated in a wheat germ cell-free protein synthesizing system and the products of translation were electrophoresed with unlabeled marker histones on acetic acid-urea polyacrylamide gels. No preliminary purification to separate the histones from other translation products was carried out prior to electrophoresis. The gel was prepared for fluorography and used to expose Kodak RP-54 X-ray film which had been pre-exposed according to the method of Laskey and Mills (25) so that the darkening was approximately proportional to the amount of radioactivity in the gel. Exposed film was then scanned using a Joyce-Loebel densitometer. The results for regions labeled H4(1) and H4(2) are shown in Fig. 2. It should be noted that the amount of [^{3}H] lysine label incorporated into hot acid-resistant, trichloroacetic acid-precipitable material was proportional to the amount of RNA added to the translation mixture. A similar procedure was followed to assign the coding properties for other bands shown in Fig. 1. It can be seen that for both bands designated H4 (Fig. 1), the majority of the labeled translation product co-migrates with marker H4 histone (Fig. 2a and 2b). Based on the assumption that each of the protein products has a similar specific activity and that the area of each peak is proportional to the amount of radioactivity in the gel band, planimetric integration of the peaks indicates that approximately 95% of the translation product of band H4(2) (Fig. 1) and approximately 85% of the translation product of band H4(1) (Fig. 1) constitute H4 histone. Peak WG (Fig. 2a and 2b) is a wheat germ protein (based on its translation in the absence of added mRNA); therefore, its contribution to the total amount of protein has been subtracted in the above

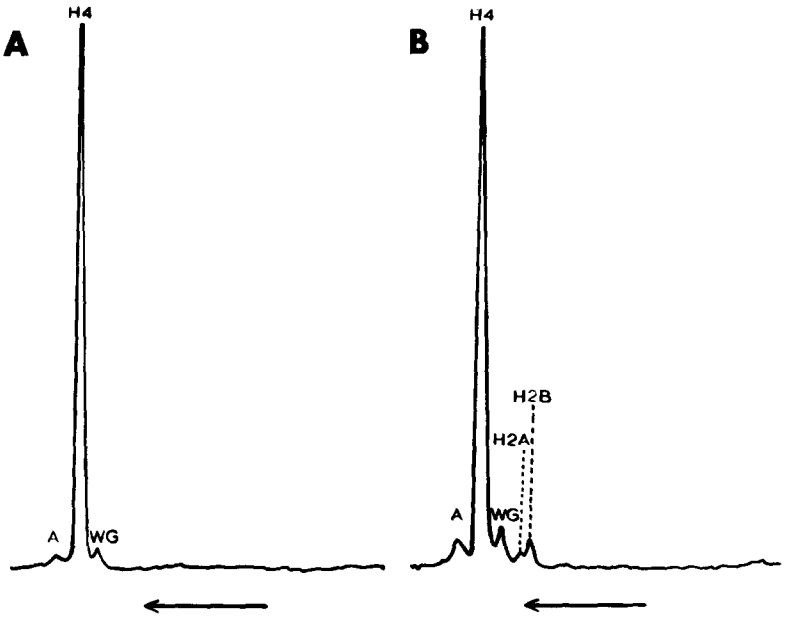

Figure 2 Acetic acid-urea acrylamide gel electrophoretic analysis of in vitro translation products of RNA extracted from bands H4(2) (Fig. 2A) and H4(1) (Fig. 2B) shown in Fig. 1. 15 µl of wheat germ in vitro translation products were electrophoresed on an acetic acid-urea acrylamide gel in the presence of marker histones, and fluorography was performed as described in Materials and Methods. The migration of marker histones is indicated.

calculations. The fast migrating material in band A may represent incomplete polypeptides.

H4 histone has not been shown to have variant forms other than those produced by post-translational modification. Therefore, our results indicate the apparent presence of two RNAs coding for proteins with identical amino acid sequences - an observation with important biological implications. It was necessary to investigate several alternative explanations for this phenomenon. Since the buffer system used in the electrophoretic fractionation shown in Fig. 1 contained 0.1 M Na^+, which would allow RNA to have considerable secondary structure, it is possible that two mRNA species have the same molecular weight but differ in secondary structure. It is also possible that band H4(1) (Fig. 1) is a complex of band H4(2) (Fig. 1) with some other RNA species. Since the RNA applied to the preparative gel (Fig. 1) had not been

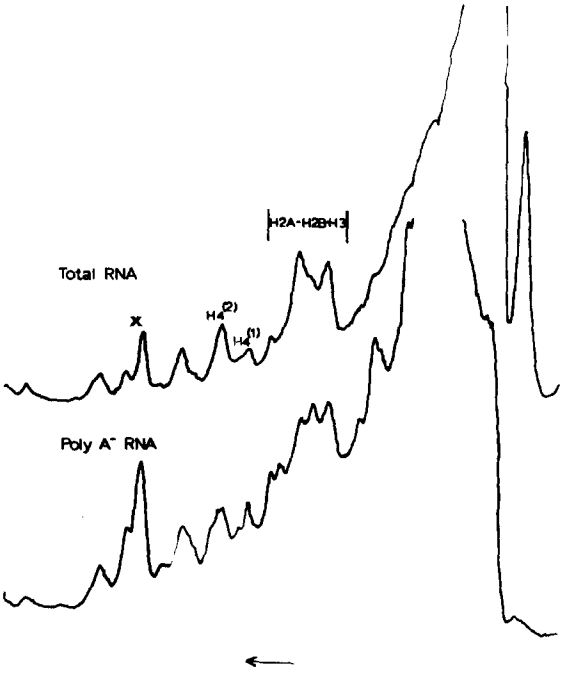

Figure 3 Comparison of 4-18 S polysomal RNA from S phase HeLa S_3 cells which was subjected to oligo dT-cellulose chromatography with total 4-18 S polysomal RNA. ^{32}P-labeled RNA (870,000 cpm of total 4-18 S RNA and 650,000 cpm of poly A⁻ 4-18 S RNA) was electrophoresed, autoradiography was performed, and densitometer tracings were made as in Fig. 1. The positions of H4 coding bands are indicated. Peak X which does not translate histone polypeptides exhibits variable recoveries from sucrose gradients.

run over an oligo dT-cellulose column before electrophoresis, the possibility exists that the difference in mobility between the two H4 histone mRNAs is caused by the presence of AMP residues at the 3' OH terminus of one of the RNA species. To test these possibilities, ^{32}P labeled polysomal RNA was isolated from S phase HeLa cells, and half of the preparation was passed twice through an oligo dT-cellulose column. Both the poly A-containing and non-poly A-containi RNAs were sedimented in 5-30% sucrose gradients and then were electrophoresed along with unfractionated 4-18 S RNA in parallel wells under the same conditions as in Fig. 1. The resulting electrophoretic profiles for total RNA and poly A minus RNA are shown in Fig. 3. In the region where histone-coding RNAs are found, there are no bands present in the total polysomal RNA sample which are

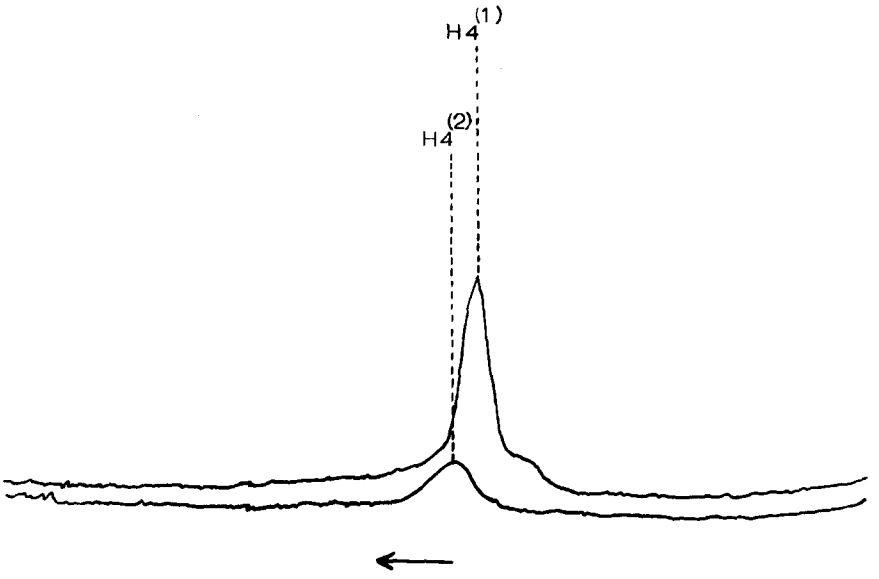

Figure 4 Electrophoretic analysis under denaturing conditions of RNAs coding for histone H4. ^{32}P-labeled RNAs extracted from an acrylamide gel similar to that shown in Fig. 1 were electrophoresed on an 8% acrylamide-98% formamide gel as described in Materials and Methods. H4 coding bands were electrophoresed on adjacent wells. Densitometric scans were superimposed to facilitate comparison.

absent in the sample from which poly A-containing RNA had been removed. In particular, the two RNA species which code for H4 histone are present in both samples. This indicates that neither RNA species contains a poly A region long enought to allow it to be retained on an oligo dT-cellulose column. Although poly A containing RNAs show large amounts of high molecular weight material in the 3% stacking gel region, there were no discreet bands of radioactivity in the lower molecular weight region (data not shown). Pinder et al. (28) have shown that RNA is denatured in 98% formamide and migrates solely as a function of molecular weight. If the two RNA species are the same molecular weight but are separated in aqueous gels because of differences in secondary structure or because of aggregation with smaller RNA species, they should co-migrate in formamide. When the two RNA bands were eluted from an aqueous gel and rerun on parallel wells of an 8% acrylamide - 98% formamide gel, it can be seen in Fig. 4 that the two bands have distinctly different mobilities.

We have also observed that the two H4 histone mRNAs are not present on polysomes of S phase HeLa cells treated with cytosine arabinoside, an inhibitor of DNA synthesis.

There are several possible explanations for the apparent differences in molecular weight between the two RNA species which code for histone H4. One is that the smaller molecular weight RNA represents a cleavage product of the higher molecular weight species. Another possibility is that since there are 30-40 copies of the histone genes in human cells (2), the different RNAs could represent transcripts from different copies of the gene. T1 ribonuclease fingerprint mapping of H4 variants from sea urchins suggests that there is not a simple precursor-product relationship between the larger and smaller RNAs (17). It remains to be seen whether the same is true in HeLa cells. The presence of multiple RNAs coding for H4 histone in both developing sea urchin embryos and in continuously dividing HeLa cells raises the question of whether there are differences in the transcriptional and translational control of the different messages. Alternatively, if the two messages are regulated identically, examination of similarities and differences in their sequence could illuminate the role of these RNA sequences in translation.

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