REITERATION FREQUENCY OF HISTONE CODING SEQUENCES IN MAN

Michael C. Wilson, Marialuisa Melli and Max L. Birnstiel

Institut für Molekularbiologie II
der Universität Zürich
Winterthurerstrasse 266A
8057 Zürich, Switzerland

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Summary

A "10S"-RNA fraction with the characteristics of histone messenger was isolated from pulse labeled synchronized HeLa cells. Two properties indicate that this fraction consists of histone messenger sequences. First, its appearance in the cell cytoplasm is dependent upon continued DNA replication; and second, it is capable of cross hybridization with isolated histone DNA of sea urchin. The rate of hybridization of this RNA with an excess of human DNA suggests that there are 10-20 copies of each histone coding sequence in the human haploid genome.

Introduction. Several messenger RNAs have been isolated from eukaryotes and measurements of the complementary sequences in the DNA have been made by molecular hybridization (1-7). With the exception of the histone mRNA, the number of complementary sequences of these messengers has been found to be low, between 1 and 5. The histone mRNA coding sequences in sea urchin (Psammechinus miliaris), on the other hand, have been estimated to have a reiteration of 1000 (7,8). In addition, evidence from cross hybridization experiments has suggested that the DNA sequences complementary to the 9S histone mRNA of sea urchin are reiterated in Drosophila (9) and Xenopus (7) genomes. Here we have used the homologous messenger RNA extracted from HeLa cells to investigate the extent of reiteration of the histone DNA sequences in Man.

Materials and Methods. Cell culture: HeLa cells were grown in suspension culture in Eagle's minimum essential medium with Earle's salts supplemented with 5% each of calf serum and feotal calf serum. For histone messenger RNA preparation, cells were synchronized with a double thymidine block (2mM) as described by Galavazi, et al. (10). The block was released by washing the cells in warm spinner salts and resuspending them into warm fresh media to a concentration of 4-5 x 10^6 cells/ml.

As measured by ^{3}H-thymidine incorporation in a 10 fold more dilute culture, the cells were shown to reach the peak of S-phase 4.5 hours after release. To inhibit further DNA synthesis in some experiments cytosine arabinoside was added 15 minutes after resuspension to a concentration of 40 ug/ml. The RNA was labeled by adding ^{3}H-uridine (200 μCi/ml, 25 Ci/mMol) 45-60 minutes after release and the cells were further incubated for an additional 60 minutes before harvesting.

To prepare heterogeneous nuclear RNA nonsynchronized cells were grown for two hours in the presence of 200 μCi/ml ^{3}H-uridine and a low dose of actinomycin D (0.05 μg/ml) to inhibit ribosomal RNA synthesis.

RNA preparation: Cells from suspension culture were washed in ice cold spinner salt solution, isotonic buffer (0.14 M NaCl, 0.01 M TRIS pH 7.5, 0.001 M $MgCl_2$) and finally resuspended in hypotonic RSB (0.01 M NaCl, 0.01 M TRIS pH 7.5, 0.0015 M $MgCl_2$) for five minutes before lysis with 0.5% Nonidet P-40 (Shell Oil Co.). To prepare histone mRNA total cytoplasmic RNA was extracted with phenol/chloroform as described by Perry, et al. (11). The RNA was layered on 15-30% sucrose density gradients (0.1 M NaCl, 0.01 M TRIS pH 7.5, 0.001 M EDTA) and centrifuged at 2°C for 28-30 hours at 25,000 revs/min in the Spinco SW 27 rotor. The region between the 18 and 5S RNA peaks was pooled, precipitated with alcohol, and loaded on 6% polyacrylamide gels (8,12).

Heterogeneous nuclear RNA was isolated from Nonidet P-40 prepared nuclei as described by Melli, et al. (13).

Hybridization procedures: Filter hybridization was carried out according to Birnstiel, et al. (14). DNA excess hybridizations were conducted according to the precedure of Melli et al. (15). The DNA used in the latter experiments, prepared from human placenta (16) was sonicated to an average single stranded molecular weight of 150,000 daltons. Conditions of hybridization are stated in the figure legends.

Results and Discussion. Preparation of histone messenger RNA: Pulse labeled cytoplasmic RNA, prepared from synchronized HeLa cell cultures released into S-phase, was fractionated on sucrose density gradients. The material sedimenting between 6 and 14S was pooled and analysed on 6% polyacrylamide gels. Figure 1 shows that this RNA may be separated into several discrete components. It has been previously demonstrated

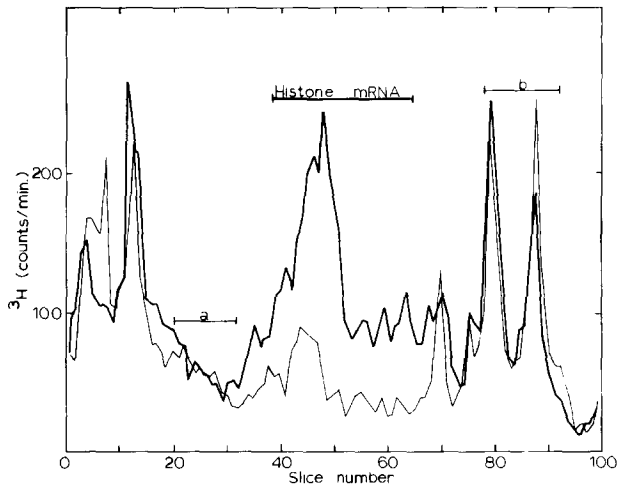

Figure 1. Polyacrylamide gel electrophoresis fractionation of 6-14S RNA. 6% gels were run at 3 mA/gel in 3.6 mM phosphate buffer, 0.2% SDS (8,12) for 6.5 hours. Slices of 1 mm were eluted in 1 ml of 0.6 M lithium acetate pH 6.0, 0.5% SDS, 5 μg/ml E.coli soluble RNA and aliquots of 20 μl counted. The heavy line refers to the RNA preparation from synchronized cells; the fine tracing represents the RNA extracted from a cytosine arabinoside (40 μg/ml) treated culture. Migration is from left to right. Under these conditions 18S rRNA migrates to about fraction 7; the 4 and 5S RNAs have run out of the gel.

that the appearance of histone mRNA in the cytoplasm is dependent upon continued DNA replication (17). Accordingly, we observe a marked inhibition of a few distinct RNA species in cells where DNA synthesis has been blocked by cytosine arabinoside. Although the DNA synthesis is effectively halted by the drug, a complete inhibition of these species is not achieved. This observation is in agreement with other workers (Gallowitz, personal communication), and implies that the suppression of DNA synthesis cannot completely abolish the appearance of newly synthesized histone mRNA in the cytoplasm. However, the presence of minor RNA species not affected by the cytosine arabinoside, and migrating in the same position as the histone messengers cannot be entirely ruled out.

To further confirm that the cytosine arabinoside sensitive RNA is indeed histone messenger RNA, we have cross hybridized it with isolated histone DNA of sea urchin (18). Table 1 indicates that the specific hybridization (cpm hybridized/ug DNA) of the HeLa histone mRNA fraction with purified histone DNA is 38 times greater than

Table 1. Standard reactions of 15,000 cpm/ml of the indicated RNA fractions were incubated with two filters of 10 μg each of total Psammechinus miliaris DNA and two filters of 0.2 μg each of purified histone DNA(18) at 60°C for 6 hours in 4 x SSC. The filters were washed, RNAased and counted according to Birnstiel, et al.(14). The HeLa RNA fractions were obtained from synchronized cells not treated with cytosine arabinoside. The fractions refer to Figure 1: Histone messenger RNA; slices 38-62; a & b, slices 20-26 and 78-92. Fractions a and b represent RNA species not subject to inhibition by cytosine arabinoside treatment and therefore presumably not histone messenger RNA. The latter fractions, a and b, were incubated separately and the results averaged.

Cross hybridization of HeLa RNA fractions to sea urchin DNA

RNA fraction	Sea urchin DNA hybridized	Specific hybridization (cpm hybridized/μg DNA)	Hybridization ratio Histone DNA/Total DNA
HeLa histone mRNA	Histone DNA	97.9	38
	Total DNA	2.6	
Region a&b (Fig.1)	Histone DNA	7.9	3.5
	Total DNA	2.25	
Sea urchin 9S histone mRNA	Histone DNA	1317	46.7
	Total DNA	28.2	

that obtained with total sea urchin DNA. Other fractions, not inhibited by the cytosine arabinoside, do not hybridize significantly to the sea urchin histone DNA. As the homologous control reaction with sea urchin 9S histone mRNA gives a ratio of specific hybridization similar to that found with the cytosine arabinoside sensitive RNA, we conclude that a) sequence homology exists between the HeLa histone mRNA and sea urchin histone DNA, and b) that this RNA fraction hybridizes specifically to those sequences enriched for in purified histone DNA.

These lines of evidence taken together strongly suggest that this fraction of the cytoplasmic RNA is that which codes for histone protein.

Hybridization in DNA excess: To determine a standard allowing comparison of kinetic rate, and thereby reiteration frequencies, of RNA hybridization with an excess of DNA, we have chosen the heterogeneous nuclear RNA of HeLa. The RNA complementary to the kinetically slow and presumably unique sequences of the DNA (15) was isolated free from reiterated material

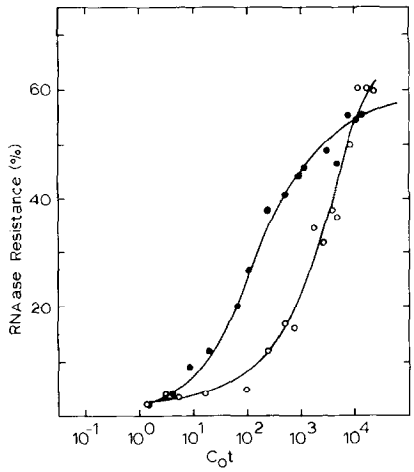

Figure 2. DNA excess hybridization experiments. Heterogeneous nuclear RNA (o-----o) was hybridized (2,600cpm/mg DNA) in 2 x SSC at 67°C. Histone mRNA (●-----●) hybridization (500 cpm/mg DNA) was in 2 x SSC at 75°C. In the latter experiment, 5.3% of the RNA input has been subtracted for zero time RNAase resistance.

by hybridizing the reiterated RNA species to exhaustion on filters as described by Pagoulatos and Darnell (19). The RNA remaining in solution, that is those RNA sequences not annealed to the immobilized DNA, was then hybridized in DNA excess. The hybridization (Figure 2), though not reaching a plateau value, has been taken as a second order reaction with an approximate $Cot_{1/2}$ of 2500. This Cot value was then taken as a kinetic standard for hybridization of RNA to unique DNA.

With this standard in mind, we have hybridized the histone mRNA under conditions considered to be in DNA excess. The curve obtained (see Figure 2) clearly follows the second order kinetic expected of uniformly reiterated species with a $Cot_{1/2}$ of 150. It is noteworthy that the hybrid obtained at a Cot of 800 has a T_m of 87°C in 1 x SSC (data not shown), a value consistent with the reported base analysis of HeLa histone mRNA indicating a G+C content of 54% (20). The rate of hybridization of the histone mRNA is of the order of 10 to 20 times faster than that found with the kinetically slow heterogeneous nuclear RNA. We therefore estimate that the DNA sequences complementary to the HeLa histone messenger RNA, characterized by its a) sensitivity to inhibition of DNA synthesis by cytosine arabinoside, b) homology to the isolated histone DNA of sea urchin, and c) kinetically uniform behaviour in DNA excess hybridization experiments, are reiterated 10-20 fold.

Interestingly, similar results indicative of a low reiteration of histone coding sequences in the mouse have been obtained in our laboratory by Dr. E. Jacob. This suggests that although the histone genes in Man and in mouse are repetitive, the high degree of reiteration present in the sea urchin is not a general characteristic of the mammalian genome.

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