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Purification of histone messenger ribonucleoprotein particles from HeLa cell S-phase polysomes. Characterization of associated proteins

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ABSTRACT

We have purified HeLa histone mRNA from polysomes of S-phase cells which had been synchronized by hydroxyurea treatment. This mRNA was shown to direct the *in vitro* synthesis of all five histones which amount to at least 90-95% of its total translational activity. Polysomal histone mRNP was also purified and identified by cell-free translation and hybridization to a clone of histone DNA from *E. esculentus*. The protein moiety of this mRNP contained three prominent species of molecular weight 86,000, 73,000 and 53,000 daltons. The presence of the 73,000 species previously assessed to be bound to poly(A) is discussed in view of the fact that histone mRNA does not contain a poly(A) tail. As globin mRNA, histone mRNA as well as histone mRNP were translated with equal efficiency in cell-free extracts from either S-phase or hydroxyurea blocked HeLa cells.

INTRODUCTION

The existence of proteins associated with mRNA to form ribonucleoprotein particles (mRNP) in the polysomes of eucaryotic cells has been first established by Perry and Kelley[1] and Henshaw[2]. Such particles have then been described in a wide variety of cells and tissues (for review see reference 3).

The protein-complement of mRNPs has been analysed either in differenciated systems highly enriched in one mRNA species[4-8] or in dividing cells containing an highly heterogenous population of mRNAs[9-12]. Further information on the structure and protein composition has been obtained by the introduction of oligo(dT) cellulose or poly(U)-sepharose chromatography[13-17]. All mRNPs analysed by this method contain poly(A)$^+$mRNAs. At least one of mRNP protein has been shown to be tightly bound to this poly(A) sequence[18].

Poly(A)$^-$ mRNA which amounts to 20-30% of total polysomal mRNA[19] has also been found in RNP structures after release from polysomes[12,20] although nothing has yet been reported with respect to their protein composition.

Histone mRNA is so far the only identified example of poly(A)$^-$ mRNA[21] and it is known to be active only during S-phase in cultured cells[21]. This

situation prompted us to investigate whether histone mRNA could be released from S-phase polysomes from HeLa cells. Having established this point by demonstrating the presence in mRNP of histone mRNA both by cell-free translation assay and hybridization with a cloned histone DNA probe, we then proceeded to purify histone mRNP and analyse their associated proteins by comparison of those present in mRNPs containing poly(A)$^+$ mRNAs.

MATERIAL AND METHODS

Cell culture, synchronization and fractionation

HeLa cells S3 strain were grown in suspension as previously described[22]. Large scale synchronization was achieved with hydroxyurea[23] and monitored by cell counting, (^{3}H) thymidine incorporation and mitotic index measurement. Labeling conditions were indicated in the legends to figures. Cells were harvested, broken and the post-mitochondrial supernatant prepared as described[11] was centrifuged through a double 1.5 ml cushion of 15% and 30% w/w sucrose in buffer A. The 30% sucrose cushion was eventually adjusted to 500 mM KCl (buffer A_{500}) in order to obtain washed polysomes. Centrifugation was for 5 hours at 40,000 rpm in a Spinco SW41 rotor at 4°C and yielded a pellet of polysomes.

RNA extraction and anlysis

The above polysomal pellet was resuspended in 10 ml buffer B and extracted three times with water-saturated phenol at room temperature. RNA was ethanol precipitated and dissolved in a small volume of water. Sucrose gradient fractions containing histone mRNP were extracted in the same way.

Sucrose gradient centrifugation of deproteinized RNA was at 30,000 rpm for 17 hours at 20°C in the Spinco SW41 rotor using 5-20% w/w gradients of sucrose in buffer C.

Electrophoresis of RNA was through 10 cm long, 2 mm thick slab gels containing 6% acrylamide, 0.2% bisacrylamide, 8 M urea and 0.05% SDS in the buffer system described by Peacok and Dingman[24]. After electrophoresis, RNA was eluted from gel slices for 60 hours in 2.5 ml water and radioactivity determined in a Triton X100 based scintillation mixture.

Hybridization with cloned histone DNA

DNA from phage λ27 containing a 6 kilobases $EcoR_I$ fragment of histone DNA from *Echinus esculentus* was kindly donated to us by Dr. E. Southern. Propagation was accomplished in E coli K12 C600 host in the L3 facility of the Pasteur Institute (Dr. P. Kourilsky). DNA was recovered from the phage by phenol extraction then denatured and loaded onto Schleicher and Schüll

B6 nitro-cellulose filter according to Gillespie and Spiegelman[25]. Filters were incubated in 2XSSC for 24 hours at 65°C in the presence of (^{3}H) labeled mRNP and 500 µg/ml proteinase K then processed without RNAse treatment as described by Melli et al.[26].

Cell-free protein synthesis

HeLa cells harvested and washed as previously described[11] were homogenized with a Dounce glass homogenizer in the presence of 100 µM hemin. Nuclei and mitochondria were sequentially removed by centrifugation and the post-mitochondrial supernatant (S30) was passed through a 1.5 x 30 cm Sephadex G25 column equilibrated with buffer D and treated with nuclease from *S. aureus* prior to use[27]. Globin mRNA and crude initiation factors from reticulocytes were prepared as described by Schreier and Staehelin[28]. Each 50 µl reaction mixture contained : 10 µl nuclease-treated S30 supplemented with 1/20 volume of a 6 mg/ml solution of initiation factors, 10 µl of a mix containing 5 mM ATP, 1.5 mM GTP, 50 mM creatine phosphate, 0.25 mg/ml creatine kinase, 0.5 mM of each amino acid except lysine and 2 µCi of (^{3}H) lysine (20 Ci/mmole, CEA, Saclay) in buffer D, RNA and all necessary additions to adjust the final ionic concentration to that of buffer D. Acid-precipitable material was assayed on 5 µl aliquots according to Mans and Novelli[29].

Sodium dodecylsulfate gel electrophoresis

Proteins from mRNP or from cell-free systems were precipitated by 10% trichloracetic acid and analysed respectively on 12.5% or 17% acrylamide gels as previously described[17]. Fluorography was carried out according to Bonner and Laskey[30].

Buffers

Buffer A : 10 mM Tris-HCl pH = 7.4 ; 10 mM KCl ; 3 mM Mg Cl_2 ; 7 mM β-mercaptoethanol. Buffer B : 10 mM Tris-HCl pH = 7.4 ; 3 mM EDTA ; 100 mM NaCl ; 0.5% SDS. Buffer C : 10 mM Tris-HCl pH = 7.4 ; 1 mM EDTA ; 10 mM NaCl ; 0.1% SDS. Buffer D : 30 mM Hepes pH = 7.4 ; 3 mM MgOAC ; 130 mM KOAC ; 5 mM β-mercaptoethanol. Buffer E : 10 mM Tris-HCl pH = 7.4 ; 30 mM EDTA ; 10 mM KCl ; 7 mM β-mercaptoethanol.

RESULTS

Characterization of histone mRNA

RNA extracted from S-phase polysomes was centrifuged through a sucrose gradient and each fraction assayed for stimulation of protein synthesis in the HeLa cell-free system made dependent upon exogenous message by nuclease

treatment (Figure 1). A peak of activity appears in the 6-10S region. Gel electrophoresis of proteins synthesized across this peak revealed that around 90-95% of counts incorporated in the HeLa as well as the wheat germ system comigrate with authentic HeLa histones (Figure 2). It should be noted that the HeLa system does allow the synthesis of H_1 (Figure 2) thereby establishing the presence of its message which appears to be localized on the faster migrating side of the histone mRNA peak of Figure 1. Conversely, it does not seem to be the case in wheat germ as previously observed in this[31] as well as in the reticulocyte[32] system although it might only reflect incomplete precipitation of H_1 by 10% trichloracetic acid.

A striking characteristic of histone mRNA is its abrupt disappearance

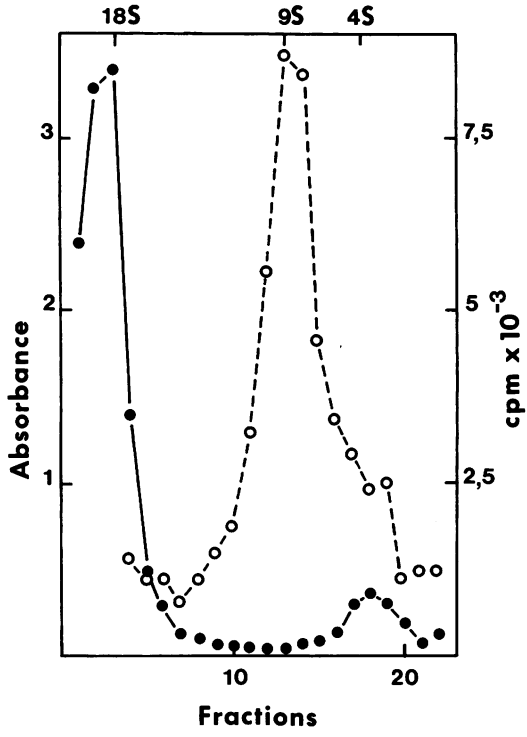

Figure 1 : Sucrose gradient profile of messenger activity of S phase RNA from synchronized cells.

3 x 10^9 synchronized HeLa cells were grown into S phase for 3 hours after release of the hydroxyurea block. Polysomal RNA was extracted and centrifuged in a sucrose gradient as described in Materials and Methods. Each fraction was ethanol precipitated twice, dissolved in 100 µl water and 5 µl aliquots were assayed in the HeLa cell free protein synthesis system. •—— • A_{260}nm ; o- - -o (^{3}H) lysine incorporation in 5 µl of reaction mixture.

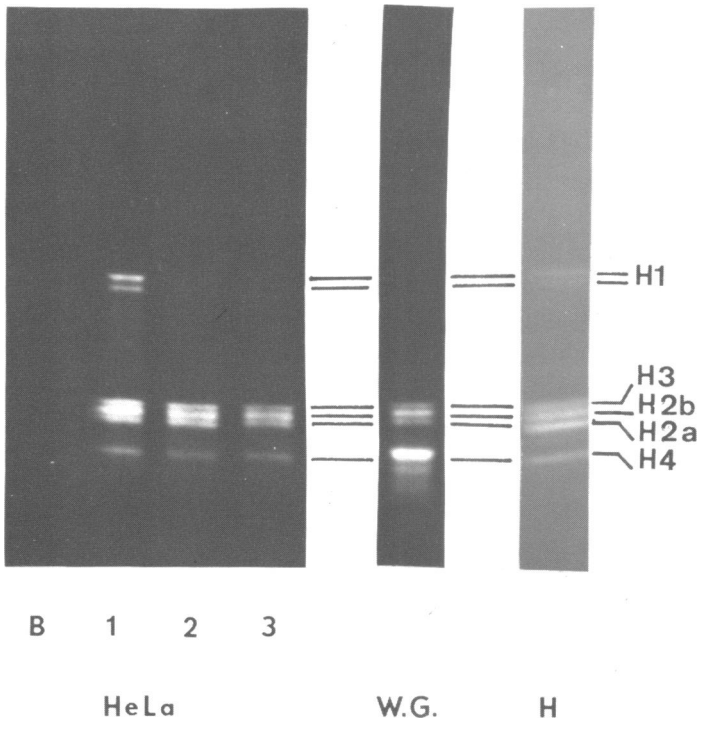

Figure 2 : SDS-polyacrylamide gel electrophoresis of cell-free translation products of S phase polysomal mRNA.
40 µl aliquots of selected cell-free reaction mixtures from Figure 1 were electrophoresed on 17% acrylamide gels as described in Materials and Methods. HeLa : proteins synthesized in the HeLa cell-free system in the presence of either no added mRNA (B) or respectively fractions 11 (1), 13 (2) and 15 (3). WG : proteins synthesized in the wheat germ cell-free system in the presence of fraction 13. (H): in vivo (^{3}H) lysine labeled histones extracted with HCl as described by Breindl and Gallwitz (32). Fluorography was according to Bonner and Laskey (30).

from the cytoplasm when DNA synthesis is impaired. We have indeed verified that inhibition of DNA synthesis by hydroxyurea results in the total disappearance of histone mRNA (Figure 6).

On all these grounds, it is therefore established that mRNAs for all histone species are actually present within the 6-10S region of polysomal RNA from HeLa cells engaged in DNA synthesis.

Characterization of histone mRNPs from S-phase polysomes

Considering its sedimentation coefficient of 8-10S (see above) which

makes it one of the smallest cellular mRNAs, histone mRNA can be expected to belong to an mRNP around 15-20S. In order to detect histone mRNA sequences within the total mRNP population present in S phase cells, we have used as an hybridization probe a 6 kilobases $EcoR_I$ fragment of histone DNA from *E. esculentus* cloned in λ27 phage vector.

Cells were labeled with (^3H) uridine during one hour after the beginning of S phase. Polysomes were treated with EDTA in order to separe mRNP from ribosomal subunits then centrifuged on a sucrose gradient under conditions where all mRNP sediment in gradient. The fractions were analysed for their total acid-precipitable radioactivity as well as for their ability to hybridize to the histone DNA clone under DNA excess conditions[26]. Under these conditions only mRNA is labeled and we have observed that the pattern of messenger was roughly superimposed with that of labeled RNA (data not shown). As shown in Figure 3 hybridization occurs exclusively in the region of the gradient containing the smallest mRNPs sedimenting as a rather heterogenous population centered around 18S. This heterogeneity probably reflects the presence of the five different messages. This result clearly demonstrates that the mRNPs coding for histones do not aggregate under the present experimental conditions. Furthermore, it shows that centrifugation can be employed to separe histone mRNP from the ribosomal subunits and from the bluk of the other mRNPs.

In order to further demonstrate the presence of histone mRNA sequences in mRNP, the latter were directly added to an *in vitro* protein synthesizing system totally dependent upon added messenger RNA (Figure 4). The majority of the translational activity corresponds to material sedimenting in the 15S region. Two different concentrations of each mRNP fraction were used to insure that protein synthesis was linear with respect to mRNP input : such was the case in all experiments reported here. In non synchronized cells, template activity was reduced 4 to 5 fold in the 15S region (data not presented). It should be noted that mRNP can promote protein synthesis with very good efficiency. Stimulation factors up to 20 fold above the background of endogenous incorporation can be observed. RNPs sedimenting at the position expected for histone mRNP are highly enriched in mRNA activity.

The products of cell-free translation in the HeLa system were analysed by SDS gel electrophoresis after precipitation with trichloracetic acid. Under these conditions, all synthesized proteins are displayed and the result should reflect the complexity of the template used provided no discriminatory mechanism operates at the translational level. Results presented in Figure 5 clearly show that the material sedimenting around 15S contain

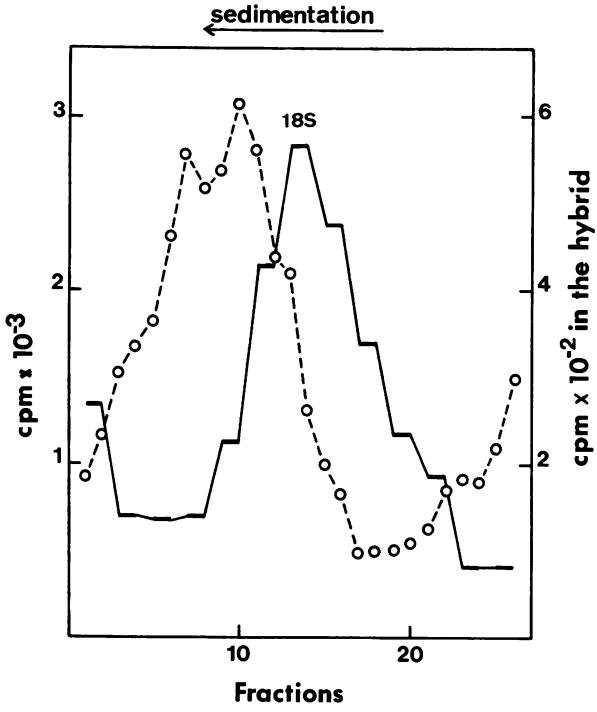

Figure 3 : Localization of histone mRNA sequences in mRNP from S phase polysomes. 3×10^9 synchronized cells were concentrated to 5×10^6 cells/ml by centrifugation at 30 minutes after the beginning of S phase and labeled for one hour with (^{3}H) uridine at 5 µCi/ml. Polysomes prepared as described in Materials and Methods were dissolved at a concentration of 2-5 mg RNA/ml in buffer E containing 30 mM EDTA and centrifuged through a 10-50% w/w sucrose gradient in buffer E at 4°C for 18 hours at 37,000 rpm in a Spinco SW41 rotor. 50 µl aliquots of each fraction were assayed for total acid-insoluble radioactivity (o- - -o). Fractions were pooled two by two and hybridized to 1 µg λ27 DNA as described in Materials and Methods (solid line).

a majority of histone message. mRNP coding for the different histones were partially resolved. H_1 histone mRNP sediments mainly around 20S whereas the bulk of the other histone mRNPs sediment between 13-16S. Furthermore, fractions C and D code almost exclusively for histones. The mRNP nature of these histone mRNA containing structures was clearly confirmed by their low density (1.42 g/cm^3) in CsCl density gradient (results not shown).

(^{3}H) uridine labeled mRNP were fractionated as in Figure 4 and cuts C and D were pooled, phenol extracted and analysed by acrylamide gel electrophoresis (Figure 6). A broad peak appeared between low molecular weight material among which 5S RNA and larger species like rRNA. This pattern

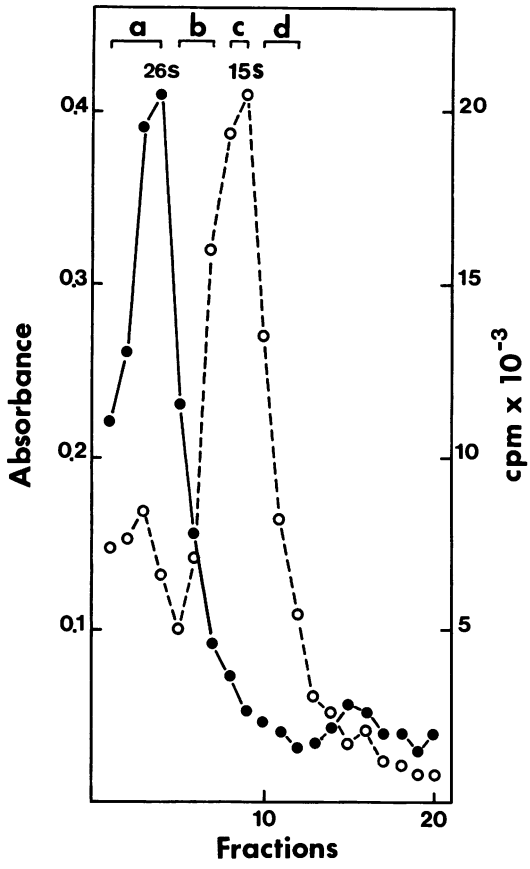

Figure 4 : Sedimentation pattern of translational activity of mRNP from S phase polysomes.
3 x 10^9 synchronized cells were harvested 3 hours after beginning of S phase. Polysomes were prepared and centrifuged through 15-30% w/w sucrose gradient in buffer E at 4°C for 16 hours at 37,000 rpm, collection was started above the four bottom fractions in order to avoid washing the pellet into highter fractions. 25 µl aliquots of each fraction were assayed in standard HeLa cell-free system. ●——● $A_{260}{}^{nm}$; o- - -o (^3H) lysine incorporation in 5 µl of reaction mixture.

clearly overlapped that of 6-10S RNA extracted from S phase polysomes and the presence of histone mRNA in the middle peak could be unambiguously identified by its disappearance after treatment with hydroxyurea[32]. The rather broad size distribution of this peak most likely reflects the presence of at least four of the histone messengers (that for histone H_1 was largely discriminated against by excluding the heavy side of the peak

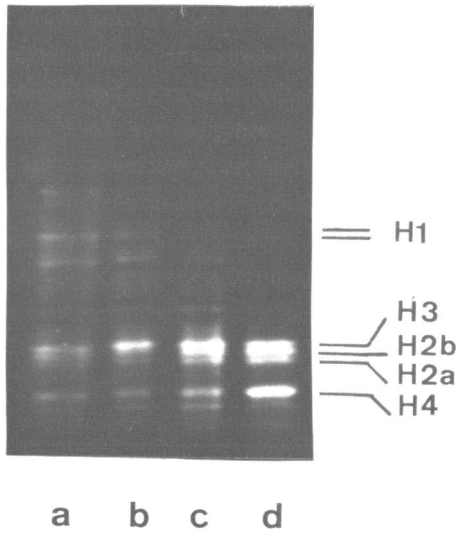

Figure 5 : SDS-polyacrylamide gel electrophoresis of cell-free translation products of S phase polysomal mRNP.
Cell free products obtain with mRNP fractions pooled as indicated in Figure 4 were electrophoresed and fluorographed as described in Figure 2.

in order to minimize contamination by other species).

In order to further purify histone mRNPs, the entire peak from an experiment similar to that reported above was concentrated 8 fold by ultra-filtration through an Amicon PM30 membrane. Loss of material was only about 10% and corresponded to a similar decrease in template activity. The concentrated material was then recentrifuged on a similar sucrose gradient. Figure 7 shows that the material sedimenting at 15S retains all the template activity. Gel analysis of cell-free synthesized proteins (Figure 8) reveals that only histones were synthesized (Track b) as was the case with purified mRNA (Track a).

Protein composition of the histone mRNP

The protein complement of histone mRNP from Figure 7 was investigated by SDS gel electrophoresis. Gel (a) (Figure 9) shows the presence of a number of proteins in the 15S region after two cycles of centrifugation with three predominant polypeptides of 53,000, 73,000 and 86,000 daltons. Especially noteworthy is the presence of the 73,000 protein which is supposed to be specifically bound to the poly(A) tail of mRNA[18]. This band became relatively even more abundant when polysomes had been washed with high

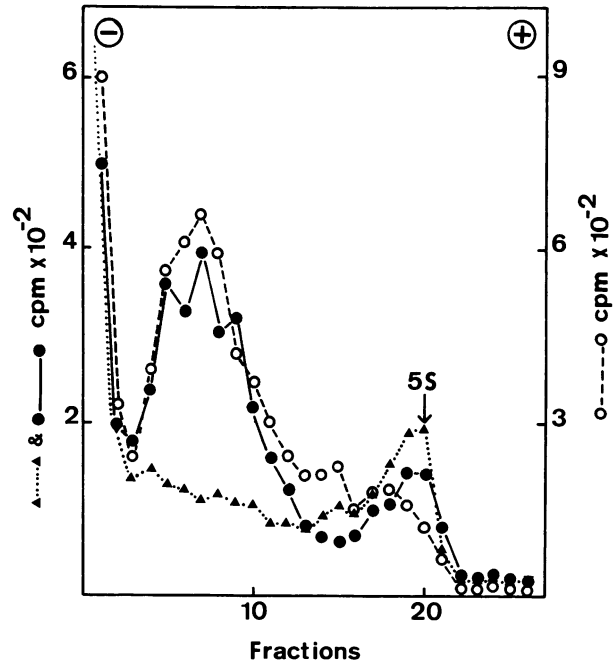

Figure 6 : Acrylamide gel analysis of RNA from mRNP purified by sucrose gradient. Polysomes were prepared and centrifuged on sucrose gradient as in Figure 4 except that cells had been exposed to 0.5 µCi/ml of (^{3}H) uridine during the 3 hours after release of the synchronization block. mRNP fractions corresponding to cuts C and D of Figure 4 were pooled and RNA extracted and electrophoresed as described in Materials and Methods (o---o). 6-10 S RNA was prepared from cells labeled for one hour during S phase in the absence (● —●) or presence (▲·····▲) of 5 mM hydroxyurea according to Breindl et al. (32) and run in parallel.

salt prior to mRNP isolation (under these conditions, the density of histone mRNP increases to 1.56-1.58 g/cm^3).

We have also analysed the proteins present in the two regions flanking the 15S peak from Figure 7. The faster migrating peak corresponds to the small ribosomal subunit which sediments at 26S in this EDTA containing gradient as shown by the presence of 18S rRNA (not presented) and of ribosomal proteins (Figure 9, gel e). The slower migrating region was shown to contain a wide spectrum of polypeptides including a very prominent band at 35,000 (Figure 9, gel d) which must correspond to L3 ribosomal protein known to be released along with 5S rRNA by EDTA treatment of ribosomes[8]. Comparison of these patterns with that of histone mRNP shows little although

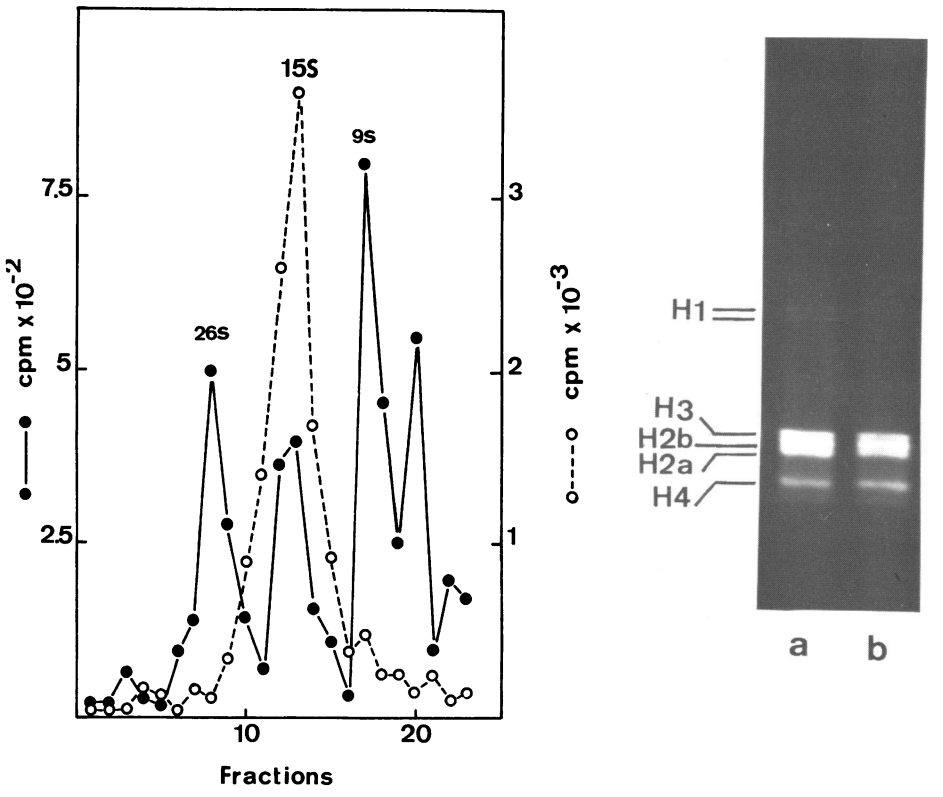

Figure 7 : Second cycle of sucrose gradient sedimentation of Histone mRNP. Cells were labeled as in Figure 6. mRNP were obtained as in Figure 4 and the entire (fractions b,c,d) peak was concentrated by ultra-filtration, recentrifuged on a sucrose gradient and analysed in the same way. o- - -o (^{3}H) lysine incorporated as in Figure 4. •——• *in vivo* labeled RNA.

Figure 8 : Comparison of in vitro translation products of purified histone mRNA and mRNP. Cell-free translation in the HeLa system was carried out and analysed using respectively : a) 2.5 µl of histone mRNA (fraction 13 from Figure 1) and b) 25 µl of histone mRNP (fraction 13 from Figure 7).

detectable contamination of the latter.

The histone mRNP share some proteins with poly(A)$^+$ mRNP. Polypeptides of molecular weight 53,000 and 73,000 are common to both preparations.

Comparison of template activity of histone mRNP in HeLa cell-free system obtained either from S phase cells or cells where DNA synthesis was blocked

A rapid decline of histone messenger activity is known to occur after

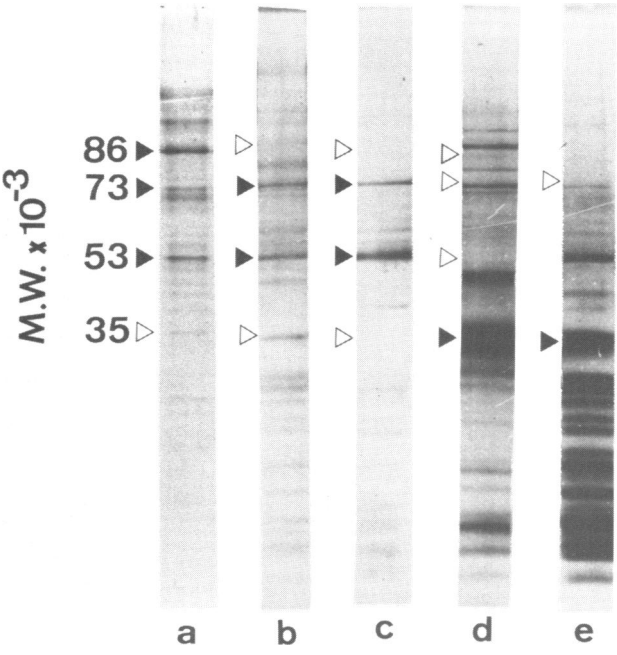

Figure 9 : Polypeptide composition of histone mRNPS.
a) 15S histone mRNPs (fractions 12-14 of Figure 7) ; b) 15S histone mRNPs prepared as in Figure 7 except that polysomes had been previously washed with 0.5 M KCl (see Materials and Methods) ; c) total poly(A)$^+$ polysomal mRNPs washed with 0.5 M KCl and prepared by poly(U) sepharose chromatography as previously described (17) ; d) 10S RNPs (fractions 17-19 of Figure 7) ; e) 26S RNPs (fractions 7-9 of Figure 7). Sample preparation and electrophoresis on 12.5% acrylamide gels containing sodium dodecylsulfate were as described in Materials and Methods. Triangles indicate proteins referred to in the text, the size which was calibrated with markers (not shown).

cessation of DNA synthesis either at the end of S phase[33] or as a result of inhibition by hydroxyurea[32]. The possibility that some factor present in cells where DNA synthesis is impaired might be responsible for this phenomenon has been addressed by Breindl and Gallwitz[32] by comparing the ability of S-phase or hydroxyurea treated HeLa cell free extracts supplemented with rabbit reticulocyte initiation factors to translate purified globin and histone mRNA as well as tobacco mosaic virus RNA. No difference was found between these two systems with respect to the stimulatory effect of the various mRNAs used. We have repeated and confirmed this experiment with purified globin and histone mRNA (Table 1). Furthermore, the same

Cell-free extract from	Template activity of		
	globin mRNA	Histone mRNA	Histone mRNP
S-phase cells	100%	100%	100%
Hydroxyurea-treated cells	99 ± 12%	99 ± 16%	94 ± 8%

Table 1 : Comparison of the template activity of mRNA and mRNP in HeLa Cell-free systems prepared with S-phase or hydroxyurea-treated cells.

Globin mRNA was prepared as described in Materials and Methods. Histone mRNA and mRNP were prepared as described respectively in Figures 1 and 7 (fraction 13). In two cases, histone mRNP were prepared from polysomes washed with 0.5 M KCl. Synchronized cells were grown for 3 hours after the beginning of S-phase. One half of the suspension was harvested and postmitochondrial supernatant prepared as described in Materials and Methods. The other half was incubated with 5 mM hydroxyurea for 30 min. and the postmitochondrial supernatant prepared as above. Protein synthesis assays were carried out as described in Materials and Methods. The activity in the cell-free system from synchronized cells was taken as 100%. The results summarise height experiments.

lack of difference was observed when comparing naked histone mRNA to histone mRNP whether or not washed by 0.5 M KCl.

These findings suggest that proteins associated with histone mRNA have not been able to restore the *in vivo* conditions leading to the rapid disappearance of histone message. The explanation for this negative result must therefore lie either in the loss or destruction of a required component during cell-free extract preparation or in the use of non homologous initiation factors. Unfortunately, this second possibility is not yet amenable to experimental test due to our present inability to obtain an entirely homologous HeLa cell-free system endowed with sufficient translational capability.

DISCUSSION

We have purified histone mRNA from polysomes of S-phase HeLa cells to such an extent that histone message accounted for at least 95% of the total mRNA content as follows from the results of *in vitro* translation where no significant background as well as no other discrete polypeptide species can be detected.

Histone mRNP have also been purified from S-phase HeLa polysomes after

release by EDTA treatment. These mRNP migrate at 15S and exhibit the typical low density in cesium chloride. Their content in histone mRNA was established by hybridization with cloned histone genes from the sea urchin *E. esculentus* and by *in vitro* translation. The same level of purity as with mRNA was achieved. It should be recalled that only RNA species with messenger activity are taken into account by *in vitro* translation assays ant that therefore no absolute value for the purity of histone mRNA can be inferred from the present data.

The protein composition of purified histone mRNA has been investigated by comparison with that of total polysomal mRNP. Three major species of molecular weights 86,000, 73,000 and 53,000 daltons were found to stand out above a background of minor ones. The relative proportion of the 86,000 protein was largely decreased after washing of the polysomes by high salt. Especially interesting was the association with histone mRNA which lacks poly(A)[21] of the 73,000 protein so far reputed to be specific for this sequence[18]. Its presence in histone mRNP cannot be accounted for by contamination by mRNP containing poly(A)$^+$ mRNA as we have clearly shown the absence of any other messenger in significant abundance. This observation raises the possibility that this protein might not be specific for the poly(A) sequence by itself but rather to some other feature more general to all kinds of mRNA. Along this line, at least four alternative explanations can be thought of : 1) the 73,000 protein might be only related to the 3' end of the molecule regardless of its sequence. In this context, it should be kept in mind that capped 5' ends are actually also of the 3' type due to the inverted position of the 7 methyl G[34] ; 2) the 73,000 protein might be specific for the heteropolymeric 3' trailer region i-e adjacent to poly(A) in poly(A)$^+$ mRNA. In this latter case, a common feature should be found at this location in both poly(A)$^+$ mRNA and histone mRNA. The oligonucleotide AAUAAA has actually been found in the trailer region of several mammalian mRNAs[35] and in H_{2B} but not H_{2A} or H_3 mRNA from *S. purpuratus*[36]. In this respect it would be of value to know whether all five histone mRNPs contain this protein, an information that the present data are unable to provide. 3) the 3' end of all mRNAs could share a common secondary structure recognizable by the 73,000 protein. Too scant information along this line is available by now to allow further speculations. 4) A last possibility would be that histone mRNAs contain a short stretch of oligo(A) not long enough to qualify them for oligo(dT) or poly(U) binding[37] while still sufficient for binding this 73,000 protein. Such a possibility must be taken seriously in view of the

fact that histone mRNA from xenopus oocytes does indeed contain poly(A)[38].

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